



Molecular detection and genotypes of *Enterocytozoon bieneusi* in farmed mink (*Neovison vison*), blue foxes (*Alopex lagopus*), and raccoon dogs (*Nyctereutes procyonoides*) in Xinjiang, China

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ABSTRACT

Enterocytozoon bieneusi is a zoonotic pathogen that infects a variety of hosts including humans, livestock, wildlife, companion animals, and birds, as well as being abundant in the environment. Humans and nonhuman animals could be infected with *E. bieneusi* via consumption of food or water that contains zoonotic and host-adapted genotypes. In this study, 288 fecal specimens were collected from farmed minks, blue foxes, and raccoon dogs, in Xinjiang, China. *Enterocytozoon bieneusi* was examined by PCR amplification based on sequence analysis of the internal transcribed spacer (ITS) region. The overall infection rate of *E. bieneusi* was 4.9% (14/288), with mink samples showing the highest infection rate (5.6%, 12/214), followed by blue foxes (2.9%, 1/35), and then raccoon dogs (2.6%, 1/39). Six *E. bieneusi* genotypes were identified, including D (n = 5), PigEBITS7 (n = 4), EbpA (n = 2), CAM5 (n = 1), WildBoar3 (n = 1), and a novel genotype XJMI-1 (n = 1). Phylogenetic analysis showed that all *E. bieneusi* genotypes belonged to group 1, which composed of over 300 genotypes and most of them have been identified in human and variety of animals, suggesting a risk of zoonotic transmission from farmed wildlife to humans.

1. Introduction

Enterocytozoon bieneusi is a genetically diverse zoonotic parasite with a wide host range, including humans, livestock, and wild animals (Li and Xiao, 2021). Hosts infected with *E. bieneusi* typically display chronic diarrhea, and in immunocompromised individuals, wasting syndrome, caused by intestinal malabsorption (Lobo et al., 2012; Matos et al., 2012). Transmission routes of *E. bieneusi* are not fully understood; however, contaminated soil, feces, food, and water, are the main sources of *E. bieneusi* infection. Direct contact with infected humans or animals may also be an important transmission route (Li et al., 2019).

Based on sequence analyses of the internal transcribed spacer (ITS) region of the rRNA gene, over 500 *E. bieneusi* genotypes have been identified from humans, animals, and water (Li and Xiao, 2021). Phylogenetic comparative analyses clustered those genotypes into 11 distinct genetic groups with different host ranges. Groups 1 and 2 contain 90% of human-pathogenic genotypes (most of which have been identified in animals), whereas the remaining groups (3–11) comprise

host-adapted genotypes associated with specific animal species (Li et al., 2020a).

Mink, foxes, and raccoon dogs are commonly farmed for their fur in China, as of 2015, the total number of domestic breeding is about 70.38 million (of which 32.4 million are mink, 17.08 million are foxes and 20.9 million are raccoon dogs). In China, Xinjiang Uygur Autonomous Region of China (hereinafter referred to as Xinjiang) was suitable for the breeding of minks, foxes and raccoon dogs because of the temperate zone continental arid climate. However, there is limited data on the presence and genetic characterization of *E. bieneusi* in these three species. This study aimed to determine the prevalence and genotype of *E. bieneusi* in farmed mink (*Neovison vison*), blue foxes (*Alopex lagopus*), and raccoon dogs (*Nyctereutes procyonoides*) in Xinjiang, and to assess the potential zoonotic risk of *E. bieneusi*.

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Table 1
Prevalence of *Enterocytozoon bieneusi* in farmed minks, blue foxes and raccoon dogs in Xinjiang.

Collection site	Animal	Age	No. Positive/ No. specimens (%)	<i>Enterocytozoon bieneusi</i> genotypes (n)
Bole	Mink	<6 months	9/119 (7.6)	D (5), PigEBITS7 (3), NXJMI-1 (1)
		>6 months	3/95 (3.2)	EbpA (1), PigEBITS7 (1), CAM (1)
	Subtotal		12/214 (5.6)	D (5), EbpA (1), PigEBITS7 (4), CAM5 (1), XJMI-1 (1)
Barkol Kazakh	Blue foxes	<6 months	0/15 (0)	-
		>6 months	1/20 (5.0)	WildBoar3 (1)
	Subtotal		1/35 (2.9)	WildBoar3 (1)
Raccoon dogs		<6 months	0/20 (0)	-
		>6 months	1/19 (5.3)	EbpA (1)
	Subtotal		1/39 (2.6)	EbpA (1)
Total			14/288 (4.9)	D (5), EbpA (2), PigEBITS7 (4), WildBoar3 (1), CAM5 (1), XJMI-1 (1)

2. Materials and methods

2.1. Ethics approval

Appropriate permission was obtained from the farm managers before fecal specimen collection. No permits were required for the described field studies. The protocol was reviewed and approved by the Ethics Committee of Tarim University.

