J Vet Res 66, 373-379, 2022
DOI:10.2478/jvetres-2022-0049

High prevalence of *Plasmodium* infection in fighting cocks in Thailand determined with a molecular method

Kotchaphon Vaisusuk[1], Wasupon Chatan[2], Tossapol Seerintra[2], Supawadee Piratae[3]✉

[1]Department of Veterinary Technology and Veterinary Nursing, Faculty of Agricultural Technology,
Rajabhat Maha Sarakham University, Maha Sarakham 44000, Thailand
[2]Faculty of Veterinary Sciences, [3]One Health Research Unit, Faculty of Veterinary Sciences,
Mahasarakham University, Maha Sarakham 44000, Thailand
supawadee.p@msu.ac.th

Received: April 22, 2022 *Accepted: September 2, 2022*

Abstract

Introduction: Avian malaria caused by *Plasmodium* and the malaria-like parasites of the genus *Haemoproteus* has been regularly described in multiple regions worldwide. These parasites significantly affect many avian taxa, including domestic chickens and fighting cocks. There are limited epidemiological studies of these blood parasites in vertebrate hosts, especially in Thailand. **Material and Methods:** This study used microscopic examination of blood samples and PCR amplification exploiting primers for nucleotide sequences of *Plasmodium* or *Haemoproteus* species based on the cytochrome *b* gene to determine the occurrence of *Plasmodium* spp. in fighting cocks. **Results:** Examination of 249 blood samples of fighting cocks revealed that 41.37% (103/249) were positive for malaria by microscopic examination and 88.76% (221/249) were positive by DNA amplification. Sequencing and DNA analysis of 61 PCR products revealed that infection by *Plasmodium juxtanucleare* was the most common avian malaria in fighting cocks in Thailand followed by infections by *Plasmodium gallinaceum*; however, *Haemoproteus* infection was not discovered. **Conclusion:** This study indicated that plasmodiasis is widespread in fighting cocks in Thailand although the prevalence was not clearly determined; therefore, prevention and control strategies for these protozoa should be improved, especially those for avoiding vector exposure and eliminating mosquito breeding sites.

Keywords: avian malaria, fighting cock, *Plasmodium*, molecular detection, Thailand.

Introduction

Avian malaria is a parasitic disease of birds caused by blood protozoa of the *Plasmodium* genus. Avian malaria infections are highly prevalent in tropical and subtropical regions of the world. In Thailand, two types of *Plasmodium* have been reported in chickens: *Plasmodium gallinaceum* in domestic chickens (*Gallus gallus domesticus*) (25) and *Plasmodium juxtanucleare* in Burmese red junglefowl (*Gallus gallus spadiceus*) (40). At least 55 morphological datasets and DNA sequences of avian *Plasmodium* have been described (41, 44). The principal vector of these parasites are the Culicidae mosquitoes including *Culex*, *Aedes*, *Culiseta*, *Anopheles*, *Psorophora*, *Mansonia*, *Aedeomya* and *Coquillettidia* (22, 36, 44). Avian *Plasmodium* develops within blood cells causing plasmodiasis and asymptomatically invades various organs through its ability to develop exoerythrocytically all over the body including in the brain, eyes, heart, lungs, skeletal muscles, and other organs (43). Generally, avian clinical signs include depression, anorexia, fever, weight loss, declines in food consumption, anaemia, green feces and death (2, 47). However, clinical manifestations may vary from asymptomatic to high morbidity depending on the lineage of the parasite, parasitaemia and immune status and host species (7). Although most infected chickens show mild to moderate clinical signs (25), in some non-adapted avian species high-severity infections occur (15).

Plasmodium gallinaceum is highly prevalent in Asia and Africa and may cause mortality of 80%–90% among domestic chickens, while *P. juxtanucleare* is endemic in Asia, Africa and South America (26, 47). Although infections with *P. juxtanucleare* result in mild to moderate clinical signs (18), high parasitaemia can cause anaemia, diarrhoea, weight loss and death (37). Despite the extent of the disease burden, there are insufficient epidemiological data on these blood parasites, as well as on the molecular identification of infections within the vertebrate host. Epidemiological studies are essential to describe the avian malaria infection and identify particular pathogens in poultry, including fighting cocks, which are commonly raised for a popular sport, for sale, and for local consumption.

