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Epidemiological investigation and genotypes of *Enterocytozoon bieneusi* in 11 captive Rhesus macaque populations

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ABSTRACT

Enterocytozoon bieneusi is an obligate intracellular parasite and the most common pathogen of microsporidiosis in humans and animals. In this study, a total of 198 fecal samples were collected from 11 captive populations of Rhesus macaque in Chinese zoos, to investigate the prevalence and analyze the zoonotic potential of *E. bieneusi* by genotype of the Internal Transcribed Spacer (ITS) gene on the rRNA of *E. bieneusi* via nested PCR. Results showed that the average infection rate of *E. bieneusi* in the 11 populations was 13.6%, and the highest infection rate was 56.5% in the population of Xinjiang Tianshan Zoo. Seven genotypes were identified including 2 known genotypes (D and CM1) and 5 novel genotypes (Mul1, Mul2, Mul3, Mul4 and Mul5). Phylogenetic analysis revealed that the novel genotypes Mul2, Mul3, Mul4 and Mul5 belonged to Group 1 showed the zoonotic potential . These findings extend the distribution of *E. bieneusi* genotypes and provide baseline data for controlling *E. bieneusi* infection.

1. Introduction

Enterocytozoon bieneusi (Microsporidia, Enterocytozoonidae) is a common zoonotic pathogen and an obligate intracellular parasite (Han and Weiss, 2017), also have a wide range of hosts including human and domestic animals. The symptoms caused by microsporidiosis is self-limiting diarrhea, and 90% of microsporidiosis patients are infected by *E. bieneusi* (Zhu and Niu, 2004). The primary mode of infection is the ingestion of contaminated food or water and direct oral–fecal contact. In recent years, *E. bieneusi* has been continuously found in humans, livestock, pets and water, which attracting public health concern (Diao et al., 2014).

PCR amplification and sequencing techniques are considered the best tool for genotype and species identification The Internal Transcribed Spacer (ITS) region of ribosomal RNA (rRNA) genes is highly diverse , and widely used in the genotyping of *E. bieneusi* (Thellier and Breton, 2008). According to phylogenetic analysis, over 500 genotypes of *E. bieneusi* were divided into 11 groups, most of the genotypes in Group1 were zoonotic and the genotypes in other groups showed host specificity in varying degrees (Li et al., 2019). More than 30 genotypes considered to be zoonotic genotypes, such as D, WL11, Peru10, EbpC and CM17 (Sulaiman et al., 2003a; Sulaiman et al., 2003b; Wang et al., 2013; Yu et al., 2017).

At present, there is no effective prevention and treatment method for *E. bieneusi* infection, therefore the epidemiological study of the parasite helps to formulate the preventive measures. For the nonhuman primates (NHPs), more than 50 genotypes of *E. bieneusi* have been described and most of them are zoonotic such as A, D, Type IV, BEB6, I (Li et al., 2019). In China, *E. bieneusi* in NHPs has been increasingly reported in recent years. Chen et al. detected 9 genotypes of *E. bieneusi* in crab-eating macaques (*Macaca fascicularis*) in a commercial facility, and 6 of the genotypes have been previously seen in NHPs as well as humans (Chen et al., 2019). 116 of 411 free-range Rhesus macaque were positive for *E. bieneusi* in a popular public park in the People's Republic of China with 4 known genotypes and 2 new genotypes (Ye et al., 2012). Zhong et al. reported the infection rate of NHPs in southwest China and identified 3 zoonotic genotypes: D, PigEBITS7, and SC02 (Zhong et al., 2017).

Rhesus macaque (*Macaca mulatta)* is widely distributed in china, mostly live captive populations in the zoos to the amusement of visitors,

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also have a high risk of potentially infecting people with *E. bieneusi*. In this study, we investigated the prevalence of *E. bieneusi* in 11 Rhesus macaque populations from zoos in China and performed genotype of ITS gene to analyze the zoonotic to provide basic data for further study of the infectious and epidemic conditions about *E. bieneusi*.

