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First report of *Enterocytozoon bieneusi* and *Cryptosporidium* spp. in peafowl (*Pavo cristatus*) in China

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ABSTRACT

Enterocytozoon bieneusi and *Cryptosporidium* spp. are important pathogens causing diarrhea in humans and animals. However, few studies have been conducted on the infection of *E. bieneusi* and *Cryptosporidium* spp. in peafowl up to now. The purpose of the present study was to determine the prevalence and the involved genotypes of *Cryptosporidium* spp. and *E. bieneusi* in peafowl in Beijing and Jiangxi Province, China. In total, 258 peafowl fecal samples were collected. Overall, both *Cryptosporidium* spp. and *E. bieneusi* had the same prevalence, i.e. 6.59% (17/258). Higher infection rates of *E. bieneusi* and *Cryptosporidium* spp. were found in the adolescent peafowl. The prevalence of *E. bieneusi* in Beijing and Jiangxi Province was 5.23% and 8.57% respectively. For *Cryptosporidium* spp., the prevalence was 4.58% and 9.52% in Beijing and Jiangxi Province, respectively. Three zoonotic genotypes of *E. bieneusi* were confirmed, including two known genotypes, genotype Peru 6 and D, and one novel genotype, JXP1. Two avian specific species/genotypes of *Cryptosporidium*, Avian genotype III and Goose genotype I, were identified. To our knowledge, this is the first report of *E. bieneusi* and *Cryptosporidium* spp. occurrence in peafowl in China. The findings suggest that peafowl could be reservoirs of *E. bieneusi* and *Cryptosporidium* spp. which could be potentially transmitted to humans and other animals, and the present survey have implications for controlling *E. bieneusi* and *Cryptosporidium* spp. infection in peafowl.

1. Introduction

Cryptosporidiosis and microsporidiosis, caused by *Cryptosporidium* spp. and *Enterocytozoon bieneusi* respectively, are two important emerging infectious parasitoses in humans and animals. Humans and other susceptible hosts can be infected through accidently ingesting food and water contaminated with oocysts and spores (Ben Ayed et al., 2012; Jedrzejewski et al., 2007; Galván et al., 2013). Infected hosts show clinical symptoms depending on their different health status. In general, the clinical symptoms of immunocompromised individuals are more severe (Checkley et al., 2015; Wang et al., 2013).

Genotypes of *E. bieneusi* have been identified by analyzing the sequence of the internal transcribed spacer (ITS) of the rRNA gene and more than 240 genotypes have been identified up to now (Santin and Fayer, 2009). The genotypes of *E. bieneusi* have been divided into 9 groups (group 1–9) according to phylogenetic analysis, including a zoonotic group and other host specific groups (Zhang et al., 2018b). For *Cryptosporidium* spp., more than 30 species and 40 genotypes have been characterized by analysing the small subunit (SSU) rRNA gene (Holubova et al., 2016; Yang et al., 2016). Some species have been detected in humans (Ryan et al., 2016). In addition to *Cryptosporidium baileyi*, *Cryptosporidium galli* and *Cryptosporidium meleagridis* which are specific for birds (Current et al., 1986; Ryan et al., 2003; Slavin, 1955), also other genotypes of *Cryptosporidium*, such as goose genotypes I–IV and avian genotypes I–V, have been detected in different avian species (Ryan, 2010).

Because of the ornamental and edible value, the peafowl (*Pavo cristatus*) are commonly kept as pets and bred in captivity in many countries, including China. Many pathogens, such as avian influenza virus (Li et al., 2017), avian poxvirus (Khan et al., 2009), infectious bronchitis coronavirus (Liu et al., 2005), *Ascaridia galli* (Teixeira et al., 2012), Newcastle disease virus (Kumar et al., 2013), *Toxoplasma gondii* (Tian et al., 2012) and *Histomonas meleagridis* (Michelazzo et al., 2017), have been detected in peafowl, but the infection data of *E. bieneusi* and *Cryptosporidium* spp. are still relatively limited. To our knowledge, only one study concerning *Cryptosporidium* spp. infection in *Pavo cristatus* has been reported in Brazil (Nakamura et al., 2009), and no information about the prevalence of *E. bieneusi* in peafowl is available worldwide. The aim of the present study was to investigate the prevalence of *Cryptosporidium* spp. and *E. bieneusi* in peafowl from Beijing and Jiangxi

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2. Materials and methods

2.1. Ethics statement

This work was approved by the Animal Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences. The procedures of collecting feces from peafowl were strictly in line with good animal practices required by the Animal Ethics Procedures and Guidelines of the People's Republic of China.

