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New genotypes and molecular characterization of *Enterocytozoon bieneusi* in pet birds in Southwestern China

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ABSTRACT:

Enterocytozoon bieneusi, a unicellular enteric microsporidian parasite, can infect humans and a wide range of animals throughout the world. Although *E. bieneusi* has been identified in many animals, there is no information regarding the genotypes of *E. bieneusi* in pet birds in China. Birds are important sources of emerging infectious diseases that affect humans, and immunosuppressed individuals can be exposed to potential zoonotic agents shed by birds. The aim of the present study was to determine the prevalence and genotypic diversity of *E. bieneusi* in pet birds, as well as assessed its zoonotic potential. A total of 387 fecal samples were collected from Psittaciformes (n = 295), Passeriformes (n = 67), and Galliformes (n = 16) from four pet markets in Sichuan province, Southwestern China. The overall prevalence of *E. bieneusi* in pet birds was 25.1% based on nested polymerase chain reaction analysis of the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene (Psittaciformes, 21.7%; Passeriformes, 37.3%; Galliformes, 50.0%). Eight genotypes of *E. bieneusi* were identified, including five known genotypes (D, SC02, BEB6, CHB1, and MJ5) and three novel genotypes (SCB-I, SCB-II, and SCB-III). In phylogenetic analysis, genotypes D and SC02 and one novel genotype SCB-II were clustered within group 1, genotype BEB6 was classified within group 2, and the remaining genotypes (CHB1, MJ5, SCB-I, and SCB-III) clustered with group 10. To the best of our knowledge, this is the first report of *E. bieneusi* infection in pet birds in China. Genotypes D, SC02, and BEB6 that have been previously identified in humans, were found in pet birds in this study, suggesting that these pet birds can be a potential source of human microsporidiosis in China.

1. Introduction

Microsporidia, classified as fungi, are unicellular and obligate intracellular eukaryotes regarded as emerging opportunistic human pathogens (Matos et al., 2012). To date, approximately 17 species within nine genera of microsporidia have been identified in humans, among which *Enterocytozoon bieneusi* is the most common (Dengjel et al., 2001). Since *E. bieneusi* was first detected in patients with human immunodeficiency virus in 1985, an increasing number of hosts have been reported as susceptible to this pathogen (Desportes et al., 2010). Most human hosts are thought to have acquired *E. bieneusi* through fecal-oral transmission of spores from infected hosts via contaminated water or food, and other routes such as inhalation of spores for respiratory tract infections were also confirmed (Ayinmode et al., 2011; Graczyk et al., 2007). The clinical signs of *E. bieneusi* infection in healthy individuals include self-limiting diarrhea, malabsorption, and wasting (Xu et al., 2011). In contrast, in immunocompromised patients such as those with acquired immunodeficiency syndrome, this infection can cause life-threatening diarrhea (Lin et al., 2013).

E. bieneusi isolates are usually characterized genetically by sequencing the internal transcribed spacer (ITS) region of the rRNA gene (Santin and Fayer, 2010; Zhang et al., 2018a). To date, molecular epidemiological surveys of *E. bieneusi* have identified more than 474 genotypes from a wide range of hosts (Li et al., 2019). More than 52 genotypes have been identified in humans and 103 exclusively from animals (Zhang et al., 2018b). However, other *E. bieneusi* genotypes can

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be found in different species of animals and humans (e.g. D, CAF1, EbpC, Type IV, and WL11) (Mehlhorn, 2015). Since the first report of *E. bieneusi* in birds (chickens, *Gallus gallus*) in Germany (Reetz et al., 2002), more than 25 genotypes of *E. bieneusi* from 12 different countries have been identified in various birds (Zhao et al., 2016). Genotypes A, D, Peru6, Type IV, Peru8, EbpA, J, Peru6-var, BEB6, Henan-IV, and Peru11 identified in humans and birds are regarded as having zoonotic potential. In contrast, genotypes M, E, L, CHN-B1, CHN-B2, CHN-B3, CC-1, and Col 01-07 were only identified in birds and thus are considered as having less zoonotic potential.

