



Species-specific Nested PCR for Detecting *Plasmodium gallinaceum* in Fresh Chicken Blood

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Abstract

Avian malaria, epidemic in Thailand's chicken farms, is caused by *Plasmodium gallinaceum*. The standard method for detecting avian malaria is microscopic diagnosis, but parasites may go undetected in light infections. Therefore, the objective of this study was to detect *P. gallinaceum* DNA in infected fresh-blood samples by nested polymerase chain reaction (nested PCR). Primers were designed from small subunit ribosomal RNA (SSUrRNA) genes, with genus-specific primers for initial amplification, and species-specific primers for nested amplification. The PCR conditions were optimized using *P. gallinaceum* DNA as templates. The optimum conditions for the 1st and 2nd amplifications were different at the annealing stages, using 60 °C and 45 °C, respectively. The first amplification resulted in unobservable PCR products at 420 bp of the target fragment in all diluted blood samples. These products were continued in the 2nd amplification, when the species-specific 120 bp-DNA fragment of *P. gallinaceum* was found. This nested PCR condition was sensitive to detecting 0.0000085% parasitemia or 0.2 infected red cells/μl of the lowest blood dilution in the study. The results suggest that this assay had high sensitivity and species-specificity using whole blood samples without DNA extraction.

Keywords: *Plasmodium gallinaceum*, detection, nested PCR, whole blood

Introduction

Avian malaria, caused by *Plasmodium gallinaceum*, affects broilers and egg-layers in Thailand [1]. The conventional parasite-detection technique is microscopic diagnosis of Giemsa-stained blood smears. However, this may be

unsuitable for samples with low parasitemia (< 1% infection), where the detection of infected red blood cells can be difficult.

Molecular techniques, such as PCR and nested PCR, have been proven sensitive, specific, and useful for detecting low levels of malaria parasitemia [2,3]. Genes of malarial parasites have been utilized as molecular tools for parasite detection. Use of the small subunit ribosomal RNA (SSUrRNA) gene as a DNA target is another diagnostic advancement,

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since it leads amplification of both conserved (within the genus *Plasmodium*) and variable regions, thus representing species differentiation [4]. Based on specific primers for the *P. gallinaceum* gene, the PCR assay is a highly sensitive technique for detecting infected blood. However, further research is required to develop more specific tests among the *Plasmodium* species [5]. To date, nested PCR has proven more specific than PCR [6]. DNA normally needs to be extracted from samples for PCR detection, and this preparatory process takes time, making it inappropriate for routine work. Mercier *et al* have proved a simple and efficient method to amplify DNA directly by PCR from whole blood samples, without purification [7]. Hence, the potential and sensitivity of using fresh infected blood with *P. gallinaceum* in nested PCR reaction directly, without DNA extraction, was examined in this study.

Materials and methods

Malarial parasite and laboratory animal cultivation

CSTH43 (originating from Chachoengsao Province, Thailand) isolate of the parasite *P. gallinaceum* was used in the study. Parasite maintenance, monitoring and harvesting were conducted in three-week-old male chickens with the same breed and housing conditions. The *Pg*-positive blood samples from chickens with approximately 85% parasitemia were collected from the jugular vein and were heparinized, as previously described by Saiwichai *et al* [8].

DNA extraction

Blood controls were washed 3 times before extraction, as follows. Each 1.5 ml of heparinized blood control was centrifuged at 3,214g (Varifuge 20 RS; Heraeus Sepatech), at 4°C for 5 min. The supernatant was removed, and PBS solution (0.005 M Na₂HPO₄, 2 M NaCl, pH 7.4) was added until the same volume was reached. The samples were then gently mixed and centrifuged, as above. Supernatants were removed and the pellets obtained for DNA extraction. The *Pg*-positive and *Pg*-negative control DNA templates for PCR

reactions were obtained by extraction of the pellets using a QIAamp DNA Minikit (QIAGEN®), according to the manufacturer's instructions.

Blood dilutions

Heparinized blood from infected chickens was used to make a 10-fold blood dilution of 200 µl volume with PBS (20 µl blood : 180 µl PBS). The 7 blood dilutions, used to vary the DNA content, were 10, 10², 10³, 10⁴, 10⁵, 10⁶, and 10⁷ times. These samples were kept at 4°C and mixed gently before use as DNA templates in the nested PCR reaction. Uninfected blood (undiluted blood) was used as negative blood control.