2.2. Sample collection

Between November 2017 and June 2018, a total of 258 fresh fecal samples taken from cloaca of the peafowl were obtained with sterile cotton swabs, including 153 from Beijing and 105 from Jiangxi Province. At the time of sampling, no obvious clinical signs were observed. The samples fall into two categories according to the age of the peafowl, the adult (≥24 months, n = 108) and the adolescent (≥5 months or < 24 months, n = 150) (Tian et al., 2012). All of the samples were separately collected into sterile centrifuge tube, put into box filled with ice packs, and then transported to the laboratory immediately.

2.3. DNA extraction and PCR amplification

The E.Z.N.A.® Stool DNA Kit (Omega Biotek Inc., Norcross, USA) was used to extract genomic DNA from 200 mg fecal samples following the manufacturer's protocol. The extracted DNA was stored at −20 °C until further PCR analysis. The small subunit ribosomal RNA (SSU rRNA) gene was amplified by nested PCR to identify *Cryptosporidium* species/genotypes as described by Nolan et al. (2010). To detect *E. bieneusi*, a fragment of ITS was amplified via nested PCR in accordance with previous methods (Buckholt et al., 2002). The obtained sequences were aligned with each other and reference sequences downloaded from the GenBank database with ClustalX 1.83 software package to differentiate *E. bieneusi* genotypes. All the primers used in this study were listed in Table 1. Both negative (reagent-grade water) and positive controls (DNA extracted from the *E. bieneusi* PtEbIX genotype and *C. baileyi*) were included in each amplification to ensure the accuracy of the results. The secondary PCR products were detected by 2% agarose gel electrophoresis with GoldView™ (Solarbio, China) stained.

The nucleotide sequences generated in present study have been deposited in GenBank under accession numbers MK168300-MK168302 (*E. bieneusi*) and MK168303-MK168304 (*Cryptosporidium* spp.).

2.4. Sequencing and phylogenetic analyses

The secondary PCR products were bi-directionally sequenced by the Sino Geno Max Company (Beijing, China). Each PCR product was sequenced three times to ensure that the sequencing results were correct. Chromatograms of the forward and reverse sequences were manually confirmed and the sequences were assembled with Lasergene SeqMan software (DNASTAR, Madison, Wisconsin, USA).

Nucleotide sequences obtained in the present study were aligned with reference sequences available in GenBank database with the ClustalX 1.83 software package was implemented to determine the genotypes of *E. bieneusi* and *Cryptosporidium* spp. Phylogenetic analysis was implemented with MEGA 6.0 (Tamura et al., 2013) using the Neighbor-joining algorithm in a Kimura2-parameter model, and the branch reliability was analyzed using a bootstrap of 1000 replicates (Zhang et al., 2018a).

2.5. Statistical analysis

The $\chi 2$ test was used to compare the prevalence under SPSS 19.0 (SPSS Inc., Chicago, USA). Differences were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Prevalence of E. bieneusi and Cryptosporidium spp

In our study, DNA sequences of the two parasites were determined by nested PCR. The overall prevalence of both *Cryptosporidium* spp. and *E. bieneusi* was 6.59% (17/258). Higher infection rates of *E. bieneusi* and *Cryptosporidium* spp. were found in the adolescent peafowl, but only the difference in *Cryptosporidium* spp. infection was significant ($P < 0.05$). The prevalence of *E. bieneusi* in Beijing and Jiangxi Province was 5.23% and 8.57% respectively. For *Cryptosporidium* spp., the prevalence was 4.58% and 9.52% in Beijing and Jiangxi Province, respectively. According to the statistical results, no correlation between the two parasitic protozoa infection and gender ($P > 0.05$) was found. Moreover, in none of the positive samples a co-infection of *Cryptosporidium* spp. and *E. bieneusi* was found in all positive samples (Table 2).