In China, *E. bieneusi* has been found in humans, various animals (e.g., belonging to the orders Carnivora, Artiodactyla, Perissodactyla, Rodentia, and Primates), and wastewater (Qi et al., 2018; Wang et al., 2018), but only limited reports have described this pathogen in birds in China (Li et al., 2014; Zhao et al., 2016). Small pet birds are popular companions and have a close relationship with humans, especially the elderly. Birds may be sources of emerging zoonotic diseases in Asia, and companion animals have been incriminated as the source of *E. bieneusi* infection in humans (Li et al., 2019). Nevertheless, there has been no research conducted on the prevalence and zoonotic implications of *E. bieneusi* carried by small pet birds in Southwest China. Therefore, in this study, we aimed to investigate the prevalence and genotypes of *E. bieneusi* in pet birds and to assess the zoonotic potential of this pathogen.

2. Materials and methods

2.1. Ethics statement

This study complied with the guidelines of the Regulations for the Administration of Affairs Concerning Experimental Animals and was approved by the Animal Ethical Committee of Sichuan Agricultural University. No animals were harmed during the sampling process. Permission was obtained from the China Giant Panda Protection and Research Center for the collection of fecal specimens. All the procedures were conducted in accordance with the approved guidelines.

2.2. Fecal sample collection

During the period from January 2017 to August 2018, 387 fresh fecal specimens were collected from pet birds from Sichuan province, Southwestern China. Six different bird species belonging to the orders Psittaciformes, Passeriformes, and Galliformes were evaluated, including 265 budgerigars (*Melopsittacus undulatus*), 39 red-headed lovebirds (*Psittacula agapornis*), six mynas (*Acridotheres cristatellus*), 30 munias (*Lonchura striata*), 31 zebra finches (*Taeniopygia guttata*), and 16 quails (*Coturnix coturnix*) (Table 1). All birds were kept individually in small cages and bird ages ranged from 30 to 360 days. Approximately 30–50 g fecal samples were collected from the bottom of each cage after defecation by using sterile disposal latex gloves and then immediately placed into individual disposable plastic bags. All fecal specimens were stored at 4 °C until processing.

2.3. DNA extraction

Fecal specimens were washed three times in distilled water with centrifugation at 3,000 × *g* for 10 min to remove potassium dichromate. DNA was extracted from 200 mg fecal specimens using an E.Z.N.A. Stool DNA Kit (Omega Biotek, Norcross, GA, USA), according to the manufacturer's instructions. The extracted DNA was stored at −20 °C.

2.4. PCR amplification

A nested PCR targeting a ~392-bp fragment of the ITS rRNA sequence was used to determine the genotypes of *E. bieneusi*. The primers were EBITS3 (5′-GGTCATAGGGATGAAGAG-3′) and EBITS4 (5′-TTCG AGTTCTTTCGCGCTC-3′) for the primary PCR and EBITS1 (5′-GCTCT

Table 1
Prevalence and genotypes of *Enterocytozoon bieneusi* in different pet bird species.

Order	Common name (Scientific name)	No. of examined	No. of positive	Prevalence (%)	95% confidence intervals	Genotypes (n)
Psittaciformes	Budgerigar (*Melopsittacus undulatus*)	265	54	20.4%	15.5–25.2	D (20), SC02 (18), BEB6 (9), CHB1 (3), SCB-I (3), SCB-II (1)
	Red-headed Lovebird (*Psittacula agapornis*)	39	10	25.6%	11.9–39.3	SC02 (7), D (3)
Subtotal		295	64	21.7%	17.0–26.4	D (23), SC02 (25), BEB6 (9), CHB1 (3), SCB-I (3), SCB-II (1)
Passeriformes	Myna (*Acridotheres cristatellus*)	6	1	16.7%	0–46.5	CHB1 (1)
	Munia (*Lonchura striata*)	30	18	60%	42.5–77.5	D (10), BEB6 (5), MJ5 (3)
	Zebra finch (*Taeniopygia guttata*)	31	6	19.4%	5.4–33.3	D (4), SCB-III (2)
Subtotal		67	25	37.3%	25.7–48.9	D (14), BEB6 (5), MJ5 (3), SCB-III (2), CHB1 (1)
Galliformes	Quail (*Coturnix coturnix*)	16	8	50%	25.5–74.5	D (4), SC02 (4)
Total		387	97	25.1%	20.7–29.4	D (41), SC02 (29), BEB6 (14), CHB1 (4), MJ5 (3), SCB-I (3), SCB-II (1), SCB-III (2)

