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Microscopic and molecular detection of Haemoproteus spp in wild and domestic pigeons, Iraq

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ABSTRACT
Background: Haemoproteus spp. is the most common blood parasite in birds, which identified in several geographical areas, worldwide causing variable health impacts.
Aim:This study was conducted to investigate Haemoproteus spp. in wild and domestic pigeons and the effect of sex and months on the infection rate by traditional methods. Also, it aims to identify the genetic diversity of Haemoproteus spp. targeting the Cytochrome b gene.
Materials and methods:A total of 180 pigeons; 90 wild and 90 domestic, were selected and subjected to collection of blood that examined microscopically; and then, the positive sampleswere testedfurthermore molecularly by the nested PCR. Phylogenetic analysis was conductedin some positive PCR products.
Results:Microscopic examination of 180 stained blood smears revealed that 18.89% of study pigeons were infected with Haemoproteusincluding 15% wild and 3.89% domestic pigeons. Within mature erythrocytes, pigmented gametocytes of Haemoproteus were appeared markedly as black or violet small granules, free in cytoplasm and extend along the nuclei of erythrocytes. Relation to months, the total results were increased significantly in October and December but decreased in February. Concerning to sex of study animals, the total results showed that females having a higher rate of infection than males. However, no significant variation was seen between the results of male and female pigeons in both wild and domestic birds.
Conclusion: The prevalence of Haemoproteus spp. in wild pigeon was higher than domestic pigeon, with presence a significant association between infection, month and sex of study animals. The results of nested PCR assay were greatly supported the obtained findings of microscopy, suggesting that molecular assays can be applied directly to identify the prevalence of Haemoproteus genus and its species among different birds. Moreover studies in other Iraqi localities and birds are necessary to estimate the prevalence rate of parasitic infections.

Keywords: Blood parasites, Columba livia, Polymerase chain reaction (PCR), Phylogenetic analysis, Season, Sex

INTRODUCTION

Haemoproteus spp. is the most common blood parasite in birds, especially nondomestic birds, which belongs to Haemoproteidae Family in the Aconoidasida Order under the Apicomplexa Phylum (Golemansky, 2015; Nourani et al., 2018). The lifecycle of this genus is heteroxenous, with the asexual phase occurring in tissue cells and erythrocytes of the vertebrate hosts and the sexual phase occurring in hematophagous Diptera (Matta et al., 2022; Himmel et al., 2024). Worldwide, there are more than 170 species have been reported in the Haemoproteus genus which identified mostly in free-living ducks, quail and turkeys but less frequently in other commercial flocks probably because of limited vector exposure or very specific feeding habits of invertebrate vectors (Oliveira et al., 2020; Harl et al., 2024).
Birds are usually asymptomatic; however,Haemoproteus infection may be more often significant than previously thought based on increasing reports documenting decreased host fitness, nestling mortalities, fledging success, and delayed recovery in infected birds versus uninfected birds (Fletcher et al., 2019; Himmel et al., 2021). Diagnosis is made by examination of stained blood smears and observation of large, pigmented gametocytes in mature erythrocytes that partially or occasionally completely encircle the nucleus without displacing it (Mantilla et al., 2016). Merozoites are not seen in the peripheral blood; while, schizogony can be seen histologically within the endothelial cells of the lung, liver, and spleen (Bahramiet al., 2020; Duc et al., 2021). Over the past four decades, the development and application of molecular diagnostic techniques has initiated a revolution in the diagnosis and monitoring of infectious diseases since these techniques were allowed to identification and differentiation the phenotypic characteristics as well as the biotyping and susceptibility

testing of different pathogens (Altindiş andKahramanKilbaş, 2023). Recently, various nucleic acid methods such as polymerase chain reaction (PCR) have been useful for rapid detection of the etiologic agents of different diseases directly from the clinical samples without the need to culture,and for unculturable or fastidious microorganisms (Gharban and Yousif, 2020; Salahet al., 2020; ALani and Yousif, 2023; Hassan, 2023).The sequencing applications,developed during the last few decades, has demonstrated as a valuable tool that opened a new era in the diagnosis and monitoring of infectious diseases (Al-Abedi and Al-Amery, 2021;Al-Khalaf et al., 2022; Abdhalla and Al-Gburi, 2023;Hussein and Hamad, 2024).