2. Materials and methods

2.1. Specimen collection

In this study, a total of 198 fecal samples of Rhesus macaque were collected from 11 captive populations of the zoos in China, from July2016 to May 2018: Lhasa Zoo (XZ, n = 12), Xi'an Qinling Zoo (SX, n = 6), Fuzhou Zoo (FJ, n = 13), Guangzhou Zoo (GD, n = 15), Nanjing Hongshan Zoo (NJ, n = 12), Nanning Zoo (GX, n = 7), Xinjiang Tianshan Zoo (XJ, n = 23), Harbin Zoo (HLJ, n = 19), Lanzhou Zoo (GS, n = 21), Xining Wildlife Zoo (QH, n = 36), Guiyang Wildlife Zoo (GZ, n = 34) (Fig. 1). The abbreviations of 11 populations are shown in Table 1. Then, the fecal samples were placed into clean plastic bags marked with relative information, shipped in ice box to Zoology laboratory of Sichuan Agricultural University for storage at −80 °C until DNA extraction.

2.2. DNA extraction and PCR amplification

The genomic DNA of each fecal samples was isolated by using the Genomic DNA extraction Kit (MOBIO, USA) following the instructions. After testing concentration of the extracted DNA, the eligible DNA specimens were stored at - 20 °C.

The extracted DNA was detected for *E. bieneusi* by nested PCR amplification of 390bp nucleotide including the complete internal transcribed spacer (ITS) region. The primers and cycling parameters employed for these reactions were described in previous study (Buckholt et al., 2002; Karim et al., 2015). Each specimen was analyzed twice using 2 μl of extracted DNA per PCR. 2 × PCRmixture (CoWin Biosciences Company) was used for all PCR amplifications. All secondary PCR products were examined by electrophoresis (160 V, 20min) on 1.5% agarose gels containing ethidium bromide.

Table 1
Prevalence and ITS genotype distribution of E. bieneusi in rhesus macaques in different zoos of China.

Groups	Sampling site	No. of tested	No.of Positive	Infection rate	Genotypes (no.)
XZ	Lhasa Zoo	12	2	16.70%	D (2)
SX	Xi'an Qinling Zoo	6	0	0	
FJ	Fuzhou Zoo	13	0	0	
GD	Guangzhou Zoo	15	2	13.30%	D (2)
NJ	Nanjing Hongshan Zoo	12	0	0	
GX	Nanning Zoo	7	1	14.20%	D (1)
XJ	Xinjiang Tianshan Zoo	23	13	56.50%	CM1 (5), D (2), Mul1 (6)
HLJ	Harbin Zoo	19	1	5.30%	Mul4 (1)
GS	Lanzhou Zoo	21	1	4.80%	CM1 (1)
QH	Xining Wildlife Zoo	36	4	11.10%	D (3), Mul5 (1)
GZ	Guiyang Wildlife Zoo	34	3	8.80%	D (1), Mul2 (1), Mul3 (1)

2.3. Sequencing and phylogenetic analyses

All positive secondary PCR products were sequenced in both directions at Tsingke Biotech (Chengdu, China). The original sequencing was edited manually with software SeqMan of DNAstar7.0 and aligned with known reference sequences available in GenBank using the Basic Local Alignment Search Tool (BLAST) to determine the genotypes of *E. bieneusi* (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