Avian malaria is traditionally diagnosed by blood smear examination based on parasite morphology, which is not expensive but very difficult because of the small size of agents and their morphological variations. This method is not sensitive to low parasitaemia and more difficult to evaluate by an inexperienced examiner. Recently, a molecular method has become common and widely used in laboratories. This method, PCR, has higher specificity and sensitivity to *Plasmodium* infection in avian hosts, despite low sample parasitaemia or sample collection in an early stage of infection (11, 13, 35, 24).

In the present study, we demonstrated the molecular detection of avian malaria in samples from fighting cocks in Maha Sarakham province, Thailand. The first step of amplification used primers for the *Leucocytozoon/Plasmodium/Haemoproteus* genera and the second step used *Plasmodium/Haemoproteus*-specific primers and was followed by sequencing, which has been widely used to detect the infection and identify and evaluate the genetic diversity of haemosporidian parasites (8, 39, 42, 45). The objectives of this study were to determine *Plasmodium* infections by blood smear examination and PCR in naturally infected fighting cocks in Maha Sarakham, Thailand. We also identified the *Plasmodium* species and conducted a phylogenetic analysis based on the cytochrome *b* gene.

Material and Methods

Sample collection. Blood samples were collected from 249 fighting cocks in Maha Sarakham province, Thailand. The sample size was calculated to include the appropriate number of samples from an infinite population by settling a 95% confidence level, 5% margin of error and approximately 20% sample proportion (10). Approximately 0.1–0.5 mL of blood was obtained by venepuncture from the brachial vein, collected in sterile tubes with ethylenediaminetetraacetic acid and transported on ice to the laboratory at the Faculty of Veterinary Sciences of Mahasarakham University.

Microscopic examination. Blood samples were screened for *Plasmodium* and *Haemoproteus* infections by a thin blood-smear technique performed on the same day as blood collection. The thin blood smears were air dried entirely for 10 s and fixed with 100% methanol for 5 min. Blood films were stained with 10% Giemsa's solution for 15 min, then observed in monolayer fields under a light microscope (17). Blood smears are first scanned at low magnification (400×) for 15–20 min to distinguish erythrocytic stages. Then, if parasites were present, high magnification (1,000×) was used to analyse morphological traits and identify avian *Plasmodium* spp. High magnification was also used to take photographs. Blood remaining after the blood smear procedure was stored at −20°C until DNA extraction.

DNA extraction and PCR. DNA was extracted from 25 μL of anticoagulated blood samples using a GF-1 blood DNA extraction kit (Vivantis Technologies, Selangor, Malaysia) following the manufacturer's protocol. DNA concentrations and purity were determined by exposing the DNA to ultraviolet light in a NanoDrop 2000 Spectrophotometer at a wavelength of 260 nm using the pre-programmed applications for dsDNA (Thermo Fisher Scientific, Waltham, MA, USA). Each extracted DNA sample was examined for *Plasmodium* and *Haemoproteus* infection by a nested-PCR method using specific primers targeting a mitochondrial cytochrome *b* fragment of approximately 480 bp of the parasite as previously described. For the first PCR, HaemNFI (5′-CAT ATATTAAGAGAAITATGGAG-3′) and HaemNR3 (5′-ATAGAAAGATAAGAAATACCATTC-3′) primers were used, which amplify the DNA belonging to *Plasmodium*, *Haemoproteus* and *Leucocytozoon* spp. (13). For the second PCR, HaemF (5′-ATG GTG CTT TCG ATA TAT GCA TG-3′) and HaemR2 (5′-GCA TTA TCT GGA TGT GAT AAT GGT-3′) primers were used, which amplify the DNA from *Plasmodium* and *Haemoproteus* spp. (4).

In the first PCR, approximately 10–50 ng of the extracted DNA was used as a template to amplify a fragment of the cytochrome *b* gene in a 25 μL reaction containing 1 μL of each primer (10 μmol/L), 1.5 mM $MgSO_4$, 0.2 mM deoxynucleotide triphosphate, 1× PCR buffer and 1 U of *Taq* Polymerase (Vivantis Technologies). The reaction conditions comprised 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 50°C and extension for 45 s at 72°C using a PCR thermocycler (Biometra, Göttingen, Germany). In the second PCR, the reaction mixture and conditions were the same as those in the first amplification, except for the primers and the PCR product from the first amplification being used as the DNA template. PCR master mixes containing only the primers with no DNA template served as the negative control. The PCR products were run in a 1% agarose gel stained with ViSafe Red Gel Stain (Vivantis Technologies) and visualised under ultraviolet light to check for positive amplifications.

