Special Issue:
Advancements in Animal Health and Production in Low and Middle-Income Countries

Detection of Avian Malaria (*Plasmodium* spp.) in Domestic Local Breed Chickens (*Gallus gallus domesticus*) Using Real Time PCR

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Abstract | *Plasmodium* spp. are parasites often referred to as avian malaria parasites a type of infectious haemoprotozoan disease. The majority of the described species reported in birds live in tropical and subtropical countries, having been adapted for transmission globally to become cosmopolitan. This study was conducted to detect the infection of *Plasmodium* spp. in domestic local breed chickens (*Gallus gallus domesticus*) by examining 45 birds purchased from different regional markets in Baghdad city using Real-Time PCR. The total infection rate was 15.55% (7/45), which was divided into males 15% (3/20) and females 16% (4/25), with a significant difference (P ≤ 0.01). Al-Karkh district market showed a highly significant (P≤0.01) infection 18.18% (4/22), compared to Al-Rusafa district market, 13.03% (3/23). In conclusion, *the Plasmodium* parasite has a high prevalence in both sexes of domestic local breed chickens (*Gallus gallus domesticus*). These finding highlight the important of continued surveillance and assessment of appropriate disease control and management policies.

Keywords | *Plasmodium*, Malaria, Domestic chickens, Real-time PCR, Avian

Received | July 12, 2025; **Accepted** | August 18, 2025; **Published** | September 03, 2025
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Citation | Ibrahim RM (2025). Detection of avian malaria (*Plasmodium* spp.) in domestic local breed chickens (*Gallus gallus domesticus*) using real time PCR. J. Anim. Health Prod. 13(s1): 293-299.
DOI | https://dx.doi.org/10.17582/journal.jahp/2025/13.s1.293.299
ISSN (Online) | 2308-2801

INTRODUCTION

Haemosporidian protozoan parasites that infect mammals, birds, reptiles, and amphibians (Valkiūnas, 2005). The greater majority of avian *Plasmodium* spp. description was reported in birds that live in tropical and subtropical countries and have been adapted for transmission to become cosmopolitan (Valkiūnas and Iezhova, 2018). *Plasmodium* species found in poultry such as *P. durae*, *P. gallinaceum*, *P. relictum*, and P. *juxtanucleare* (Santiago-Alarcon *et al*., 2020; Zajac *et al*., 2021). They are causing many diseases, such as avian malaria (Beadell *et al*., 2004; Hellgren *et al*., 2004), which may lead to severe mortalities in domestic, wild, and zoo birds (Ferrell *et al*., 2007). Avian *Plasmodium* spp. has a much broader host range and frequently occurs in several avian families (Krizanauskiene *et al*., 2006). Of the 13 blood-feeding families, four (Culicidae, Simuliidae, Ceratopogonidae, and Hippoboscidae) are known to be involved as vectors of avian haemosporidian parasites, and an overview of their morphology, biology, importance, taxonomy, and diversity is presented (Ibáñez-Bernal *et al*., 2020). The life cycle of a parasite requires two hosts, typically a bird and a female mosquito, which most often belong to the genus *Culex* (*or Culicoides midges*) (Atkinson and van Riper III, 1991; Valkiūnas, 2005). It proliferates as clones, similar to haploid in bird hosts, and through asexual reproduction in vectors (Desser and Bennett, 1993). The clinical symptoms of the disease differ according to species. The mild symptoms, such as paleness of the comb, wattle,

and lethargy, and the infection of *P. juxtanucleare* can be moderate, mild, or asymptomatic (Kissinger *et al*., 2002; Vaisusuk *et al*., 2022) and/or may exhibit lethargy, white diarrhea, and convulsions (Tattiyapong *et al*., 2016), and in older chickens, it is mild paleness of the comb, wattle, and lethargy (Dhamayanti *et al*., 2023; Mohsen *et al*., 2024).