2.2. Specimen collection

A total of 288 fecal specimens were collected from 214 minks (*Neovison vison*) on a farm (more than 10,000 minks) in Bole City, 35 blue foxes (*Alopex lagopus*), and 39 raccoon dogs (*Nyctereutes procyonoides*) on a farm (roughly 600 foxes and 500 raccoon dogs) in Barkol Kazakh Autonomous County in Xinjiang, Northwest China. Age information of those animals was counted, including 119 young (<6 months) and 95 adult (>6 months) mink, 15 young and 20 adult blue foxes, 20 young and 10 adult raccoon dogs. The mink was mainly fed frozen chicken and fish, whilst the foxes and raccoon dogs were fed with cooked viscera and intestines from cattle or sheep. Each adult animal has one per cage, and young animals have three to five cages. Each stool counts as a single sample. Fresh feces specimens (20–30 g) were collected with sterile gloves and placed into a clean sampling bag. None of the animals had diarrhea at the time of sampling, and all specimens were transported to a laboratory and stored at 4 °C. DNA was extracted within one week after specimen collection.

2.3. DNA extraction and PCR amplification

Genomic DNA was extracted from approximately 200 mg of a fecal specimen using the E.Z.N.A.® Stool DNA Kit (Omega Biotek Inc., Norcross, GA, USA), following the manufacturer’s instructions. *E. bieneusi* was identified using nested PCR amplification of the ITS region as described by Sulaiman et al. (2003). The 2 × EasyTaq PCR SuperMix (TransGene Biotech Co. Ltd., Beijing, China) was used in the PCR amplification. Each PCR analysis contained positive (DNA from dairy cattle-derived genotype I) and negative controls (distilled water).

Table 2
Prevalence of *Enterocytozoon bieneusi* in farmed minks, blue foxes and raccoon dogs in China.

Animal	Collection site	No. Positive/ No. samples (%)	<i>Enterocytozoon bieneusi</i> genotypes (n)	References	
Mink	Heilongjiang	5/79 (6.3)	D (2), Peru 11 (1), NCM-1 (1), NCM-2 (1)	Zhang et al. (2018)	
	Hebei	10/31 (32.3)	D (5), EbpC (4), Peru11 (1)	Zhang et al. (2018)	
	Jilin	7/67 (10.5)	D (3), NCM-1 (4)	Zhang et al. (2018)	
	Liaoning	8/90 (8.9)	D (2), EbpC (3), Peru11 (3)	Zhang et al. (2018)	
	Shandong	0/31 (0)	-	Zhang et al. (2018)	
	Heilongjiang	10/257 (3.9)	EbpC (3), HLJM-1 (2), HLJM-2 (1), Peru11 (7)	Cong et al. (2018)	
	Jilin	13/302 (4.3)	EbpC (3), Peru11 (6), HJLM-1 (1)	Cong et al. (2018)	
	Xinjiang	12/214 (5.6)	D (5), EbpA (1), PigEBITS7 (4), CAM5 (1), XJMI-1 (1)	This study	
	Subtotal	65/1071 (6.1)	CAM5 (1), D (17), EbpA (1), EbpC (13), HLJM-1 (3), HLJM-2 (1), NCM-1 (5), NCM-2 (1), Peru 11 (18), PigEBITS7 (4), XJMI-1 (1)		
	Foxes	Jilin	7/91 (7.7)	D (2), NCF2 (2), Peru8 (1), Type IV (2)	Zhang et al. (2016)
Heilongjiang		5/70 (7.1)	CHN-DC1 (2), NCF2 (1), Peru8 (1), Type IV (1)	Zhang et al. (2016)	
Hebei		25/141 (17.73)	D (2), NCF1 (3), NCF2 (10), NCF3 (1), NCF4 (1), NCF6 (1), NCF5 (2), NCF7 (1), Peru8 (2), Type IV (2)	Zhang et al. (2016)	
Heilongjiang		44/191 (27.7)	D (44)	Yang et al. (2015)	
Heilongjiang		12/73 (16.44%)	D (6), CHN-F1 (1), EbpC (5)	Zhao et al. (2015)	
Jilin		6/37 (16.22%)	D (6)	Zhao et al. (2015)	
Xinjiang		1/35 (2.9)	WildBoar3 (1)	This study	
Subtotal		100/638 (15.7)	CHN-DC1 (2), CHN-F1 (1), D (60), EbpC (5), NCF1 (3), NCF2 (13), NCF3 (1), NCF4 (1), NCF5 (2), NCF6 (1), NCF7 (1), Peru8 (4), Type IV (5), WildBoar3 (1)		
Raccoon dogs		Heilongjiang	2/49 (4.1)	D (1), CHN-R1 (1)	Zhao et al. (2015)
		Heilongjiang	17/162 (10.5)	CHN-DC1 (2), D (14), WildBoar3 (1)	Yang et al. (2015)
	Heilongjiang	1/40 (2.5)	CHN-DC1 (1)	Xu et al. (2016)	
	Hebei	22/54 (40.74)	CHN-DC1 (1), CHN-F1 (3), NCF2 (15), NCR1 (2)	Xu et al. (2016)	
	Jilin	34/110 (30.91)	CHN-DC1 (7), CHN-F1 (6), D (5), NCF2 (11), NCR2 (5)	Xu et al. (2016)	
	Liaoning	11/72 (15.28)	CHN-F1 (1), D (4), NCF2 (6)	Xu et al. (2016)	
Shandong	0/29 (0)		Xu et al. (2016)		

