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Molecular detection of the avian malaria parasite *Plasmodium gallinaceum* in Thailand☆

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ABSTRACT

Avian malaria is one of the most common veterinary problems in Southeast Asia. The standard molecular method for detection of the avian malaria parasite involves the phenol–chloroform extraction of parasite genomic (g)DNA followed by the amplification of parasite gDNA using polymerase chain reaction (PCR). However, the phenol–chloroform extraction method is time-consuming and requires large amounts of samples and toxic organic solvents, thereby limiting its applications for parasite detection in the field. This study aimed to compare the performance of chelex-100 resin and phenol/chloroform extraction methods for the extraction of *Plasmodium gallinaceum* gDNA from whole avian blood that had been dried on filter papers (a common field sampling method). The specificity and sensitivity of PCR assays for *P. gallinaceum* cytochrome B (*cytb*) and cytochrome oxidase subunit I (*coxI*) gene fragments (544 and 588 bp, respectively) were determined, and found to be more sensitive with gDNA extracted by the chelex-100 resin method than with the phenol/chloroform method. These PCR assays were also performed to detect *P. gallinaceum* in 29 blood samples dried on filter papers from domestic chickens in a malaria endemic area, where the reliable identification of seven field isolates of *P. gallinaceum* was obtained with an accuracy of 100%. The analysis of *cytb* and *coxI* gene nucleotide sequences revealed the existence of at least two genetically distinct populations of *P. gallinaceum* in Thailand, both of which differed from the reference strain 8A of *P. gallinaceum*. In conclusion, the chelex-100 resin extraction method is a simple and sensitive method for isolating gDNA from whole avian blood dried on filter paper. Genomic DNA extracted by the chelex method could subsequently be applied for the PCR-based detection of *P. gallinaceum* and DNA sequencing. Our PCR assays provide a reliable diagnostic tool for molecular epidemiological studies of *P. gallinaceum* infections in domestic chickens and wild birds.

Abbreviations: *coxI*, cytochrome oxidase subunit I gene; *cytb*, cytochrome B gene; iRBCs, Plasmodium gallinaceum-infected red blood cells; PCR, polymerase chain reaction.

☆ *Note*: Nucleotide sequence data reported in this paper is available in the GenBank® database under the accession numbers KP025674 and KP025675.

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1. Introduction

The unicellular parasite *Plasmodium gallinaceum* is a causative agent of avian malaria and is one of the important protozoa pathogens of poultry production systems (Ruff, 1999). Avian malaria is a common cause of poor meat quality and poor egg production. Moreover, the disease is often fatal with a mortality rate of up to 80–90% (Permin and Juhl, 2002; Williams, 2005), and so it can contribute to substantial economic loss to the poultry industry. The disease is highly prevalent in tropical countries, with a high transmission rate in Southeast and South Asia (Garnham, 1966; Valkiunas, 2005). To prevent the spread of avian malaria diseases, it is critical to establish assays for parasite detection that are rapid, low-cost and reliable. The most commonly used method for avian malaria diagnosis is light microscopic examination of blood films, which is also an accepted practice for the diagnosis of human malaria infections (Warhurst and Williams, 1996). The major advantages of this technique are that it is low-cost and also preferred for an accurate estimation of the parasite load in infections (or parasitaemia). However, the reliability of the blood smear examination is also dependent on the quality of the microscope and staining reagents, and the skills of the technician (Ngasala et al., 2008). For accurate estimation of the parasite density, samples must contain a sufficient number of parasites. Microscopic examination of blood samples with low parasitaemia levels can be labor-intensive and time-consuming.

In addition, a clinical diagnosis is routinely employed in field studies. Clinical signs associated with avian malaria include a pale crest, green stools and lack of appetite (anorexia) (Williams, 2005), although these symptoms are not necessarily malaria-specific. The disease may run a very acute course and cause death within a week (Permin and Juhl, 2002). In addition, the exoerythrocytic stage development in the brain may also cause cerebral capillary blockage and the infected animals may die in the second or third week (Frevert et al., 2008; Macchi Bde et al., 2010), but it is not the same type of cerebral malaria as that produced by *Plasmodium falciparum* infection in humans. Nevertheless, some infected adult chickens may develop asymptomatic infections, resulting in misdiagnosis.