Nucleotide sequencing and analysis. Selecting dispersed sampling sites, we selected 63 PCR amplicons which showed a sharp and intense band of the target gene in agarose gel electrophoresis to purify and sequence directly. This was carried out using the forward primer (HaemF) at the commercial sequencing company (ATGC Co., Pathumthani, Thailand). The obtained nucleotide sequences were visualised, manually adjusted and compared for similarity to sequences deposited in GenBank, using the BLAST program hosted by NCBI (https://www.ncbi.nlm.nih.gov/) and MalAvi (4). Sequence electropherograms were also checked for quality, length and double or multiple nucleotide peaks. The sequences of the partial mitochondrial cytochrome *b* gene of *Plasmodium* and *Leucocytozoon* in this study were then deposited in the GenBank database.

Multiple sequences were aligned using ClustalW in the BioEdit program (12). The DnaSP6 program was used to identify the number of haplotypes from cytochrome *b* sequences of *Plasmodium* and *Leucocytozoon* (34) in this study. Phylogenetic relationships between the cytochrome *b* haplotypes in this study and 23 related sequences from different geographical distributions (Thailand and neighbouring countries) in the GenBank and MalAvi databases were established using the maximum likelihood method and the Kimura 2-parameter model in MEGA X (19). In addition, *Leucocytozoon caulleryi* was used as an outgroup for phylogenetic analysis.

Results

The microscopic examination showed that 41.37% (95% CI: 35.18–47.76 %) or 103 out of 249 samples were positive for *Plasmodium* spp. Mild or no clinical signs were observed in infected cocks. In Giemsa-stained blood smears, trophozoites and meronts of *Plasmodium* were observed in the cytoplasm of red blood cells (Fig. 1).

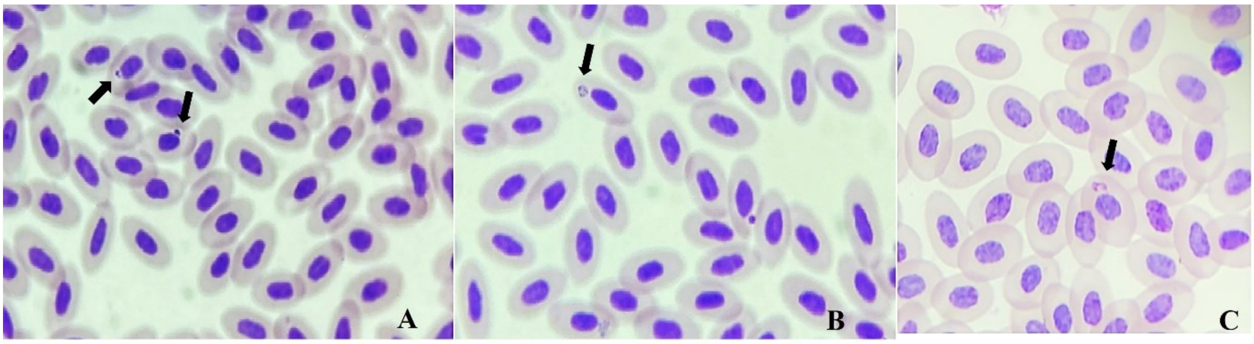

Fig. 1. Blood stage of *Plasmodium* spp. found in fighting cocks in Maha Sarakham, Thailand
A – trophozoites of *P. juxtanucleare*; B – meronts of *P. juxtanucleare*; C – meronts of *P. gallinaceum.* Arrows indicate the parasite in each case

