

EVALUATION OF TWO NESTED PCR-BASED DIAGNOSTIC ASSAYS FOR *PLASMODIUM FALCIPARUM* INFECTION

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ABSTRACT

Objective: The majority of malaria cases and deaths are caused by *Plasmodium falciparum*. The rapid and accurate diagnosis is very important for malaria treatment and control. The aim of this study was to evaluate two nested polymerase chain reaction (PCR)-based methods (protocol A and protocol B) for *P. falciparum* infection, diagnosis in Thailand.

Methods: A total of 90 dried blood spot samples were investigated. The samples composed of *P. falciparum*-, *Plasmodium vivax*-infected blood and normal human blood samples. The microscopic examination was used as gold standard.

Results: The results showed the sensitivity of 100/83.33%, specificity of 100/100%, and accuracy of 100/94.44% for protocol A and protocol B, respectively. The analytical sensitivity of protocol A and protocol B was 0.625 and 6.25 parasites/ μ l, respectively. The comparison among microscopic examination, protocol A and protocol B by statistical analysis, found that they were not a significant difference. The agreements between each method were good. The kappa value between protocol A and protocol B was 0.87, protocol A and microscopy was 1.00, and protocol B and microscopy was 0.87.

Conclusion: The results demonstrated that protocol A should be used for further development of *P. falciparum* diagnosis in Thailand, especially in case of low parasitemia such as asymptomatic infection and for screening blood donors.

Keywords: Nested polymerase chain reaction, *Plasmodium falciparum*, Malaria, Thailand.

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INTRODUCTION

Malaria is a major public health problem worldwide, especially in tropical and subtropical areas. Approximately 3.2 billion people are at risk of malarial infection and deaths [1]. Malarial infection is a mosquito-borne disease caused by the protozoan parasite belonging to the *Plasmodium* genus, which human malaria consists of five species are *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi*. *P. falciparum* could result in a severe and complications in malaria patients, such as cerebral malaria [2]. In Thailand, several reports demonstrated that the *P. falciparum* has high rates of antimalarial drug resistance and it is a major of morbidity and mortality [3,4].

The accurate and early diagnosis of malaria is useful for treatment, prevention, and reducing malaria transmission. The gold standard of laboratory diagnostic method for malarial infection is based on microscopic examination of Giemsa-stained thick and thin blood smears. However, the limitations of microscopy are the requirement of skill and experience of the investigators. In addition, low sensitivity (100-200 parasites/ μ l of blood), time-consuming, and irregularity in species identification have been reported [5]. Hence, mixed infections have been found to misdiagnose. Alternative methods, such as rapid diagnostic tests (RDTs), were developed for detection of parasite-specific antigens in the blood malaria patients. RDTs based on detecting histidine-rich protein 2 (pfHRP2), an antigen-specific only *P. falciparum*, and non-falciparum malaria have been developed to target the conserved region of Plasmodium lactate dehydrogenase (pLDH) [6-8]. However, RDTs have low sensitivity for low-level parasitemia cases. Furthermore, they have shown the false-positive responses due to the persistence of the pfHRP2 antigen in the blood [9].

Polymerase chain reaction (PCR) has been extensively used for malaria diagnosis. Several PCR-based assays include conventional

PCR [10], multiplex PCR [11], loop-mediated isothermal amplification (LAMP) [12], nested PCR [13-16], semi-nested PCR [17], and real-time PCR [18]. PCR has shown to be good alternative methods, because of the high sensitivity and specificity. These methods could be used for identification of malaria parasite and could detect malaria parasites in mixed infections and submicroscopic parasitemia better than microscopic examination and RDTs [19,20]. Nested PCR is considered to be a sensitive and specific method for detecting the malaria parasite, and it is also valuable in epidemiological survey, as previously described [13]. Due to various primer sets were reported in various areas with varying sensitivity and specificity [13,21]. Hence, in the present study, we compared the sensitivity, specificity, and accuracy of two nested PCRs using light microscopic examination as the reference method. The two nested PCRs based on 18S ribosomal RNA (18S rRNA) gene, using two different primer sets for detecting *P. falciparum* in dried blood samples obtained from malaria patients.

