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Occurrence and Zoonotic Evaluation of *Cryptosporidium spp.* and *Enterocytozoon bieneusi* in Dogs and Cats in Wenzhou, Southern China

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ABSTRACT

Background: *Cryptosporidium spp.* and *Enterocytozoon bieneusi* are important enteric diarrheal pathogens commonly found in various animals and humans and have significant implications for public health. The purpose of present study was to investigate the prevalence, species and genotypes and zoonotic evaluation of the two parasitic pathogens in dogs and cats in Wenzhou, southern of China.

Methods: 89 fecal samples collected from 51 dogs and 38 cats were used to investigate the positive rates of *Cryptosporidium spp.* and *Enterocytozoon bieneusi* using the nested polymerase chain reaction amplification on methods, species and genotypes of them were conformed by sequencing and alignment.

Result: The positive rates of *Cryptosporidium spp.* and *Enterocytozoon bieneusi* were 7.8%(4/51), 17.6%(9/51) in dogs and 7.9%(3/38) ,10.5%(4/38) in cats, respectively. Sequence analysis revealed that *Cryptosporidium canis*, genotypes PtEb IX, CD9 and NED3 of *E. bieneusi* were confirmed in dogs, *Cryptosporidium felis*, Type IV in cats, respectively. *C canis, C. felis* and Type IV of *E. bieneusi* genotype belong to the zoonotic pathogens.The zoonotic species/genotype of these two pathogens identified in pets in this study indicated the potential zoonotic risk in pets-to- humans transmission. The effective interventions should be taken to prevent the spread of these enteric zoonotic pathogens.

Key words: Cats, *Cryptosporidium*, Dogs, *Enterocytozoon bieneusi*, Genotypes.

INTRODUCTION

Cryptosporidium spp. and *Enterocytozoon bieneusi* are the most common enteric protists in humans, farms, companion animals and wild animals, mainly causing gastrointestinal issues, such as acute or chronic diarrhea, abdominal pain, malabsorption and irritable bowel syndrome, particularly in the hosts with compromised immune systems or getting sick (Feng *et al.*, 2018; Prasertbun *et al.*, 2019; Li *et al.*, 2022; Jiang *et al.*, 2023; Alarcón-Zapata *et al.*, 2023). The most important reasons for these pathogens infection are due to the fecal-oral transmission route, including the intimate contact with high-risk hosts infected with zoonotic pathogens and ingestion of fecal-contaminated food or water (Ryan *et al.*, 2018; Li *et al.*, 2019; Gururajan *et al.*, 2021; Swain *et al.*, 2018).

Currently, at least 45 species and over 120 genotypes of *Cryptosporidium* have existed in animals and humans (Ryan *et al.*, 2021; Yang *et al.*, 2021), *C. canis* and *C. felis* are among the five zoonotic *Cryptosporidium* species,while the rest are host-specific pathogens(Yang *et al.*, 2021). Similarly, there are over 500 *E. bieneusi* genotypes which are classified into 13 groups with divergent genetic diversity (Li and Xiao, 2020; Meng *et al.*, 2023). Type IV, C and D genotypes in group 1 are commonly reported in animals and humans, suggesting the zoonotic importance (Santín and Fayer, 2011). BEB4, I and BEB6 genotypes in group 2 have been observed in broad range of hosts, indicating an increasing zoonotic risk(Li *et al.*, 2019). However, the most genotypes in group 3-11 are host-specific, with low zoonotic potential (Li *et al.*, 2020; Yang *et al.*, 2023).

There are many records about *Cryptosporidium spp.* and *E.bieneusi* present in a variety of companion animals,including dogs and cats. While the most common identified species are *Cryptosporidium canis* in dogs and *C. felis* in cats (Barbosa *et al.*, 2023), other species, like *C. muris*, *C. parvum*, *C. ubiquitum* and *C. hominis* were sporadically detected in these companion animals (Xu *et al.*, 2016). Type IV, D, PtEb IX and EbpC were the