(continued on next page)

Table 2 (continued)

Animal	Collection site	No. Positive/ No. samples (%)	<i>Enterocytozoon bienersi</i> genotypes (n)	References
	Shandong	23/356 (6.5)	CHG1 (1), D (8), Peru8 (3), Type IV (11)	Ma et al. (2020)
	Xinjiang	1/39 (2.6)	EbpA (1)	This study
Subtotal		111/911 (12.2)	CHN-DC1 (11), CHN-F1 (10), CHN-R1 (1), CHG1 (1), D (32), EbpA (1), NCF2 (33), NCR1 (2), NCR2 (5), Peru8 (3), Type IV (11), WildBoar3 (1)	
Total		278/2620 (10.6)	CAM5 (1), CHG1 (1), CHN-F1 (11), CHN-DC1 (13), CHN-R1 (1), D (109), EbpA (2), EbpC (18), HLJM-1 (3), HLJM-2 (1), NCF1 (3), NCF2 (46), NCF3 (1), NCF4 (1), NCF5 (2), NCF6 (1), NCF7 (1), NCM-1 (5), NCM-2 (1), NCR1 (2), NCR2 (5), Peru8 (7), Peru 11 (18), PigEBITS7 (4), Type IV (16), WildBoar3 (2), XJMI-1 (1)	

2.4. Sequence and phylogenetic analyses

The positive PCR amplicons of *E. bienersi* were sent to GENEWIZ (Suzhou, China) for bidirectional sequencing to ensure accurate results. Obtained nucleotide sequences were compared with reference sequences downloaded from the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/>) to determine genotypes using the Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Clustal X 2.1 (<http://www.clustal.org/>). The neighbor-joining (NJ) method (Kimura 2-parameter model) was used to reconstruct phylogenetic trees using the software Mega 7.0. The evolutionary distance was computed using the Kimura 2-parameter model, and the reliability of branches was assessed by a bootstrap analysis using 1000 replicates. Representative nucleotide sequences obtained in the study were submitted to GenBank under accession numbers: MW465662 - MW465667.

3. Results and discussion

E. bienersi has been commonly detected in humans, and domestic and wild animals (Li et al., 2020b). Free-ranging park/domestic wildlife can host common zoonotic genotypes, such as D, Type IV, Peru8, and Peru1, 1 showing a high potential for cross-species transmission of *E. bienersi* between humans and wildlife (Santín and Fayer, 2011). In this study, 288 fecal specimens from farmed mink, blue foxes, and raccoon dogs were tested for *E. bienersi* using nested PCR. Our results showed that the overall infection rate of *E. bienersi* in all specimens was 4.9% (144/288),