MATERIALS AND METHODS

Blood sample collection

A total of 90 blood samples consisted of *P. falciparum*-infected blood (n=30), *P. vivax*-infected blood (n=30), and normal human blood (n=30). The samples were collected from Mae Hong Son, Tak, Kanchanaburi and Yala provinces, Thailand. Thick and thin blood films were prepared from each sample for Giemsa staining, 200 μ l of EDTA whole blood was dropped on Whatman filter paper, the dried blood spot (DBS) samples were stored in ziplock plastic bags at room temperature before transporting to a laboratory, and then, PCR determinations were assessed. The study was approved by the Ethics Committee of Rangsit University.

Microscopic examination

Both thick and thin blood films were stained with 3% Giemsa solution (Merck) for 40 minutes. Species identification and the parasite

density were examined by microscopy using the ×100 oil immersion objective lens. The parasite density (parasites/μl) was detected by counting 1,000 red blood cells from each positive thin blood film, and the number of red blood cell density was estimated as 5×10^6 for the parasites per microliter blood calculation (<https://www.cdc.gov/dpdx/diagnosticprocedures/blood/microexam.html>).

DNA isolation from DBSs

Parasite DNA was extracted from DBS, 200 μl of phosphate buffered saline (pH 7.2) was added in each sliced DBS for 10 minutes at room temperature, and then, 20 μl of proteinase K solution (25 mg/ml) was added. After that, DNA was extracted using blood DNA extraction kit, Fermentas Gene JET™ (Thermo Fisher Scientific), following manufacturer's instructions. Purified DNA was eluted in 50 μl of elution buffer and stored at -20°C for the molecular technique processing.

Nested PCR for *P. falciparum* detection

In this study, the *P. falciparum* amplification by a nested PCR assay from two different sets of primers based on 18S rRNA genes was investigated. The first nested PCR (protocol A) was performed as previously described by Snounou *et al.*, 1993 [13]. For the nest1 PCR reaction, 1 μl of DNA was used in a total volume of 20 μl; the reaction mixture contained 10×PCR buffer with MgCl₂, 125 μm of dNTP, 250 μm of each primer (Table 1), and 0.4 U of Taq DNA polymerase (i-Taq iNtRON Biotechnology/Korea). The PCR amplification conditions were as follows: Initial denaturation at 95°C for 5 minutes; 24 cycles of 94°C for 1 minute, 58°C for 2 minutes, and 72°C for 2 minutes with the final extension at 72°C for 5 minutes. A volume of 1 μl of PCR amplicon from nest1 PCR was used as DNA template for nest2 PCR. Conditions and PCR reaction used for the second amplification were identical to that of the nest1 PCR, except that second round PCR amplification was conducted over 30 cycles.

The second nested PCR (protocol B) was carried out with primers as previously described by Mahajan *et al.*, 2012 [21]. The PCR reaction was set up in a final volume of 25 μl containing of 1 μl of DNA template, 10×PCR buffer, 125 μm of dNTP, 0.4U Taq DNA polymerase, and 250 μm of each primer (Table 1). Cycling PCR conditions for both nest1 and nest2 amplifications were as follows: Initial denaturation at 94°C for 30 minutes; 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds, 20 and 35 cycles of nest1 and nest2 PCR, respectively, and the final extension at 72°C for 7 minutes.

In this study, distilled water was the negative control and DNA extracted from *P. falciparum* K1 culture was used as the positive control. The PCR products were determined by 1.2% agarose gel electrophoresis, visualized under ultraviolet light after staining with ethidium bromide.

Detection limit of nested PCR methods

Limit of detection of two nested PCRs (protocol A and protocol B) was performed to detect *P. falciparum* using the *P. falciparum* K1 culture (6.25×10^4 parasites/μl). The 10-fold serial dilution (10^{-1} - 10^{-6}) was performed to containing 6.25×10^3 , 6.25×10^2 , 62.5, 6.25, 0.625, and 0.0625 parasites/μl, and then, DNA was extracted from each dilution according to the protocol (Thermo Fisher Scientific). One microliter of each DNA sample was as used as DNA template for both nested PCR amplification. The limit of detection was the lowest number of

parasitemia that positive by PCR tested. In addition, DNA samples isolated from *P. falciparum* (6.25×10^2 parasites/μl), *P. vivax*, *P. malariae*, *P. ovale*, and human normal blood were also evaluated for the analytical specificity of both nested PCRs.