A different traditional diagnostic methods are used for detection of *Plasmodium* spp. which is the only undergo schizogony in circulating blood cells, that presence inside the erythrocytes and the both schizonts and gametocytes help in diagnoses of the parasite infections (Atkinson and van Riper III, 1991), such as blood smears or by using recent developmental methods (molecular technologies) as a more reliable (Fallon *et al*., 2003). Over the last two decades, molecular methods for studying mitochondrial DNA sequence variation have become an integral part of avian haemosporidian research. These methods have primarily been used for identification of the parasites and tentative phylogenetic reconstructions, allowing to compare data across the globe (Bensch and Hellgren, 2020), for an indication of the high diversity (Ricklefs *et al*., 2007; Kim and Tsuda, 2010) and identify the species of the parasites by sequencing of the amplified DNA (Bensch *et al*., 2009). Most investigations into avian malaria have been primarily based on material collected from naturally infected birds, and important data on ecology, distribution, molecular biology, diversity, and phylogeny have been accumulated since the end of the 20th century (Beadell *et al*., 2006; Hellgren *et al*., 2007). The model for malaria research is represented in chickens due to the severity of the disease, its pathology, and the unique model it provides for understanding the evolution of the parasite and its ecology in both field and laboratory settings (Macchi *et al*., 2010).

To the best of our knowledge, no previous molecular real time PCR analysis has been conducted to detect avian malaria (*Plasmodium* spp.) in domestic local breed chickens (*Gallus gallus domesticus*). This study not only offer a molecular detection system but also highlight the need of continued monitoring of the infection in birds.

MATERIALS AND METHODS

Blood samples

Forty-five local breed chickens (*Gallus gallus domesticus*) were purchased from different local markets in Baghdad city during the period from 1/10/2021 to 1/5/2022. About 1-2 ml from jugular vein was collected in EDTA (Ethylene diamine tetra acetic acid) tubes (Al-Daraji *et al*., 2008) and they were kept in deep freezer at -20 °C until analysis by real time polymerase chain reaction in the Laboratory of Microbiology Department, College of Veterinary Medicine, University of Baghdad.

DNA extraction from blood

Approximately 25 µL of blood was transferred into a 1.5 mL tube. The freezing-thawing was used for DNA extraction, added 200 µl buffer CL, 20 µl Proteinase K, and 5 µl RNase A solution into the sample tube and mixed vigorously, incubated the lysate at 56°C using a preheated heat block for 10 ~ 30 min., added 200 µl of Buffer BL into the upper sample tube and mixed thoroughly, then incubated at 70°C for 5 min., centrifuged at 13000 rpm for 5 min, and carefully transferred 350 ~ 400 µl of the supernatant into a new 1.5 ml tube and centrifuged. Added 200 µL of absolute ethanol to the lysate in the 1.5 mL tube, then centrifuged. The mixture was then applied to the spin column and centrifuged at 13,000 rpm for 1 min. Discard the filtrate and place the spin column in a new 2 ml collection tube. Added 700 µl of buffer WB to the Spin column and centrifuged for 1 min.at 13000 rpm. Placed the column into a new 2.0 mL collection tube and then centrifuged for 1 minute. Discarded the flow-through and collection tube altogether. Placed the spin column into a new 1.5 ml tube and added 30-100 µl of Buffer CE directly onto the membrane. Incubated for 1 min at room temperature and then centrifuged for 1 min at 13000 rpm to elute.

The primers preparation

The lyophilized, designed primers (*AMA 1* gene) with their IDs were dissolved in free ddH_2O to give a final concentration of 100 pmol/µL (stock solution) and stored at -20 °C. Prepared 10 pmol/µl concentration as working primer suspended as shown in the table below:

and the optimum conditions for gene detection were illustrated below:-

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	95°C	5 min	1 cycle
2-	Denaturation -2	95°C	45 Sec	35 cycle
3-	Annealing	52°C	45 Sec	
4-	Extension-1	72°C	1min	
5-	Extension -2	72°C	5 min.	1 cycle

Primer	Sequence	Primer sequence	Tm	GC %	Size of product (bp)	Reference
(AMA-1) gene	F	5'- GTCTTTGAATTAAGTGCTTCGGA - 3'	57.39	39.13	200 bp	Primer design (Id: LT969560.1)
	R	5'- TGTGTTACGGTGTTATAATTTCCCC - 3'	59.35	40.00		

Gel electrophoresis

Agarose (1.5%) was used to determine the DNA according to Sambrook *et al.* (1989).