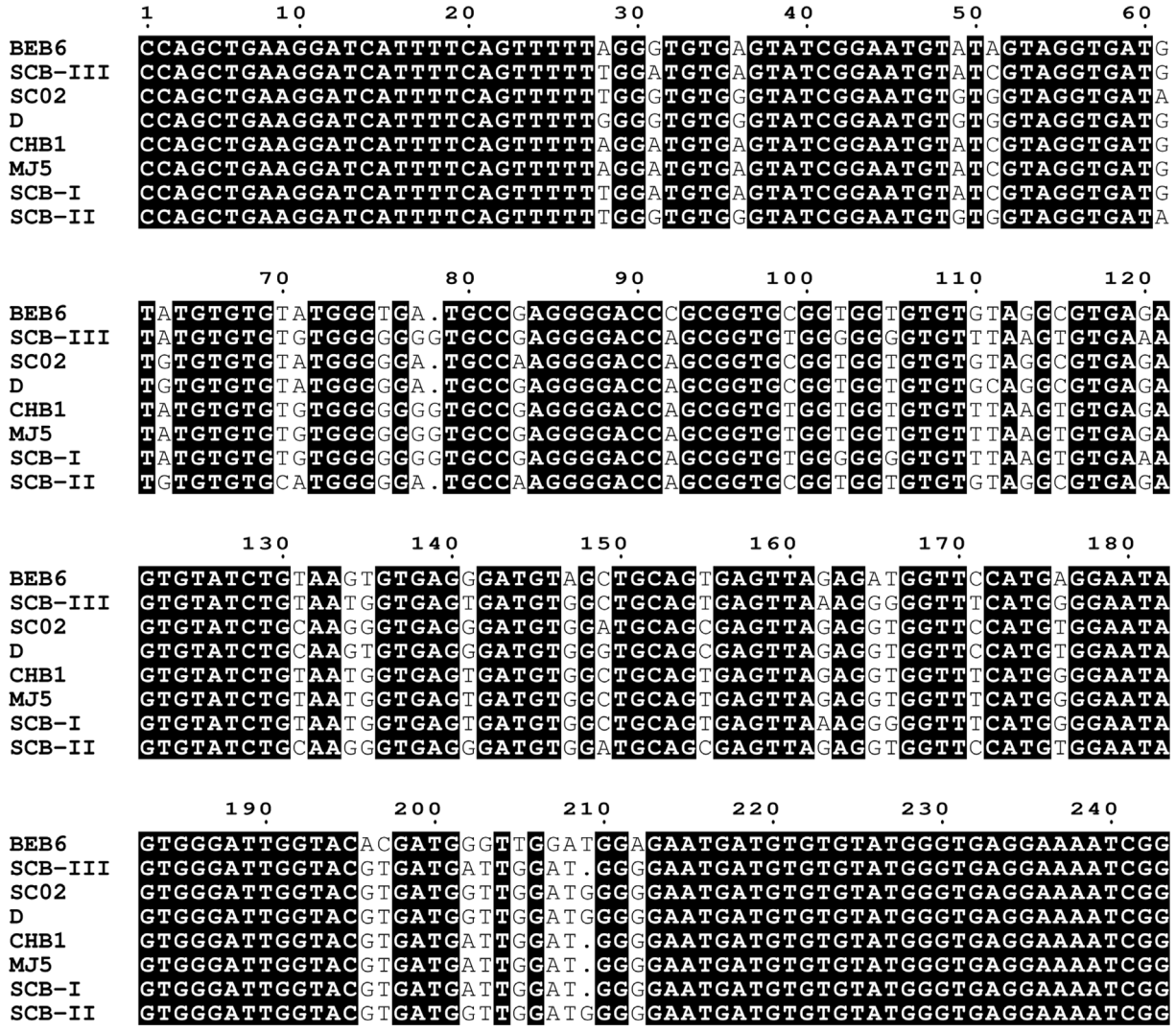

Fig. 1. Sequence variation in the ITS region of the rRNA gene of *Enterocytozoon bieneusi* isolates from pet birds. The ITS sequences of five known genotypes (D, SC02, BEB6, CHB1, and MJ5) and the three novel genotypes (SCB-I, SCB-II, and SCB-III), identified in this study, were aligned with each other.