To assess the phylogenetic relationship of the genotypes identified in

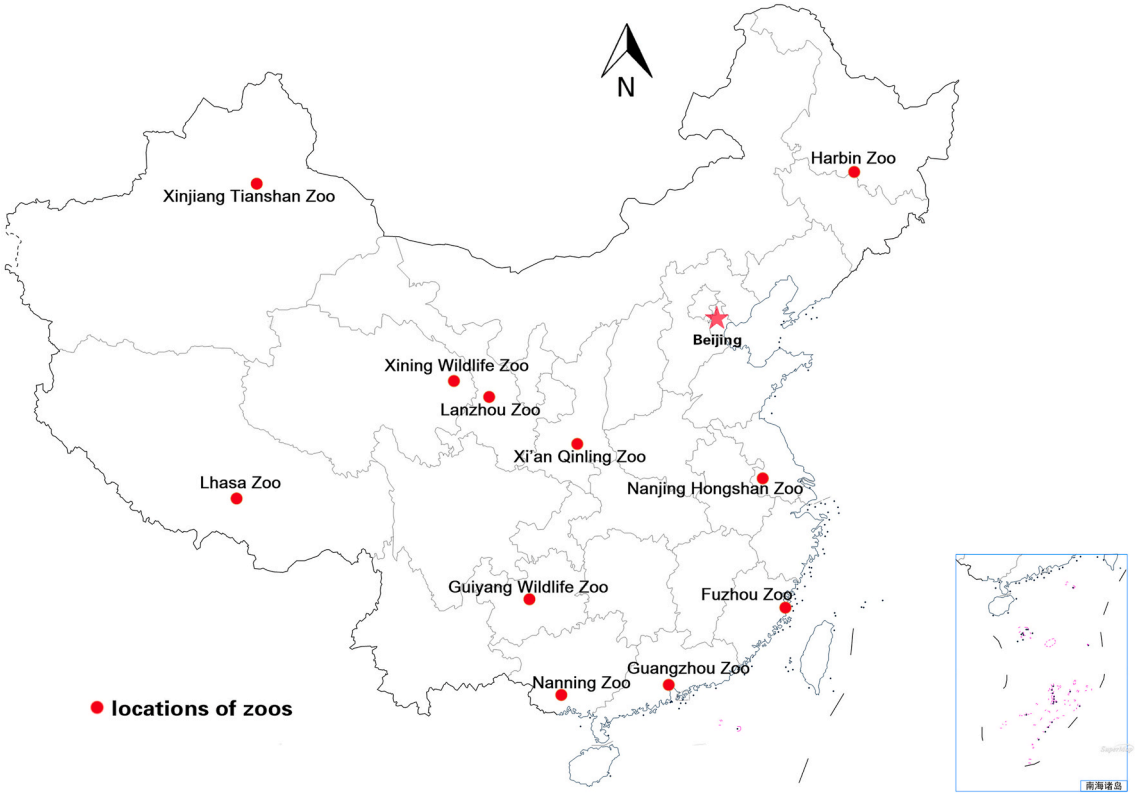

Fig. 1. Specific locations at which specimens were collected in this study.

this study to reference sequences previously published in GenBank, a neighbor-joining tree was constructed using Mega 5.1 (http://www.megasoftware.net/) based on genetic distances calculated by the Kimura 2-parameter model. The robustness of cluster formation was assessed by using bootstrapping analysis with 1000 replicates.

2.4. Statistical analysis

The $\chi 2$ test implemented in SPSS Statistics was used to compare differences in overall *E. bieneusi* infection rates among the 11 Rhesus monkey populations. Differences with $P < 0.05$ were considered significant.

2.5. Nucleotide sequence accession numbers

Representative nucleotide sequences from this study were deposited in the GenBank under accession numbers MT796853 to MT796859.

3. Results

3.1. Prevalence of E. bieneusi in Rhesus macaques

27 of the 198 fecal specimens were positive for *E. bieneusi* in 8 of the 11 Rhesus macaque populations (Table 1). The average infection rate in the 11 populations was 13.6% (27/198). The highest infection rate was 56.5% (13/23) found in the population of Xinjiang Tianshan Zoo (XJ), it was significantly higher than other populations. The second was Lhasa Zoo (XZ) with the infection rate of 16.7% (2/12). The infection rate of the population in Lanzhou Zoo (GS) was the lowest4.8% (1/21). Others from 5.3% to 14.2% (HLJ: 5.3%, GZ: 8.8%, QH: 11.1%, GD: 13.3%, GX:14.2%). The differences in the infection rates among 11 Rhesus macaque populations were statistically significant ($P < 0.001$).

3.2. Genetic characterization and genotype distribution of E. bieneusi

In the ITS nucleotide sequence analysis, 7 genotypes were identified from the 27 *E. bieneusi*-positive specimens, including 2 known genotypes: D (n = 11) and CM1 (n = 6), and five novel genotypes named Mul1 (n = 6), Mul2 (n = 1), Mul3 (n = 1), Mul4 (n = 1) and Mul5 (n = 1) (Table 1). Nucleotide sequence analysis revealed that, within the 243-bp region of the ITS gene sequence of *E. bieneusi,* genotype Mul1 had one Single Nucleotide Polymorphisms (SNP) (substitution: C to T) relative to genotype CAF4 (DQ683749); Mul2 had two SNP (substitution: C to G, T to C), Mul5 had two SNP (substitution: G to A both), Mul3 had five SNP (insertion: tow T and G. substitution: A to G, C to T) and Mul4 had six SNP (same as Mul3 but had one more substitution: A to G) relative to genotype D (MK478054) respectively.