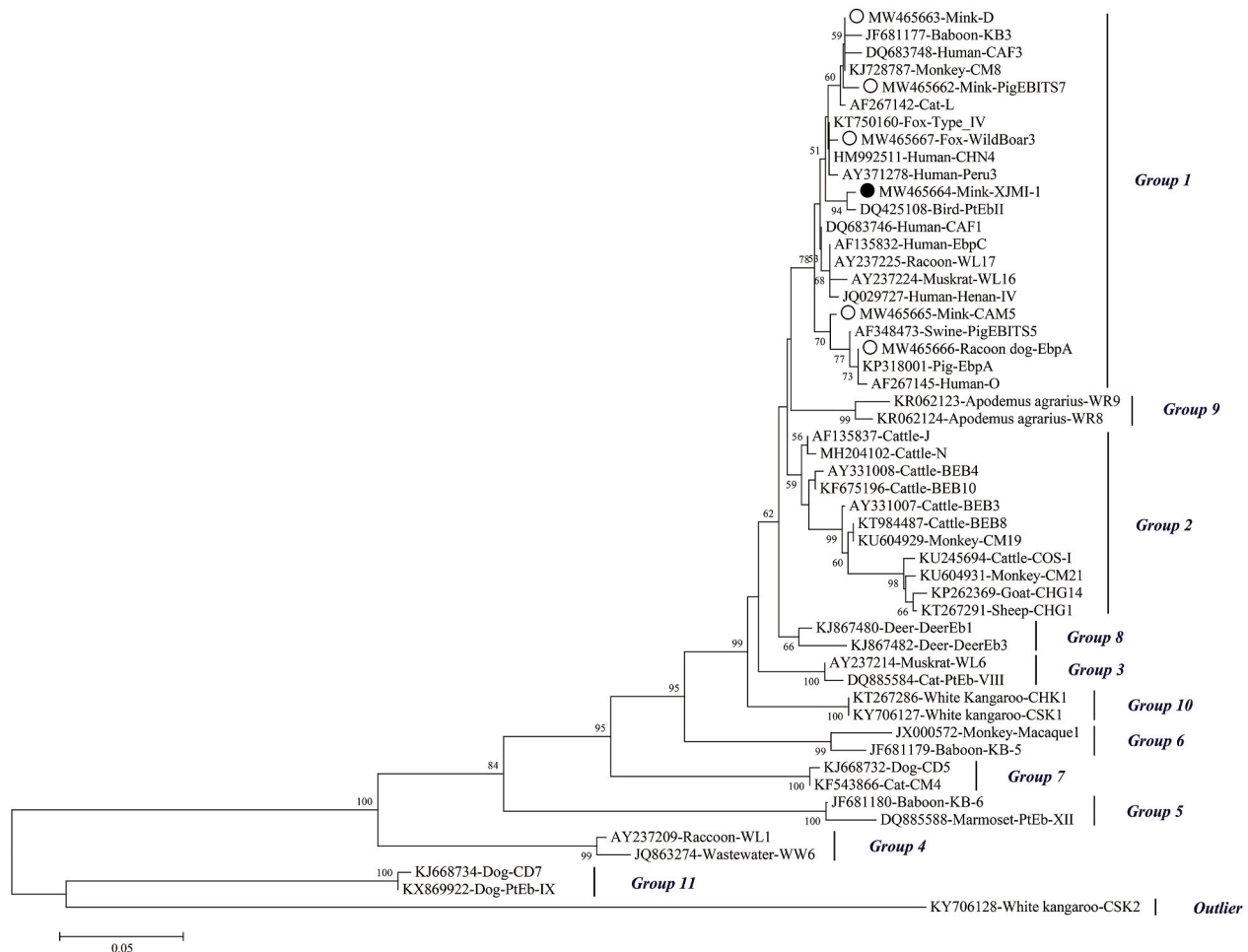


Fig. 1. Phylogenetic relationships of the *E. bienersi* genotypes. The relationships were inferred using NJ analysis of the ITS rRNA gene and the values generated greater than 50% are shown beside the nodes. Genotypes with hollow circles and filled circles are known and novel genotypes identified in this study, respectively.

