Polish Journal of Veterinary Sciences Vol. 28, No. 1 (2025), 143–150

DOI 10.24425/pjvs.2025.154022

Original article

Prevalence and molecular characterization of *Cryptosporidium* spp. from domestic pigeons (*Columba livia domestica*) in Anhui, China

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Abstract

Cryptosporidium spp. is a protozoan parasite of many vertebrates worldwide including avian hosts, causing gastroenteritis and diarrhea. Studies have been conducted on *Cryptosporidium* spp. in some avians, however, there is no information on *Cryptosporidium* spp. in pigeons from Anhui Province, China. To investigate the prevalence and assess the transmission burden of *Cryptosporidium* species in domestic pigeons, a total of 376 fecal samples were collected. The acid-fast staining and nested PCR amplification methods reveal a *Cryptosporidium* prevalence rate of 5.05% (19/376) and 1.86% (7/376), respectively. Furthermore, molecular characterization was identified as *Cryptosporidium meleagridis*. As this study is the first report on *Cryptosporidium* spp. in domestic pigeons in Anhui Province, we expect it to provide baseline information for further studies.

Keywords: *Cryptosporidium meleagridis*, Pigeon, PCR, prevalence, Phylogenetic analysis

Introduction

Cryptosporidium spp. constitutes a broad genus of protozoan parasites ubiquitously present across a wide spectrum of vertebrate hosts including birds (O'Donoghue et al. 1987, Holubová et al. 2016, Wang et al. 2021), fishes (Moratal et al. 2020, Golomazou et al. 2021, Couso-Pérez et al. 2022), reptiles (Upton et al. 1989, Pedraza-Díaz et al. 2009, Garcia et al. 2023), amphibians (Ryan 2010, Hallinger et al. 2020), humans (Dąbrowska et al. 2023) and other mammals (Xiao et al. 2004, Gattan et al. 2023). It is known that *Cryptosporidium* spp. can lead to enteritis, manifested as severe diarrhea, emesis and anorexia in hosts (Kirkpatrick 1985). Specific species such as *C. parvum* in mammals (Xiao et al. 2004, Gattan et al. 2023), particularly in humans (Xiao et al. 2008), *C. hominis* in primates (Cama et al. 2007, Gattan et al. 2023) and *C. meleagridis* in birds (Aa et al. 2015) are the most typical zoonotic pathogens.

C. meleagridis has been one of the main species in transmission of human cryptosporidiosis which causes public health concerns worldwide (Xiao et al. 2008). It also manifests as a digestive disease in birds, and it affects a very large number of avian species across all continents except Antarctica (Aa et al. 2015). Since the initial report of *C. meleagridis* in turkeys (*Meleagris gallopavo*) (Slavin 1955), it has subsequently been identified in different species of avian hosts, including feral monk parakeets (Briceño et al. 2023), broiler chickens (Lin et al. 2022, Feng et al. 2022), eared doves (Seixas et al. 2019), and pet birds (Dong et al. 2021, Liao et al. 2021), which indicates widespread infection via cross-species transmission (Yuan et al. 2014). Noteworthy, *C. meleagridis* was distinguished as the third confirmed zoonotic agent within the *Cryptosporidium* spp. Current reports have highlighted that *C. meleagridis* has a wide range of hosts, including humans, particularly vulnerable populations such as children (Tzipori 1988, Messa et al. 2021) and immunocompromised patients (Cama et al. 2007, Sannella et al. 2019, Mohamed et al. 2022), with sporadic cases in adults (Mulunda et al. 2020).

Despite *C. meleagridis* being globally prevalent and causing significant health implications for animals and humans, information on the prevalence and characteristics of *Cryptosporidium* spp. in pigeons remains limited. In China, pigeons are a significant part of the culinary landscape and widely bred (Zhang et al. 2024). Therefore, considering the influence of *Cryptosporidium* on the pigeon industry and the infection of *Cryptosporidium* in pigeons and people, we conducted an investigation on *Cryptosporidium* infection in pigeon farms in Anhui Province. This study aims to investigate the prevalence and molecular characteristics of *Cryptosporidium* species in pigeons from Anhui, China, contributing valuable insights into the field of zoonotic diseases and exhibiting the identification of *C. meleagridis* in domestic pigeon feces in Anhui Province.

