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Prevalence and new genotypes of *Enterocytozoon bieneusi* in wild rhesus macaque (*Macaca mulatta*) in China: A zoonotic concern

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ABSTRACT

Enterocytozoon bieneusi is a zoonotic pathogen with a wide range of animal host. There are only few reports of *E. bieneusi* infection in wild Chinese rhesus macaques. Here, we determined the prevalence of *E. bieneusi* in nine wild rhesus macaque populations and assessed their zoonotic potential by performed genotype of ITS gene. A total of 324 fecal samples of rhesus macaque were collected in nine geographical populations from five Chinese provinces (Sichuan, Chongqing, Qinghai, Tibet and Hainan). 38 samples (11.7%) were found to be infected with *E. bieneusi*, and 11 genotypes were identified including three known genotypes (D, EbpC and SCC-2) and eight novel genotypes named Mul6~13. Genotype D (63.2%) was the most prevalent, being observed in seven populations except XZ-2 and QH, and other genotypes were identified only in a single area. According to the phylogenetic analysis, Mul6~9, Mul11~13 and zoonotic genotype D were clustered into Group 1, indicating that these genotypes may be potentially zoonotic. Among nine populations, population SC-3 had the highest infection rate (26.3%), and the lowest was the wild QH population without infection, but the difference of infection rate among the nine populations is not significant. It is concluded that, rhesus macaque populations are generally infected *E. bieneusi* in many areas of China, and there may be a risk of cross infection with *E. bieneusi* in some areas found having zoonotic genotypes, and these areas should be paid more attention to prevent.

1. Background

Microsporidia are obligate intracellular parasites belonging to the fungi as either a basal branch or a sister group and 17 species have been demonstrated to cause infections in humans, of which *Enterocytozoon bieneusi* is the commonest pathogens (Han et al., 2021). As a common zoonotic pathogen in human beings, *E. bieneusi* have a wide range of domestic and wild hosts i.e. mammals, birds and amphibians (Li et al., 2019b). 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) (Matos et al., 2012). The symptoms depend on the type of population: in immunocompromised patients, *E. bieneusi* can produce chronic diarrhoea, wasting syndrome, cholangitis and rhinitis; in immunocompetent individuals it can produce self-limited diarrhoea and malabsorption (Han et al., 2021). The precise mechanism by which this pathogen induces disorder has been not established yet (Zhang et al., 2021). The ingestion of contaminated food or water and direct oral–fecal contact are considered the primary mode of its infection (Fayer and Santin-Duran, 2014). 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 (Wang et al., 2020). 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 (Feng et al., 2011; Yang and Rothman, 2004). 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

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(Zhang et al., 2021). A comprehensive phylogenetic analysis of 471 valid ITS genotypes was conducted, 11 major genetic groups were recognized and named as Groups 1 to 11: most genotypes in Group 1 such as genotypes D, EbpC, and IV are zoonotic; genotypes in group 2 were once considered to be adapted to ruminants, but subsequent studies have identified those genotypes in humans suggesting its zoonotic potential; genotypes in Group 3 to 11 appear to be more host-specific, but the impact on public health still need more proof (Li et al., 2019b). A recent study proposed clustered these genotypes SCC-1–3 and RS01~2 which infected rodents into Group 12 (Wang et al., 2020).

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 (Jiang XL and Ma, 1991). Meanwhile, the semi-wild populations, living in natural environment but closely contacting with humans, are frequently feed by tourists or managers as compared to the wild populations. It may increase the risk of parasitic infection. Li et al. summarized the prevalence and genotype distribution of *E. bieneusi* in NHPs worldwide, most of the genotypes belong to Group 1 and Group 2, but other are included in Group 5 (KB-6, PtEb XII), Group 6 (gorilla 3, KB-5, Macaque1) and Group 7 (XH, CM4, CM18); among them, genotype D, IV, Macaque3 and EbpC are most frequently found in *M. mualtta*. 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 (Chen et al., 2019; Karim et al., 2015; Ye et al., 2012; Zhao et al., 2021; Zhong et al., 2017).

The aim of our study was to examine the infection of *E. bieneusi* in wild and semi-wild 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 a theoretical basis for further research of *E. bieneusi* infection.