In Iraq, few available studies have been conducted to detect the presence of Haemoproteusspp. in pigeons (Ali et al., 2017; Wahhab et al., 2017; Abdullah et al., 2018). Therefore, this study was conducted to investigate Haemoproteus spp. in wild and domestic pigeons and the effect of sex and months on the infection rate by traditional methods. Also, it aims to identify the genetic diversity of Haemoproteus spp. targeting the Cytochrome b gene.

MATERIALS AND METHODS

Ethical approval

The current study was licensed by the Scientific Committee of the Department of Parasitology in the College of Veterinary Medicine (University of Baghdad, Iraq)

Samples

A total of 180 pigeons; 90 wild and 90 domestic, were selected randomly from different areas in Baghdad province (Iraq) during the 1st day of October (2023) until the 30th day in April (2024). About1-2mlofwingveinbloodwascollectedfromeachbirdusing the disposable syringe intoEDTA-anticoagulant plastic tubes and transported cooled to be frozen for molecular assay.Also, a drop of each fresh blood sample was used to prepare the slides for microscopy. Additional data related to sex of study animals and months of samples collection were recorded.

Microscopy

After fixing the slides of blood smears with absolute methanol for 3-5 minutes, all slides were stained by diluted Giemsa, and then examined by the light microscope (Olympus, Japan)underthe oil immersion lens at 100× (Al-Abedi and Al-Al-Amery, 2020).

Molecular assay and phylogeny

After thawing of frozen blood using the water bath (Kottermann, Germany) at 37°C, all positive blood samples by microscopy were mixed well using the vortex. For DNA extraction, a total 200µl of whole blood of each sample were used in following the Blood Protocol procedure of G-Spin™Total DNA Extraction Kit (iNtRON Biotechnology, Korea). For DNA amplification, two sets of primers weredesigned as described by other researchers (Harl et al., 2020; Taroda et al., 2020), and provided by the Scientific Researcher Company (Iraq), (Table 1).

Table 1: Primers of conventional PCR used for detection of Haemoproteus spp. parasite

Nested PCR	Primer Sequence (5'-3')		Size product
1st round	F	CATATATTAAGAGAANTATGGAG	617 bp
	R	ATAGAAAGATAAGAAATACCATTC	
2nd round	F	ATGGTGCTTTCGATATATGCATG	525 bp
	R	GCATTATCTGGATGTGATAATGGT	

The PCR MasterMixtubes were prepared using the ready to use GoTaq® Green PCR MasterMixKit (Promega, USA) at a final volume of 25 µl. The nested PCR steps of ThermoCycler (Bio-Rad, USA) conditions were includedtwo rounds as described below (Table 2).

Table 2: Conditions of Thermocycler system for conventional nested PCR

PCR Step	1st round			2nd round		
	Temperature	Time	Cycle	Temperature	Time	Cycle
Initial Denaturation	95ºC	5 min	1	95ºC	5 min	1
Denaturation	95ºC	30 sec	35	95ºC	30 sec	35
Annealing	55ºC	30 sec		57ºC	30 sec	
Extension	72ºC	2 min		72ºC	2 min	
Final extension	72ºC	5 min	1	72ºC	5 min	1

Hold	4ºC	Forever	-	4ºC	Forever	-

The PCR products were subjected for electrophoresis in 1.5% Agarose gel stained with ethidium bromide at 100 Volt and 80mA for 1 hour, and visualized using UV transilluminator (ATTA, Korea).

Phylogeny

PCR products of five positive samples were sent for molecular sequencing in the Macrogen Company (South Korea). Sequencing data received by email were subjected for phylogentic analysis using the MEGA-6 Software, and then local strains were confirmed in GenBank-NCBI.

Statistical analysis

Data of this study were documented in the Microsoft Office Excel Software (version 2007) and analyzed using the GraphPad Prism Software (version 6.0.1) to estimate significances at P<0.05 (Al-Taee et al., 2023).

RESULTS

Microscopic examination of 180 stained blood smears revealed that 34 (18.89%) pigeons were infected with Haemoproteus spp. involving 27 (15%) wild pigeons and 7 (3.89%) domestic pigeons (Figures1).

