Record of New Genotypes of Enterocytozoon Bieneusi in Wild Populations of Rhesus Macaque (Macaca Mulatta) in China

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Abstract

Background: *Enterocytozoon bieneusi* is a zoonotic pathogen with a wide range of animal host. In China, there are only a few reports of *E. bieneusi* infection in wild populations of Rhesus macaque. Here, we determined the prevalence of *E. bieneusi* in 9 populations of Rhesus macaque and assessed their zoonotic potential.

Methods: A total of 324 fecal samples of Rhesus macaque were collected from 9 populations in 5 provinces (Sichuan, Chongqing, Qinghai, Tibet and Hainan) in China, and performed genotype of ITS gene to analyze the zoonotic potential.

Results: 38 of the 324 (11.72%) specimens from wild Rhesus macaques were infected with *E. bieneusi*. 11 genotypes were identified including 3 known genotypes: D (n= 24), EbpC (n= 4) and SCC-2 (n= 1); 8 novel genotypes named Mul6 (n= 1), Mul7 (n= 1), Mul8 (n= 1), Mul9 (n= 1), Mul10 (n= 2), Mul11 (n= 1), Mul12 (n= 1) and Mul13 (n= 1). According to the phylogenetic analysis, Mul6, Mul7, Mul8, Mul9, Mul11 Mul12 and Mul13 were clustered into Group 1, while Mul10 were clustered into Group 5.

Conclusions: To the best of our knowledge, this is the first study reporting the prevalence and genotypes of *E. bieneusi* in several wild populations of Rhesus macaque in China. It is concluded that, population of Rhesus Macaques is likely to prone of *E. bieneusi* transmission in many regions of China, which found the zoonotic genotypes D and EbpC and the novel genotypes with zoonotic potential, it should be paid more attention to prevent.

Background:

Enterocytozoon bieneusi (Microsporidia, Enterocytozoonidae) is an obligate intracellular parasite[1]. As a common zoonotic pathogen in human beings, *E. bieneusi* have a wide range of domestic and wild hosts i.e. mammals, birds and amphibian [2]. It can also induce opportunistic infections in humans at any stage e.g. children, elder people, travelers, patients (AIDS patients, immunocompromised patients, organ transplant recipients, cancer patients) [3]. The classical symptom is self-limiting diarrhea. The precise mechanism by which this pathogen induces disorder has been not established yet [4]. The ingestion of contaminated food or water and direct oral–fecal contact are considered the primary mode of its infection [5]. In recent years, some reports have documented the contamination of vegetables and fruits with *E. bieneusi*, it is a keen subject of public health concern[6]. In future, the new research in epidemiological study of *E. bieneusi* will help to formulate epidemic prevention measures.

At present, the detection of *E. bieneusi* is mainly based on PCR, which not only has higher specificity and sensitivity but also the advantage of further analysis to identify genotypes[7, 8]. Genotype of *E. bieneusi* is mainly based upon the Internal Transcribed Spacer (ITS) region of ribosomal RNA (rRNA) genes, as it is highly diverse and have detected approximately 600 genotypes[4]. According to phylogenetic analysis, 571 genotypes were divided into 11 groups[2]. Most genotypes in Group 1 or Group 2 (such as genotypes D, EbpC, and IV) showed host adaptation and wide geographical distribution, which are a major risk of

zoonotic disease[2]. By comparison, genotypes in Group 3 to 11 appear to be more host-specific, but the impact on public health still need more proof[2].

Non-human primates (NHPs) have a high genetic similarity with humans, it makes them beneficial models for biomedical research, among them, Rhesus macaque (*Macaca mulatta*) is a common model animal used widely for research purpose. In China, the wild populations of Rhesus macaque are widely distributed[9]. Meanwhile, the semi-domestic populations, live in natural environment have close contact with humans, as they frequently feed by tourists or managers as compared to the wild populations. It may increase the risk of parasitic infection in monkeys and humans in the natural environment. At present, the infection reports of *E. bieneusi* in NHPs (i.e. Rhesus macaque) in China mainly focus on captive population in laboratories, zoos and farms, while, very few research have been reported on wild populations[10–14].

