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CRYPTOSPORIDIUM SPP.: CONVENTIONAL AND MOLECULAR DETECTION IN WILD PIGEONS (*COLUMBA LIVIA*) IN BABYLON PROVINCE, IRAQ

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Abstract

Cryptosporidium is one of the most prevalent protozoan parasites causing a disease in a wide range of birds. Wild pigeons are found in abundance around us and maybe infected or harbor many species of *Cryptosporidium* that can be transmitted to other animals or humans. This study was aimed to discover the prevalence of *Cryptosporidium* spp. in wild pigeons that inhabit in Babylon province, Iraq. A total of one hundred fecal samples were collected from wild pigeons and subjected to two diagnostic methods: staining by modified Ziehl–Neelsen-technique and molecular analyses using nested-PCR for amplification of fragments of the 18S rRNA gene. The total positivity for *Cryptosporidium* detection from both microscopical examination and nested-PCR results was 6% (6/100) and 11% (11/100) respectively. Sequencing of all positive amplified nested PCR results allowed the identification of *Cryptosporidium baileyi* 9/11 (81.81%) and *C. parvum* 2/11 (18.18%). Nested PCR was more sensitive for the detection of *Cryptosporidium* spp. than the staining method. In conclusion, the high prevalence rate of *Cryptosporidium baileyi* and less *C. parvum* in wild pigeons indicating the ability of these birds to harbor the infection and transmit these parasites to other birds or humans. These results provide baseline data to help for more studies of molecular epidemiology and control of *Cryptosporidium* infection in poultry in Iraq.

***Keywords*:** wild pigeons; *Cryptosporidium*; diagnosis; Nested PCR; Babylon

Introduction

Cryptosporidium the enteric parasite is one of the most globally distributed agents causing gastrointestinal sicknesses in humans and many other vertebrates (Pumipuntu and Piratae, 2018). Food and water contaminated by *Cryptosporidium* oocysts are the most important resources for human and animal infections (Xiao, 2010). Moreover, the parasite can be transmitted to humans by direct contact with infected animals (Ryan, 2010; Slapeta, 2013). *Cryptosporidium* infection in birds may exhibit symptoms of digestive signs, respiratory disease, and renal disorders (Nakamura and Meireles, 2015). To date five avian *Cryptosporidium* spp. have been recognized in birds which are *C. meleagridis* causing enteritis (Slavin, 1955), *C. baileyi* mostly associated with respiratory tract infection (Current *et al*., 1986; Van Zeeland *et al*., 2008; Molina-López *et al*., 2010), *C. galli* developed in epithelial cells of the proventriculus (Ryan *et al*., 2003), *C. avium* detected in the large intestine (Holubová *et al*., 2016) and *C. proventriculi* infects the proventriculus and ventriculus (Holubová *et al*., 2019). Besides, 20 genotypes of *Cryptosporidium* have been described in 30 different avian species around the world (Ryan, 2010; Holubová *et al*., 2019). Mammal-specific *Cryptosporidium* spp., including *C. hominis C. parvum*, *C. andersoni*, *C. muris* and *C. canis* have been informed infrequently in birds (Ng *et al*., 2006; Abreu-Acosta *et al*., 2009; Qi *et al*., 2014; Helmy *et al*., 2017; Oliveira *et al*., 2017; Ferrari *et al*., 2018). Pigeons live side by side with humans and other animals in nature. They are seen in more regions of the world except for the poles. Pigeons act as a potential carrier that spreads the infection to other domestic and wild birds and play a role in zoonosis (Piasecki, 2006; Gibbs *et al*., 2010). Infections of pigeons with *Cryptosporidium* have been informed in several regions of the world, such as in Turkey (Özkul and Aydin, 1994), Spain (Abreu-Acosta *et al*., 2009; Cano-Terriza *et al*., 2015), Iran (Radfar *et al*. 2012; Mirzaghavami *et al*. 2016), China (Qi *et al*., 2011; Li *et al*., 2015). Until recently, in Iraq, only one study recognized *Cryptosporidium* spp. in pigeons using PCR in Al-Qadisiya province (Jasim and Marhoon, 2015), while other studies have been reported the occurrence of cryptosporidiosis in pigeons and other birds with a preliminary microscopy-based diagnosis only (Al-Mahmood, 2011; Al-Khayat and Al- Zubaidi 2015; Al- Zubaidi *et al*., 2018). Therefore, this study was performed to investigate the prevalence of possible *Cryptosporidium* spp. in fecal samples of wild pigeons in Babylon province, Iraq using molecular tools, and to obtain information about the effects of some factors such as gender on parasitic infections.