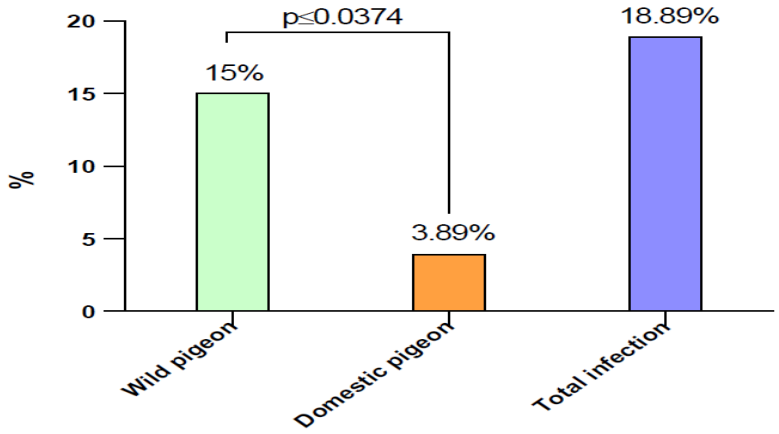

Figure 1: Total positive results of microscopic examination among 180 pigeons

Within the mature erythrocytes, pigmented gametocytes of Haemoproteus spp. were appeared markedly as black or violet small granules that free in cytoplasm and extend along the nuclei of erythrocytes (Figure 2).

Figure (2): Gametocytes of Haemoproteus spp. within the mature erythrocytes of infected pigeons

Relation to study months, the total positive results were increased significantly (p≤0.034) in October (33.33%) and December (26.67%) and decreased significantly in February (6.67%) when compared to other months; January (13.33%), March (13.33%) and April (20%), (Table 3). In addition, the results of wild pigeonwere shown a significant increase (p≤0.0334) in Haemoproteus infection in October (23.33%), December (20%) and April (16.67%) when compared to other study months; January (13.33%), February (6.67%), March (10%) and April (16.67%). Among domestic pigeon, parasitic infection was increased significantly (p≤0.0381) in October (10%) and December (6.67%) but decreased significantly in January (0%), February (0%), March (3.33%) and April (3.33%).

Table 3: Distribution of Haemoproteus spp. infection among study months

Month	**Total No.**	**Pigeon**		**Total infection**
		Wild	**Domestic**	**No. (%)**
October	30	7 (23.33%) *	3 (10%) *	10 (33.33%) *
December	30	6 (20%) *	2 (6.67%) *	8 (26.67%) *
January	30	4 (13.33%)	0 (0%)	4 (13.33%)
February	30	2 (6.67%)	0 (0%)	2 (6.67%)
March	30	3 (10%)	1 (3.33%)	4 (13.33%)
April	30	5 (16.67%)	1 (3.33%)	6 (20%)
p-value		0.0334	0.0381	0.034
Significance * (P<0.05), No significance (p>0.05)				

In comparison between the positive results of wild and domestic pigeons at each study month, no significant differences (p>0.05) were found at all study months; October (7.78% and 3.33%, respectively), December (667% and 2.22%, respectively), January (4.445 and 0%, respectively), February (2.22% and 0%, respectively), March (3.33% and 1.11%, respectively), and April (5.56% and 1.11%, respectively), (Table 4).

Table 4: Results of positive Haemoproteus infection among the wild and domestic pigeons throughout the study period

Pigeon	Total No.	October	December	January	February	March	April
Wild	90	7 (7.78%)	6 (6.67%)	4 (4.44%)	2 (2.22%)	3 (3.33%)	5 (5.56%)
Domestic	90	3 (3.33%)	2 (2.22%)	0 (0%)	0 (0%)	1 (1.11%)	1 (1.11%)
p-value		0.0756	0.0865	0.0551	0.0619	0.0838	0.061
Significance * (P<0.05), No significance (p>0.05)							

Concerning the sex of study animals, the results of total infection detected that females were having a significant (p≤0.0382) higher rate of infection (19.87%) than males (13.79%). However, no significant variation (p>0.05) was seen between the results of male and female pigeon in both wild (13.79% and 15.23%) and domestic (0% and 4.64%) birds (Table 5).

Table 5: Association of positive Haemoproteus spp. infection to sex of study animals

Sex	Total No.	Pigeon		Total infection
		Wild	**Domestic**	**No. (%)**
Male	29	4 (13.79%)	0 (0%)	4 (13.79%)
Female	151	23 (15.23%)	7 (4.64%)	30 (19.87%) *
p-value		0.0671	0.0573	0.0382
Significance * (P<0.05), No significance (p>0.05)				

In comparison between both sexes, although no significant differences (p≤0.0549) were detected between wild and domestic males (4.44% and 0%, respectively), rate of infection was significantly (p≤0.0329) higher in wild females (25.56%) than domestic females (7.78%), (Table 6).