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predominant *E. bieneusi* genotypes in dogs (Cao *et al.*, 2021), while genotypes I, Type IV and BEB6 were widely distributed in cats (Karim *et al.*, 2014; Wang *et al.*, 2021). There have been much research on the prevalence and zoonotic potential of *E. bieneusi* and *Cryptosporidium spp.* broadly conducted in dogs and cats in various countries, including regions of China such as Xinjiang (Cao *et al.*, 2021), Henan (Karim *et al.*, 2014), Guangdong (Wang *et al.*, 2020) and Shanghai (Xu *et al.*, 2016). Wenzhou, a region known for its economic vitality in southern of China, has a population of over 9 million humans and 500 thousand of pets. The warm and humid climate in Wenzhou provides favorable conditions for the dissemination of these pathogens. However, there is scarce information available about the occurrence, species of *Cryptosporidium spp.* and *E. bieneusi* genotypes in these companion animals in Wenzhou. Therefore, the purpose of this survey was to ascertain the prevalence, species of *Cryptosporidium spp.* and *E. bieneusi* genotypes in dogs and cats and to evaluate potential risk for public health.

MATERIALS AND METHODS

Specimen collection, DNA extraction and nested amplification

From September in 2023 to August in 2024, there were 89 fresh fecal samples collected from dogs and cats in Wenzhou, China. Among these, 51 were from dogs and 38 from cats, containing 11 fecal samples from dogs and 9 from cats aged <12 months respectively. Each fecal sample was collected from the dogs or cats' rectum with the sterile plastic gloves and immediately transferred to laboratory of Wenzhou Vocational College of Science and Technology under the cool condition and then placed into plastic tube containing 2.5% potassium dichromate, finally kept at 4°C for DNA extraction. To remove the large fecal particles and potassium dichromate, each fecal sample was sieved through wire screen and washed until the supernatant was clean. Genomic DNA was isolated from processed fecal materials of each sample in accordance with the manufacturer's instructions (Omega, Norcross, GA, USA). The obtained DNA samples were kept at -20°C.

A nested PCR amplification and DNA sequencing for the SSU rRNA gene of *Cryptosporidium spp.* were used to detect the positive rate and species compositions in dogs and cats, as previously reported (Ghebremichael *et al.*, 2023). Similarly, a nested PCR techniques and sequence analysis of *Enterocytozoon bieneusi* ITS region were used to examine the positive rate and genotypes in fecal samples (Yu *et al.*, 2023). The nested PCRs were conducted in a 25 µL reaction systems: 2.5 µL of 10 × PCR Buffer, 0.15 µL rTaq enzyme (TaKaRa Co., Ltd., Beijing, China), 2 µL dNTP, 2.0 µL $MgCl_2$,1 µL of primers (10 µM each), 1 µL of obtained DNA sample and 15.35 µL of nuclease-free water. The obtained secondary PCR products were performed by electrophoresis and visualized with the nucleic acid staining.

Sequence analysis

The positive obtained PCR products were sent to Sangon Biotech (Shanghai, China) for bi-directional sequencing. Each sequence obtained from the positive samples in present study was firstly assembled and edited using the tools online and then compared with the nucleotide sequence database in GenBank. To assess the relationships of *Cryptosporidium spp.* and *E. bieneusi* found in this study, phylogenetic trees were performed using MEGA V11.0 based on the maximum likelihood (ML) method and Bootstrap analysis calculated with 1000 replicates.

RESULTS AND DISCUSSION

In this investigation, out of the 51 fecal samples analyzed, 4 (7.8%, 4/51) tested positive for *Cryptosporidium spp.* and 9 (17.6%, 9/51) tested positive for *E. bieneusi* in dogs. While the positive rates of *Cryptosporidium* varied in different sites, age groups and deworming statuses, no significant differences were observed. Similarly, although the positive rates of *E. bieneusi* also varied, with notably high rates in the group without deworming (42.9%, 3/7), the differences for positive rates of *E. bieneusi* were not statistically significant among the different sites, age groups and deworming statuses. Notably, no positive samples for *E. bieneusi* were found in dogs from pet market (Table 1).

3 out of 38 fecal samples (7.9%, 3/38) tested positive for *Cryptosporidium spp.* and 4 samples (10.5%, 4/38) tested positive for *E. bieneusi* in cats. While the positive rates for *Cryptosporidium* and *E. bieneusi* varied based on site, age and deworming status, the highest positive rate for *Cryptosporidium spp.* was observed in hospitals (11.8%, 2/17) and for *E. bieneusi* in no deworming group (22.2%, 2/9). However, no significant differences were found among different sites, age groups and deworming statuses. Surprisingly, cats in pet market and aged<12 months were neither infected with *Cryptosporidium spp.* nor *E. bieneusi* (Table 2).