Real time PCR

The KAPA SYBR FAST qPCR Kit is supplied as a 2X master mix with integrated antibody-mediated hot start, SYBR Green I fluorescent dye, $MgCl_2$, dNTPs, and stabilizers. The optimum conditions of the RT-PCR cycling program for gene detection are as follows:

No.	Phase	Tm (°C)	Time	No. of cycles
1-	Enzyme activation	95	5 min.	Hold
2-	Denaturation -2	95	0.2 Sec.	40
3-	Annealing	56	0.2 Sec.	
4-	Extension-1	72	0.2sec.	
5-	Final Extension -2	72	2 min.	1
6	Melting curve	90	0.15 sec.	1

Statistical analysis

Statistical analysis was performed according to Al-Mohammed *et al.* (1986) using the Chi-square test to detect significant differences in the effect of specific epidemiological parameters (sex and location) of the study at a significance level of P ≤ 0.05 or 0.01.

RESULTS

The total infection rate of *Plasmodium* spp. in local breed chickens (*Gallus gallus domesticus*) was 15.55% (7/45) in Baghdad city (**Figures 1** and **2**).

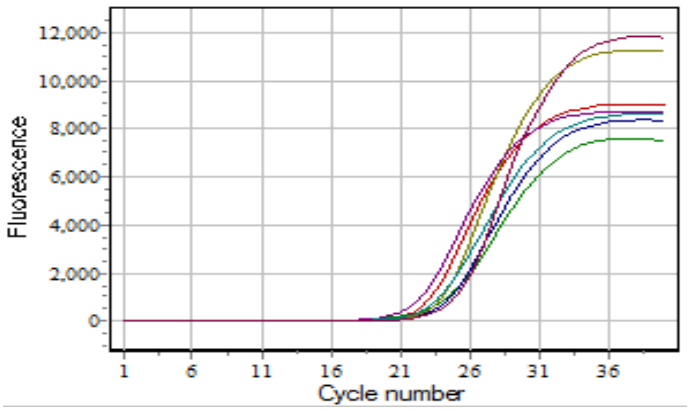

Figure 1: The infection rate of *Plasmodium* spp. in local breed chickens (*Gallus gallus domesticus*) in Baghdad city, as determined by RT-PCR.

The infection rate among males was 15% (3/20), while among females it was 16% (4/25), with a significant difference (P ≤ 0.01) (Table 1).

According to the area, a highly significant (P ≤ 0.01) infection rate was observed in the Al-Karkh district market at 18.18% (4/22), compared to the Al-Rusafa district market at 13.03% (3/23) (Table 2).

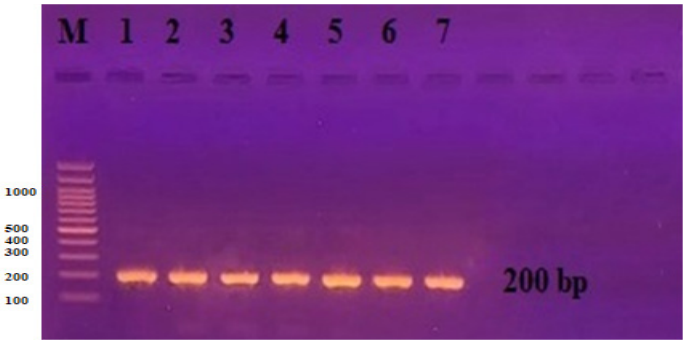

Figure 2: DNA products were electrophoresed on 1.5% agarose at 5 volts/cm² for 1:30 hours. M: DNA ladder with positive lanes of the band size at bp 200.

Table 1: The infection rate of *Plasmodium* spp. in local breed chickens (*Gallus gallus domesticus*) at Baghdad city.

Sex	No. of chickens examined	Positive (%)
Males	20	3(15)
Females	25	4(16)
Total	45	7(15.55)
χ^2	10.23 ** P ≤ 0.01	

Table 2: The infection rate of *Plasmodium* spp. in local breed chickens (*Gallus gallus domesticus*) at Baghdad city according to the district markets.