Table 6: Results of positive Haemoproteus infection among the wild and domestic pigeons in relation to sex of study animals

Pigeon	Total No.	Male	Female	p-value
Wild	90	4 (4.44%)	23 (25.56%)	0.0194
Domestic	90	0 (0%)	7 (7.78%)	0.0418
p-value		0.0549	0.0329	-
Significance * (P<0.05), No significance (p>0.05)				

Targeting the Cytochrome b gene, further molecular testing of 51 positive samples by microscopyrevealed that the total infection rate of Haemosporidium spp. was 56.86% (No: 29) including 45.1% (No: 23) wild and 11.76% (No: 6) domestic pigeons (Figures 3, 4).

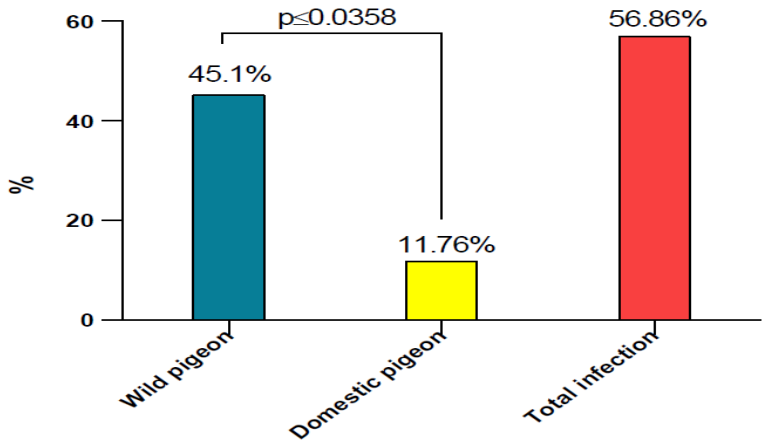

Figure 3: Total positive results of PCR assayamong 51 positive samples by microscopy

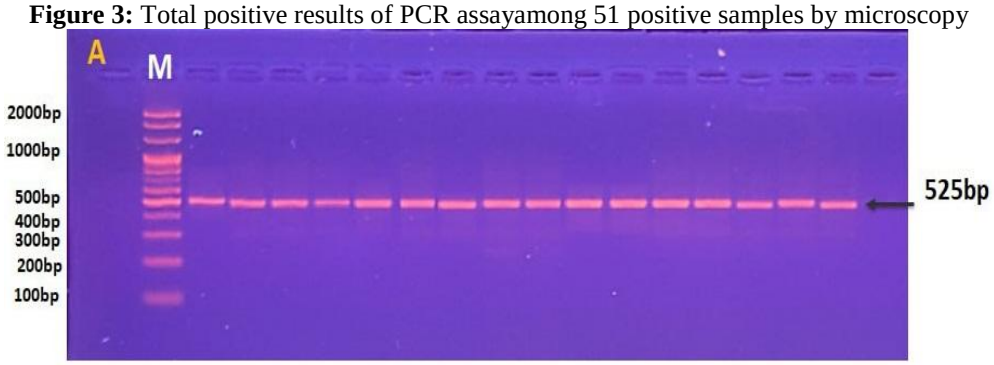

Figure 4: Agarose-gel electrophoresis (2nd round) of positive PCR products at 100 Volt and 80 mA for 1 hour.

The DNA sequencing method was carried out based onmitochondrial Cytochrome b gene in local wild and domestic Haemoproteus isolates and the NCBI-Genbank related isolates.The phylogenetic tree, constructed using the evolutionary distances,found that the local Haemoproteus isolates of wild (IQ-Wild No.1, IQ-Wild No. 2,and IQ-Wild No. 3) as well as the domestic (IQ-Domestic No.1, IQ-Domestic No. 2, IQ-Domestic No. 4, and IQ-Domestic No. 5) pigeons were closely related to NCBI-BLAST Haemoproteus sp. (MT513730.1) at a genetic homology sequence identity ranged from 99.64-99.84% and a genetic distance ranged 0.15-0.35% (Figure 5, Table 7).