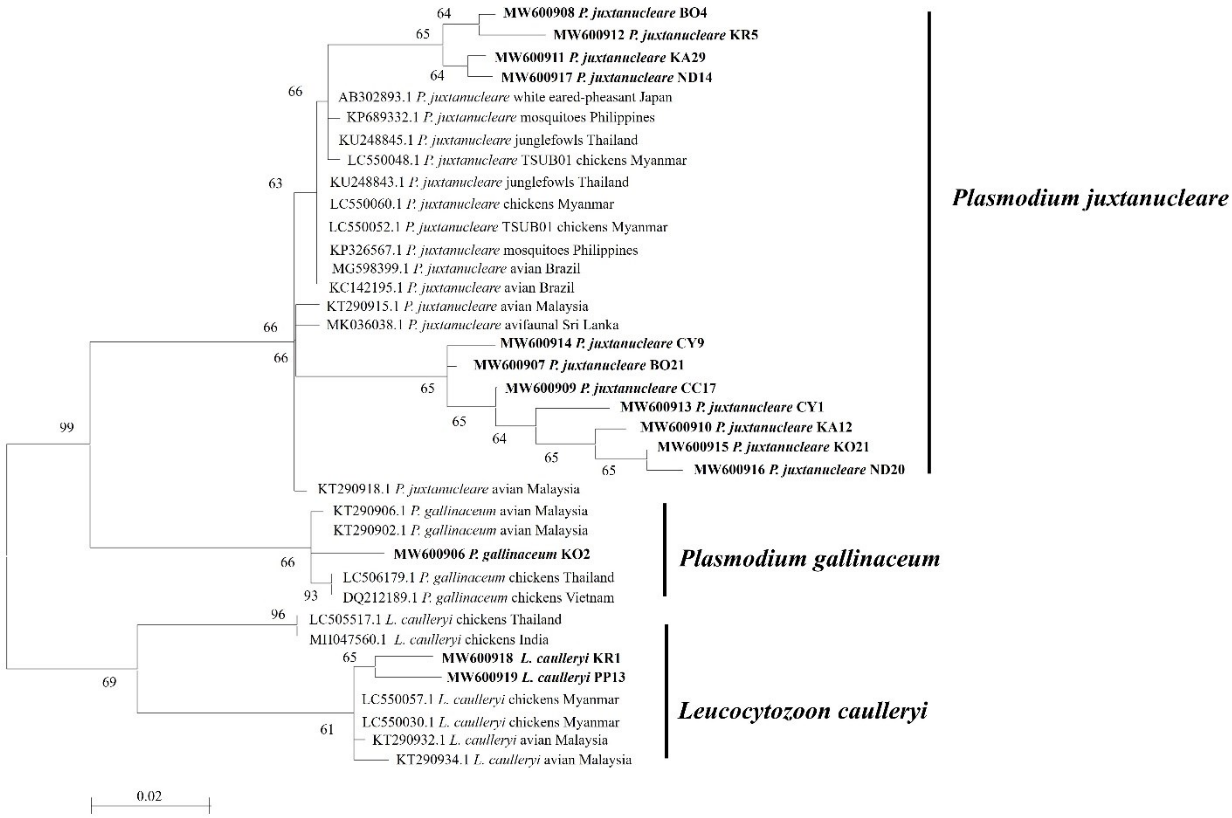

Fig. 2. Phylogenetic tree based on partial cytochrome *Plasmodium* and *Leucocytozoon b* gene sequences from Maha Sarakham (indicated in bold typeface) together with 23 related sequences from different distributions in Thailand and neighbouring countries in the GenBank and MalAvi databases. Sequences were compared with the maximum likelihood method. The cytochrome *b* gene of *Leucocytozoon caulleryi* from this study and related sequences in the database were used to root the tree. The percentage of trees in which associated taxa clustered together is shown next to the branches

Table 1. Haplotypes of 61 cytochrome *b* sequences from fighting cocks in Thailand and National Center for Biotechnology Information BLAST results

Haplotype number (n)	Sample IDs	(GenBank accession number)	Closest sequences in NCBI GenBank (% similarity)
CAN1 (21)	BO3, BO13, BO21, CC10, CY2, CY17, CY18, CY24, KA1, KA13, KA20, KA30, KO7, KO19, ND17, PP5, PP10, PP16, WP16, WP20, WP22	MW600907	*Plasmodium juxtanucleare* MG598396.1 (99–100%) MG598393.1 (99–100%) MG598392.1 (99–100%)
CAN2 (1)	BO4	MW600908	*Plasmodium juxtanucleare* KU248845.1 (99.8%)
CAN3 (3)	BO6, CC17, CC28	MW600909	*Plasmodium juxtanucleare* MG598396.1 (99–100%)
CAN4 (19)	BO15, BO23, CC9, CC18, CY10, KA2, KA12, KO11, KR3, KR7, KR19, KR21, KR23, ND11, PP1, PP3, PP8, WP6, WP28	MW600910	*Plasmodium juxtanucleare* MG598396.1 (99–100%) MG598393.1 (99–100%) MG598392.1 (99–100%)
CAN5 (3)	CC1, KA29, ND8	MW600911	*Plasmodium juxtanucleare* KU248845.1 (99–100%) AB250415.1 (99–100%)
CAN6 (3)	CC2, KO18, KR5	MW600912	*Plasmodium juxtanucleare* KU248845.1 (99–100%) AB250415.1 (99–100%)
CAN7 (1)	CY1	MW600913	*Plasmodium juxtanucleare* MG598396.1 (99.4%)
CAN8 (1)	CY9	MW600914	*Plasmodium juxtanucleare* MG598396.1 (99%)
CAN9 (1)	KO2	MW600906	*Plasmodium gallinaceum* LC506179.1 (99%) LN835294.1 (99%) KP025675.1 (99%)
CAN10 (3)	KO21, KO23, WP10	MW600915	*Plasmodium juxtanucleare* MG598396.1 (99–100%)
CAN11 (1)	KR1	MW600918	*Leucocytozoon caulleryi* LC550057.1 (99.8%)
CA12 (2)	ND5, ND20	MW600916	*Plasmodium juxtanucleare* MG598396.1 (99–100%)
CAN13 (1)	ND14	MW600917	*Plasmodium juxtanucleare* KU248845.1 (100%)
CAN14 (1)	PP13	MW600919	*Leucocytozoon caulleryi* LC550057.1 (99.8%) KT290932.1 (99.6%)