Primer design

The small-subunit ribosomal RNA (SSUrRNA) sequences of avian malarial parasites were retrieved from GenBank (<http://www.ncbi.nlm.nih.gov>), including *P. gallinacuem* (*Pg* : M61723), *P. lophurae* (*Pl* : X13706), *P. juxtanucleare* (*Pj* : AF463547), *P. relictum* (*Pr* : AF145399) and *P. elongatum* (*Pe* : AF463506), which infect chickens, ducks, chickens, birds and ducks, respectively. To design the primers, all sequences underwent multiple alignments by ClustalW (<http://www.ebi.ac.uk/Tools/clustalw2/>).

Nested PCR and amplicon evaluation

Amplification was performed in a thermal cycler (GeneAmp® PCR system 2700, Applied Biosystems), for 25 cycles in the first amplification and 35 cycles in the second, with a reaction mixture of 0.2 µM of each primer, 2.5 mM MgCl₂, 2.5 U/100 µl DNA polymerase, and 200 µM dNTP. PCR conditions were: 30 sec denaturation at 95°C, 30 sec annealing at 45-60°C, and extension at 72°C. Five minutes were used for pre-denaturation and final-extension steps. The primer sets for the first amplification were PF and PR (outer primers); while the second amplification was NF and NR (nested primers). PCR products were evaluated by 1.5% agarose gel electrophoresis, as described by Saiwichai *et al* [9], and the sizes of the DNA fragments were calculated by comparison with a standard marker (100 bp DNA ladder: Promega).

Results

Several SSUrRNA sequences of avian malaria parasites, *P. gallinaceum*, *P. lophurae*, *P. juxtanucleare*, *P. relictum*, and *P. elongatum*, from GenBank database, were aligned using the ClustalW program (Fig 1). Conserved and polymorphic

regions were identified. Two conserved regions were selected and used for outer primer design, both forward and reverse primers, PF (1,468th to 1,492th nucleotide) and PR (1,885th to 1,909th nucleotide), respectively (Fig 2). Two highly polymorphic regions were chosen and used as

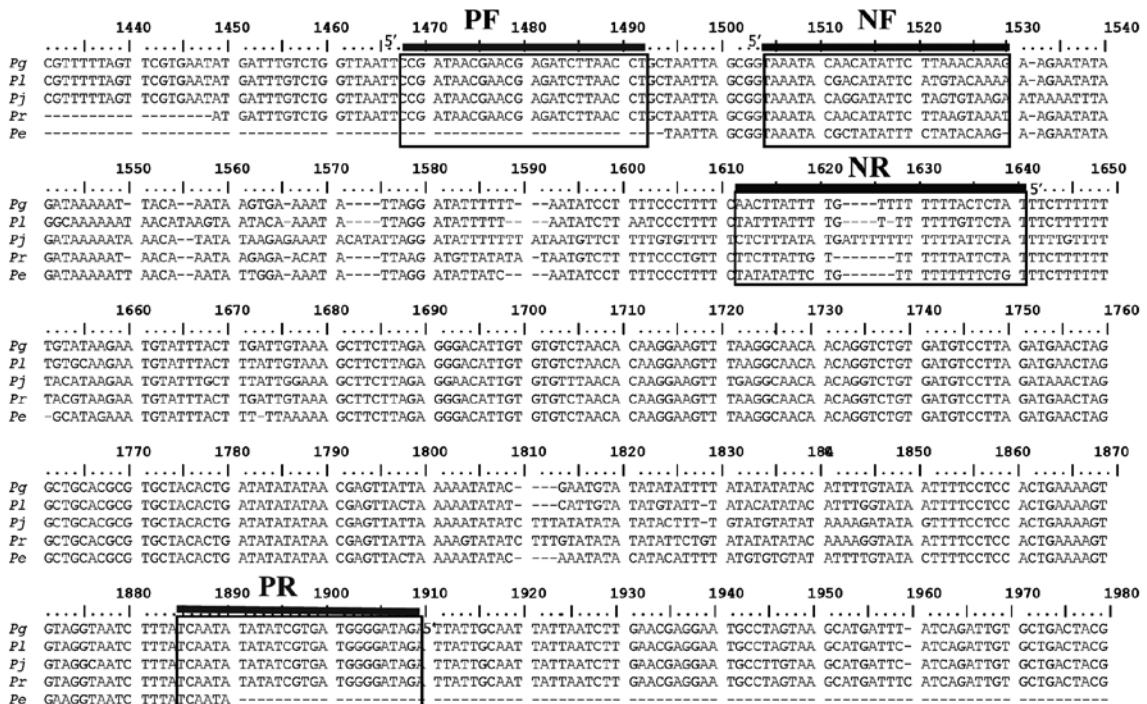


Fig 1 Multiple alignment of SSUrRNA sequences from several avian malarial parasites, and position of outer primers for the 1st amplification (PF and PR) and nested primers for the 2nd amplification (NF and NR). Pg = *P. gallinaceum*, Pl = *P. lophurae*, Pj = *P. juxtanucleare*, Pr = *P. relictum* and Pe = *P. elongatum*

Outer primers:

PF 5'	CCG	ATA	ACG	AAC	GAG	ATC	TTA	ACC	T	3'
PR 5'	CTA	TCC	CCA	TCA	CGA	TAT	ATA	TTG	A	3'

Nested primers:

NF 5'	TAA	ATA	CAA	CAT	ATT	CTT	AAA	CAA	AG	3'
NR 5'	ATA	GAG	TAA	AAA	AAA	CAA	AAT	AAG	TT	3'

Fig 2 Sequences of the designed primers; outer (1st amplification) and nested (2nd amplification).