Materials and Methods

Area of study

The study was carried out in Babylon province, middle Euphrates region, located 100 Km south of the capital Baghdad, Iraq. The samples collected from different areas after permission from the environmental organization from January 2019 to October 2019. A total of one hundred samples (61 males and 39 females) were taken. Stool samples were collected after trapping the wild pigeons, about ten pigeons per month. Each fecal sample transferred into a clean, labeled plastic container and kept at 4ºC until being processed.

Sample procedure

Each fecal sample was divided into 2 parts: 1st part has been screened using modified Ziehl–Neelsen-staining technique, and examined microscopically under 400 × magnifications for the detection of *Cryptosporidium* oocysts (Fig.1). The 2nd part was subjected to DNA analysis and PCR tests.

DNA extraction

The genomic DNA was extracted from the fecal samples with the commercial kit (Presto™ stool DNA extraction kit, Geneaid, Taiwan) using a bead-beating

disruption process followed by isolation/purification as stated by the manufacturer's instructions. The eluted DNA sample was preserved at –20 °C before it was used in the molecular investigation.

Cryptosporidium PCR screening

A nested PCR line was used to amplify a partial region of the small ribosomal subunit rRNA gene using primers F1 (5′- AAACCCCTTTACAAGTATCAATTGGA-3′) and R1 (5′- TTCCTATGTCTGGACCTGGTGAGTT-3′) for primary amplification and F2 (5′- TGCTTAAAGCAGG CATATGCCTTGAA-3′) and R2 (5′-AACCTCCAATCT CTAGTTGGCATAGT-3′) for secondary amplification (Bialek *et al*., 2002). The reactions contained a volume of 20 µL of PCR premix with 1 U of Top DNA polymerase, 250 µM of dNTPs, 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, 1.5 mM of MgCl2, 5 µL of target DNA in the PCR and 2.5 µL of DNA in the nested PCR. Samples were subjected to initial DNA denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 52°C and extension at 72°C, each for 1 minute. Amplified products of PCR were detected by agarose gel electrophoresis, visualized by ethidium bromide stain.

Sequencing analyses

The positive PCR products of wild pigeons were sequenced in both directions using the secondary PCR primers (Macrogen Inc. Geumchen, Seoul, South Korea). *Cryptosporidium* species were determined by alignment with known reference sequences available in Gen-Bank using BLAST (https://www.ncbi.nlm.nih.gov). Representative nucleotide sequences were deposited in Gen- Bank under accession numbers MT308760- MT308768 and MT308769-MT308770 to represent *C. baileyi* and *C. parvum* respectively.

Phylogenetic analyses

A specific comprehensive *Cryptosporidium* tree was constructed in this study according to the protocol described by Sarhan *et al.* (2019) with several modifications. The observed parasite variants were compared with their neighbor homologous reference sequences using the NCBI-BLASTn server (Zhang *et al*., 2000). Then, the blast results of the observed variants were combined and aligned together using a Clustal Omega based tools. A neighbor-joining tree was generated using the Simple Phylogenetic tree EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute) server (ebi.ac.uk/Tools/phylogeny/simple_phylogeny/). Subsequently, the full inclusive tree, including the observed variant, was visualized as a polar cladogram using iTOL (Interactive Tree of Life) tool (Letunic and Bork, 2019). The parasite sequences of each classified phylogenetic group/clade in the comprehensive tree were colored appropriately.

Statistical analysis

Data were analyzed using the SPSS version 23 and Chi-square test, differences were considered statistically significant at $P < 0.05$.

Results and Discussion

In six of the 100 pigeons screened 6%, *Cryptosporidium* spp. oocysts were detected using the staining method (Fig.1). Nested PCR recorded a higher positivity rate for *Cryptosporidium* spp. was 11% (11/100) (Table 1). There was an insignificant difference ($P > 0.05$) between nested PCR and microscopic analysis for detecting *Cryptosporidium* spp. Moreover, females had a higher *Cryptosporidium* prevalence (15.38%, 6/39) than males (8.19%, 5/61) but statically, with an insignificant ratio at ($P > 0.05$).

