



Molecular Detection of Avian Malaria (*Plasmodium gallinaceum*) in Local Domesticated Breed Chickens (*Gallus gallus domesticus*) in Baghdad

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A B S T R A C T

This study was conducted to investigate the prevalence of avian malaria (*Plasmodium gallinaceum*) in the local domesticated breed chickens (*Gallus gallus domesticus*) that were purchased from the local markets in Baghdad city, using 100 blood samples which were collected from the wing vein, and kept in EDTA-K2 tubes for conventional PCR analysis during the period extended from 1 /10 / 2018 till 31/ 3 / 2019. Total infection rate was 18% (18/100), which were divided into males 20.00% and in females 16.00%. The eight isolates were recorded in the GenBank under accession numbers ID: MN082405.1, MN082406.1, MN082407.1, MN082408.1, MN082409.1, MN082410.1, MN082411.1, and MN082412.1 with identity 99.20 - 99.87% and with other isolates (United Kingdom and USA) 99.34 - 99.88 %. In conclusion, *Plasmodium gallinaceum* may have a moderate spread in local domesticated breed chicken at Baghdad.

Keywords: *Plasmodium*, chickens, PCR, haemosporidian, *Gallus*

INTRODUCTION

Haemosporidian infections are protozoa parasites that causing diseases by a group of parasites that infect mammals, reptiles, birds, and amphibians (1). The diseases are induced by an unknown number of haemozoin species (2), and most often only *Plasmodium* spp. are referred to as avian malaria parasites (3), that as a haemoparasitic infectious (4-6). The great majority of described avian *Plasmodium* species were reported only in

birds that live in tropical and subtropical countries and which have adapted for transmission globally and have become cosmopolitan (7). It may be severe mortal in zoos and for domestic birds (8). It is referred that *P. gallinaceum*, *P. juxtannuclear* and *P. durae* appeared to be the most dangerous for poultry causing up to 90% mortality (9). Life cycle of these parasites need two hosts undergo in vectors invertebrate host, sexual reproduction (10), *Culex* mosquitoes (11) that only develop in females (1, 12) and in vertebrate host that is asexually (13).

There are different diagnostic methods used for the detection of the haemosporidian parasites. Traditional method detects the infection by microscopic examination of blood smears only, while the development of new molecular technology such as polymerase chain reaction (PCR) has made detection for these parasites more reliable and faster (4) as it can indicate the high diversity of parasites (14, 15) reaching to the species level by sequencing the amplified DNA (16).

Erythrocyte invasion by *Plasmodium* parasites requires apical membrane antigen-1 (AMA-1) and rhoptry neck protein 2 (RON2) interactions. These proteins of *P. gallinaceum* are evolutionarily and structurally conserved and the suggesting may play essential roles for invading host erythrocytes. In addition, a high level of genetic variation was found in *ama-1* of avian *Plasmodium* species and these findings have implications for investigating (17).

Due to the important effects of avian malaria on economic loss and mortalities, and their prevalence is not well known by using molecular technique (PCR) in local breed domestic chickens in Baghdad city, this study was conducted.

MATERIALS AND METHODS

Animals, Areas and Periods of the Study

All procedures conducted in this study was reviewed and approved by the scientific committee in the College of Veterinary Medicine, University of Baghdad in accordance with the ethical standards of animal welfare.

One hundred local breed chickens (*Gallus gallus domesticus*), 50 males and 50 females, were randomly purchased from the local markets of Baghdad city (Baghdad Aljadida, Abu-Ghurib, and Al-Baia) during the period from 1/10/2018 until 31/3/2019.

Blood Samples

About 1-2 mL of wing blood samples were collected from each bird by a sterile syringe and kept in EDTA (Ethylene diamine tetra acetic acid, K₂) tubes (18) and stored in -20 °C until for PCR analysis.

DNA Extraction

Genomic DNA from the whole 100 blood samples was extracted using a G-spin DNA extraction kit (Cat. No.17045, Life Science, Korea). Concentration and purity (absorbance at 260/280 nm) of extracted DNA were checked using a Nanodrop spectrophotometer (Thermo Scientific, USA).

Primers

Primers used according to Lauron et al. (17) for the partial amplification (875 bp) of the *ama-1* gene are shown in Table 1. The lyophilized primers were dissolved in nuclease free water to give a final concentration of 100 pml/μL as a stock solution and kept at -20 °C. Then, a concentration of 10 pmol/μL was made as working primers, in which 10 μL of the stock solution was added to 90 μL of deionized distilled water (ddH₂O) to reach a final volume of 100 μL (Integrated DNA Technologies Company).