The most common genotype was D (40.7%, 11/27), detected in 6 Rhesus macaque populations (XZ, GD, GX, XJ, QH, GZ), and existed separately in XZ, GD and GX populations. Another known genotype CM1 (6/27) was only detected in groups XJ and GS. In addition to the CM1 (n = 5), genotype D (n = 2) and the new genotype Mul1 (n = 6) have been identified in the XJ population together. The novel genotypes were found in different populations respectively, except Mul2 and Mul3 in group GZ together. All the populations with novel genotypes were detected in genotype D, except Mul4 which was found in the group HLJ alone. The number of the individual infected with the new genotype was only one in the populations which both have genotype D and novel genotypes, except for Mul1 (n = 6). Besides, no mixed infections involving different genotypes were identified in this study.

3.3. Phylogenetic analysis

As the phylogeny of ITS sequences shown (Fig. 2), genotypes D, CM1, Mul2, MuL3, MuL4, and Mul5 belonged to Group 1 and the novel genotypes were distributed in a subgroup 1a with genotype D. The other new genotype Mul1 were clustered in Group 5.

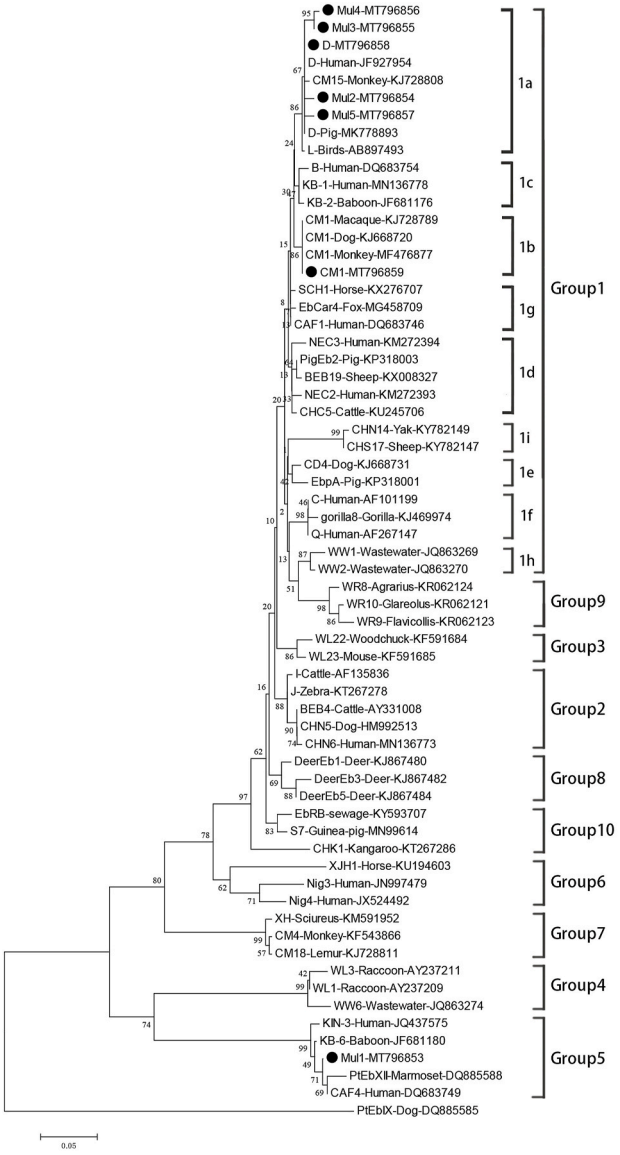

Fig. 2. Phylogenetic relationship among the *Enterocytozoon bieneusi* groups. The relationship between the *E. bieneusi* genotypes identified in this study and other known genotypes deposited in GenBank was inferred by neighbor-joining analysis of ITS sequences based on genetic distance using the Kimura-2-parameter model. The numbers on the branches represent percent bootstrapping values from 1000 replicates, with more than 50% shown in the tree. Each sequence is identified by its accession number, genotype designation, and host origin. Genotypes marked with black dot are identified in this study.