All 4 *Cryptosporidium*-positive samples in dogs were successfully sequenced and identified as *C. canis*. Among the 4 *C. canis* isolates identified in present study, 3 were identical to the reference sequence (MN696800.1) from dogs in Guangzhou, southern China, with one nucleotide deletion.Meanwhile, there were two nucleotide deletions, a C to G substitution and two C to A nucleotide substitutions in the remaining isolate compared with this reference sequence (MN696800.1). Out of the 3 positive samples were sequenced for *Cryptosporidium spp.*, only *C. felis* was identified. Among the 3 *C. felis* isolates, one was identical to a reference sequence from UK (OP935207.1) with 100% homology. One was 99% identical to an isolate from a cat in China (KJ194110.1), with one single nucleotide substitution, the last isolate had 99% similarity to another reference sequence (KX168415.1) (Fig 1).

Of the 9 *E. bieneusi*-positive samples sequenced successfully in dogs, three known genotypes, namely

PtEb IX, CD 9 and NED 3genotypes, were identified using the ITS gene locus sequencing method. PtEb IX (66.7%, 6/9)was the most predominant genotype found in dog isolates across all groups except the pet market. CD9 (22.2%, 2/9) was detected in samples from shelters and hospitals, while genotype NED3 (11.1%, 1/9) was only found in one specimen. Type IV was the only genotype identified in the 4 positive samples analyzed for *E. bieneusi* in cats (Fig 2).

Analysis from the perspective of concurrent infections, there was one positive-sample co-infected with *C. canis* and NED3 genotype of *E. bieneusi* in dogs, two positive-samples were concurrently infected with Type IV genotype of *E. bieneusi* and *C. felis* in cats, the remaining positive-samples were mono-infections.

Cryptosporidium spp. and *E. bieneusi* are crucial zoonotic pathogens widely found in humans and various animals across different regions. Dogs and cats, commonly kept as pets in many households, have close contact with humans and could serve as important reservoirs of zoonotic pathogens, posing a potential risk for human health.

The results of this investigation revealed that the positive rate of *Cryptosporidium spp.* was 7.8% (4/51) in dogs and 7.9% (3/38) in cats respectively, which was higher than that in Poland (3.4%, 2%) (Piekara-Stêpińska *et al.*, 2021), Egypt (1.8%, 6.0%) (Elmahallawy *et al.*, 2023), Yunnan in China (4.6%, 0.6%) (Wang *et al.*, 2021), but was consistent with that in Brazil (7.8%, 5.4%) (de Oliveira *et al.*, 2021), in Turkey (7.14%, 9.09%), (Ipek, 2023), in Saudi Arabia (8.5%) (Malk, 2021), Guangdong (6.9%, 6.2%) (Li

Table 1: Occurrence, distribution, species of *Cryptosporidiumspp.* and genotypes of *E. bieneusi* genotypesin dogs in Wenzhou, southern China.

Factors	No. examined	*Cryptosporidium* species		*E. bieneusi*	
		No Positive (%)	species (No.)	No. Positive (%)	Genotype (No.)
Site					
Shelters	13	1(7.7)	*C. canis*(1)	3 (23.1)	PtEbIX(1), CD9(1), NED3(1),
Hospitals	28	2 (14.3)	*C. canis*(2)	6 (21.4)	PtEbIX(5), CD9(1)
Pet market	10	1 ((7.1)	*C. canis*(1)	0	
Age					
6-12 month	11	1 (9.1)	*C. canis*(1)	2 (18.2)	PtEbIX(2)
>12 month	40	3 (7.5)	*C. canis*(3)	7 (17.5)	PtEbIX(4), CD9(2), NED3(1)
Deworming					
Yes	44	3 (6.8)	*C. canis*(3)	6 (13.6)	PtEbIX(3), CD9(2), NED3(1)
No	7	1 (14.3)	*C. canis*(1)	3 (42.9)	PtEbIX(3)
Total	51	4 (7.8)	*C. canis*(4)	9 (17.6)	PtEbIX(6) CD9(2), NED3(1)