Table 1 PCR products for outer and nested primers, tested at different annealing temperatures using *Pg*-DNA template.

Annealing temperature (°C)	Occurrence of PCR products with different primers	
	PF-PR	NF-NR
40	+	+
45	+	+
50	+	+
55	+	-
60	+	-

Table 2 Products from nested-PCR, detecting varied whole-blood samples.

10-fold blood dilutions: infected red blood cells (%)	Occurrence of PCR products
10 : 8.5	+
10 ² : 0.85	+
10 ³ : 0.085	+
10 ⁴ : 0.0085	+
10 ⁵ : 0.00085	+
10 ⁶ : 0.000085	+
10 ⁷ : 0.0000085	+

nested primers, both forward and reverse primers, NF (1,505th to 1,530th nucleotide) and NR (1,612th to 1,641th nucleotide) (Fig 2).

When we matched sequences in the BLAST program on the GenBank database, these two outer primer sequences (PF and PR) showed promising results, matching evenly with all 4 human, primate, and other malarial parasites (*P. yoelii*, *P. brasilianum*, *P. knowlesi*, *P. reichenowi*, *P. inui*, *P. simium*, *P. cynomolgi*, and *P. berghei*). BLAST revealed a complete match only with the *P. gallinaceum* SSUrRNA sequence (accession number M61723), when queries were conducted by NF and NR primer sequences.

Each pair of primers was optimized for PCR using *P. gallinaceum* DNA, which was extracted as a DNA template by amplifying at different annealing temperatures—40, 45, 50, 55, and 60°C. All annealing temperatures produced 420

bp PCR products for the first amplification using PF-PR primers, while 40, 45, and 50°C, were all optimum annealing temperatures for the second amplification by NF-NR primers, producing 120 bp PCR products (Table 1). The first amplification at 60°C, and the second at 45°C annealing temperature using *Pg*-extracted DNA as reaction template, were selected as optimal conditions in this study, and adopted for parasite detection in PBS-diluted, *Pg*-infected, fresh blood. The PCR products were unobservable from the first amplification with the outer primers (Fig 3), but revealed clear 120 bp bands in the second amplification with nested primers (Fig 4). Nested PCR sensitivity was also observed from different dilutions of infected blood samples (85% parasitemia) in this study. Nested PCR could detect the lowest dilution, 10⁷ (equal to 0.0000085% infected red blood cells), as shown in Table 2 and Fig 4.

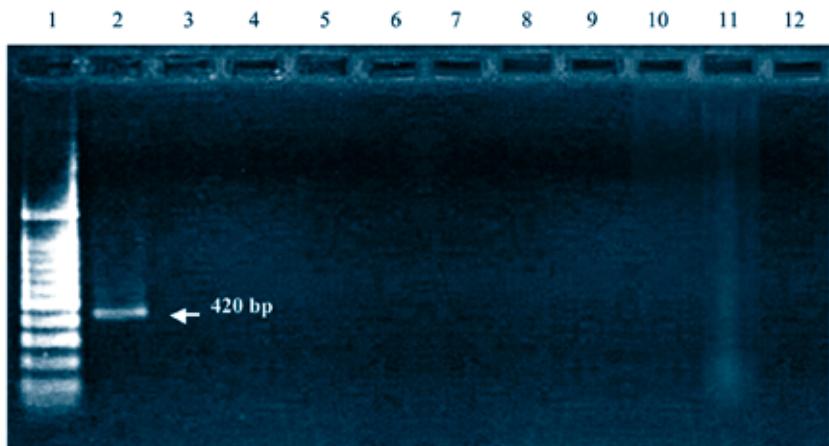


Fig 3 Agarose gel electrophoresis showing PCR products from different dilutions of *Pg*-positive fresh blood samples, detected by outer primers in the first amplification. Lane: 1 = DNA marker, 2 = positive control (*Pg* DNA), 3 = dilution 10^7 , 4 = dilution 10^6 , 5 = dilution 10^5 , 6 = dilution 10^4 , 7 = dilution 10^3 , 8 = dilution 10^2 , 9 = dilution 10, 10 = undiluted *Pg*-blood, 11 = uninfected whole blood, and 12 = negative control.