GAATATCTATGGCT-3′) and EBITS2.4 (5′-ATCGCCGACGGATCCAA GTG-3′) for the secondary PCR (Buckholt et al., 2002). TaKaRa Taq DNA Polymerase (TaKaRa Bio, Tokyo, Japan) was used for all PCR amplifications. The cycling conditions for both primary and secondary PCRs were: 94 °C for 5 min; followed by 35 cycles of 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 1 min; followed by 72 °C for 10 min. Positive and negative controls with no DNA added were included in all PCR tests. All secondary PCR products were subjected to electrophoresis on 1% agarose gels and were visualized after staining with ethidium bromide.

2.5. Nucleotide sequencing and analysis

The secondary PCR products of the predicted size (approximately 392 bp) were directly sequenced by Life Technologies (Guangzhou, China) using a BigDye® Terminator v3.1 cycle sequencing kit on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Nucleotide sequence accuracy was confirmed by sequencing two separate PCR products. Nucleotide sequences obtained in the present study and reference sequences downloaded from GenBank were aligned with each other using Clustal X 2.0 (http://www.clustal.org/) to determine the genotypes. Representative nucleotide sequences were deposited in GenBank with the following accession numbers: MK301522-MK301529. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

2.6. Phylogenetic analysis

Phylogenetic analyses were performed using sequences obtained in the present study and published sequences obtained from GenBank. The substitution model that best fit the dataset was selected using the Akaike Information Criterion (AIC) implemented in ModelFinder (Kalyaanamoorthy et al., 2017). A maximum likelihood (ML) phylogenetic tree was constructed in PhyML version 3.0 (Guindon et al., 2010), with 1000 bootstrap replicates and the nearest neighbor interchange (NNI) branch search algorithm. Finally, the phylogenetic trees were displayed using TreeView (Page, 2002).

2.7. Statistical analysis

The prevalence of *E. bieneusi* were compared using the chi-square test and 95% confidence intervals. All results were considered statistically significant at $p < 0.05$. The analysis was performed using SPSS version 22.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Prevalence of E. bieneusi in pet birds

In the present study, 97 out of 387 fecal specimens from pet birds

Fig. 2. Phylogenetic tree based on the internal transcribed spacer (ITS) sequences obtained in this study in relation to published sequences from GenBank using ML methods. *Enterocytozoon bieneusi* genotypes identified in the present study are indicated in bold-type, and genotypes PtEbIX (DQ85585) and CD8 (KJ668735) from dogs were used as outgroups.

(25.1%) were positive for *E. bieneusi*. All the tested pet markets were *E. bieneusi*-positive, and prevalence ranged from 10% to 29.1% with no difference among them ($P > 0.05$, $df = 3$). The highest prevalence of *E. bieneusi* was observed in munias (60%, 18/30), followed by quails (50%, 8/16), red-headed lovebirds (25.6%, 10/39), budgerigars (20.4%, 54/265), zebra finches (19.4%, 6/31), and mynas (16.7%, 1/6) (Table 1). However, the differences among these species was significant ($P < 0.05$, $df = 5$).

3.2. Genetic characterization and genotype distributions of *E. bieneusi* in pet birds

Analysis of the nucleotide sequences of the ITS region of *E. bieneusi* revealed that the 97 *E. bieneusi*-positive isolates obtained here belonged to eight genotypes, including five known genotypes (D, SC02, BEB6, CHB1, and MJ5) and three novel genotypes (SCB-I, SCB-II, and SCB-III). Genotype D was the predominant (42.3%, 41/97) and was present in all bird species except for mynas. followed by genotypes SC02 (29.9%, 29/97) in budgerigar, red-headed lovebird, and quail; BEB6 (14.4%, 14/97) was identified in budgerigar and munia. Additionally, genotype

Table 2
Prevalence and genotypes of *Enterocytozoon bieneusi* in birds from different countries.