Statistical analysis

The evaluations of each test were performed with the results of microscopy as the gold standard. Sensitivity, specificity, positive and negative predictive value, and accuracy of the test were calculated using two-by-two tables. Kappa statistic was used for comparing the agreement against which might be expected by chance with ranged from 1 (perfect agreement), -1 (complete disagreement), and 0 indicated that no agreement.

RESULTS

Detection limit and cross-amplification of nested PCR

For the detection limit of nested PCR methods by protocol A and protocol using a DNA template extracted from *P. falciparum* K1 culture, the results demonstrated that the protocol A was more sensitive than protocol B. According to the protocol A could detect *P. falciparum* K1 culture as low as 0.625 parasite/μl, whereas the protocol B could amplify at 6.25 parasite/μl. Both nested PCR methods amplifying of 18S rRNA gene of *P. falciparum* showed the PCR product size of 205, 287 bp for protocol A and B, respectively.

In this study, we determined the specificity of both nested PCR methods using the DNA of other *Plasmodium* spp. From clinical samples (*P. vivax*, *P. malariae*, and *P. ovale*), *P. falciparum* K1 culture and human normal blood. Both nested PCR targeting the 18S rRNA gene could amplify only the DNA of *P. falciparum* K1 culture and showed no cross-amplification with other parasites and human normal blood DNA.

Diagnostic sensitivity and specificity of nested PCR to detect *P. falciparum* in clinical samples

A total of 90 DBS samples in the present study were obtained from blood of 60 patients who diagnosed with clinical symptoms, and microscopy from blood film that was confirmed with 30 samples of *P. falciparum* and 30 samples of *P. vivax* infections and 30 samples of human normal blood. Microscopic examination showed the average of parasitemia of 364.29 parasites/μl (range 1.08-2183 parasites/μl) for the *P. falciparum* infection, and parasitemia in *P. vivax* infection was 30.80 parasites/μl (range 0.0017-172.5 parasites/μl).

Table 2 shows the sensitivities, specificities, and positive predictive values (PPV) and negative predictive values (NPV), and accuracies of two nested PCR methods compared with the microscopic examination to detect *P. falciparum* in clinical specimens. The protocol A showed 100% of the high diagnostic sensitivity, specificity, PPV, NPV, and accuracy. In addition, the diagnostic sensitivity and specificity of protocol B were 83.33% and 100%, respectively. Moreover, the tests of PPV, NPV, and accuracy for protocol B were 100%, 92.31%, and 94.44%, respectively. When protocol A and protocol B were compared, the protocol A displayed more sensitivity of detection than the protocol B. 25 samples were detected by nested PCR using protocol B with showing 5 samples false negative. In addition, the comparison between microscopy, protocol A, and protocol B through statistical analysis was not different at the significant value ($p < 0.05$). The measure agreement

Table 1: Sequences of the primers used in this study

Methods	Primer sets	Primer sequence (5'-3')	References
Protocol A	rPLU5	CCT GTT GTT GCC TTA AAC TTC	[13]
	rPLU6	TTA AAA TTG TTG CAG TTA AAA CG	
	rFAL1	TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT	
	rFAL2	ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC	
Protocol B	pPIF1	GAA CGA GAT CTT AAC CTG CTA A	[21]
	pPIrR1	TCA GCA CAA TCT GAT GAA TCA T	
	Pf-F	ACA TAG GTA ACT ATA CAT TTA TTC AGT	
	Pf-R	AGC ATC AAA GAT ACA AAT ATA AGC A	

Table 2: The diagnostic performance of two nested PCR methods to detect *P. falciparum* compared to the microscopic examination

nPCR	Microscopy		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
	Pf+	Pf-					
Protocol A							
Pf+	30	0	100	100	100	100	100
Pf-	0	60					
Protocol B							
Pf+	25	0	83.33	100	100	92.31	94.44
Pf-	5	60					

nPCR: Nested polymerase chain reaction, Pf+: *Plasmodium falciparum* positive, Pf-: *Plasmodium falciparum* negative, PPV: Positive predictive value, NPV: Negative predictive value

of kappa values showed good agreements of 1.00, 0.87, and 0.87 for protocol A versus microscopy (Table 3), protocol B versus microscopy (Table 4), and protocol A versus protocol B (Table 5), respectively.