Materials and Methods

Sample collection

Among the several types of poultry raised in this region, domestic pigeons hold a significant place, being commonly reared by many residents. Between September and December 2023, a total of 376 fecal samples were collected from pigeon farms across nine prefecture-level cities in Anhui Province. The pigeon populations on these farms ranged from approximately 2,500 to over 3,000 birds, with varying numbers of pigeon houses. All pigeons in the sampled lofts were in perfect health. The specific data for each pigeon farm are presented in Table 1. From each farm, 1% of the pigeons were randomly and evenly selected for sampling and subsequent analysis (Fig.1). In the sampling regions, pigeons were reared under intensive farming conditions, which posed challenges in acquiring fecal samples statistically. The fecal samples were collected only from adult pigeons (>6 months) and were stored at 4°C to ensure preservation of their integrity.

Microscopic examinations

The samples were examined for *Cryptosporidium* oocysts using the sucrose flotation technique. Briefly, 1 g of feces was suspended in 9 ml of saturated sucrose solution (Specific gravity 1.2) in a 15 ml centrifuge tube and centrifuged at 1300 g for 5 min. A sucrose solution was then added to the 15 ml tube, which was then left at room temperature for 30 min. The tube was then filled to create a meniscus by adding drops of solution and putting a glass slide over the tube for 5-10 min. After that, the coverslip was put on a glass slide and examined for oocysts under a light microscope using a 40× objective lens (Fujino et al. 2006). Thereafter, oocysts of *Cryptosporidium*-positive samples were transferred from the surface of the suspension to a microscopic slide, and air dried before staining with Acid-Fast Bacillus (AFB) Stain Kit (Modified Kinyoun's Method) (Solarbio Life Science, Beijing, China) according to the manufacturer's instructions, then rinsed again with tap water and air dried. After drying, the stained glass slides were examined for oocysts under alight microscope using an oil immersion objective lens (×100) (Zaglool et al. 2013). The remain-