3.2. Genetic characterization of E. bieneusi and Cryptosporidium spp

In our studies, three genotypes of *E. bieneusi* were confirmed by sequencing the second PCR products of ITS region, including two known genotypes, genotype Peru 6 and D, and one novel genotype, termed JXP1. Genotype D was discovered in peafowl from Beijing, and genotype Peru 6 and JXP1 were from Jiangxi Province. Among the seventeen *Cryptosporidium*-positive fecal samples, 2 species/genotypes of *Cryptosporidium*, Avian genotype III and Goose genotype I, were revealed by amplifying the SSU rRNA gene. The Avian genotype III was found in peafowl from Beijing, and the other was from Jiangxi Province (Table 3).

3.3. Phylogenetic analysis

The *E. bieneusi* genotypes were separated into 9 distinct groups on the basis of the ITS1/5.8S/ITS2 sequences, and the group 1 was further divided into 8 subgroups (group1a-group1i) (Zhang et al., 2018b). The

Table 1
Primers used for identification of *E. bieneusi* and *Cryptosporidium* spp. in the present study.

Parasite	Primer	Sequence (5′-3′)	Reference
E. bieneusi	EBITS3	GGTCATAGGGATGAAGAG	Buckholt et al. (2002)
	EBITS4	TTCGAGTTCTTTCGCGCTC	
	EBITS1	GCTCTGAATATCTATGGCT	
	EBITS2.4	ATCGCCGACGGATCCAAGTG	
Cryptosporidium spp.	XF2f	GGAAGGGTTGTATTTATTAGATAAAG	Nolan et al. (2010)
	XF2r	AAGGAGTAAGGAACAACCTCCA	
	pSSUf	AAAGCTCGTAGTTGGATTTCTGTT	
	pSSUr	ACCTCTGACTGTTAAATACRAATGC	

Table 2
The occurrence of *E. bieneusi* and *Cryptosporidium* species/genotypes in peafowl in Beijing and Jiangxi Province, China.

Factors	Category	*E. bieneusi*			*Cryptosporidium* spp.		
		No. tested/No. positive	Prevalence (%) (95% CI)	*P*-value	No. tested/No. positive	Prevalence (%) (95% CI)	*P*-value
Region	Beijing	8/153	5.23 (1.66–8.80)	0.288	7/153	4.58 (1.23–7.92)	0.115
	Jiangxi	9/105	8.57 (3.13–14.01)		10/105	9.52 (3.82–15.23)	
Age	Adult	4/108	3.70 (0.08–7.32)	0.113	3/108	2.78 (0.83–2.87)	0.036
	Adolescent	13/150	8.67 (4.11–13.22)		14/150	9.33 (4.62–14.04)	
Gender	Female	8/119	6.72 (2.16–11.29)	0.936	7/119	5.88 (1.59–10.17)	0.672
	Male	9/139	6.47 (2.33–10.62)		10/139	7.19 (2.84–11.54)	
Total		17/258	6.59 (3.54–9.64)		17/258	6.59 (3.54–9.64)	

isolate BJP-18 (MK168302) identified in Beijing was clustered into group1a, JXP1 (MK168300) and JXP-41 (MK168301), were clustered together in group1b (Fig. 1). Phylogenetic tree of *Cryptosporidium* spp. was constructed based on the SSU rRNA gene. The two isolates of *Cryptosporidium* spp. recovered in the present study were grouped with the genotype Goose I (AY504516) and Avian genotype III (KU885387) with 99% bootstrap support respectively (Fig. 2).

4. Discussion

E. bieneusi and *Cryptosporidium* spp. are important pathogens that can cause severe watery diarrhea in humans and animals globally. To explore the role of peafowl in the epidemiology of these pathogens above, we characterized the prevalence and genotypes of *E. bieneusi* and *Cryptosporidium* spp. in peafowl from Beijing and Jiangxi Province, China.