Various polymerase chain reaction (PCR)-based assays have been developed for diagnosis of human malaria parasites in the laboratory (Cordray and Richards-Kortum, 2012; Moody, 2002). These molecular methods are efficient for increasing the sensitivity and specificity of the malaria diagnosis. Thanks to the availability of genome sequences of *P. gallinaceum* deposited at the Wellcome Trust Sanger Institute website, this approach could be established and applied for the detection of avian malaria. A reliable high quality genomic (g)DNA preparation is needed to ensure high PCR performance. Generally, the most optimized methods for gDNA extraction use either fresh or frozen whole blood obtained by pricking a finger or using venipuncture. But, in epidemiological studies, the collection and storage of blood on filter papers is more practical (Chaorattanakawee et al., 2003; Singh et al., 1996). The collection of blood samples on filter papers has proven to be cheaper and this technique does not require refrigerated reagent storage. In the present study, our goals were to determine an optimal gDNA extraction method and to develop PCR-based assays for the detection of avian malaria from dried blood samples on filter papers. Two methods that are routinely used for the extraction of blood with human malaria from filter papers are phenol/chloroform extraction (Fortes et al., 2011; Scopel et al., 2004; Simpalipan et al., 2014) and chelex-100 resin extraction (Baidjoe et al., 2013; Bereczky et al., 2005; Gadalla et al., 2013; Hwang et al., 2012; Miguel et al., 2013; Ogouyemi-Hounto et al., 2013). This study was conducted to assess the performance of each method for preparation of *P. gallinaceum* gDNA from dried avian blood samples on filter papers.

Currently, mitochondrial genes have been extensively employed in PCR-based detections of the human malaria parasites, including cytochrome oxidase subunit I (*coxI*) and cytochrome B (*cytb*) (Aldritt et al., 1989; Haanshuus et al., 2013; Isozumi et al., 2015; Polley et al., 2010). The mitochondrial genome usually has higher copy numbers than the nuclear genome, and approximately, 15 haploid copies of the mitochondrial genome are found in *P. gallinaceum* (Joseph et al., 1989). Because these genes are also shown to be highly conserved, they are often used as targets for species identification and are useful for phylogenetic studies (Ekala et al., 2007; Farrugia et al., 2011; Perkins, 2008). Here, we developed two PCR-based assays for the amplification of *coxI* and *cytb* gene fragments to detect *P. gallinaceum* DNA from the whole avian blood samples dried on filter papers. Our assays would facilitate the rapid and reliable detection of *P. gallinaceum* and be useful for large-scale epidemiological surveys of *P. gallinaceum* infection levels in natural populations.

2. Materials and methods

2.1. Malaria parasite and avian host

The avian malaria parasite *P. gallinaceum* isolate Pg22/2012MU was a laboratory line, originally adapted from a field isolate from Chacheongsao province, Thailand. The avian hosts were female domestic chickens (*Gallus gallus domesticus*), infected at 4 weeks of age. The animals were purchased from a commercial hatchery. The origin and the maintenance of the parasite and the avian hosts were performed as described previously (Kumnuan et al., 2013). All experiments were performed in accordance with the approved animal study protocol (no. 1431065) and animal care & use regulations of Chulalongkorn University.

2.2. Preparation of P. gallinaceum infected blood on filter papers

Blood stage parasites of *P. gallinaceum* were collected from infected chickens. The infected blood was then mixed with uninfected blood to produce inoculums containing 10^4, 10^3, 10^2 and 10 infected red blood cells (iRBCs) per μl, equivalent to a 0.2%, 0.02%, 0.002% and 0.0002% parasitaemia, respectively. Then 10 μl of each blood inoculum was spotted onto DNase-free Whatman 3MM filter paper (Brentford, UK). Thus, the total numbers of the parasite