Table 1. Primers used for amplification hypervariable domain I region of *ama-1* gene

Primers	Sequence (5'-3')	Product size	Reference
<i>Pg_AMA1F1</i>	F GATTTAGGTGAAGATGCAGAAGT	875 bp	(17)
<i>Pg_AMA1R1</i>	R TTAATTAACATGTTGGTTTACAT		

PCR

The PCR reaction mixture involved the use of iNtRONSMaxime PCR PerMix kit. The components of the kit included: i-Taq DNA Polymerase 5 U/μL, dNTP mixture 2.5 mM, reaction buffer 1x, gel loading buffer1x. The PCR working solution in a final volume of 25 μL included: 5 μL of Taq PCR PreMix, 10 pmol/μL (1 μL) of each forward and reverse primers, 1.5 μL of the DNA templates as well as 16.5 μL of nuclease-free water.

Maxime Premix Kit (i-Taq) (Cat No.25025) was used for PCR reaction. with an optimal condition as follows: initial denaturation (94 °C, 4 min, 1 cycle), followed by 30 cycles of denaturation (94 °C, 30 sec), annealing (50 °C, 1 min.); extension 1 (72 °C, 1.2 min), and final extension for 1 cycle (72 °C, 5 min). The PCR product was then run out on a 2% agarose gel using 1× TBE, and visualized by SYBR Safe (ABM, Canada) staining under UV light.

Sequencing of Gene and Sequence Arrangement

The PCR amplicons were sent for sequence (8 samples) to Macrogen company (Korea). The sequencing was performed by National Instrumentation Center for Environmental Management, biotechnology lab using DNA sequencer 3730XL (Applied Biosystem). Homology search was conducted using Basic Local Alignment Search Tool (BLAST-NCBI) program, which is available at the National Center, Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>). The phylogenetic tree diagrammatic was done by molecular evolutionary genetic analysis (MEGA) software version 6.0Sequences (10, 21).

Statistical Analysis

Chi-square was used to determine the effect of sex in the infection rate at a significant ($P < 0.05$) difference level (20).

RESULTS

Infection Rate

Using the conventional PCR, our result indicated that the total infection rate of *Plasmodium* in local breed domestic chickens (*Gallus gallus domesticus*) was 18% (18/100) which divided in males 20% (10/50) and females 16% (8/50) ($P < 0.01$) (Table 2, Figure 1).

Table 2. Infection rate of *Plasmodium gallinaceum*

Sex	No. samples examined	Positive	%
Males	50	10	20
Females	50	8	16
Total	100	18	18
χ^2		20.56*	

* $P < 0.01$

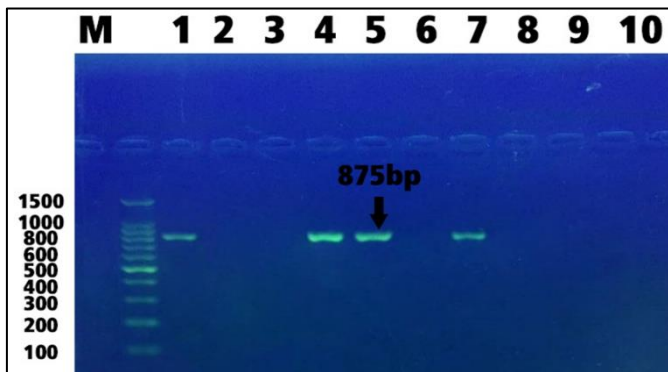


Figure 1. Agarose gel (2%) electrophoresis with SYBR Safe stain shows amplification of 875 bp fragment of *ama-1* gene. PCR product was emigrated at 5 volt/cm², for one and half h and visualized under UV light. Lane M: DNA ladder (100 bp); Lanes 1, 4, 5, and 7 positive cases

Phylogenetic Analysis

The phylogenetic relationship of the eight local isolates of *Plasmodium gallinaceum* was between 99.20-99.87% and between these isolates and the world isolates between 99.34-99.88% (Figure 2). These isolates were recorded in the GenBank under accession numbers MN082405.1, MN082406.1, MN082407.1, MN082408.1, MN082409.1, MN082410.1, MN082411.1, MN082412.1.

DISCUSSION

Haemosporidian infections are group of diseases caused by protozoan parasites that infect different animals including birds, their community, and may reflect a large-scale of geographical variations (1,22). Eggs and meat of the family use are exposed to parasitic diseases such as haemosporidians (23). Shadan (24) observed that *Plasmodium* spp. was the most prevalent haemoparasites 52.63% (70/133), Flayyih (25) recorded an infection was 9.86% in turkey. These infection rates were differered from the present result (18%) in which it was less or higher and that may be due factors related to the sampling effort, location, poultry breed, immune status, habitat type, season, the abundance of arthropod vectors responsible for transmitting the parasites (26), behavior and diet (27). The great majority of described avian *Plasmodium* species were reported only in birds that live in tropical and subtropical countries or in Holarctic migrants wintering in the same regions, indicating that transmission of these pathogens occur mainly in countries with warm climates. The malaria parasites, which have adapted for transmission globally and have become cosmopolitan; Molecular diagnostics using general primers (the main diagnostic tool currently used in wild life malariology) is often insensitive in distinguishing of avian *Plasmodium* spp.co-infections, which are common and even predominate in many bird populations (7).

