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Diagnosis of *Plasmodium gallinaceum* in Infected Mosquitoes by Multiplex PCR

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Abstract

The aim of this project was to use multiplex PCR as a detective reaction for *Plasmodium gallinaceum* in *Aedes aegypti* mosquitoes. Laboratory-reared female mosquitoes were divided into two groups–groups 1 and 2–and allowed to feed on the blood of parasitized and non-parasitized chickens, respectively. The engorged female mosquitoes were kept individually in vials at -20°C. The DNA was extracted from clotted blood from the mosquitoes' stomachs using QIAamp® Blood Mini Kit (Qiagen). Multiplex PCR was used to detect *P. gallinaceum* from DNA samples with two forward primers; FP1 (5' ACT TGA CCG ATT GTT CCT CAT CGC CTT T 3') and FP2 (5' AGT TCG TGA ATA TGA TTT GTC TGG T 3'), together with reverse primer RP1 (5' TTG TTG CCT TAA ACT TCC TTG TGT T 3'). From the experiment, the PCR condition 95°C for 30 sec, 55°C for 30 sec and 72°C for 3 min, gave the most satisfactory result. Two distinct bands of 279 and 1,527 bp were only shown in *P. gallinaceum*-positive samples. This technique, therefore, could be used for mosquito surveillance for avian malaria.

Keywords: *Plasmodium gallinaceum*, mosquito, multiplex PCR

Introduction

Avian malaria is caused by more than 20 species of *Plasmodium*, and the natural hosts of these parasites may be domestic chickens and jungle fowl. The Thai poultry industry faces a serious problem of avian malaria transmission from *Plasmodium gallinaceum* [1]. The disease could conventionally be diagnosed by a clinical diagnosis showing anemia, high fever, loss of body weight, and eventual death [2,3]. However, some infected chickens are asymptomatic, usually resulting in misdiagnosis [4]. The cheapest method used for the parasite screening has been microscopic examination of thin blood film. This method requires blood samples from suspected chickens, which may produce stress in broilers and reduce egg production in layers. Monitoring parasites in the mosquito vectors that feed on chickens may be

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an alternative approach for assessing *P. gallinaceum* infection on a farm, or for the early detection of infected vectors around clean farms, assist with planning preventive measures. In this study, a multiplex-PCR technique was developed for detecting P. *gallinaceum* in infected mosquitoes.

Materials and methods

Mosquito rearing

The *Aedes aegypti* (Liverpool strain) mosquitoes used in this study were reared in the Veterinary Science Insectarium, Chulalongkorn University, under 12:12 h dark:light cycle, at a temperature of 25°C and 80% relative humidity. Dechlorinated tap water was used to wet hay for 3 h before use for hatching mosquito eggs. Yeast extract, egg yolk, and liver were provided to encourage successful larval growth and metamorphosis. Mosquitoes were fed on 10% sucrose in water from moist cotton-bud sticks placed inside the cages. Prior to blood feeding, the sucrose solution was removed for 3 h. On the 6th day or earlier, the emergent adult mosquitoes were fed with 10% sucrose solution for 3 days and then used for further study.

Parasite and chicken preparation

The parasites used in this study were *P. gallinaceum* isolate NJTH39 (Nong Chok District, Bangkok, Thailand, 1995). Parasites, and parasitemia in the chickens, were maintained as described by Saiwichai *et al* [5]. Mosquitoes were allowed to feed on a 1%-parasitemic chicken for 5 min. From the same chicken, 1 ml blood was obtained (using heparin as anticoagulant) for comparison. The culture blood containing *P. falciparum* was provided by the Malaria Research Unit, Institute of Medical Research, Chulalongkorn University. The engorged mosquitoes were sucked up by air tube, transferred individually to 1.5 ml microcentrifuge tubes, then immediately frozen at -20°C, and stored until needed. The mosquitoes' stomachs were dissected under a light stereoscope on a clean glass slide in PBS solution (0.05 M Na_2HPO_4, 2 M NaCl, pH 7.4), then used for extracting DNA.

DNA preparation

The QIAamp DNA blood mini kits (QIAGEN®) was used to extract and purify total DNA from the blood samples, including blood samples from the dissected mosquitoes' stomachs, the infected chicken, the cultivated *P. falciparum* (K1 strain), and the uninfected chickens' blood, which served as a negative sample.