In our study, we detected, for the first time, the prevalence (6.59%) of *E. bieneusi* in peafowl, which was similar to some studies performed in other birds (Bart et al., 2008; Lallo et al., 2012; Pirestani et al., 2013). In contrast to our result, much higher prevalence was found in studies conducted by Li et al. (2014) in chicken, Lobo et al. (2006) in pigeon and Tavalla et al. (2018) in exotic birds. Lower infection rates were identified in pigeon and Brazilian captive birds investigated by Slodkowica-Kowalskaet et al. (2013) and da Cunha et al. (2017) respectively. *Cryptosporidium* spp. has been detected in a wide range of domestic and wild avian hosts worldwide (Nakamura and Meireles, 2015). In China, *Cryptosporidium* spp. has been reported in ruddy shelduck (Amer et al., 2010), quails (Wang et al., 2012), ostriches (Qi et al., 2014), domestic pigeons (Li et al., 2015), parrots (Zhang et al., 2015), Java sparrows (Yao et al., 2017) and chickens (Liao et al., 2018). In the present study, we first detected the prevalence of *Cryptosporidium* spp. in peafowl in China. In Beijing, the infection rate was 4.58%, while in Jiangxi Province, the infection rate was 9.52%. This differences may be caused by climatic conditions, management level and differences in age composition of peafowl in the two locations. To our knowledge, there is no other reports on *Cryptosporidium* spp. infection in peafowl other than studies conducted by Nakamura et al. (2009). In studies performed in other birds elsewhere, infection rates of *Cryptosporidium* spp. vary from 0.82% to 43.9% (Baroudi et al., 2013; Li et al., 2015a; Maca and Pavlasek, 2015). Though infection rate varies with geographical location, avian species and detection methods, the number of samples may also be the causation of the differences. Moreover, different hosts are unequally susceptible to various genotypes of the parasites, which may also be responsible for the difference in infection rates. In addition, higher prevalence of *E. bieneusi* and *Cryptosporidium* spp. in the adolescent peafowl might be caused by their naive immune status.

Three genotypes of *E. bieneusi* were identified based on analyzing the ITS region, including two known genotype, Peru6 and D, and one novel genotype, JXP1. All the positive samples detected in Beijing belong to genotype D. In Jiangxi Province, the predominant genotype was JXP1, followed by genotype Peru 6 (Table 3). Genotype D has been widely found in mammals, including humans (Kicia et al., 2016; Prasertbun et al., 2017; Wang et al., 2017), non-human primates (Karim et al., 2014), Suidae (Nemejc et al., 2014; Zhao et al., 2014), ruminants (Huang et al., 2017; Zhao et al., 2015), canids (Li et al., 2015b; Zhang et al., 2016), felines (Li et al., 2016; Xu et al., 2016), rodents (Perec-Matysiak et al., 2015; Yang et al., 2016) and equines (Deng et al., 2016; Qi et al., 2016; Yue et al., 2017). However, genotype D was reported only in a few avian species, such as chicken (Rodrigues da Cunha et al., 2016), pigeon (Pirestani et al., 2013), common crane and red-crowned crane (Zhao et al., 2016), rook (Perec-Matysiak et al., 2017), falcon (Mueller et al., 2008) and swan goose (*Anser cygnoides*) (da Cunha et al., 2017), and our findings constitute the first report of the genotype in peafowl. Genotype Peru6 was mainly reported in humans (Bern et al., 2005; Lobo et al., 2012), cattle (Santin et al., 2005), sheep and goats (Zhao et al., 2015). Pigeon and lovebird also can be infected by the genotype (Luisa Lobo et al., 2006; Zhao et al., 2016). To our knowledge, this is the first report that peafowls were infected with the genotype Peru6. Together, both genotype Peru 6 and D can infect a wider range of host species, including peafowl, thus may cause microsporidiosis to the host. Moreover, the novel genotype (JXP1) of *E. bieneusi* identified in our study should be further explored to reveal its danger to the host, especially to human beings. Phylogenetic analysis was performed based on the ITS sequence of *E. bieneusi*. Both genotype Peru 6 and the novel genotype JXP1 obtained in the present study were grouped into group 1b, and genotype D was clustered into the group 1a. Both group 1b and group 1a belong to group 1, the most important human-pathogenic group. These results suggest that peafowl are potential source of human and animal microsporidiosis.

Table 3
Distribution of *E. bieneusi* genotypes and *Cryptosporidium* species/genotypes in peafowl in this study.