DISCUSSION

Microscopy is known to be the gold standard for malaria detection. However, this method has low specificity and sensitivity. Moreover, misdiagnosis of mix parasite infection has been reported [22]. Currently, the detection of *P. falciparum* infection has required the method that has high sensitivity and specificity, especially for a screening of the asymptomatic patients and blood donation [21]. PCR has been developed to detect the malaria parasite for increasing the sensitivity and specificity, including semi-nested PCR [17], nested PCR [13,23,15], and real-time PCR [18].

This study was conducted to evaluate the efficacy of nested PCR for detection of *P. falciparum* in Thailand, which protocol A as described by Snounou *et al.*, 1993 [13], while the protocol B was carried out as described by Mahajan *et al.*, 2012 [21]. The result of this study indicated that the nested PCR protocol A was more efficacy and superior for detection of *P. falciparum* over protocol B. The detection limit of this protocol was good and similar as found in previous nested PCR studies [5,23-25].

While both nested PCRs of protocol A and B used the same samples and DNA extraction method and were tested under appropriate conditions with the same reagents. Nested PCR of protocol A showed that it is useful for detecting asymptomatic infection, which is difficult to detect by microscopy because of low parasitemia [26].

In the present study, 5 samples were unable to detect *P. falciparum* by nested PCR protocol B in *P. Falciparum* positive by microscopy, but the nested PCR protocol A could be detected. This may be an effect of low malaria parasite presented in the samples, and it may be possible that the DNA templates of these 5 samples have some sequence variation, so the primer was not annealed with the DNA templates. In addition, several studies were described inconsistencies in malaria positive when reaction was performed using the nested PCR including lower of DNA yield from blood spot and presence of blood inhibitors such as hemoglobin, hem, immunoglobulin G, and lactoferrin [27,28].

The results revealed that the protocol A had a high sensitivity and specificity. Our result was similar to that of a previous report that used the nested PCR as described by Snounou *et al.*, 1993 [13], mentioned by Anthony *et al.*, 2013 [14]. A study reported by Li *et al.*, 2014 [15], demonstrated that the nested PCR using blood filter paper samples had more sensitivity than microscopy for detecting *Plasmodium* infections. A report by Yentur Doni *et al.*, 2016 [16], revealed that nested PCR has 100% sensitivity and 97.2% specificity.

Moreover, the present study was able to detect mixed infections (*P. falciparum* and *P. vivax*) in one sample by both nested PCRs, which were positive only *P. vivax* detected by microscopy. This suggested that it is possible for microscopic misdiagnosis occurs due to the dominance of one species over the other species [29,30].

Table 3: Comparison between nested PCR protocol A and microscopy results

Microscopy	PCR-protocol A		Total	Kappa value/p value
	Positive	Negative		
Positive	30	0	30	1.000/0.000
Negative	0	60	60	
Total	30	60	90	

PCR: Polymerase chain reaction

Table 4: Comparison between nested PCR protocol B and microscopy results

Microscopy	PCR-protocol B		Total	Kappa value/p value
	Positive	Negative		
Positive	25	5	30	0.870/0.000
Negative	0	60	60	
Total	25	65	90	

PCR: Polymerase chain reaction

Table 5: Comparison of two nested PCR (protocol A versus protocol B) results

PCR-protocol A	PCR-protocol B		Total	Kappa value/p value
	Positive	Negative		
Positive	25	5	30	0.870/0.000
Negative	0	60	60	
Total	25	65	90	

PCR: Polymerase chain reaction

In conclusion, we demonstrated that nested PCR by protocol A was sensitive and specific to diagnose malarial infection caused by *P. falciparum*, especially for submicroscopic infections. Furthermore, both nested PCRs can also be used in mixed infection detection and could be improved for screening blood donation. Due to its high sensitivity, this method could be useful for the epidemiological studies of malarial infection in Thailand.

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