Primer design

The small subunit ribosomal RNA (SSUrRNA) sequences of birds were retrieved from GenBank (http://www.ncbi.nlm.nih.gov), including *P. gallinaceum* (infected chicken), *P. lophurae* (infected duck), and *P. relictum* (infected birds) with GenBank accession numbers M61723, X13706, and AF145399, respectively. The homologous sequences among three species of avian malaria parasites and *P. falciparum* (infecting man, accession number: M19172) were aligned using Clustal W multiple alignment program. The conserved regions of the gene were selected for genus-specific primer design. The variable regions were selected for *P. gallinaceum* species-specific primer design.

DNA amplification and PCR product evaluation

A multiplex-PCR technique was used to detect *P. gallinaceum* from the DNA samples, using two forward primers and a reverse primer. Amplification for 30 cycles was performed in a thermal cycler (TC-I; Perkin Elmer Cetus), with the following reaction mixture and PCR condition: 0.1 µM each forward primer, 0.2 reverse primer, 200 µM dNTP, 2.5 U/100 µl, 2.5 mM $MgCl_2$, and denaturation at 95°C for 30 sec; extension at 72°C for 3 min; annealing was conducted at different temperatures (45, 50, 55, and 60°C) for 30 sec.

The PCR products were resolved on agarose gel (1.5%) in TBE (0.045 M Tris-borate, 0.001 M EDTA) at 100 volts for 30 min, then stained with ethidium bromide (10 ng/ml) and visualized under UV transillumination.

Results

Reared *Ae. aegypti* mosquitoes normally became adults within 7 days. Female adult mosquitoes were fed on chicken blood on day 3 post-emergence, and some became engorged within 5 min after the chicken was placed inside the cage. The mosquito stomachs were easily dissected after freezing at -20°C. Using gel electrophoresis, the DNA extracted from mosquito stomach, engorged with chicken blood, were resolved and seen as a smear pattern (data not shown).

The primers, designed by alignment of the SSUrRNA gene (Fig 1), were 2 forward primers; FP1 (5′ ACT TGA CCG ATT GTT CCT CAT CGC CTT T 3′) and FP2 (5′ AGT TCG TGA ATA TGA TTT GTC TGG T 3′), and a reverse primer RP1 (5′ TTG TTG CCT TAA ACT TCC TTG TGT T 3′). The best DNA amplification condition was denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 3 min. Two distinct bands of 279 (*Plasmodium* genus-specific primers; FP2-RP1) and 1,527 bp (*P. gallinaceum* species-specific primers; FP1-RP1) were PCR products obtained from the same reaction (Fig 2). Gel electrophoresis of multiplex PCR products showed blood samples from the infected chicken and mosquito stomachs, with 2 bands of DNA (279 bp, and 1,527 bp), but the *P. falciparum* sample showed only the 279 bp band. On the other hand, the PCR product from the uninfected chicken-blood samples, mosquito stomach with uninfected chicken blood, and negative control, did not show these DNA bands.

Discussion

An adequate supply of yeast extract, egg yolk and liver was provided for the successful larval growth and metamorphosis of the *Ae. aegypti* mosquitoes. Most mosquitoes became adult within 7 days, as previously reported [6]. Mosquitoes were obtained from those that emerged on day 6 or earlier, because they were larger than those emerging on day 7. The adult females were fed 10% sucrose solution for 3 days,