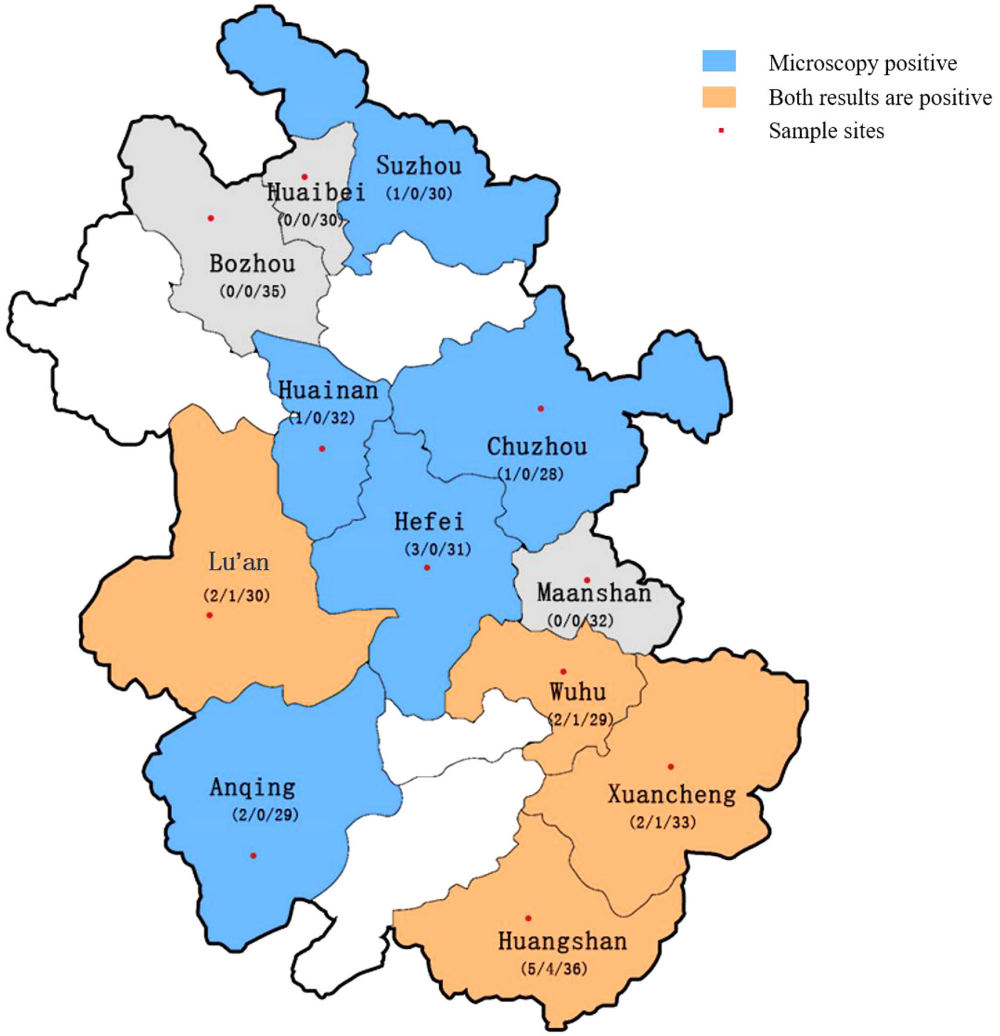

Fig. 1. Geographical distribution of sampling sites of domestic pigeons in partial regions of Anhui Province in the present study. Sampling locations are marked with "·". Regions with microscopy positive results are represented in blue, while regions with both microscopy and PCR positive results are represented in orange. Additionally, in the figure, we have indicated the microscopy-positive rate, PCR-positive rate, and the number of samples for each region: (microscopy positive samples / PCR positive samples / total number of samples).

Table 1. Prevalence and infection of *Cryptosporidium* in pigeons.

Location	No. pigeons	No. houses	No. sample	Prevalence and Prevalence rate (%)	
				Microscopy	PCR
Hefei	3144	2	31	3 (9.68)	0
Huainan	3196	2	32	1 (3.13)	0
Xuancheng	3302	2	33	2 (6.06)	1 (3.03)
Huangshan	3568	3	36	5 (13.89)	4 (11.11)
Chuzhou	2784	1	28	1 (3.57)	0
Bozhou	3540	3	35	0	0
Suzhou	2990	1	30	1 (3.33)	0
Anqing	3048	2	30	2 (6.67)	0
Lu'an	2968	1	30	2 (6.67)	1 (3.33)
Maanshan	3230	2	32	0	0
Wuhu	2880	1	29	2 (6.90)	1 (3.48)
Huaibei	3042	2	30	0	0
Total	37692	22	376	19 (5.05)	7 (1.86)

ing samples were kept at 4°C until DNA extraction (Elliot et al. 1999).

DNA extraction

Genomic DNA was extracted from these samples using a TIANamp Stool DNA Kit (TIANGEN, Beijing, China), following the prescribed protocol provided by the manufacturer. Subsequently, all extracted DNA samples were meticulously stored at -20°C to maintain their stability and prevent degradation until further analytical use.

Nested PCR analysis of the 18S rRNA

For PCR reactions, Platinum Taq High Fidelity polymerase (Invitrogen, Carlsbad, CA, USA) and *Cryptosporidium*-specific primers, as previously described, were used. The initial PCR targeted a 1325 bp fragment with the following specific primer sequences: forward primer (5'-TTCTAGAGCTAATACATGCG-3') and reverse primer (5'-CCCATTTCCTTCGAAACAG GA-3'). The subsequent PCR, aimed at amplifying an 835 to 838 bp fragment, used the product of the first PCR as the DNA template, with primer sequences of the forward primer 5'-GGAAGGGTTGTATTTATT AGATAAAG-3') and the reverse primer (5'-AAG GAGTAAGGAACAACCTCCA-3') (Xiao et al. 1999).

The amplification reaction was conducted in a 25 µl volume, comprising 2.5 µl KOD buffer, 2.5 µl dNTPs (2 mM each), 0.5 µl of KOD, 1.5 µl of MgSO4 (all reagents from the KOD-PLUS kit, TOYOBO Co., Ltd., Osaka, Japan), 1 µl of DNA, and 16 µl of double distilled water, with 0.5 µM of each primer. This setup was nearly identical for both the primary PCR and nPCR (nested PCR). The primary PCR included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, and extension at 72°C for 90 sec, with a final extension at 72°C for 7 min. The nested PCR differed only in the extension time within the cycles, which was 1 min at 72°C.

PCR products were purified using a 1.5% agarose gel (agarose from BioSharp®, Anhui, China) and the secondary PCR products were purified using the Fast-Pure Gel DNA Extraction Mini Kit (Vazyme Biotech Co., Ltd., Jiangsu, China) following the manufacturer's instructions. The purified products were cloned in a 5 min TM TA/Blunt-Zero Cloning Kit (Vazyme Biotech Co., Ltd., Jiangsu, China) and transformed into *Escherichia coli* DH5α cells. Clones were then selected on Luria-Bertani (LB) agar plates containing 50 µg/ml ampicillin and incubated in a constant temperature incubator at 37°C for 12-18 hr. Selected colonies were harvested using an inoculating loop for subsequent analysis by agarose gel electrophoresis.