2. Methods

2.1. Specimen collection

Totally 324 fecal samples of rhesus macaque were collected from nine populations in five provinces (Sichuan, Chongqing, Qinghai, Tibet and Hainan) in China from 2016 to 2018 (Fig. 1). Including 3 semi-wild 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 locations of all populations are showing in Table 1. Sichuan Baiyu, Sichuan Sertar, Tibet Jomda, Tibet Gongbo'gyamda and Qinghai Yushu belong to the Qinghai-Tibet Plateau region, has high-altitude climate characteristics of low temperature, low oxygen and high ultraviolet. Except QH population which living in primordial forest and difficult to contact with human, the home range of other 4 wild populations are close or overlap with villages and pastoral areas. The 3 semi-wild populations in Hainan Nanwan peninsula, Chongqing Fengjie and Sichuan Xichang are living in natural scenic spots with wild living status but have close contact with humans and are frequently feed by tourists or managers. Hainan is low-altitude tropical monsoon climate, Sichuan Xichang is tropical plateau monsoon climate and Chongqing Fengjie is humid monsoon climate. Fresh fecal samples were taken immediately after defecation using sterile disposable gloves during tracking and direct observation, avoiding repeatedly sampling the same individual and moving along with the troop of rhesus macaque to gather as many samples as possible. Fecal samples were stored at ice box until transported to laboratory 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 QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) following the instructions. Finally, the DNA specimens were stored at - 20 °C until PCR analyses.

E. bieneusi was identified by using nested PCR amplification: 390bp nucleotide containing partial small subunit (SSU) gene, the complete ITS region (243 bp), and partial large subunit (LSU) gene. The primers and

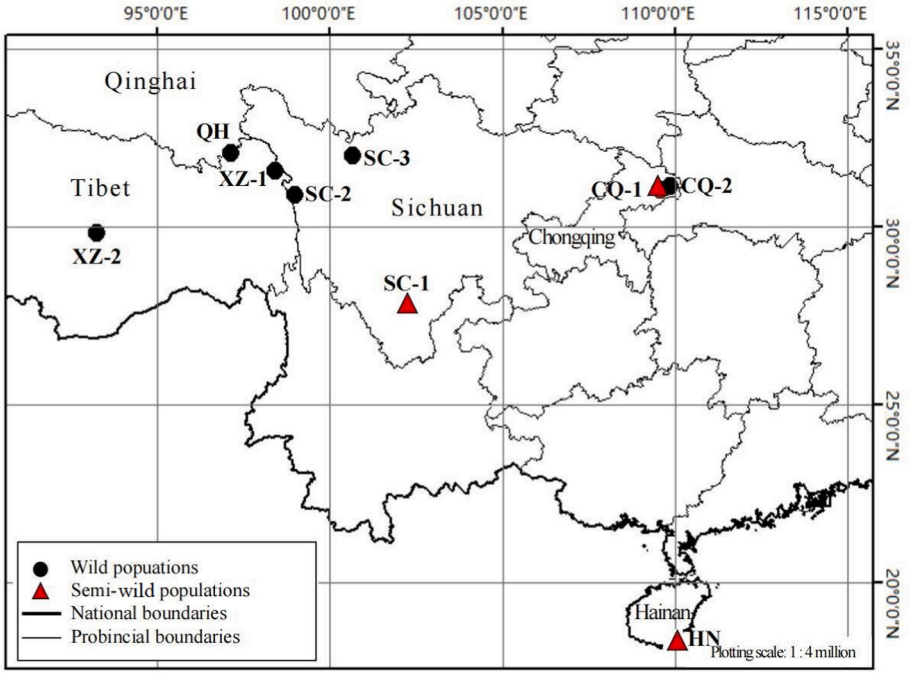

Fig. 1. Specific locations at which specimens were collected in this study.