Districts markets	No. of chickens examined	Positive (%)
Al-Rusafa	23	3(13.04)
Al-Karkh	22	4(18.18)
Total	45	7(15.55)
χ^2	11.20 ** P≤0.01	

DISCUSSION

Blood parasite infections in poultry, such as *those caused by Plasmodium*, pose a serious threat to the poultry industry due to their potential to cause significant economic losses (Dhamayanti *et al.*, 2023). Morphological identification using microscopic examination of blood films remains an important tool for malaria diagnostics. It is particularly valuable when applied in conjunction with PCR as a diagnostic tool (Valkiūnas and Iezhova, 2018). PCR is used as a molecular marker, which is sensitive for distinguishing different parasite species and their lineages, and is essential for identifying cryptic *Plasmodium* species (Palinauskas *et al.*, 2015).

Microscopic examination revealed that 17/105 positives (16.19%) were positive for blood parasite infection. Trophozoites, erythrocytic meronts, and microgametocytes of *Plasmodium* were found in blood samples. Based on

morphological examination, the species found in the samples was closely related to *P. juxtanucleare*. Polymerase chain reaction examination revealed that 21 out of 60 samples were positive for *Plasmodium* (35%). The *Plasmodium* species identified from the sequenced samples were proven to be *P. juxtanucleare*, which is endemic to Thailand, and it is closely related to the samples (99.64%–100%), with a genetic distance of 0%–1%.

Additionally, age, population, and cage type were not found to be significantly associated with *Plasmodium* infection (Dhamayanti *et al*., 2023). The adaptation of chicken immunity to *P. juxtanucleare* infection may influence the severity of infection (Vaisusuk *et al*., 2022). Chicken immunity can also be affected by cage type; chickens in battery cages showed lower immunity due to chronic stress compared to those in enriched cages. On the other hand, chickens kept in enriched cages and those that are free-range tend to have a higher chance of contracting the disease (Bhanja and Bhadauria, 2018; Hofmann *et al*., 2020). In chickens that survive *Plasmodium* infection, they still experience low parasiteemia even when the parasiteemia level does not increase (Paulman *et al*., 2005). Samples that underwent PCR showed more positive results than those that underwent microscopic examination, and polymerase chain reaction has higher sensitivity than microscopic examination (Dhamayanti *et al*., 2023).

The thickness of the band on the agarose gel for each sample indicated the level of parasitemia. The lowest parasitaemia level was 0.21%, represented by the thinnest band. In contrast, the highest parasiteamia level among the positive samples was 0.64%, represented by the thickest band, and the thickness of the band on the agarose gel for each sample indicated the parasiteamia level (Dhamayanti *et al*., 2023). Bensch *et al*. (2009) demonstrated a correlation between the thickness of the band on an agarose gel and the rate of erythrocyte infection, with the thinnest band observed at 0.04% and the thickest at 1.93%.

The host-specificity of avian malaria parasites is diverse: some parasites can infect hosts from multiple families or even multiple orders; others are restricted to a single avian family or even species (Ricklefs and Fallon, 2002; Fallon *et al*., 2005; Loiseau *et al*., 2012).

Of the 55 samples tested using PCR analysis, 39 (71%) were positive for *Plasmodium* spp., and 28/40 (62%) blood smears were positive for *Plasmodium* spp. This is the first report from New Zealand in which specific *Plasmodium* spp. mixed infections have been found in introduced birds. Co-infections with several cosmopolitan *Plasmodium* lineages were identified, as well as the first report in New Zealand of an exotic avian *Plasmodium* sp. lineage, in Australian magpies. Whilst the role of introduced birds in maintaining and spreading pathogenic avian malaria in New Zealand is unclear, there is a potential infection risk to native birds, especially where distributions overlap (Schoener *et al*., 2019).