The aim of our study was to examine the infection of *E. bieneusi* in wild or semi-domestic Rhesus macaque populations in 5 provinces of China by genotype of ITS gene. It was also hypothesized that *E. bieneusi* infection may transmit from Rhesus macaque to human beings. In future, this study could provide basic data for further research on disease transmission from *E. bieneusi* to other animals.

Methods

Specimen collection

Total 324 fecal samples of Rhesus macaque were collected from 9 populations in 5 provinces (Sichuan, Chongqing, Qinghai, Tibet and Hainan) in China (Fig.1). Including 3 semi-domestic populations: HN (n= 46), CQ-1 (n= 44) and SC-1 (n= 29); and 6 wild populations: SC-2 (n= 58), SC-3 (n= 19), CQ-2 (n= 30), XZ-1 (n= 28), XZ-2 (n= 30) and QH (n= 40). The fecal samples were placed into clean plastic bags, then shifted in to the ice box and transported to laboratory for storage at -80℃ until DNA extraction.

DNA extraction and PCR amplification

The genomic DNA of each fecal samples was isolated by using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) following the instructions. Finally, the perfect DNA specimens were stored at - 20℃ until PCR analyses.

The DNA was analyzed of *E. bieneusi* by using nested PCR amplification of 390bp nucleotide along with partial small subunit (SSU) gene, the complete ITS region (243 bp), and partial large subunit (LSU) gene. The primers and cycling parameters employed for these reactions were described in previous study[15]. Each specimen was analyzed twice using 2 μl of extracted DNA per PCR. 2×PCR mixture (CoWin Biosciences Company) was used for all PCR amplifications. Finally, all secondary PCR products were examined by electrophoresis in 1.5% agarose gels containing ethidium bromide.

Sequencing and phylogenetic analyses

Table 1. Prevalence and ITS genotype distribution of *E. bieneusi* in different Rhesus macaque populations.

	Population	Location	Sample	Positive	Infection rate	Genotypes (no.)
Semi-wild	HN	Hainan Nanwan peninsula	46	7	15.20%	D (6), Mul13 (1)
	CQ-1	Chongqing Fengjie	44	7	15.90%	D (6), Mul6 (1)
	SC-1	Sichuan Xichang	29	2	6.90%	SCC2 (1), D (1)
wild	CQ-2	Chongqing WuShan	30	2	6.70%	D (1), Mul7 (1)
	SC-2	Sichuan Baiyu	58	6	10.34%	D (4), Mul9(1), Mul8(1)
	SC-3	Sichuan Sertar	19	5	26.30%	D (3), Mul10 (2)
	XZ-1	Tibet Jomda	28	4	14.30%	D (3), Mul11 (1)
	XZ-2	Tibet Gongbo'gyamda	30	5	16.70%	EbpC (4), Mul12 (1)
	QH	Qinghai Yushu	40	0	0	
	Total		324	38	11.72%	

All appropriately sized PCR products were sequenced at Tsingke Biotech (Chengdu, China). We performed bidirectional sequencing to verify sequence accuracy. The sequencing results were aligned with known reference sequences available in GenBank using the Basic Local Alignment Search Tool (BLAST) and edited manually with SeqMan of DNAstar7.0 to determine the genotypes of *E. bieneusi*.

For assess the phylogenetic relationship of the novel and known genotypes, a neighbor-joining tree was constructed using Mega X software (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.

Statistical analysis

The $\chi 2$ test was implemented in SPSS Statistics to compare the difference of *E. bieneusi* infection rates in different populations. Difference with $P < 0.05$ was considered significant.

Nucleotide sequence accession numbers

Obtained Representative nucleotide sequences were deposited in the GenBank under accession numbers MT796858, MZ787960, and MZ772486 to MZ772494.

