IDENTIFICATION OF HUMAN MALARIA PARASITES AND DETECTION OF MIXED INFECTION IN THAI PATIENTS BY NESTED PCR

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Abstract. The species-specific nested PCR previously described by Snounou and others, for detecting the four species of human malaria parasites, is evaluated in the current study testing 40 blood samples from malaria patients admitted during July-September, 2003, at the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Thailand. Parasite DNA of each blood sample was extracted and purified by QIAamp, DNA mini kit. Nested PCR was performed using genus-specific primers for the first PCR cycle and species-specific primer for the second cycle. Thin and thick smears were also made, stained with Giemsa, and examined by expert microscopists. Only one of 40 samples (2.5%) was identified as *Plasmodium malariae* infection by both microscopy and nested PCR. Twenty blood samples (50%) were identified as *Plasmodium falciparum* infections by both methods. However, 19 blood samples (47.5%) were reported as *Plasmodium vivax* infections by microscopic methods, whereas nested PCR could detect a mixed infection of *Plasmodium vivax* and *Plasmodium falciparum* in one sample taken from a young girl with 8 ameboid trophozoites of *P.vivax* per 200 white blood cells. These results demonstrated that the nested PCR assay surpasses microscopy and also offers a clear advantage in the detection of mixed infections, which is important not only for successful medical treatment, but also for the study of malaria epidemiology.

INTRODUCTION

Approximately 300 million people worldwide are affected by malaria and between 1 and 1.5 million infected people die every year. Previously extremely widespread, malaria is now mainly confined to Africa, Asia, and Latin America (Anonymous, 2003). Thailand is situated in Southeast Asia and lies in the heart of tropical monsoon forests which present a diversity of overlapping ecological niches and the most efficient malaria vector. The incidence rate of malaria is 3.5/1000 population/year (Kalra and Orlov, 1992). However, the core problem is in forested rural areas along the borders, especially with Myanmar and Cambodia, where multidrug-resistance, difficult terrain, security problems, highly mobile populations, and the presence of efficient vectors, are prominent features (Wernsdorfer, 1994). The predominant parasite species is *Plasmodium falciparum*, with peaks higher than *Plasmodium vivax* (Zahar, 1996). Accurate diagnosis is clearly essential for successful treatment.

Microscopy has historically been the mainstay for diagnosing malaria. A clinical diagnosis of malaria currently depends on visualization of parasites by light microscopy of Giemsa-stained thick and thin blood smears. This procedure is cheap and simple, but it is labor-intensive and requires personnel who are well-trained in the morphological differentiation of the *Plasmodium* species (Payne, 1988). When parasite levels are very low, and in detection of mixed infections, the information obtained by microscopy is restricted. Polymerase Chain Reaction (PCR) technology has been developed to defeat this limitation leading to proper treatment, and increasing our knowledge and understanding of this disease (Tirasophon *et al*, 1991; Barker *et al*, 1992).

In this study, we evaluated species-specific nested PCR previously described by Snounou *et al (*1993a) for detecting the four species of human malaria parasites in blood samples obtained from malaria patients admitted to the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Thailand.

MATERIALS AND METHODS

Sample collection and microscopic examination

Blood samples were taken from forty patients aged 16-50 years, admitted July–September, 2003 at the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Thailand. This study

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was approved by the Ethics Committee of Faculty of Tropical Medicine, Mahidol University and informed consent was obtained from all patients. Blood films and DNA extraction were prepared from venous blood collected in EDTA tubes. Both thick and thin blood films were stained with Giemsa and analyzed for the presence of parasites and parasite species by expert microscopists. Parasites were quantified by counting the number of parasites per 1,000 red blood cells in a thin blood film and counting the number of parasites per 200 white blood cells in a thick blood film.

DNA preparation

For PCR assay, parasite DNA was extracted and purified by QIAamp® DNA mini kit. Two hundred µl of packed red cells were added to 20 µl proteinase K and 200 µl lysis buffer, immediately vortexed for 15 seconds. After incubation for 10 minutes at 56 ˚C, DNA was precipitated with 200 µl absolute ethanol. The mixture was applied to a QIAamp® spin column and after centrifugation, the column was washed with 500 µl Buffer AW1 and Buffer AW2, respectively. DNA was eluted with 200 µl Buffer AE and kept at -20 ˚C until PCR assays were performed.