Country	Host	No. of examined	No. of positive	Prevalence (%)	Genotypes (n)	Reference
Germany	Chicken	8	2	25.0%	J (2)	Reetz et al. (2002)
Spain	Pigeon	124	19	15.3%	ND	Haro et al., 2005
	Pigeon		7		Col 01 to Col 07 (one each)	Haro et al., 2006
	Ostriches	17	1	5.9%	Type IV (1)	Kašičková et al., 2009
Portugal	Various bird	83	24	28.9%	Peru6 (17), Peru6-var. (2), Peru6 and Peru6-var. mixed infection (2)	Lobo et al., 2006
US	Feral pigeon	10	10	100%	ND	Graczyk et al. (2007)
Netherlands	Pigeon	331	18	5.4%	ND	Bart et al., 2008
United Arab Emirates	Falcon		6[d]		D (6)	Müller et al. (2008)
Czech Republic	Exotic bird	287	61	21.3%	EbpA (31); A (24)	Kašičková et al., 2009
Peru	Chicken		1		Peru 8 (1)	Feng et al., 2011
Brazil	Various bird	196	11	5.6%	EbpA (11)	Lallo et al. (2012)
	Chicken	151	24	15.9%	D (14), Peru11 (8), Type IV (1), Peru6 (1)	Cunha et al. (2016)
	Captive bird	85	3	3.5%	Peru6 (2), D (1)	Cunha et al. (2017)
Poland	Pigeon	139	2	1.4%	ND	Słodkowicz-Kowalska et al. (2013)
Iran	Pigeon	147	13	8.8%	D (6), J (4), M (3)	Pirestani et al. (2013)
	Exotic bird	816	103	12.6%	D (57), M (39), L (5), E (2)	Tavalla et al. (2017)
China	Chicken	14	2	14.3%	Henan-IV (1); CC-1 (1)	Li et al. (2014)
	Various bird	194	43	22.2%	Peru6 (29), BEB6 (5), D (3), EbpA (1), CHN-B1 (1), CHN-B2 (3), CHN-B3 (1)	Zhao et al. (2016)

ND: *E. bieneusi* genotype not determined.

CHB1 (4.1%, 4/97) was found in two bird species (budgerigars and mynas), whereas genotype MJ5 (3.1%, 3/97) was only found in munias.

Genetic polymorphism was observed among the novel genotypes. The novel genotypes SCB-I (MK301527) and SCB-III (MK301529) differed from genotype CHB1 (KU825466) and had eight and six single nucleotide polymorphisms (SNPs), respectively. In contrast, genotype SCB-II (MK301528) had only one SNP when compared with genotype SC02 (KY950533). The base variation of the genotypes within the 243 bp of the ITS sequence is presented in Fig. 1.

3.3. Phylogenetic relationship of *E. bieneusi*

Phylogenetic analysis of the ITS sequences of all *E. bieneusi* genotypes detected here and reference genotypes published previously shows that genotypes D, SC02, and SCB-II were clustered into group 1. Genotype BEB6 was clustered into group 2 and the remaining four genotypes (CHB1, MJ5, SCB-I, and SCB-III) belonged to group 10 (Fig. 2).

4. Discussion

This is the first study showing that pet birds may be infected with *E. bieneusi*, with some zoonotic genotypes identified in pet birds and humans in China suggesting that pet birds can be a direct or indirect source of infection to humans. Direct fecal-oral transmission is likely to occur in the system tested here owing to the close relationship among humans and some bird species shedding spores of zoonotic *E. bieneusi* genotypes. This may be the case for Psittaciformes birds that are commonly kept indoors. These birds can also contaminate water and food, therefore acting as potential indirect sources for human infection.