Sequencing and phylogenetic

The amplicons of the 18S rRNA gene were sequenced bidirectionally by Tsingke Biotech Co., Ltd. (Beijing, China). The resulting sequences were analyzed using ChromasPro V1.33 (www.technelysium.com.au/ChromasPro.html) and DNA star (https://www.dnastar.com/software/). These sequences were then subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the species and ascertain their high similarity and homology with known sequences of *Cryptosporidium* spp. in GenBank. Bootstrap values were generated from 1,000 replicates to validate the data.

After sequencing, all sequences underwent multiple alignments and were analyzed using MEGA 11 software (http://www.megasoftware.net/). Phylogenetic trees for the 18S rRNA gene were constructed using the Neighbor-Joining method in MEGA 11 software (Kumar et al. 2016, Tamura et al. 2021) to elucidate the evolutionary relationships. The robustness of the phylogenetic trees was verified through bootstrap analysis with 1,000 replicates, ensuring the accuracy and reliability of the phylogenetic inferences.

Nucleotide sequence accession number

The partial 18S rRNA nucleotide sequences obtained in this study have been deposited in the GenBank database under accession numbers (PP204060-204066).

Statistical analysis

Data analysis was performed using SPSS 26, and results were considered to be statistically significant when p-values were <0.05.

Results

Positive rates of *Cryptosporidium* spp. in pigeons

Out of the 376 pigeon fecal samples collected, 19 samples (5.05%) were positive for *Cryptosporidium* by acid-fast staining and 7 (1.86%) were positive for *Cryptosporidium* infection by nested PCR. The results obtained using these two methods showed significant differences ($\chi^2=4.821$; $p=0.028$).

The samples from Huangshan showed the highest infection rate by acid-fast staining (13.89%, 5/36). Fecal samples from Maanshan and Bozhou exhibited no evidence of *Cryptosporidium* spp. infection.

The positive rates in other areas compared with the Huangshan rate were 9.68% (3/31, χ^2=0.023, p=0.879), 3.13% (1/32, χ^2=1.285, p=0.257), 6.06% (2/33, χ^2=0.458, p=0.499), 3.57% (1/28, χ^2=0.946, p=0.331), 3.33% (1/30, χ^2=1.114, p=0.291), 6.67% (2/30, χ^2=0.300, p=0.584), and 6.90% (2/29, χ^2=0.252, p=0.616), indicating that the prevalence of *Cryptosporidium* in pigeons in other areas is different from that in Huangshan (p>0.05). Not all samples that tested positive under microscopic examination were the same as nested PCR results. The fecal samples from Huangshan exhibited the highest infection rate (11.11%, 4/36). In contrast, seven geographical areas showed a zero percent infection rate, while the rates in Xuancheng, Lu'an and Wuhu were 3.03% (1/33, χ^2=0.686, p=0.407), 3.33% (1/30, χ^2=1.114, p=0.291), and 3.48% (1/29, χ^2=0.468, p=0.494) (Table 1), also indicating that the prevalence of *Cryptosporidium* in pigeons in other areas is different from that in Huangshan (p>0.05).

Sequence and phylogenetic analyses

A sequence analysis of the SSU rRNA gene indicated the existence of only one *Cryptosporidium* species in pigeons. The sequences (PP204061, PP204065, PP204066) had 100% genetic similarity with sequences previously published in GenBank DQ656356 (Meamar et al. 2007) from humans in Iran. The sequence (PP204060) had a C to A (the 29 base site) single nucleotide substitution (SNP) compared with the reference sequence DQ656356, the sequence (PP204063) had a C to T (the 285 base site) SNP compared with the reference sequence DQ656356, the sequence (PP204064) had the T to G (the 114 base site) and G to C (the130 base site) two nucleotide substitutions compared with the reference sequence DQ656356, the sequence (PP204062) had a 98.57% similarity to DQ656356 with 10 base substitutions (the 37, 38, 70, 143 base site was T to A, the 46 base site was T to C, the 67, 244 base site was G to A, the 71, 79, 246 base site was C to T) and 2 base deletions (C at the 73 base site and G at the 75 base site).