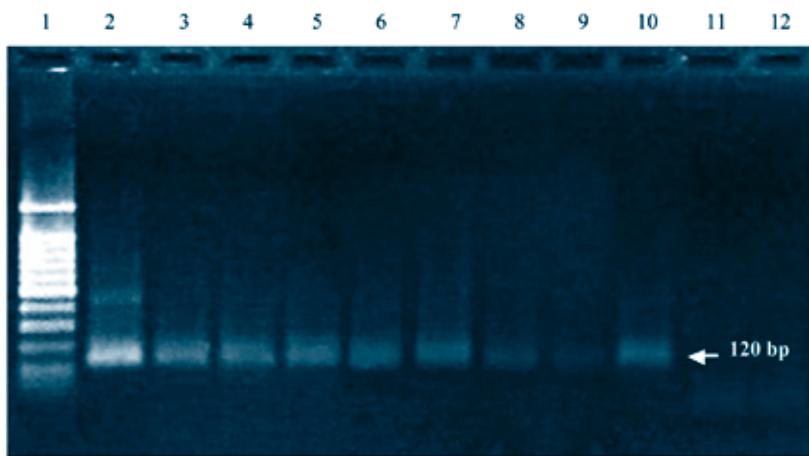


Fig 4 Agarose gel electrophoresis, showing PCR products from the 2nd amplification by nested primers, detecting DNA templates from the 1st PCR products. Lane: 1 = DNA marker, 2 = positive control (*Pg* DNA), 3 = dilution 10^7 , 4 = dilution 10^6 , 5 = dilution 10^5 , 6 = dilution 10^4 , 7 = dilution 10^3 , 8 = dilution 10^2 , 9 = dilution 10, 10 = undiluted *Pg*-blood, 11 = uninfected whole blood, and 12 = negative control.

Discussion

Since the outer primer sets PF and PR were designed from the conserved regions of many SSUrRNA sequences of several species of avian malarial parasites by running multiple alignments, the 1st amplification revealed PCR amplicons at all annealing temperatures. The primer data for base

length (base) : GC content (%) : Tm (°C) of PF, PR, NF and NR were 25:44:56, 25:36:53, 26:19:47, and 26:15:45, respectively. Our primer qualifications were as almost identical to the general notes on PCR primer design by Prezioso [10]. However, the outer primers did not show good results for all blood-sample dilutions when compared with the

P. gallinaceum DNA sample. This might be due to the very low malarial DNA content in each dilution and also contamination with the blood contents. The nested primers (NF and NR) were designed from variable regions specific for *P. gallinaceum*, confirmed by running BLAST in the GenBank database, which matched only with the SSUrRNA of this parasite. It seems that the nested primers are specific to *P. gallinaceum* DNA due to PCR products (unobservable) from the first amplification being specifically complemented with these primers. Then, the DNA target fragment, 120 bp, can be seen in all dilutions.

We also detected the specificity of this two-primer set using *P. falciparum* DNA as a template. The PCR product was found in the first amplification by the outer primers (specific for the genus *Plasmodium*), while no product was found in the second amplification by nested primers (specific for *P. gallinaceum*) with this DNA template (data not shown). Polymerase chain reaction (PCR) is a highly specific method for the detection of malaria [11]. Since PCR sample preparation is time-consuming, this experiment aimed to determine the possibility and sensitivity of using fresh blood as template. From our 10-fold dilution of *Pg*-infected blood, the lowest dilution that elucidated a positive product was 10^7 (0.0000085% parasitemia). One microliter of chicken blood contains $\sim 2.3 \times 10^6$ red cells [12], so that 0.0000085% is $[0.0000085 \times (2.3 \times 10^6)]/100$ equals 0.195, or about 0.2 infected red cell/1 μ l of fresh blood as PCR template, which can provide positive detection by nested PCR. The 85% parasitemia of infected blood of this experiment contained a high density of mature schizonts, and the occurrence of the lowest parasite numbers at 0.2 infected red blood cell may be caused by free merozoites, which are released quickly by very mature schizonts [13].

The detection of human malaria by nested PCR from DNA template can reproducibly detect a single parasite from 10 μ l of blood (0.000002% parasitemia, 5×10^6 RBC/ μ l of blood) [14]. Our study on avian malaria revealed that nested PCR can detect the parasite quickly by skipping the

DNA extraction step in low-density cases, such as 0.2 infected red cells/1 μ l of blood. Thus, a single avian malaria parasite per 5 μ l of blood may be adopted for efficient direct parasite detection. The results of this study can be used in endemic areas requiring numerous sample detections in fresh chicken blood.

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