The first case of microsporidiosis in birds caused by *E. bieneusi* was detected in chickens originating from a poultry abattoir in Germany (Reetz et al., 2002). In recent decades, *E. bieneusi* has been detected in birds from several localities worldwide (Table 2). The prevalence of *E. bieneusi* in birds in our study was similar to that reported in a study of chickens in Germany (25.0%) (Reetz et al., 2002), but higher than the prevalence previously reported from China (14.3%) (Li et al., 2014), Iran (8.8% and 12.6%) (Pirestani et al., 2013; Tavalla et al., 2017), Brazil (5.6% and 3.5%) (Cunha et al., 2017; Lallo et al., 2012), the Netherlands (5.4%) (Aldert et al., 2008), and Poland (1.4%) (Słodkowicz-Kowalska et al., 2013; Perec-Matysiak et al., 2017). Previous reports of *E. bieneusi* in other animals have demonstrated that there are differences in prevalence according to different feeding conditions. The prevalence of *E. bieneusi* may also be affected by different geographical regions, management methods, host nutritional and health status, and seasonal variations.

In the present study, a total of eight different genotypes were identified from 97 *E. bieneusi*-positive specimens from pet birds, including five previously known genotypes (D, SC02, BEB6, CHB1, and MJ5) and three novel genotypes (SCB-I–III). Genotype D was the prominent genotype (42.3%), which is consistent with previous studies in chickens in Brazil (58.3%) (Cunha et al., 2016), exotic birds in Iran (55.3%) (Tavalla et al., 2017), and falcons in the United Arab Emirates (Müller et al., 2008). Genotype D has been reported in humans in Shanghai city, Henan province, Guangxi Zhuang autonomous region, Hubei province, and Heilongjiang province in China. In addition, it also identified in a wide range of animals, including nonhuman primates, livestock, pet animal, wildlife, and birds in China. Genotype SC02 has been identified in humans in Sichuan province, and also reported in a wide range of animals, such as Tibetan blue bears, sun bears, Asiatic black bears, Northern raccoons, horses, giant pandas, and squirrels (Deng et al., 2017; Li et al., 2018). The results above indicate that birds may play a role in the transmission of *E. bieneusi* to humans and other animals by acting as a reservoir host. Future epidemiological studies of *E. bieneusi* will be preferentially focused on the different hosts in the same areas to better understand the transmission dynamics of *E. bieneusi*.

Genotype BEB6 was first reported in cattle in the eastern United States (Fayer et al., 2007), and has subsequently been identified in birds, goat, sheep, rhesus macaques, deer, and cats in China (Md Robiul et al., 2014). This genotype was also identified in a pediatric hospital in China (Wang et al., 2013). Moreover, genotype BEB6 was found to be common in raw wastewater in China (Li et al., 2012; Ye et al., 2017). Genotype CHB1 was originally identified in Ursidae, including Tibetan blue bears, brown bears, Asiatic black bears, and Malayan sun bears (Deng et al., 2017). Additionally, genotype MJ5 was identified in black bears in Yunnan province, China (Wu et al., 2018). The fact of genotypes BEB6 and CHB1 have been identified in ursidae and here in pet birds in the same areas suggests the circulation of these genotypes between these animal hosts.

The genetic relationships between the eight genotypes of *E. bieneusi* detected in the present study and other known genotypes were

determined in our phylogenetic analysis (Fig. 2). Three genotypes (D, SC02, and SCB-II) were clustered within group 1, suggesting the possibility of zoonotic transmission and public health significance. Genotype BEB6 was classified within group 2. The remaining genotypes (CHB1, MJ5, SCB-I, and SCB-III) were clustered within group 10, together with genotypes CSK1, CHK1, and CHK2 from red kangaroos (Zhang et al., 2018b). However, further molecular epidemiological studies are required to investigate the potential of these group 10 genotypes to cause microsporidiosis in humans.

5. Conclusions

This is the first to report the prevalence of *E. bieneusi* (25.1%, 97/387) in pet birds in China. Five known genotypes (D, SC02, BEB6, CHB1, and MJ5) and three novel genotypes (SCB-I, SCB-II, and SCB-III) were identified. The detection of the three known genotypes D, SC02, and BEB6, which are also known to infect humans, suggests that pet birds in the investigated regions may be a source of *E. bieneusi* infection for humans. Therefore, further studies are needed to investigate the transmission dynamics between pet birds and humans.

Conflicts of interest

The authors declare that they have no competing interests.

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