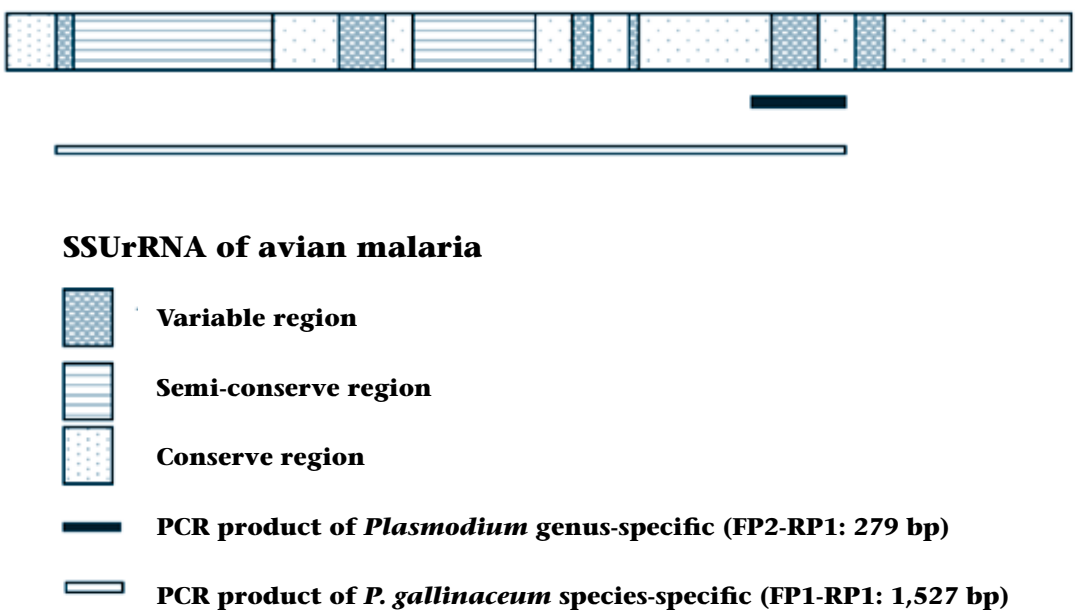

Fig 1 Schematic diagram illustrating primer design. The striated bar shows different parts of SSUrRNA (3): the dark fragment is generated from *Plasmodium* genus-specific primers; the light fragment is generated from *P. gallinaceum* species-specific primers.

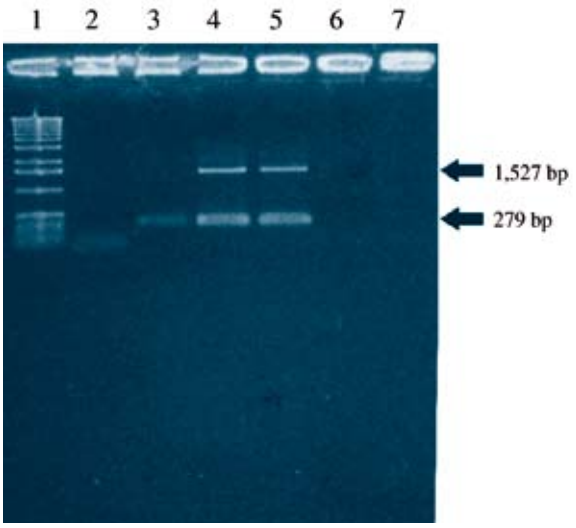

Fig 2 Gel electrophoresis of multiplex-PCR products from samples from mosquito, chicken, and *P. falciparum*: molecular weight marker (1 kb ladder), lane 1; uninfected chicken blood, lane 2; *P. falciparum* from human blood, lane 3; Pg-infected chicken blood, lane 4; mosquito with Pg-infected blood, lane 5; mosquito with uninfected blood, lane 6; negative control, lane 7.

to build strength. After emergence, they were fed with chicken blood on day 3. Engorged female mosquitoes were identified and captured within 5 min after the chicken was placed in the cage, indicating that the mosquitoes were thirsty since the sucrose solution had been removed before introducing the chicken. The mosquitoes thrust their mouthparts repeatedly through the host skin, searching for blood. *Ae. aegypti* thrust at 7-sec intervals [7].

Although microscopic examination of blood smear remains the diagnostic gold standard, the method is insufficiently sensitive and requires considerable expertise. In this study, *P. gallinaceum* was detected from various samples; genus and species levels could be diagnosed in the same reaction, indicating some improvement. A multiplex PCR for *P. gallinaceum* was developed from a set of generic primers targeting a highly conserved region of the 18S SSUrRNA gene of the genus *Plasmodium*. The primers were designed to be genus-specific; a variable region was sufficiently internally polymorphic to confirm design as a species-specific primer set [8]. In poultry coops in Thailand, fowl are sometimes suspended above large ponds, which are breeding places for mosquitoes; when mosquitoes finish feeding on the chickens, most of them rest on the walls of the chicken coop. The current study showed that blood samples in mosquito stomachs can be used for the detection of *P. gallinaceum*, indicating that multiplex PCR permits discrimination of infected from uninfected mosquitoes. It therefore constitutes a very efficient and powerful tool to improve our knowledge and surveillance of this parasite in mosquito vectors [9].

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