Results

Prevalence of *E. bieneusi* in Rhesus macaque populations

Of the 324 fecal specimens collected from the nine Rhesus macaque populations, 38 (11.72%) were positive for *E. bieneusi* (Table 1). Only QH population (n= 40) was without any infection of *E. bieneusi*. The highest infection rate was 26.3% (5/19) found in the wild population SC-3. Other recorded wild populations were: SC-2,10.34% (6/58); CQ-2, 6.7% (2/30); XZ-1, 14.3% (4/28); XZ-2, 16.7% (5/30). The infection rate in the 3 semi-domestic populations were: HN,15.2% (7/46); CQ-1, 15.9% (7/44) and SC-1, 6.9% (2/29). Moreover, the differences in the infection rates among 9 Rhesus macaque populations were nonsignificant (P >0.05).

E. bieneusi genotypes

From 38 positive specimens, 11 *E. bieneusi* genotypes were identified based sequence analysis of the ITS region. Including 3 known genotypes: D (n= 24), EbpC (n= 4) and SCC-2 (n= 1); 8 novel genotypes were following: Mul6 (n= 1), Mul7 (n= 1), Mul8 (n= 1), Mul9 (n= 1), Mul10 (n= 2), Mul11 (n= 1), Mul12 (n= 1) and Mul13 (n= 1) (Table 1). Genotype D (63.15%) was the most prevalent, it was observed in 7 populations except XZ-2 and QH. The population SC-1 had another known genotypes SCC2. Genotype EbpC was only found in XZ-2. Distribution of novel genotypes have shown in table 1.

Genetic relationships of ITS genotypes

As illustrated in Fig. 2, phylogenetic analysis revealed that genotypes Mul6, Mul7, Mul9, Mul11, Mul12 and Mul13 belonged to zoonotic Group 1 with known genotype D, whereas, novel genotypes contained 1 to 4 single nucleotide polymorphisms (SNPs) with genotype D (MT796858); Mul8 also belonged to Group 1, with 3 SNPs relative to genotype PL9 (MT497898); Mul10 was clustered in Group 5 and had 2 SNPs with genotype CAF4 (MZ502643). The base variation of the novel genotypes within the 243 bp of the ITS sequence is presented in (Fig. 3).

Discussion

E. bieneusi is the most diagnosed species in the 17 species of microsporidia which reported to cause infections in humans, domestic and wild animals[3]. The first documented transmission of *E. bieneusi* infection in human (afflicted with AIDS) and a Rhesus macaque (afflicted with simian immunodeficiency virus) was reported in 1997 [16]. At present, the research of *E. bieneusi* in wild or captive non-human primates (NHPs) has been reported all over the world. In China, researches in Rhesus macaque found that the prevalence range of *E. bieneusi* is 4.8 to 56.5%, these studies mainly focus on captive populations[13, 17, 18]. In our study, we find out the infection of *E. bieneusi* in 6 wild and 3 semi-domestic populations of Rhesus macaque from 5 provinces in China (i.e. Sichuan, Tibet, Qinghai, Chongqing and Hainan). These populations, SC-2, XZ-1, XZ-2, HN and CQ-1 had high infection rates 10.3–26.3%. Whereas, CQ-2 (6.7%) and SC-1(6.9%) had low infection rates. Food-borne transmission of *E. bieneusi* has been documented, although the contamination of vegetables and fruits with this pathogen was also reported in China[19]. The location of the four populations SC-2, SC-3, XZ-1 and XZ-2 were close to villages and pastoral areas,

where Rhesus macaque had the opportunity to consume the same water and food that the villager and free-rang livestock were used. We consider that it may cause parasitic disease among humans, livestock and wild Rhesus macaque. Previous study reported that *E. bieneusi* has spread infection in pigs, yaks and cattle in Tibet China [20–23], it is correlated to our study. Another study in eastern Qinghai found *E. bieneusi* in free-rang sheep and yaks[24], but in our study, we didn't find the infection in QH (n = 40) southern Qinghai population. This population was living in primitive forests and had little or no contact with villagers and livestock. While, CQ-2 population (6.70%) had the lowest infection rate was observed to very close with the cliffs on both sides of the Yangtze River, there may be no contact with other potential infection sources of *E. bieneusi* except water source. In the semi-wild populations, infection rate of HN population (15.2%) was almost same with the previously study in the Nanwan Monkey Island (15%)[14]. The 3 semi-wild population (CQ-1. SC-1 and HN) were living in the natural scenic spot with many tourists. The populations CQ-1 and SC-1 had similar geographical and climatic conditions, which were different from HN. But the infection rate of SC-1 (6.9%) was lowest than HN (15.2%) and CQ-1 (15.9%). In previous research the infection of *E. bieneusi* in 11 captive Rhesus macaque populations showed the significant result of infection rate in different zoos. It could be mainly due to the different environment and level of management in the zoos[17]. In the semi-wild population living in the natural scenic spot, the difference of infection rate may also be affected by many factors (i.e. Health status of the host, detection methods, sample size and the experimental design).