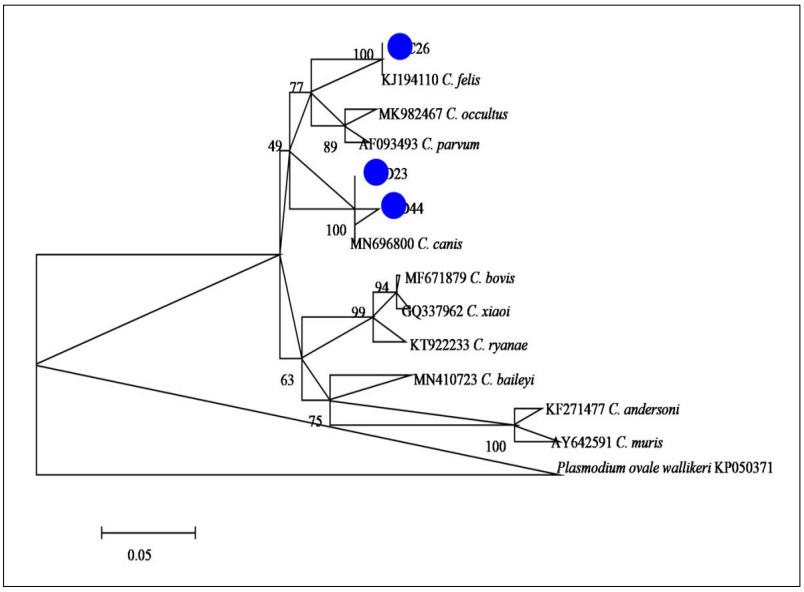

Fig 1: Phylogenetic relationship of *Cryptosporidium spp.* identified (the filled bluecircle) in present study with the nucleotide sequence downloaded in database.

et al., 2019), Shanghai (8.0%, 3.8% (Xu *et al.*, 2016) and other regions in China.The discrepancy in *Cryptosporidium* infection in pets may be related to factors, like sample sizes,detection methods, geographic locations and living conditions.

Enterocytozoon bieneusi has been increasingly found in human and broad animals, including diverse intimate companions. In this survey, the positive rate of *E. bieneusi* was 17.6% in dogs and 10.5% in cats respectively, which was comparable to the positive rate of dogs (18.8%, 136/724) and cats (14.1%, 22/156) examined respectively in Sichuan province in China (Zhong *et al.*, 2021), but was much higher than that in dogs (0.8%, 2/237) and cats(3.0%, 3/99) in Northern Spain (Dashti *et al.*, 2019) and was much lower than that in stray cats in Türkiye (50.15%, 170/339) (Erkunt *et al.*, 2023). Variations in *E. bieneusi* prevalence among dogs and cats in different studies can be attributed to various risk factors such as living habits, age, sex,sample sizes and sources of the animals.

The findings, only *C. canis* identified in dogs and *C. felis* in cats respectively in present survey, were consistent

Table 2: Occurrence, distribution, species of *Cryptosporidium spp.* and genotypes of *E. bieneusi* genotypes in cats in Wenzhou, southern China.

Factors	No. examined	*Cryptosporidium* species		*E. bieneusi*	
		No. Positive (%)	Species (No.)	No. Positive (%)	Genotype (No.)
Site					
Shelters	10	1 (10)	*C. felis*(1)	2 (20.0)	TypeIV (2)
Hospitals	17	2 (11.8)	*C. felis*(2)	2 (11.8)	TypeIV (2)
Pet market	11	0		0	
Age					
6-12 month	9	0		0	
>12 month	29	3 (10.3)	*C. felis*(3)	4 (13.8)	TypeIV (4)
Deworming					
Yes	29	2 (6.9)	*C. felis*(2)	2 (6.9)	TypeIV (2)
No	9	1 (11.1)	*C. felis*(2)	2 (22.2)	TypeIV (2)
Total	38	3 (7.9)	*C. felis*(3)	4 (10.5)	TypeIV (4)