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

	Population	Location	Coordinates		NO.	Positive	Infection rate	Genotypes (no.)
Semi-wild	HN	Hainan Nanwan peninsula	18°24′44″N	109°58′00″E	46	7	15.2%	D (6), Mul13 (1)
	CQ-1	Chongqing Fengjie	31°2′27″N	109°34′39″E	44	7	15.9%	D (6), Mul6 (1)
	SC-1	Sichuan Xichang	27°50′4″N	102°16′9″E	29	2	6.9%	SCC2 (1), D (1)
wild	CQ-2	Chongqing WuShan	31°10′33″N	109°52′28″E	30	2	6.7%	D (1), Mul7 (1)
	SC-2	Sichuan Baiyu	30°55′12″N	98°58′36″E	58	6	10.3%	D (4), Mul9(1),Mul8(1)
	SC-3	Sichuan Sertar	32°0′18″N	100°38′55″E	19	5	26.3%	D (3), Mul10 (2)
	XZ-1	Tibet Jomda	31°35′13″N	98°23′46″E	28	4	14.3%	D (3), Mul11 (1)
	XZ-2	Tibet Gongbo'gyamda	29°51′30″N	93°13′31″E	30	5	16.7%	EbpC (4), Mul12 (1)
	QH	Qinghai Yushu	32°4′30″N	97°6′15″E	40	0		
	Total				324	38	11.7%	

cycling parameters employed were described in previous study (Buckholt et al., 2002). Specifically, primers were EBITS3 (5′-GGTCATAGGGATGAAGAG-3′) and EBITS4 (5′-TTCGAGTTCTT TCGCGCTC-3′) as external primers, and EBITS1 (5′-GCTCTGAATATC TATGGCT-3′) and EBITS2.4 (5′-ATCGCCGACGGATCCAAGTG-3′) as internal primers. The cycling conditions for PCRs were: 94 °C for 3 min; followed by 35 cycles of 94 °C for 15 s, 57 °C (primary PCR) or 55 °C (secondary PCR) for 15 s, and 72 °C for 15 s; followed by 72 °C for 5 min. Each specimen was analyzed twice using 2 μl of extracted DNA per PCR and 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.

2.3. Sequencing and phylogenetic analyses

All appropriately sized PCR products were sequenced at Tsingke Biotech (Chengdu, China) by performed bidirectional sequencing. 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*. The new genotypes were resequenced of twice independent PCR to ensure these genotypes are novel.

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.

2.4. 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.

2.5. Nucleotide sequence accession numbers

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

3. Results

3.1. Prevalence of E. bieneusi in rhesus macaque populations

Of the 324 fecal specimens collected from the nine rhesus macaque populations, 38 (11.7%) were positive for *E. bieneusi* (Table 1). In the 9 populations, the wild population SC-3 had the highest infection rate 26.3% (5/19), other wild populations were: SC-2,10.3% (6/58); CQ-2, 6.7% (2/30); XZ-1, 14.3% (4/28); XZ-2, 16.7% (5/30). The 3 semi-wild populations were: HN, 15.2% (7/46); CQ-1, 15.9% (7/44) and SC-1, 6.9% (2/29). Only QH population (n = 40) did not have infection of *E. bieneusi*. Moreover, the differences in the infection rates among the 9 populations were nonsignificant ($p > 0.05$).

3.2. Molecular diversity of 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). The base variation of the novel genotypes within the 243 bp of the ITS sequence is presented in Fig. 3: Mul6, Mul7, Mul9, Mul11, Mul12 and Mul13 contained 1 to 4 single nucleotide polymorphisms (SNPs) with genotype D (MT796858); Mul8 had 3 SNPs relative to genotype PL9 (MT497898); Mul10 had 2 SNPs with genotype CAF4 (MZ502643). Genotype D (63.2%) 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 known and novel genotypes have shown in Table 1.

3.3. Genetic relationships of ITS genotypes

As illustrated in Fig. 2, phylogenetic analysis revealed that among the eight novel genotypes seven were belonged to zoonotic Group 1: Mul6, Mul7, Mul9, Mul11, Mul12 and Mul13 were further sub-divided in subgroup 1a with known genotype D, and Mul8 in subgroup 1f. Mul10 was clustered in Group 5, other known genotypes SCC-2 in Group12 and EpbC in Group1.