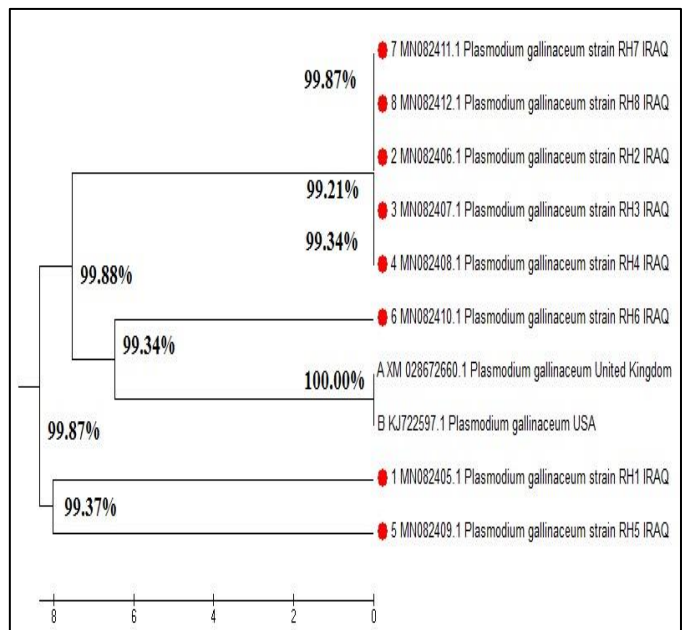


Figure 2. Neighborgoining tree *plasmodium gallinaceum ama-1* gene

In the present study use PCR as molecular markers which are sensitive for distinguishing different parasite species and their lineages, and they are essential for the identification of cryptic *Plasmodium* species (28) and it is more sensitive method for detecting the *Plasmodium* when compared with conventional methods, and the microscopy diagnosed give 50% false- negative results when confirm by PCR (9), (29). Morphological identification using microscopic examination of blood films remains an important for malaria diagnostics. It is particularly valuable if applied with PCR as based diagnostic tools. During the past 15 years numerous avian *Plasmodium* parasites were named and described using morphological features of their blood stages and molecular markers for parasite detection were developed in a handful of these descriptions (7).

In conclusion, high infection rate of *Plasmodium* is found in domesticated local breed chickens. Sex is significantly affected in the prevalence of *Plasmodium* infections in chickens and the high infection rate is found in males more than females.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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التحري الجزيئي لمالريا الطيور (*Plasmodium gallinaceum*) في الدجاج المحلي المستأنس (*Gallus gallus domesticus*) في مدينة بغداد

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الخلاصة

هدفت هذه الدراسة الى التحري عن نسبة انتشار مالريا الطيور (*Plasmodium gallinaceum*) في عرق الدجاج المحلي المستأنس (*Gallus gallus domesticus*) والتي تم شرائها من الاسواق المحلية في مدينة بغداد، من خلال استعمال مئة نموذج دم جمعت من الوريد الجناحي وحفظت في انابيب تحتوي على مانع تخثر اثلين ثنائي الامين رباعي حامض الخليك الثلجي (EDTA-K₂) لغرض التحليل بتقنية تفاعل سلسلة البلمرة التقليدي خلال المدة من 2018/10/1 ولغاية 2019/3/13. بلغت نسبة الاصابة الكلية 18%، توزعت في الذكور 20% وفي الاناث 16%. وكان التحليل التتابعي لثمانية عزلات والتي سجلت في بنك الموروثات تحت الارقام MN082411.1, MN082410.1, MN082409.1, MN082408.1, MN082407.1, MN082406.1, MN082405.1, MN082412.1. ويتطابق بين هذه العزلات تراوح بين 99.20% - 99.87% وبين العزلات الاخرى (المملكة المتحدة والولايات المتحدة الامريكية) 99.34 - 99.88%. نستنتج ان *Plasmodium gallinaceum* ينتشر بنسبة معتدلة في عرق الدجاج المحلي المستأنس في مدينة بغداد.

الكلمات المفتاحية: *Plasmodium*, PCR, Haemosporidian, دجاج Gallus، الدم