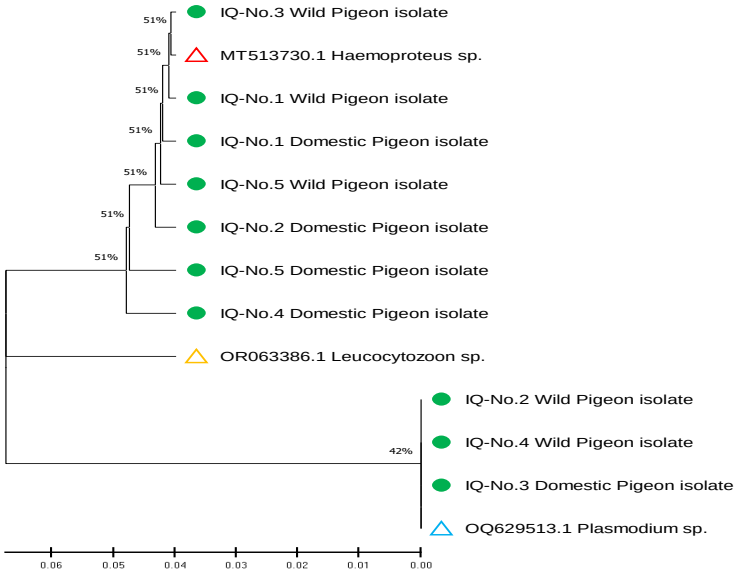

Figure 5: Phylogenetic tree analysis of the local and global NCBI-BLAST Haemoproteus spp. isolates

Table 7: Homology Sequence identity of the local and global NCBI-BLAST Haemoproteusspp isolates

Study local isolate		**NCBI-BLAST Isolate**		
Name	**Accession No.**	**Isolate**	**Accession No.**	**Identity (%)**
IQ-Wild No. 1	PP764028.1	Haemoproteus sp.	MT513730.1	99.85%
IQ-Wild No. 2	PP764030.1	Haemoproteus sp.	MT513730.1	99.64%
IQ-Wild No. 3	PP764032.1	Haemoproteus sp.	MT513730.1	99.84%
IQ-Domestic No. 1	PP764033.1	Haemoproteus sp.	MT513730.1	99.65%
IQ-Domestic No. 2	PP764034.1	Haemoproteus sp.	MT513730.1	99.84%
IQ-Domestic No. 4	PP764036.1	Haemoproteus sp.	MT513730.1	99.64%
IQ-Domestic No. 5	PP764037.1	Haemoproteus sp.	MT513730.1	99.65%

DISCUSSION

Wild pigeon (Coumbalivia) is a free living form of domestic pigeon which accompanies human settlements, and adapted well to the urban environments. Several species of parasites occur in pigeons throughout their global range, in among these are the Haemosporidae (Borges et al., 2017; Farfán et al., 2019). In the present study, the total prevalence rate of Haemoproteus infection in study pigeon was 18.89%, with significant higher rate of

infection in wild (15%) than domestic (3.89%) pigeons. In Iraq, few available studies were conducted to detect the prevalence of Haemoproteus and their related species in various birds including demonstration of H.porzanae(11.8-15.8%) and H. baghdadensis(3.6-9.1%) in avian Rallidae family in the middle, south and west of Iraq (Mohammad, 2001), Haemoproteus spp. (60%) in rock pigeon of several localities in Iraq (Al-Barwari and Saeed, 2012), blood parasites of some passeriform birds in Baghdad province (Mohammad and Al-Moussawi, 2012), H. columbae(31.72%) in feral pigeons in Sulaimani province (Ali et al., 2017), Haemoproteus spp. in wild pigeon (63.3%) in Al-Qadisiyah province (Marhoon and Alwan, 2017), H. columbaein domestic pigeons (21.42%) in Erbil province (Basha, 2017), H. columbaein domestic pigeons (10.48%) in Garmian area of Kurdistan region (Wahhab et al., 2017), and Haemoproteus spp.(28.20%) in domestic pigeons in Sulaimani province (Abdullah et al., 2018).In other countries, the prevalence of Haemoproteus infection in pigeons was 55.63% in India (Jahan and Shoeb, 2010), 50% in Iran (Borji et al., 2011), 29.41% in Italy (Scaglione et al., 2015), 55.6% in Turkey (Balkaya et al., 2016), 22.3% in Bangladesh (Zahan et al., 2018), and 62.5-100% in Indonesia (Rosyadi et al., 2021). Variation in prevalence rate of Haemoproteus infection between different studies might be attributed to reliability of diagnostic method(s), abundance of the intermediate host, management system, geographical location, and sample size. Our finding demonstrated that the wild pigeons harbor to a higher rate of infection than domestic pigeons which could because the poor management and control efforts in either the animal or immediate environment where infection or re-infection directly or indirectly.