4. Discussion

E. bieneusi is the most diagnosed species within the 17 species of microsporidia which reported to cause infections in humans, domestic and wild animals (Matos et al., 2012). The first documented experimental transmission of *E. bieneusi* infection between human (afflicted with AIDS) and two rhesus macaques (afflicted with simian immunodeficiency virus) was reported in 1997 (Tzipori et al., 1997). At present, *E. bieneusi* is reported in wild or captive non-human primates (NHPs). In Chinese rhesus macaque, the infection rates of *E. bieneusi* range from 4.8 to 56.5%, but these studies mainly focus on captive populations (Karim et al., 2014, 2015; Yu et al., 2020). In our study, we find out the infection of *E. bieneusi* in six wild and three semi-wild populations of rhesus macaque from five 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, and the contamination of vegetables and fruits with this pathogen was also reported in China (Li et al., 2019a). The location of the four wild 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 as the villager and free-rang livestock. We consider that it may cause parasitic disease

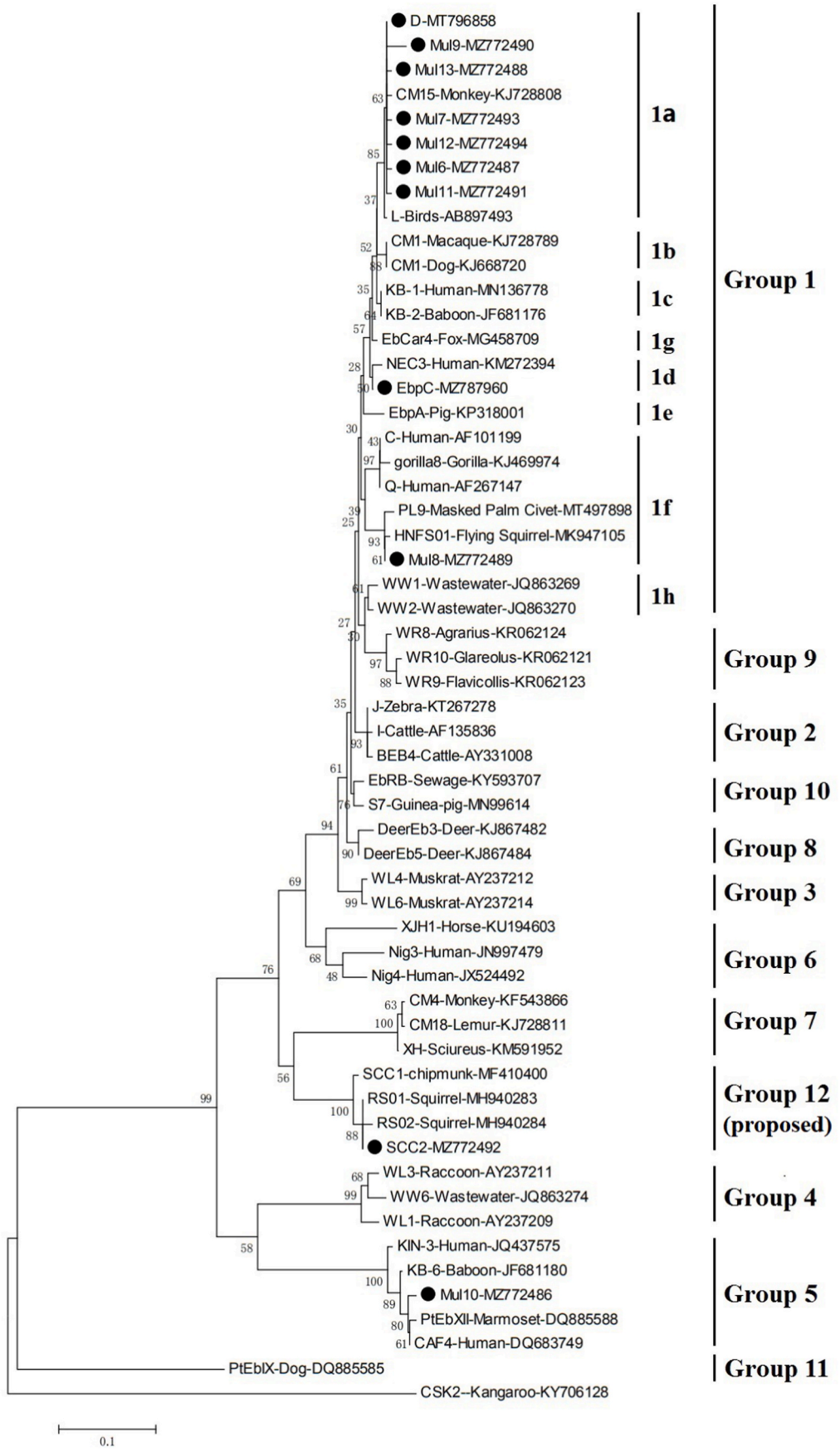

Fig. 2. Phylogenetic relationship among the *Enterocytozoon bieneusi* groups. The relationship between the *E. bieneusi* genotypes identified in this study and other known genotypes deposited in GenBank was inferred by neighbor-joining analysis of ITS sequences based on genetic distance using the Kimura-2-parameter 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.

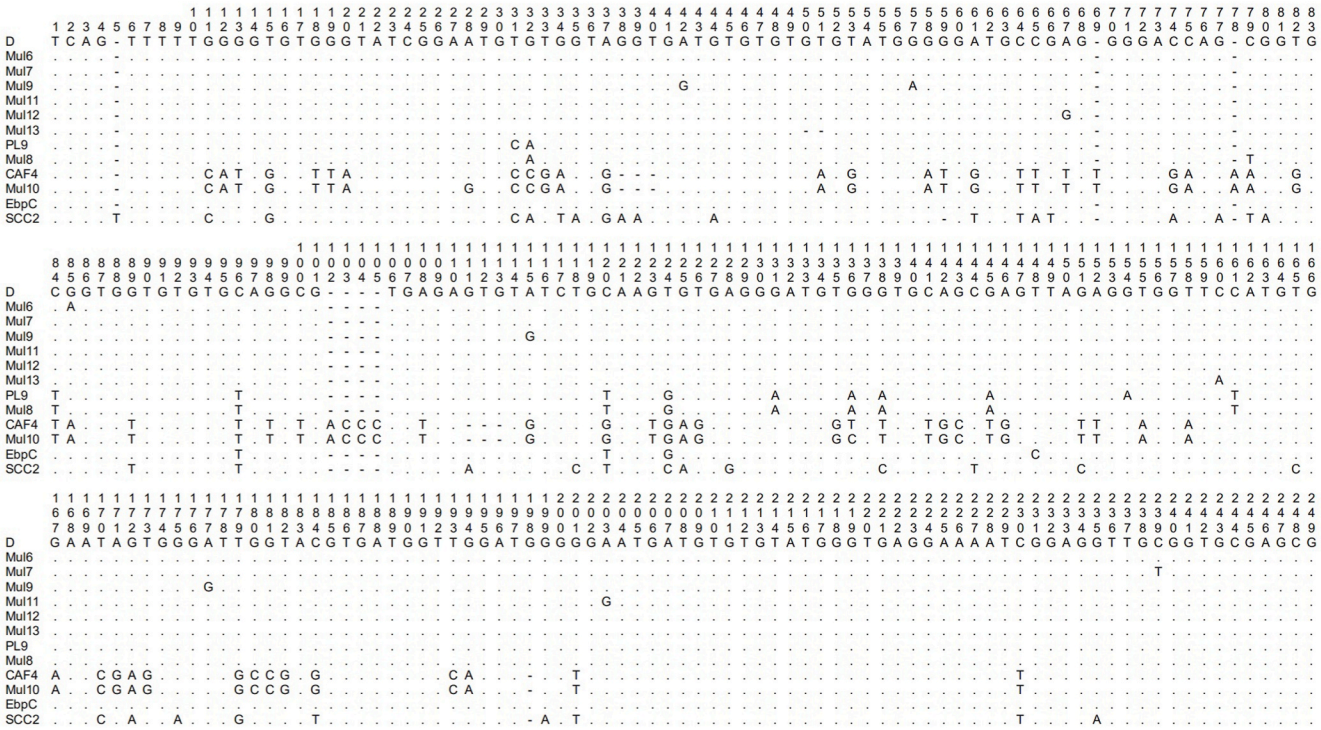

Fig. 3. Sequence variation in the ITS region of the rRNA gene of *Enterocytozoon bieneusi* isolates from rhesus macaque. The ITS sequences of 5 known genotypes (D, PL9, CAF4, EbpC, and SCC-2) and 8 novel genotypes (Mul6 to 13) identified in this study, were aligned with each other. The dots and transverse lines indicate base identities and deletions, respectively, relative to the ITS sequence of genotype D.