including 5.6% (12/214) in mink, 2.9% (1/35) in blue foxes, and 2.6% (1/39) in raccoon dogs (Table 1). Previous studies conducted in Heilongjiang, Jilin, Liaoning, Hebei, and Shandong, China, have also identified *E. bieneusi* infection in our three study species (Table 2) (Cong et al., 2018; Ma et al., 2020; Xu et al., 2016; Yang et al., 2015; Zhang et al., 2016, 2018; Zhao et al., 2015). The infection rate of *E. bieneusi* in mink (5.6%, 12/214) from Xinjiang was higher than that reported in Shandong (0%, 0/31), and lower than Hebei (32.3%, 10/31), but consistent with infection rates in Heilongjiang (6.3%, 5/79; 3.9%, 10/257), Jilin (4.3%, 13/302), and Liaoning (8.9%, 8/90) (Yang et al., 2015; Zhang et al., 2016). The infection rate that we found for blue foxes (2.9%, 1/35) from Xinjiang was lower than all of those previously reported in other areas, including Jilin (7.7%, 7/91; 16.2%, 6/37), Heilongjiang (7.1%, 5/70; 27.7%, 44/191; 16.4%, 12/73) and Hebei (17.7%, 25/141). The infection rate of *E. bieneusi* reported previously in raccoon dogs in China varied from 0 to 40.7% (Xu et al., 2016; Yang et al., 2015; Zhao et al., 2015).

The prevalence of *E. bieneusi* in mink >6 months was 3.2% (3/95), and in mink < 6 months was 7.6% (9/119). In blue foxes and raccoon dogs, *E. bieneusi* prevalence in individuals >6 months was 5.0% (1/20) and 5.3% (1/19) respectively, with no *E. bieneusi* infection found in individuals <6 months (Table 1). Previous studies in China also found that *E. bieneusi* infection rates differed in differently aged mink, foxes, and raccoon dogs (Cong et al., 2018; Xu et al., 2016; Zhang et al., 2016). The difference in *E. bieneusi* prevalence in different areas and different age categories may be related to different geographical regions, feeding conditions, sampling times, specimen size, and animal husbandry and animal welfare practices (Deng et al., 2019; Li et al., 2020a). Furthermore, the susceptibility of different animals to *E. bieneusi* infection may also be a contributing factor (Li and Xiao, 2021; Zhang et al., 2020).

We identified six *E. bieneusi* genotypes from PCR and sequencing analysis, including D (n = 5), PigEBITS7 (n = 4), EbpA (n = 2), WildBoar3 (n = 1), CAM5 (n = 1) and XJMI-1 (n = 1) (Table 1). One was found to be a novel genotype (XJMI-1). This genotype XJMI-1 showed high similarity to genotype Peru 6 (MN179309) with one base variation at position 153 (A → G). The distribution of genotypes was different among the three study species. Genotypes D (n = 5), EbpA (n = 1), PigEBITS7 (n = 4), CAM5 (n = 1), XJMI-1 (n = 1) were identified in mink, while only one genotype was identified in the other two species. These were WildBoar3 (n = 1) in foxes and EbpA (n = 1) in raccoon dogs. The *E. bieneusi* genotype previously identified in mink, foxes and raccoon dogs are shown in Table 2. The identification of the predominant zoonotic genotype D in our study aligns with findings from studies conducted on non-human primates, rabbits, captive foxes, captive raccoons, domestic cats, and other captive wildlife species (Karim et al., 2014). Genotype D has also been found in drinking water (Guo et al., 2014), showing that contaminated water could be a potential source of transmission between humans and farmed wildlife.

Our phylogenetic analysis showed that all genotypes identified in this study were clustered into group 1 (Fig. 1). Among those genotypes, genotypes D, EbpA, and PigEBITS7 have been identified in fecal specimens from humans and animals in China (Wang et al., 2018). Genotype CAM5 has been identified in cattle and camels in Xinjiang, which could potentially threaten farming livestock by contaminating drinking water or grass in Xinjiang (Qi et al., 2018; Zhao et al., 2020). Our study indicates that *E. bieneusi* genotypes are human-pathogenic and the infection could be transmitted between human and farmed animals.

In conclusion, this study showed that the infection rates of *E. bieneusi* in mink, foxes, and raccoon dogs from Xinjiang were relatively low at 5.6% (12/214), 2.9% (1/35), and 2.6% (1/39), respectively. We identified six genotypes, all from zoonotic Group 1, and genotypes D, EbpA, and PigEBITS7, which have all been identified in humans. Our findings indicate that mink, blue foxes, and raccoon dogs, maybe a potential source of *E. bieneusi* infection for other animals and humans. Contaminated water and feces may be discharged to farmland around the farm, which could cause infection in animals lived in farmland, such as mouse

and hare. Measures need to be taken to mitigate this potential threat, such as reduce animal contact with the environment and feed with cooked food, and waste water and feces should be treated to kill pathogenic before they are discharged.

Declaration of competing interest

All authors declare that they have no competing interests.

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