Our findings reported that most Haemoproteus infections were observed during October and December, and this may be due to the high presence of vectors that available at the warm months in tropical and subtropical areas as Iraq. Our findings were in agreement with that reported by other studies (Adriano and Cordeiro, 2001; Senlik et al., 2005; Gupta et al., 2011) which mentioned to the role of weather in increasing and decreasing of vectors, and interestingly in increasing the prevalence of Haemoproteus infection. Jahan andShoeb(2010) recorded that the overall highest infectivity of parasites was recorded during the summer season (82.85%) followed by spring season (59.37%) and least in the winter season (42.30%). (Zahan et al., 2018) found that the prevalence rate of Haemoproteus infection was significantly higher in the rainy season (56.1%) followed by summer (33.8%) and winter (10%) indicating that the infection is increased with presence of suitable conditions for mosquitoes reproduction. We suggested that the seasonal peaks of haemosporidian occurrence might be due to either the physiological changes during reproduction or the role of migratory birds in transmission of infection to domestic birds.

The total results of this study showed that females having a higher rate of Haemoproteus infection than males, which in agreement with the findings of Gicik and Arslan (2001) in Turkey, Guptaet al. (2011) in India,Ali et al.(2017)and Nadeem et al. (2023) in Iraq who found that the prevalence rate of H. columbae infection was higher in female (62.5%, 62.79%, 34.43% and 65.9%, respectively) than male (52.6%, 57.65%, 29.76% and 51.18%, respectively) pigeons. Ali et al.(2017) suggesting the role of female sex hormones, behavioral aspects and intrinsic physiological conditions that could make the host more or less susceptibility to the parasite. In contrast, Al-Barwari and Saeed (2012) recorded that the male pigeons were more prone to Haemoproteus infection (73.2%) than females (49.1%) suggesting that several endogenous and exogenous factors may have an accumulative influence on the parasitisation of both sexes of the pigeons by these parasites such as the host's hormones and humoral compounds, age and nutritional state, behavioral and habits as well as the season of the year and ecological and physical features of the regions.However, other studies showed that no significant differences in prevalence of Haemoproteus infection between male and female pigeons as detected 24.32% and 18.18% respectively by Basha (2017) as well as10.17% and 10.87% respectively byWahhab et al.(2017) who suggested that the absence of significant differences between the males and females of both doves and domestic pigeons might be attributed to that both sexes having the same body sensitivity to the blood vectors and parasitic infections.This agrees with the fact that in columbid birds of both sexes live in the habitat and participate in the breeding duties in a similar fashion.

Worldwide, the quantity of sequence data on avian haemosporidians of pigeons has been published remain low so far.This study showed that the study local Haemoproteus isolates of both wild and domestic pigeons were identical significantly to the NCBI-BLAST Haemoproteus sp. (MT513730.1) at a genetic homology ranged from 99.64-99.84% and a genetic distance ranged 0.15-0.35%.A number of authors confirmed that the Cytochrome blineages, morphologically belong to Haemoproteusgenus, are found and common in pigeons (Chagas et al., 2016; Nebel et al., 2020). In addition, several previous and recent studies hypothesized that >5% genetic distance in the genetic differentiation Cytochrome bgene is indicative with different morphospecies of avian haemosporidians (Hellgrenet al., 2007; de Freitas et al., 2023; Prompiram et al., 2023). These results indicate that geographical and environmental factors have little influence on the genetics of Haemoproteus in the specific ecological context in fields.

CONCLUSION

We showed that the prevalence of Haemoproteus spp. in wild pigeon was higher than domestic pigeon. Also, there was a significant association between the prevalence of infection, month and sex of study animals. The results of nested PCR assay were greatly supported the obtained findings of microscopy, suggesting that molecular assays can be applied directly to identify the prevalence of Haemoproteus genus and its species among different birds. Knowledge of other parasitic diseases should be expanded to explore pathogenic infections in new ecological and geographical distributions on endemic host species, which could provide essential information for disease prevention or species conservation.

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Authors' contribution
SMS: Collection of blood samples and data of study animals, preparation of slides and phylogenetic analysis of sequencing data. MMS: Microscopic examination of blood smears, molecular examination using the PCR assay, and statistical analysis of study results.Both authors contributed equally in writing and approving the final copy of the manuscript.

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Conflicts of interest
No

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