However, the morphology of the parasites hampered the distinction of morphospecies; therefore, the parasite species were adjusted based on the results of sequencing. In PCR detection, 221 out of 249 or 88.76% (95%CI: 84.16–92.40%) were positive when amplification was attempted with the HaemF and HaemR2 primer set which was designed for the detection of *Plasmodium* and *Haemoproteus* DNA. However, based on the results of the sequencing of nested PCR products, a PCR primer targeting *Plasmodium* and *Haemoproteus* was observed to possibly amplify DNA of *Leucocytozoon* species.

Sixty-three PCR amplicons from different sampling locations in Maha Sarakham province which showed a sharp and intensive band in agarose gel electrophoresis were selected for direct sequencing. Of these 63, 61 sequences were successfully sequenced. The sequences of the partial mitochondrial cytochrome *b* gene of *Plasmodium* and *Leucocytozoon* parasites in this study were approximately 478-bp-long (468–488 bp) fragments.

Among the 61 sequences, 58 were *P. juxtanucleare* and represented 11 haplotypes, one sequence was *P. gallinaceum* and represented one haplotype and two sequences were *L. caulleryi* and represented two haplotypes. All haplotype sequences obtained were deposited in the GenBank database under accession numbers MW600907–MW600917 for *P. juxtanucleare*, MW600906 for *P. gallinaceum* and MW600918 and MW600919 for *L. caulleryi* (Table 1). The phylogenetic analysis indicated that 11 haplotypes in this study clustered together with *P. juxtanucleare* isolates from the database, one haplotype was grouped with *P. gallinaceum* and two haplotypes were found to identify with *L. caulleryi* (Fig. 2).

Discussion

In the present study, we demonstrated the prevalence and identification of *P. juxtanucleare*, *P. gallinaceum* and *L. caulleryi* from fighting cocks in Maha Sarakham province, Thailand. The overall prevalence of *Plasmodium* spp. infection was 41.37% by microscopic detection and 88.76% by nested PCR assay. Avian malaria is widespread, especially in tropical and subtropical countries, including Thailand (7, 27). However, insufficient studies have been performed to determine the prevalence in avian hosts. In Thailand, the nested PCR prevalence of *Plasmodium* spp. was reported to be 9% in owls (30), 50% in native Thai fowl (29), and 70.59% in Burmese red junglefowl (40). The difference between prevalence rates of *Plasmodium* in avian species in Thailand might be explained by the susceptibility of hosts, the presence of potential vectors and the possibility of exposure of hosts to vectors. In a related study in nearby Myanmar, the nested PCR prevalence of *Plasmodium* spp. was reported to be 86.8%–100% in chickens in some areas (48). It is likely that *Plasmodium* spp. infection in chickens is common in mainland Southeast Asian regions. The ecological conditions deriving from the tropical climate, which is conducive to greater abundance and wider distribution of vectors and consequent spread of agents, would predict high detection rates (1, 20). Moreover, the enduring veterinary relevance of this vector-borne disease in Thailand could be explained by a lack of knowledge of vector control and insufficient vector eradication, as well as the lack of effective treatments which prevent parasites from remaining in the blood of the reservoir host for a long period before clearance.