among humans, livestock and wild rhesus macaque. Previous study reported that *E. bieneusi* has spread infection in pigs, yaks and cattle in Tibet China (Chang et al., 2020; Wu et al., 2019, 2020; Zou et al., 2019), areas in these studies are close to the population XZ-2 (in the same county: Gongbo'gyamda). There was a report of free-rang sheep and yaks infection with *E. bieneusi* in eastern Qinghai (Zhang et al., 2018), but we didn't find the infection in QH population (n = 40) which in southern Qinghai. This population was living in primitive forests and had little or no contact with villagers and livestock. While, CQ-2 population (6.7%) 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%) (Zhao et al., 2021). The 3 semi-wild population (CQ-1. SC-1 and HN) were living in the natural scenic spot where have 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 our published 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 of the zoos (Yu et al., 2020). 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 (63.2%). 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 worldwide (Li et al., 2019b). In China, genotype D (synonyms: PigEBITS9, WL8, Peru9, CEbC, and PTEb VI) has been detected in infants, HIV positive patients and HIV negative patients (Matos et al., 2012; Qi et al., 2020; Wang et al., 2013; Yu et al., 2019; Zang et al., 2021). Genotype EbpC (synonyms: CHG23, E, Peru4, SC03, WL13 and WL17) was commonly found in humans and more than 15 animal species (Breitenmoser et al., 1999; Li et al., 2016; Rinder et al., 2000; Shi et al., 2016; Sulaiman et al., 2003). According to the phylogenetic analysis, the novel genotypes clustered in Group 1-a: Mul6, Mul7, Mul9, Mul11, Mul12 and Mul13 were genetically closely related to the genotype D. Whereas, the novel genotype Mul8 was distributed in Group 1-f. Most genotypes in Group 1 are host adaptation and zoonotic (Li et al., 2019b). Therefore, we conjecture these new genotypes in Group 1 may be zoonotic. Genotype SCC-2 (n = 1) was found in population SC-1. Li et al. described 11 major groups of *E. bieneusi*, but genotypes SCC-1–3 and RS01∼2 were proposed to cluster in Group12 (Wang et al., 2020), for these genotypes were found only in rodents from China (Deng et al., 2018, 2020). Our study is the first record to find SCC-2 in rhesus macaque. It may transmit from infected rodents to rhesus macaque, but further studies are needed to understand the host range and public health importance of these genotypes. Moreover, the novel genotype Mul10 may be host-specific, it was clustered in Group 5 with three host-specific genotype KB-6, KIN-3 and PtEb-XII and one non-specific genotype CAF4, which have been thought to be human-specific, recently was reported in NHPs (Breton et al., 2007; Li et al., 2011; Lobo et al., 2006, 2014; Köster et al., 2022).

It is noteworthy that the 4 wild populations (SC-2, SC-3, XZ-1 and XZ-2) which found genotypes D, EbpC, and novel genotypes (Mul7, Mul8, Mul9, Mul11 and Mul12) were close to villages and pastoral areas. And the rhesus macaque, from 3 semi-wild populations which also found zoonotic genotypes, have more opportunities to contact with tourists and managers in scenic spot. Considering the mode of infection, there may be a risk of cross infection with *E. bieneusi*.

5. Conclusions

This study investigated the infection of *E. bieneusi* in 9 rhesus macaque populations in China. The results showed that rhesus macaque populations are generally infected *E. bieneusi* in many regions of China, and there may be a risk of cross infection with *E. bieneusi*. In future, it should be paid more attention to prevent.

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.

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Authors' contributions

YY, HX1 and XH2 conceived and designed the experiments. XL, MX, DL, QN, MZ, and JW collected the samples. XL performed the experiments. MY, XL and GY analyzed the data. MY, XL and FK wrote the paper. All authors read and approved the final manuscript.

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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Abbreviations

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

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