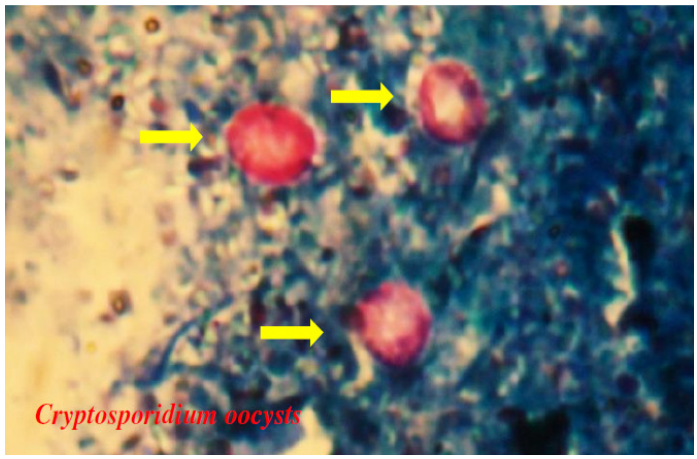

Fig.1: *Cryptosporidium* oocysts, stained by the modified Ziehl–Neelsen staining method from feces of wild pigeons

The high sensitivity of PCR in detecting *Cryptosporidium* infection has been reported by Qi *et al.* (2011) and Oliveira *et al.* (2017). The prevalence of *Cryptosporidium* spp. in pigeons varied in different countries, with the infection rate ranging from 0.8% to 26.7% in recent studies (Abreu-Acosta *et al*., 2009; Radfar *et al*., 2012; Qi *et al*., 2011; Koompapong *et al*., 2014; Jasim and Marhoon, 2015; Li *et al*., 2015; Mirzaghavami *et al*., 2016; Oliveira *et al*., 2017). In this study, the overall infection rate in pigeons was 11% (11/100), which was similar to those seen in the studies mentioned above.

Table 1 : *Cryptosporidium* spp. Prevalence (%) by microscopy and Nested PCR in wild pigeons.

Host	Fecal samples	Conventional microscopy		Molecular-Nested PCR	
Wild pigeons	Total No.	No.	%	No.	%
	100	6	6 %	11	11 %

Two *Cryptosporidium* species were identified by DNA sequencing and phylogenetic analysis in this study, the *C. baileyi* and *C. parvum*. The sequence analysis confirmed the identification of *C. baileyi and C. parvum* since 100% homology was observed with their respective species sequences reported on Gen-Bank accession numbers (MN461549.1) in China and (MT010356.1) in Uruguay respectively (Fig.2). Among the two *Cryptosporidium* species identified, the predominant *Cryptosporidium* species was *C. baileyi* has been recognized in the total positive surveyed pigeons 9/11 (81.81%). *C. baileyi* was considered the most prevalent avian *Cryptosporidium* species having been diagnosed in at least 21 avian hosts. Pigeons stated as one of the most important avian hosts that can be infected

with this parasite (Li *et al*., 2015 and Nakamura *et al*., 2015). Accordingly, the common occurrence of *C. baileyi* infection in wild pigeons in this study is in agreement with this hypothesis. Respiratory cryptosporidiosis caused by *C. baileyi* can be the main reason for morbidity and mortality in poultry, pet birds, and wild birds (Ryan, 2010). The signs in poultry are subclinical combining with poor weight gain and reduction of egg as well as meat production yield (Goodwin *et al*., 1996; Sreter and Varga 2000). The occurrence of *C. baileyi* infection in Iraq has been detected in domestic and broiler chickens, quail and feral pigeon in Al-Qadisiya province (Jasim and Marhoon, 2015). In addition to the prevalence of *C. baileyi*, our study has confirmed the presence of two samples of *C. parvum* 2/11 (18.18%). In the previous report, *C. parvum* was identified in carrier pigeon using nested PCR with 7% (7/100) of positivity (Oliveira *et al*., 2017). This study confirms our result that pigeons can be infected with this parasite. *Cryptosporidium parvum* inhabits in a large host range (more than 100 species) including livestock, wild birds, human, and other mammalian species, is responsible for most cases of cryptosporidiosis in humans and pre-weaned calves (Xiao and Ryan 2008; Fayer 2010). Depending on the physical condition, pigeons may come into contact with other animals of any nutritional levels, take infection and when the return may disseminate *Cryptosporidium* in pigeonry. Hence, pigeons could passively transmit oocysts without becoming infected. Furthermore, wild pigeons have some strange behaviors that distinguish them from domestic pigeons that are often seen in streets and squares. *Cryptosporidium parvum* transmission can occur by interaction with other birds, chickens, or from the contaminated environments due to human wastes located in the vicinity of pigeons.