In our study, genotype D was the most prevalent and observed in 7 populations with the highest infection rate (63.15%). While, another known genotype EbpC was only found in XZ-2 population. Genotype D and EbpC were commonly found in the humans, domestic and wild animals all over the world [2]. In China, genotype D (synonyms: PigEBITS9, WL8, Peru9, CEbC, and PTEb VI) has been detected in infants, HIV positive patients and HIV negative patients[3, 25–28]. Genotype EbpC (synonyms: CHG23, E, Peru4, SC03, WL13 and WL17) was commonly found in humans and more than 15 animal species[15, 29–32]. According to our phylogenetic analysis, the novel genotypes, Mul6, Mul7, Mul9, Mul11, Mul12 and Mul13, were genetically closely related to the genotype D, and all were clustered in Group 1-a. Whereas, the novel genotype Mul8 was distributed in Group 1-f. At present study, most genotypes in Group 1 are host adaptation and zoonotic[2]. Therefore, we conjecture these new genotypes in Group 1 may be zoonotic. Genotype SCC-2 (n = 1) was found in population SC-1. Genotypes SCC-1 ~ 3 and RS01 were clustered in Group12[33], these genotypes were found only in rodents from China in previous studies[34, 35]. Our study is the first record to find SCC-2 in NHPs. It may transmit from infected rodents to Rhesus macaque. Further studies are needed to understand the host range and public health importance of genotype SCC-2. Moreover, the novel genotype Mul10 may be host-specific, it was clustered in Group 5 with the host-specific genotypes CAF4, KB-6, KIN-3 and PtEb-XII which have been found only in those hosts from which they were originally reported[36–39].

It is noteworthy that the 4 wild populations (SC-2, SC-3, XZ-1 and XZ-2) which found genotypes D, EbpC, and the potentially zoonotic novel genotypes (Mul7, Mul8, Mul9, Mul11 and Mul12) were located close to villages and pastoral areas, it induces a great risk of *E. bieneusi* infection in humans and free-range livestock. Besides that, the Rhesus macaque from 3 semi-wild populations, had genotype D and

potentially zoonotic novel genotypes (Mul3 and Mul6), contained more opportunities to contact with tourists and managers of scenic spot. Considering the density of the crowd, there is always a risk of parasite transmission between humans and monkeys,

Conclusions

This study investigated the infection of *E. bieneusi* in 9 Rhesus macaque populations in China. The results showed that there were zoonotic or potentially zoonotic genotypes in these populations. Considering the degree of intimate contact, we think there may be a potential risk of *E. bieneusi* transmission in human, livestock and Rhesus macaque. In future, it should be paid more attention to prevent.

Abbreviations

PCR: polymerase chain reaction; ITS: internal transcribed spacer; NHPs: Non-human primates; SSU rRNA: the small subunit rRNA; SNPs: single nucleotide polymorphisms;

Declarations

Ethics approval and consent to participate

All applicable international, national and institutional guidelines for animal care and use were observed. In addition, the research protocol was reviewed and approved by the Research Ethics Committee of Sichuan Agricultural University.

Consent for publication

Not applicable.

Availability of data and materials

Data supporting the conclusions of this article are included within the article. Representative nucleotide sequences generated in this study were deposited in the GenBank database under the accession numbers MT796858, MZ787960, and MZ772486 to MZ772494.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YY, HX and XL conceived and designed the experiments. MX, DL, QN, MZ, and JW collected the samples. XL performed the experiments. MY and XL analyzed the data. MY, XL and FK wrote the paper. All authors read and approved the final manuscript.