Ibrahim and Al-Rubaie (2020) were reported that the prevalence of avian malaria (*Plasmodium gallinaceum*) was 18% (18/100), which divided closely between males (20.00%) and females 16.00%, with a moderate spread in the local domesticated breed chickens (*Gallus gallus domesticus*) in Baghdad city. Shadan (2013) reported that the most prevalent Haemoparasite was *Plasmodium* spp., with an infection rate of 52.63% (70/133), while Flayyih (2014) recorded an infection rate of 9.86% in Turkey. This study found no relationship between the age of the chickens, the type of cage, and the population, which may be due to the insufficient sample size. Based on microscopic and PCR examinations, the *Plasmodium* species found in layer chickens from the three districts of Yogyakarta, Indonesia, was identified as *P. juxtanucleare*, which showed a low genetic distance from *P. juxtanucleare* from Thailand and Japan (0%–1%). There was no correlation between *Plasmodium* infection and age, type of cage, or population of layer chickens, but this may be due to the insufficient sample size (Dhamayanti *et al*., 2023). Avian malaria and related haemosporidian parasites are globally distributed, with host diversity and environmental factors in tropical regions favoring the prevalence and diversity of this group of parasites (Santiago-Alarcon and Marzal, 2020). A combination of microscopic, PCR-based, and serological diagnostic methodologies provides better estimates of the proper distribution and other aspects of the biology of haemosporidians, particularly in studies on virulence, prevalence, and biodiversity (Valkiūnas and Atkinson, 2020). These parasites were monitored by microscopic analysis of thin blood smears stained with Giemsa stain (Prunk-Nern *et al*., 2014). However, it is not a reliable method when performed by non-experts due to a lack of specialized training and expertise, as it requires specific training and considerable expertise (Chen *et al*., 2013; Razzak and Al-Haqban, 2015). Despite avian malaria having been detected by microscopic examination of blood smears (Greiner *et al*., 1975; McClure *et al*., 1978; Peirce, 1981; Atkinson and Van Riper III, 1991). Differences may be related to the diversity of the vectors (*Culex*, *Aedes*, and *Simuli*um, which are abundant in rivers/or to the specimens that were collected (Cohen, 1977). Haemoparasite infections mainly depend on invertebrate vectors, usually sucking insects, to infect their avian host (Valkiūnas, 2005). In *Plasmodium* parasites that infect multiple host species through host-switching events, such as avian malaria species, these polymorphisms may contribute to a broad range of host specificity (Iyer *et al*., 2007). The effects influence the host's habitat choice, and bird species that are unable to develop resistance to these parasites may have increased vectors and abundance, which

in turn increase the chances of infection (Atkinson *et al*., 1995; 2000; Woodworth *et al*., 2005). The total infection rate, 28.33% (51/180), was recorded in *Plasmodium* spp. in chickens (*Gallus gallus domesticus*) using Giemsa-stained blood smears, with a higher infection rate in females than in males, but without significant differences (Ibrahim and A-Rubaie, 2020). Zamaura-Vilchis *et al*. (2012) found that 1.74% *Plasmodium* is found in birds. Fernandez-Davila and Phalen (2013) found 10.9% *Plasmodium* in birds. The differences between the results of the current study and the previous studies in the infection rates may be due to the diagnostic methods or to parasite biological habitat as heteroxenous parasite and need more than one obligatory host in its life cycle, which are dipteran insects transmit the infective stages between birds hosts (Atkinson and Van Riper III, 1991; Valkiünas, 2005; Angrisano *et al*., 2012).

ACKNOWLEDGEMENT

The authors express their gratitude to the College of Veterinary Medicine, Department of Microbiology, University of Baghdad for their invaluable assistance in successfully conducting the study. We acknowledge the support of time and facilities from for this study

NOVELTY STATEMENT

This study introduces a novel approach to detecting *Plasmodium* spp. infections in domestic local breed chickens (*Gallus gallus domesticus*) in Baghdad, utilizing Real-Time PCR for enhanced sensitivity and specificity. By examining 45 birds from various regional markets, the research not only addresses a significant gap in understanding avian malaria prevalence in this region but also provides crucial insights into the potential impact of such infections on poultry health and local agriculture. This work contributes to the broader field of veterinary parasitology and offers a foundation for future studies on disease management in poultry populations.

AUTHOR'S CONTRIBUTION

Rana Mohammed Ibrahim conducted the study design, data collection, analysis, and manuscript preparation. The author is solely responsible for all aspects of the work.

Generative AI or AI-assisted Technology Statement

The author(s) declare that no Genrative AI was used in the creation of this manuscript.

Conflict of interest

The authors has declared no conflict of interest.

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