Factors		*E. bieneusi* genotype (n)	*Cryptosporidium* spp. (n)
Region	Beijing	D (8)	Avian genotype III (7)
	Jiangxi	JXP1 (6); Peru6 (3)	Goose genotypeI(10)
Age	Adult	D (4)	Avian genotype III (2)
	Adolescent	D (4); JXP1 (6); Peru6 (3)	Avian genotype III (5); Goose genotypeI(10)
Gender	Female	D (7); JXP1 (3); Peru6 (1)	Avian genotype III (4); Goose genotypeI(5)
	Male	D (1); JXP1 (3); Peru6 (2)	Avian genotype III (3); Goose genotypeI(5)
Total		D (8); JXP1 (6); Peru6 (3)	Avian genotype III (7); Goose genotypeI(10)

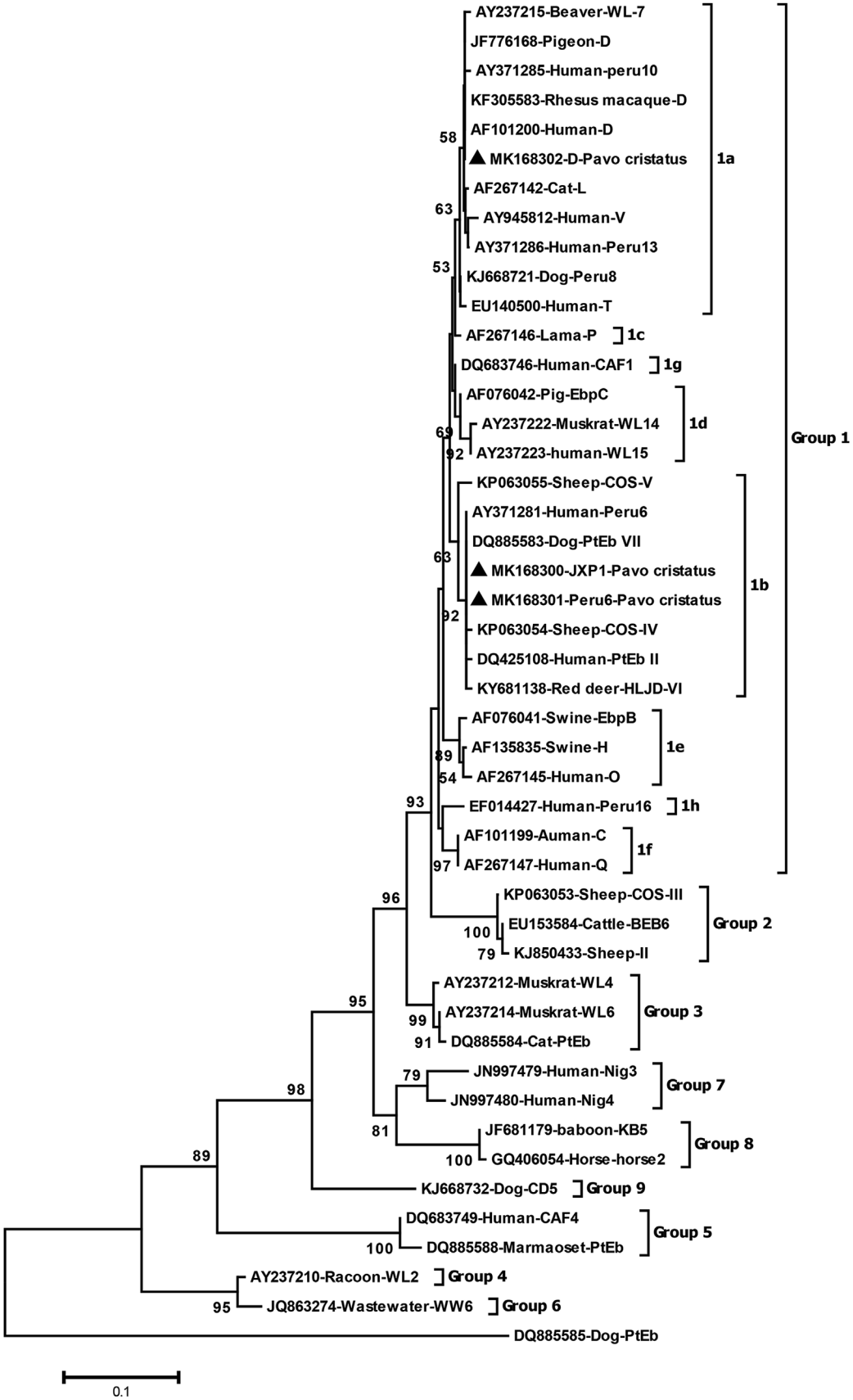

Fig. 1. Phylogenetic relationships of ITS nucleotide sequences of *Enterocytozoon bieneusi* identified in the present study and reference genotypes. The phylogenetic tree was constructed with a Neighbor-Joining method with the Kimura 2-parameter model. Bootstrap values > 50% from 1000 replicates are shown on the nodes. The *E. bieneusi* genotype PtEb (DQ885585) from dog was used as outgroup. The genotypes detected in the current study are shown with solid triangle.