Primers

Four sets of oligonucleotide primers were used in this study. Primer set rPLU6 (5′ TTAAAATTGTT GCAGTTAAAACG 3′) and rPLU5 (5′ CCTGTT GTTGCCTTAAACTTC 3′) is genus specific and was used to detect the presence of the *Plasmodium* parasite. The falciparum species specific primers were FAL1 (5′ TTAAACTGGTTTGGGAAAACCAAATA TATT 3′) and FAL2 (5′ ACACAATGAACTCAA TCATGACTACCCGTC 3′), the species-specific for *P.vivax* were VIV1 (5′ CGCTTCTAGCTTAATCCA CATAACTGATAC 3′) and VIV2 (5′ ACTTCCAAGC CGAAGCAAAGAAAGTCCTTA 3′), and the species-specific for *P.malariae* were MAL1(5′ ATAACATAGTTGTACGTTAAGAATAACCGC 3′) and MAL2 (5′ AAAATTCCCATGCATAAAA AATTATACAAA 3′) (Snounou *et al,* 1993a).

Nested PCR assay

Detection of PCR was performed as a two-step procedure. Firstly, amplification of *Plasmodium* genus-specific fragment was carried out as follows; PCR mixture containing 5 µl of PCR buffer containing Tris HCl, KCl, Mg_2Cl (Amersham®), 125 µM of each of the four dNTPs, 0.4 U Taq DNA polymerase (Amersham,), 250 nM of each oligonucleotide primer. All PCR reactions were carried out in a total volume of 20 µl. One µl of the purified template DNA was used for the first reaction, in which the fragment spanned by rPLU5 and rPLU6 is amplified. Secondly, a 1 µl aliquot from the product of the first PCR reaction was then used as a template for amplification of the *Plasmodium* species-specific fragment.

Duplex PCR was performed using both sets of primers specific to *Plasmodium falciparum* and *Plasmodium vivax* in one reaction. The duplex PCR reaction was modified by slightly decreasing each oligonucleotide primer (VIV1,VIV2 and FAL1,FAL2) to 200 nM and increasing the volume of the first PCR product to 1.5 µl.

The cycling parameters for the first amplification reaction were as follows: step1, 95˚C for 5 minutes; step 2, annealing at 58˚C for 2 minutes; step 3, extension at 72˚C for 2 minutes; step 4, denaturation at 94˚C for 1 minute; repeat 24 times for steps 2-4, then step 2, and finally step 3 for 5 minutes. The amplification cycle was stopped by reducing the temperature to 20˚C. Species-specific amplification was performed at the same as the first step PCR reaction, except 30 PCR cycles were performed (Snounou *et al,* 1993a).

Detection of PCR products

PCR products were detected by running 20 µl of DNA product on 2.5% agarose gel, which was subsequently stained with a 0.5 µg/ml ethidium bromide solution and visualized under ultraviolet transillumination.

RESULTS

Of the forty patients, 34 (85%) were male and 6 (15%) female, aged 16-50 years. Approximately 62.5, 30, and 7.5% of cases were from Tak, Kanchanaburi, and the north of Thailand, respectively. Parasitemia ranged from 0.1 to 2.6%, with one case of *Plasmodium falciparum* >10% (Table1).

All samples were identified as infected with malaria by blood film analysis. Only one sample (2.5%) was microscopically identified as *Plasmodium malariae* infection; 20 blood samples (50%) were identified as *Plasmodium falciparum* infection, and 19 (47.5%) were reported as *Plasmodium vivax* infection.

The specific size of the PCR product was obtained from each *Plasmodium* species, as follows; 205 bp for *Plasmodium falciparum,* 120 bp for *Plasmodium vivax,* and 144 bp for *Plasmodium malariae,* respectively (Fig 1).

The diagnoses obtained by nested PCR correlated closely with those obtained by Giemsa staining, except

Table1
Comparison of Giemsa staining with nested PCR assay for detection of *Plasmodium* infection in Thai patients.