4. Discussion

In the present study about infection of *E.bieneusi* in the captive NHPs from Henan, Sichuan and Guangxi China, results revealed the infection rate of Rhesus macaques was 11.9% (62/521) (Karim et al., 2014b) which was similar to the results of this study (13.6%, 27/198). However, higher infection rate of 31.1% (33/106) was reported in six zoos in eastern China (Karim et al., 2015). In Beijing Rhesus macaque laboratory, the infection rate of *E. bieneusi* in cynomolgus monkeys was 25.6% while in Rhesus macaques was only 4.2% (3/72) (Yang et al., 2017). The reasons for the differences in infection rates may be related to the environmental conditions of different populations, the health of the animals at the time of sampling and the overall sample size. Normally, to

the diet and living environment of captive animals in the zoo, there are seasonable disinfection and cleaning. And the captive animals will be given antibiotics and anti-parasite drugs according to their health status (Zhao et al., 2007). In general, captive Rhesus macaques can be treated more promptly for various diseases with human intervention. Therefore, for captive populations, the level of management about environmental hygiene and feeding conditions have a great impact on the infection rate of *E. bieneusi*. In this study, the Rhesus macaque population of Xinjiang Tianshan wild zoo (XJ) compared with other populations showed the highest infection rate (56.5%), it could be due to the poor environment and lower level of management which make the captive Rhesus macaques out of condition and the *E. bieneusi* transmission for a long time. The infection rate of the other 10 zoos (5.3%–14.2%) was significantly lower than Xinjiang Tianshan wild zoo, indicating that good standards of environmental hygiene, healthy feeding and management conditions could effectively reduce the infection of parasite (Li et al., 2015).

7 genotypes were identified in this study (D, CM1, Mul1, Mul2, Mul3, Mul4, Mul5). As the one of the most prevalent genotype in NHPs (Zhou et al., 2019), genotypes D accounts for 40.7% of positive samples (10/27) and was identified in 6 of 11 populations. In the previous reports, genotype D (synonymous with genotypes PigEBITS9, WL8, Peru9, CEbC, PTEb VI) were identified in many mammals including human, reptile and birds (Da Cunha et al., 2017; Huang et al., 2019). The reports of genotype CM1 (synonymous with genotype Macaque3) was less, it was only found in NHPs and dog at present (Karim et al., 2014a, 2014b, 2015; Yang et al., 2017).

Based on previous research, according to phylogenetic analysis of ITS region of *E. bieneusi,* the genotypes can be divided into 11 groups. The genotypes in Group1 were considered zoonotic and in group2-11 have host specificity (Li et al., 2019). In this study, all the genotypes were clustered in Group 1 except Mul1 in group 5 (Fig. 2) . Genotype D has been proven to be zoonotic (Wang et al., 2013). There is no evidence to suggest that CM1 can infect humans. The new genotypes Mul2 (n = 1) , Mul3 (n = 1) ,Mul4 (n = 1) and Mul5 (n = 1) have close genetic relationships with the human-pathogenic genotypes distributed in group 1 as is shown in Fig. 2. Besides, these new genotypes were clustered with genotype D in a subgroup 1a and have a few SNP relative to genotype D (MK478054). We consider the new genotypes in Group1 have the potential for zoonotic or cross-species transmission. In Group 5, genotype CAF4, KB6, KIN-3 and PtEb XII were suggested have strong host specificity, as their genotypes have been found only in those hosts from which they were originally reported (Breton et al., 2007; Li et al., 2011; Lobo et al., 2006; Wumba et al., 2012). Therefor we consider Mul1 have strong host specificity.

This study investigated the prevalence and genotype of *E.bieneusi* in 11 captive Rhesus macaque populations from zoos in China by nested PCR amplification of the Internal Transcribed Spacer (ITS) gene. Resultantly, 5 new genotypes were observed, among them 4 were zoonotic. There is a risk of *E. bieneusi* transmission to human, we suggest that more attention should be paid for prevent the transmission of this pathogen to humans and other animals.

Declaration of competing interest

The authors declear no conflicts of interest and are alone responsible for the content and writing of the paper.

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