Discussion

Cryptosporidium spp. has caused significant ecological disturbances and environmental challenges, concurrently precipitating substantial global economic repercussions (Wang et al. 2021). In this study, microscopic examination and PCR assay revealed an infection rate of 5.05% and 1.86%, respectively in domestic pigeons in Anhui Province. The positive rate between microscopic examination and nested PCR detection is possibly due to fecal constituents such as bilirubin, bile salts, and complex polysaccharides, which can inhibit PCR amplification (Kabir et al. 2020).

Cryptosporidium infections in intensive farming conditions can be transmitted by sharing the same water and feed sources (Ryan and Power 2012). This prevalence contrasts markedly with the reported frequencies in other regions, the infection rates in pigeons from Guangdong, China (0.82%) (Li et al. 2015) and South Khorasan, Iran (2.94%) (Radfar et al. 2012) were lower, while the infection rate in pigeons from Thailand (25%) was significantly higher. Furthermore, the infection rates of *Cryptosporidium* spp. in pigeons also vary significantly by region in this study. The variability in these rates may be ascribed to differences in sample sizes and distinct geographic environmental factors. Regional discrepancies in positivity rates also hint at the influence of diverse environmental conditions, nutritional practices, hygiene standards, and disease control measures.

The study detected *C. meleagridis* in pigeon feces, consistent with results found in domestic pigeon feces in Thailand (Koompapong et al. 2014). However, *C. baileyi*, *C. parvum, C. andersoni*, *C. muris*, *C. galli*, and *C. ornithophilus* (Holubová et al. 2024) were not detected in this study. Thus far, *C. meleagridis* has been detected in monk parakeets (Briceño et al. 2023), broiler chickens (Lin et al. 2022), foxes (Wang et al. 2022) and also in pigeons (Koompapong et al. 2014). Avian species have been identified as the natural host for *C. meleagridis*. Nevertheless, C. *meleagridis,* as zoonotic pathogen, transmission has been described from avian species to humans (Silverlås et al. 2012, Kabir et al. 2020). Although pigeons in the sampled lofts exhibited no clinical symptoms, the findings of the study suggest the potential risk of a cryptosporidiosis outbreak in the region (Robertson et al. 2020). Additionally, since the clinical symptoms of this disease are similar to other bacterial infections, such as *Salmonella*, pathogenic *Escherichia coli*, and avian *Pasteurella* (Ryan 2010), coupled with the absence of standardized disease monitoring systems on private farms and limited awareness of parasitic diseases, cryptosporidiosis may pose a significant challenge for these pigeon farms. Therefore, more attention should be paid to the detection of *C. meleagridis* to prevent zoonotic conditions in domestic pigeons in Anhui province, China. In addition, maintaining strict control over the cleanliness of water and feed, alongside regular sanitation of the environment (Bouzid et al. 2013), may significantly reduce the fecal-oral transmission of *C. meleagridis* in pigeons. This approach could be a critical strategy in lowering the infection rates in the region.

Fig. 2. Phylogenetic relationships of the SSU rRNA sequences from the *Cryptosporidium* isolates detected in this study and selected *Cryptosporidium* isolates from GenBank inferred by the neighbor-joining method.

Conclusions

In summary, our study is the first report on the prevalence and molecular characterization of *Cryptosporidium* spp. in pigeon farms in Anhui Province, China with higher prevalvence in some sampling sites which may be due to regional variation among adult pigeons. Our study recorded no other *Cryptosporidium* spp. except *C. meleagridis*.

Acknowledgements

This work was supported by the Natural Science Foundation of Anhui Province (2008085QC136), the Natural Science Research Project for Anhui Universities (2024AH050434), the National Natural Science Foundation of China (52005009) and the Natural Science Young Foundation of Anhui Agricultural University (2019zd18).

Z.C.L. and Y.L.Z. conceived and wrote the project, collected and analyzed the data, and wrote the manuscript. Z.C.L. and X.L.S. carried out data collection and verification, revised the manuscript. Y.H.Z. and A.O.O. participated in data collection and verification, and revised the manuscript. Z.Z.L. and S.H.J. performed data curation. H.F.A. and Q.X.Z. participated in data verification, and editing of results. Q.M.X. and C.S.Y. participated in the project management and revised the manuscript. Q.X.Z. and C.S.Y. managed the project and reviewed the manuscript.

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