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Figures

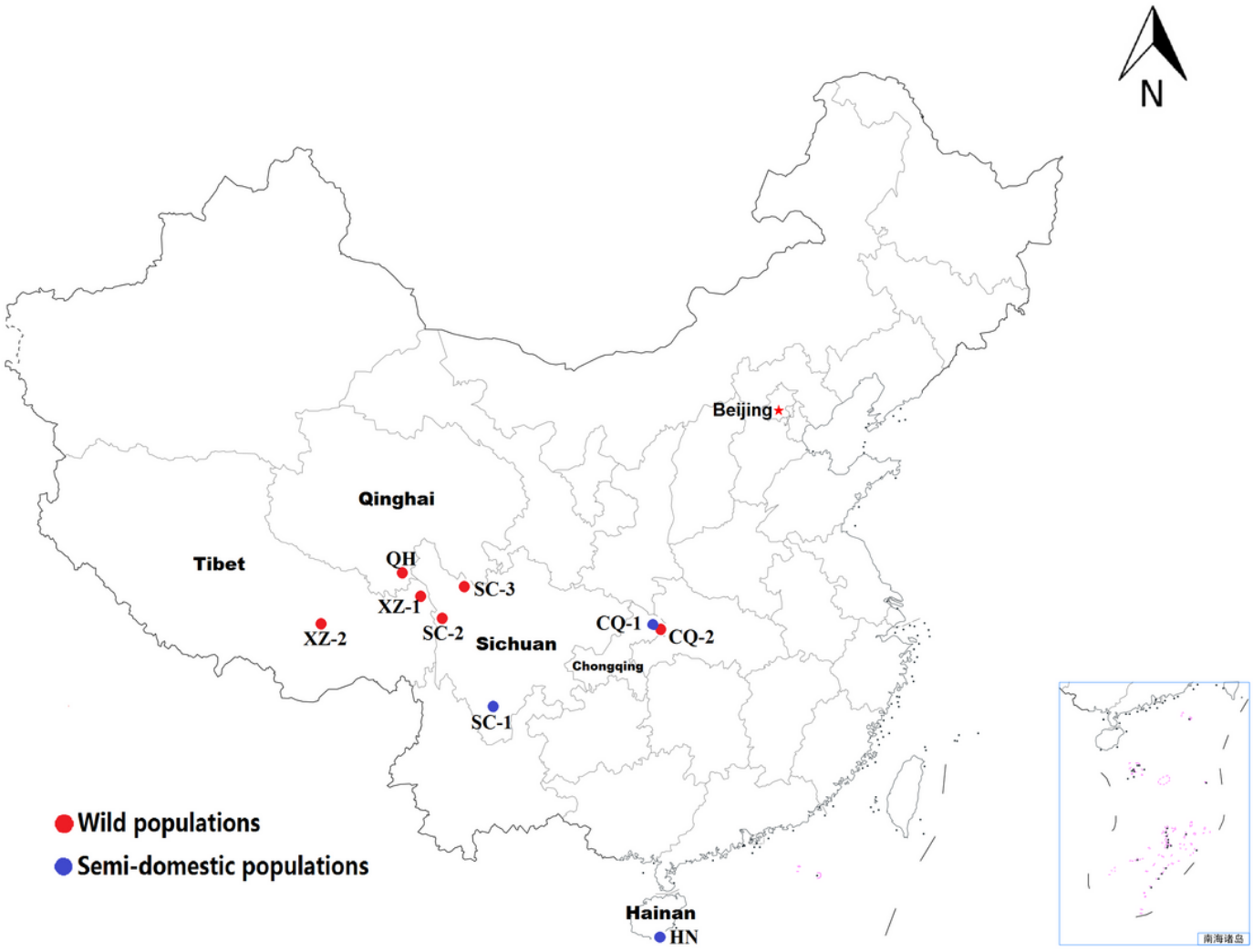

Figure 1

Specific locations at which specimens were collected in this study

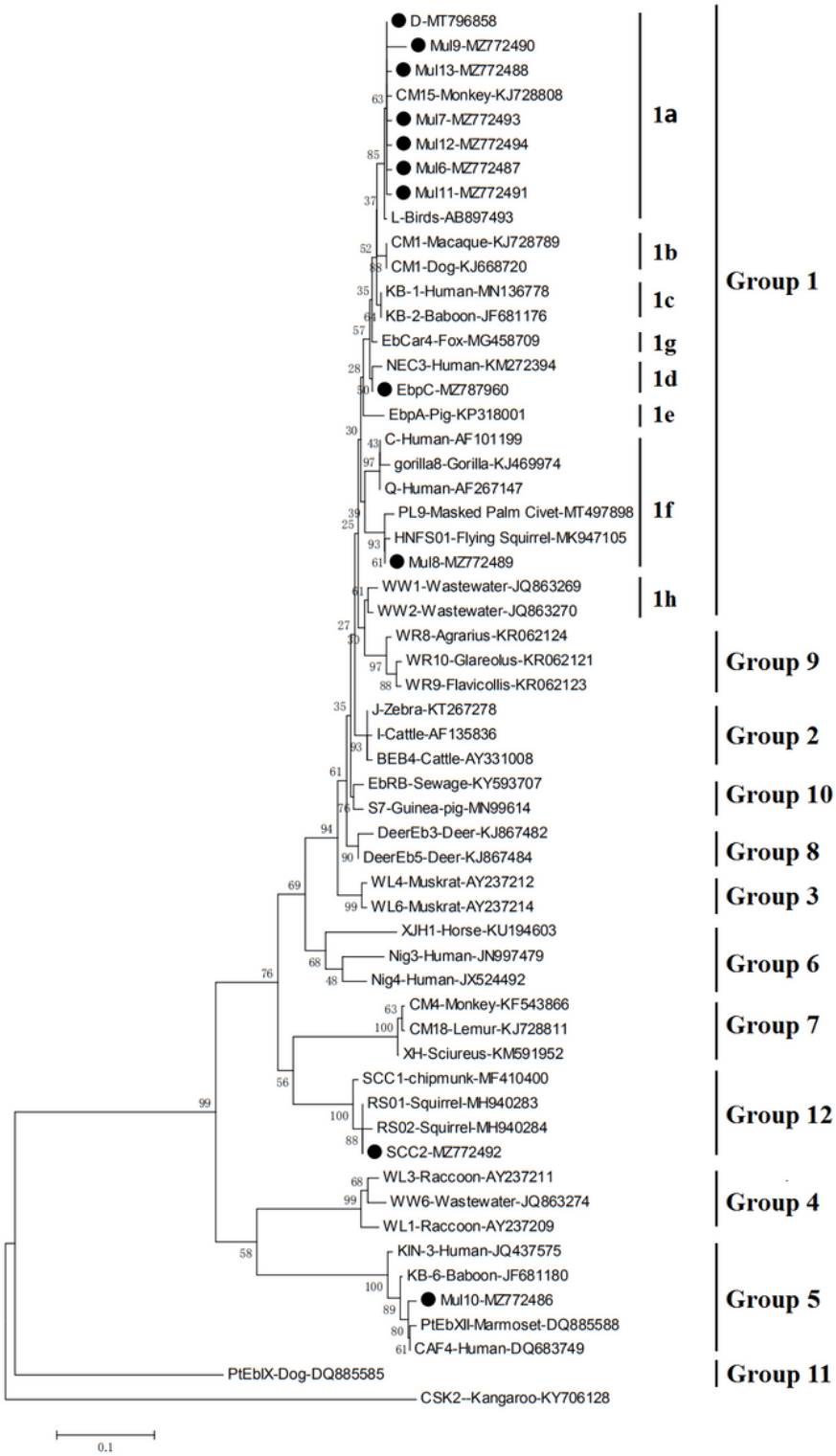

Figure 2

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.

Figure 3

Sequence variation in the ITS region of the rRNA gene of Enterocytozoon bieneusi isolates from Rhesus macaque. The ITS sequences of 5 known genotypes (D, PL9, CAF4, EbpC, and SCC-2) and 8 novel genotypes (Mul6 to 13) identified in this study, were aligned with each other. The dots and transverse lines indicate base identities and deletions, respectively, relative to the ITS sequence of genotype D.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Graphicalabstracts.png