In studies conducted overseas, the molecular prevalence of plasmodiasis in avian hosts varied from 5.9% in injured wild birds in Tokyo, Japan and the city's environs (16), through 12.1% in birds in Iran (23), 13.5% in penguins undergoing rehabilitation in Brazil (46), 18% in domestic chickens (*Gallus gallus domesticus*) in Baghdad, Iraq (14) and 20% in wild birds on Tsushima Island in Japan (39) to 100% in chickens in some areas of Brazil (32). Differences in prevalence may be explained due to the species or strain of the vector and the avian host (38), geographical and climatic conditions which affect the distribution and spread of vectors (3), the avian health management programme including vector prevention and control strategy in a given country, and also the sensitivity and specificity of the specific primers used for molecular detection (33).

Although avian malaria of the *Plasmodium* and *Haemoproteus* genera can infect a wide number of bird species and is distributed everywhere except for Antarctica (9, 41), there are insufficient epidemiological data on these blood parasites in domestic chickens in Thailand. Regarding the prevalence of *Plasmodium* spp. in fighting cocks, this study offers new knowledge of which species is dominant despite it never having been reported in village chickens or poultry farms in Thailand – *P. juxtanucleare* is this species and it plays a role in malaria spread in this region. This finding correlated with the lack of or only mild clinical signs observed in infected fighting cocks, as infections with *P. juxtanucleare* result in mild to moderate clinical signs (18). Based on microscopic examination and sequencing results, *Haemoproteus* infection was not discovered. This finding is similar to that reported in captive birds from a Brazilian megalopolis which showed that infections with *Plasmodium* spp. (overall prevalence 97.6%) predominated when compared to those with *Haemoproteus* (2.4%) parasites (8). The absence of *Haemoproteus* may be explained by the lack of potential vectors of parasites of this genus (*Culicoides* biting midges and Hippoboscidae flies) (5, 41) in this region, which requires further investigation. Although the high prevalence of *Plasmodium* spp. infections in fighting cocks indicates that these animals were reservoirs of *P. juxtanucleare*, mild or no clinical signs were observed. Adaptation of fighting cocks as hosts to *P. juxtanucleare* infections may be the reason for the actual or near asymptomaticity of these infections. These data are in accordance with a previous report indicating that high-severity cases were found mostly in some non-adapted avian species (15). This is most probably due to the host's immunity to parasites and due to the strain of the parasite (7), which defines its level of virulence, infectivity and pathogenicity. Asymptomatic infection is transmissible, and may develop symptoms later in cases of low host immunity levels. This is a concern because manifestation of such symptoms impacts animal health and production.

Sequence analysis showed that after amplifying with *Plasmodium*- and *Haemoproteus*-specific primers (4, 13), we also unintentionally amplified *Leucocytozoon caulleryi.* The *L. caulleryi* parasite is transmitted through the black fly (*Simuliidae*) and the biting midge (*Culicoides*) (21, 41), which are widespread in Maha Sarakham (31). Recently, a study reported *L. caulleryi*-infected backyard chickens in Khon Kaen province (28), where the proximity to and border with Maha Sarakham indicated this parasite is endemic in this region. Such mixed amplification unforeseen in the protocol has previously occurred in samples from chickens from Myanmar, also because of the sequence similarity of the cytochrome *b* region between *Plasmodium* and *Leucocytozoon* (48). Consequently, newly designed primers are required to amplify all *Plasmodium* or *Haemoproteus* species with high sensitivity but not detect *Leucocytozoon* or other haemosporidian parasites.

In conclusion, this study on fighting cocks in Maha Sarakham province, Thailand, describes the presence of two *Plasmodium* species, with *P. juxtanucleare* being the dominant one over by *P. gallinaceum*; it also reports infection with *Leucocytozoon caulleryi*. Our study constitutes the first molecular epidemiological survey of *Plasmodium* from an avian host in Thailand. Therefore, the implementation of molecular techniques in clinical practice for the diagnosis of *Plasmodium* is recommended. This study indicated that plasmodiasis is widespread in fighting cocks; although the frequency of infection was not clearly determined, prevention and control strategies against this protozoa must be improved. The very high prevalence of *Plasmodium* spp. suggests a necessity of further study to investigate the prevalence and distribution of vector mosquitoes and farm management in Thailand.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This research project was financially supported by Mahasarakham University (2021).

Animal Rights Statement: The study protocol was approved by the Institutional Animal Care and Use Committee, Mahasarakham University (IACUC-MSU-07/2021).

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