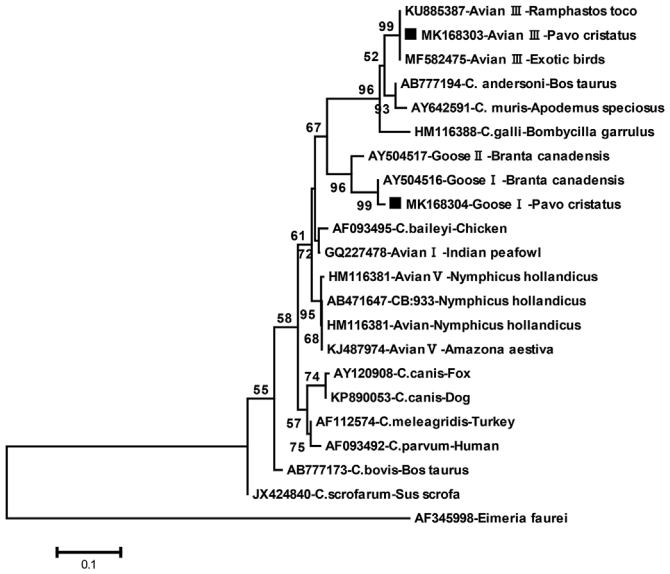

Fig. 2. Phylogenetic analysis of *Cryptosporidium* spp. using Neighbor-Joining (NJ) method based on sequences of the small subunit ribosomal RNA (SSU rRNA) gene. Bootstrap values > 50% are shown (1000 replicates). Isolates obtained in the present study are indicated by solid square. The SSU rRNA gene sequence of *Eimeria faurei* is used as the outgroup.

Molecular characterization of the SSU rRNA gene verified the presence of two genotypes/species, *Cryptosporidium* Avian genotype III and Goose genotype I. The former was found in peafowl from Beijing, and the latter was recovered in peafowl from Jiangxi Province. *Cryptosporidium* Avian genotype III was considered specific to birds and first identified in parrots in western Australia by Ng et al. (2006). After that, the genotype was isolated from birds in other countries, such as from the families Psittacidae in Brazil (Gomes et al., 2012; Nakamura et al., 2009; Novaes et al., 2018), Japan (Abe and Makino, 2010; Makino et al., 2010), China (Qi et al., 2011) and the USA (Ravich et al., 2014), from seagulls in Thailand (Koompapong et al., 2014), and from aquatic birds in Spain (Cano et al., 2016). Though the *Cryptosporidium* Avian genotype III seems to play no role in zoonotic potential, its global distribution makes it impossible to ignore its potential threat to bird health. *Cryptosporidium* Goose genotype I had been detected in Canada Geese (Zhou et al., 2004) and was identified in the feces of peafowl for the first time. Both *Cryptosporidium* Avian genotype III and Goose genotype I have not been found in humans up to now. Further research is needed to determine whether zoonotic *Cryptosporidium* species/genotypes occur in peafowl in other areas and settings.

5. Conclusions

To our knowledge, this is the first report of *E. bieneusi* infection in peafowl worldwide and first report on *Cryptosporidium* spp. occurrence in peafowl in China. Moreover, two known zoonotic genotypes, genotype Peru 6 and D, and one novel genotype JXP1 of *E. bieneusi* and two known avian adapted genotypes, Avian genotype III and Goose genotype I of *Cryptosporidium* were identified, which suggest the transmission potential of the parasites from peafowl to humans or other animals. To better understand the epidemiology of *E. bieneusi* and *Cryptosporidium* spp. in peafowl, further investigations involving more areas and larger number of samples are needed.

Conflicts of interest

The authors have no conflict of interest.

Acknowledgment

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