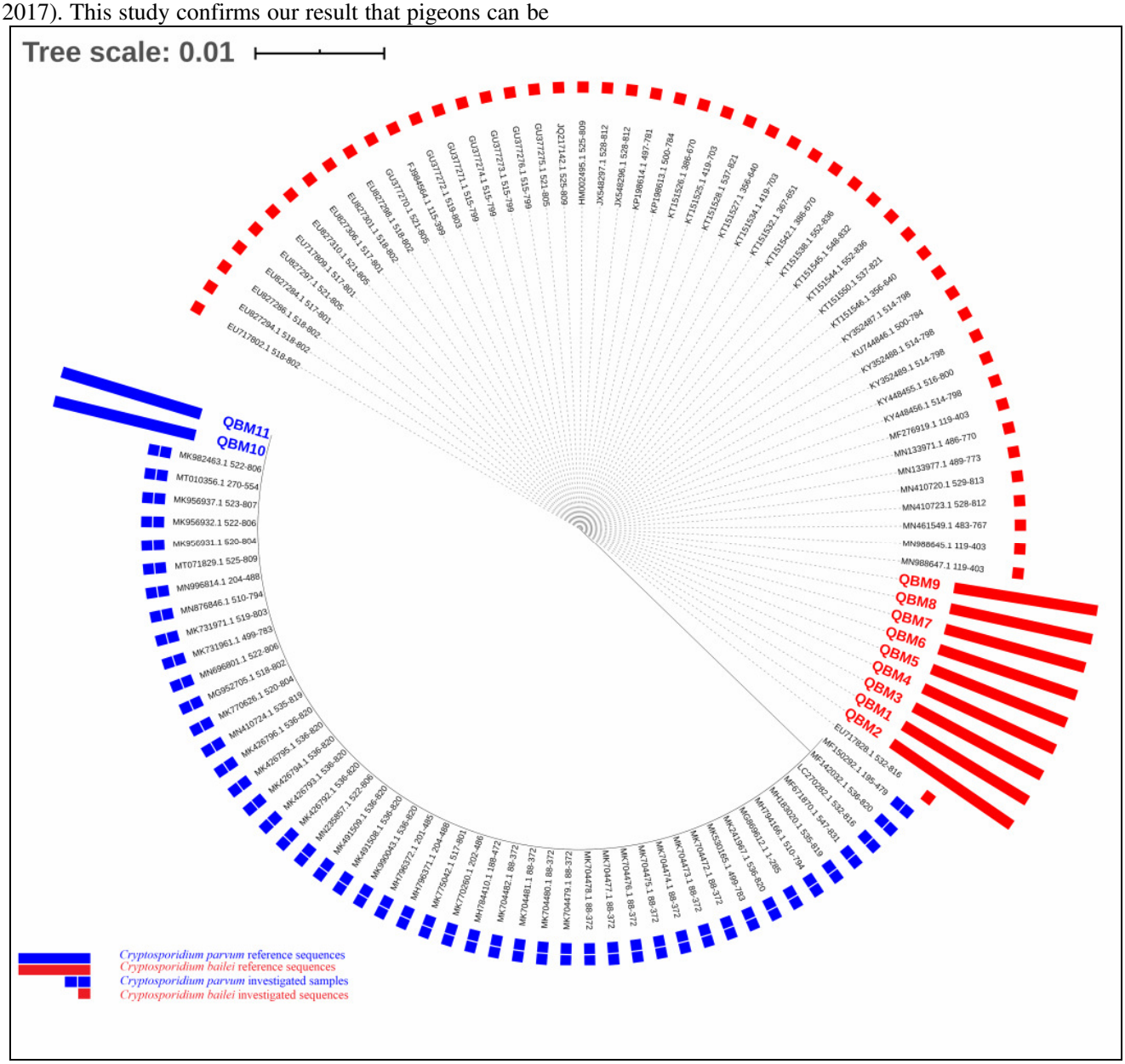

Fig. 2: Comprehensive neighbor-joining Phylogenetic tree of *Cryptosporidium* spp. detected in this study. The analyzed 18S ribosomal RNA sequences and their phylogenetic neighboring accession numbers were obtained from the NCBI Genbank database (https://www.ncbi.nlm.nih.gov). Ribosomal sequences were aligned using multiple sequence alignment and phylogenetic tree tools of the online Clustal omega server. Aligned sequences were used for the phylogenetic analysis conducted with the iTOL server (https://itol.embl.de/). The number 0.01 at the top left of the tree refers to the phylogenetic distance measure by a bootstrap scale. Blue and red colors refer to *Cryptosporidium parvum*, and *Cryptosporidium baileyi*, respectively.

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