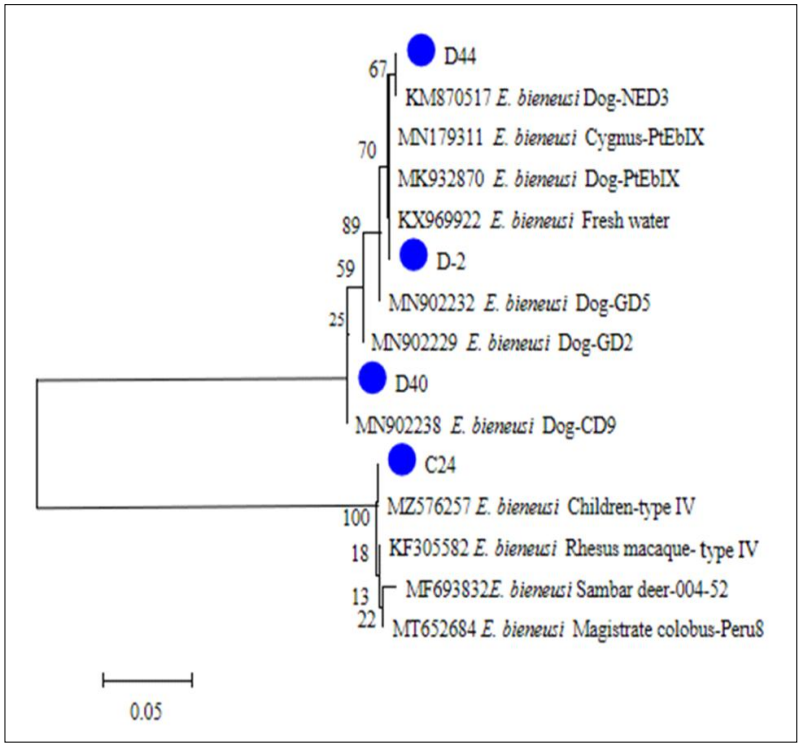

Fig 2: Phylogenetic relationship of *Enterocytozoon bieneusi* obtained (the filled blue circle)with representative *E. bieneusi* genotypes deposited in GenBank conducted based above-mentioned method.

with previous research. However, in some other recent records, there were presence of several other *Cryptosporidium* species occasionnally infecting dogs and cats, like *Cryptosporidium parvum*, *Cryptosporidium ubiquitum*, *Cryptosporidium muris* and the *Cryptosporidium* rat genotype IV (Gil *et al.*, 2017; Li *et al.*, 2019). *C. canis* and *C. felis*, generally regarded as host-specific pathogens, were commonly detected in some human cases worldwide and mainly found in immunocompromised individuals and low-income countries (Yang *et al.*, 2021). The result suggests that people should take protective measures to minimize zoonotic pathogen transmission from their pets.

There were 4 genotypes of *E. bieneusi*, PtEb IX, CD9, NED3 in dogs and Type IV in cats identified in this study. PtEb IX, which was the main *E. bieneusi* genotype and regarded as the dog-specific with widespread globally in dogs (Xu *et al.*, 2016; Wang *et al.*, 2020), was also the dominant genotype (6/9) in present survey. However, CD9 was the second dominant genotype in this survey (2/9), which was also the predominant *E. bieneusi* genotype in Sichuan province (Zhong *et al.*, 2021). NED3, identified in this study,is recognized as a dog-adapted genotype.

Type IV was the sole genotype identified in adult cats in this study, this consequence was also consistent with its prevalence in cats from other sources (Sürgeç *et al.*, 2023).This genotype, known for its pathogenicity in humans and global presence, has also been found in a wide variety of animals, such as *Lynx pardinus* (Ávalos *et al.*, 2024), foxes and raccoon dogs (Zhang *et al.*, 2021; Chen *et al*., 2023), zoo animals (Zhang *et al.,* 2021), pigeons (Holubová *et al.*, 2024) and domestic animals. Moreover, there were some reports about Type IV of *E. bieneusi* infections in humans in Shanghai (Jiang *et al.*, 2023), Ningbo (Liu *et al.*, 2023), southern China and northeast Egypt respectively (Naguib *et al.*, 2022). These findings suggest that animals carrying zoonotic Type IV genotype may serve as significant sources of human microsporidiosis. In contrast, the PtEb IX genotype was predominantly found in cats in other research (Wang *et al.*, 2020).

CONCLUSION

The molecular epidemiological survey of *Cryptosporidium spp.* and *E. bieneusi* in dogs and cats in Wenzhou, southern China, revealed positive rates of 7.8% (4/51) and 17.6% (9/51) for *Cryptosporidium spp.* and *E. bieneusi* in dogs and 7.9% (3/38), 10.5% (4/38) in cats, respectively. *Cryptosporidium canis*, genotypes PtEb IX, CD 9 and NED3 of *E. bieneusi* were confirmed in dogs, *Cryptosporidium felis*, Type IV in cats, respectively, *C. canis, C. felis* and Type IV of *E. bieneusi* genotype belong to the zoonotic pathogens. These findings suggest the need for effective interventions to minimize cross-infection of these two pathogens between pets and humans. Further research should explore the prevalence and species/genotypes of these two pathogens in pet owners, veterinarians and other animals in the same habitat and assess the potential public health risks.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the Wenzhou Vocational College of Science and Technology of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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