Sample No.	Sex	Age	Positive by microscopic method			Positive by nested PCR
			Species	Number of parasites in a thin film/100 RBCs	Number of parasites in a thick film/200 WBCs	
1	M	20	*P.vivax*	0.1	12	*P.vivax*
2	M	18	*P.vivax*	0.3	-	*P.vivax*
3	M	45	*P.vivax*	-	22	*P.vivax*
4	M	21	*P.falciparum*	0.6	-	*P.falciparum*
5	M	26	*P.falciparum*	0.2	7	*P.falciparum*
6	M	50	*P.vivax*	0.2	25	*P.vivax*
7	M	25	*P.vivax*	0.3	10	*P.vivax*
8	M	28	*P.falciparum*	1.2	-	*P.falciparum*
9	M	23	*P.falciparum*	-	26	*P.falciparum*
10	M	24	*P.vivax*	0.1	9	*P.vivax*
11	F	-	*P.malariae*	-	44	*P.malariae*
12	M	18	*P.vivax*	0.4	-	*P.vivax*
13	F	18	*P.vivax*	-	50	*P.vivax*
14	M	22	*P.falciparum*	0.2	3	*P.falciparum*
15	M	25	*P.falciparum*	1.1	-	*P.falciparum*
16	M	23	*P.falciparum*	-	13	*P.falciparum*
17	M	29	*P.falciparum*	0.3	-	*P.falciparum*
18	M	30	*P.falciparum*	0.2	-	*P.falciparum*
19	M	31	*P.falciparum*	0.3	2	*P.falciparum*
20	F	28	*P.falciparum*	0.5	2	*P.falciparum*
21	M	21	*P.falciparum*	0.4	2	*P.falciparum*
22	M	40	*P.falciparum*	2.3	-	*P.falciparum*
23	F	17	*P.vivax*	0.2	5	*P.vivax*
24	F	40	*P.falciparum*	1.7	-	*P.falciparum*
25	M	22	*P.vivax*	0.3	4	*P.vivax*
26	M	23	*P.falciparum*	0.1	-	*P.falciparum*
27	M	22	*P.vivax*	0.3	-	*P.vivax*
28	M	26	*P.vivax*	-	16	*P.vivax*
29	M	18	*P.vivax*	0.8	-	*P.vivax*
30	M	21	*P.falciparum*	2.6	-	*P.falciparum*
31	M	24	*P.falciparum*	0.4	0.1	*P.falciparum*
32	M	27	*P.vivax*	-	3	*P.vivax*
33	M	19	*P.vivax*	0.3	11	*P.vivax*
34	M	24	*P.vivax*	-	26	*P.vivax*
35	M	20	*P.vivax*	0.2	37	*P.vivax*
36	M	18	*P.vivax*	-	15	*P.vivax*
37	M	20	*P.falciparum*	0.7	14	*P.falciparum*
38	M	20	*P.falciparum*	1.4	2	*P.falciparum*
39	M	24	*P.falciparum*	10.4	2	*P.falciparum*
40	F	16	*P.vivax*	-	8	*P.vivax and P.falciparum*

that one vivax sample taken from a young girl with 8 ameboid trophozoites of *Plasmodium vivax* per 200 white blood cells by microscopy, showed mixed infection with *Plasmodium falciparum* (Fig1).

Mixed infection of *Plasmodium falciparum* and *Plasmodium vivax* was successfully detected by the duplex PCR developed in this study. The result showed the usefulness of duplex PCR, which was able

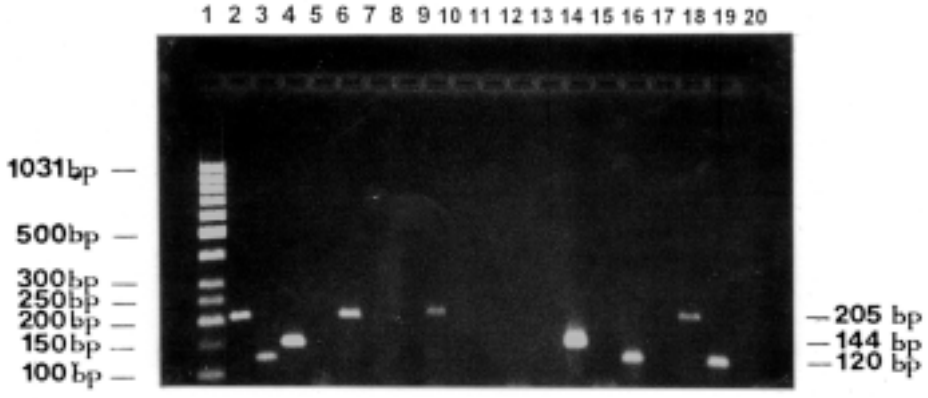

Fig 1- Nested PCR products obtained from malaria patients using species-specific primer pairs for *P. falciparum, P. vivax, P. malariae*. lane 1: marker is 50 bp ladder; lanes 2-4: positive control of *P. falciparum, P. vivax, P. malariae,* respectively; lane 5: negative control; lanes 6-8: *P. falciparum* sample; lanes 9-11: *P. falciparum* sample; lanes 12-14: *P. malariae* sample; lanes 15-17: *P. vivax* sample; lanes 18-20: mixed infection of *P. vivax* and *P. falciparum*.

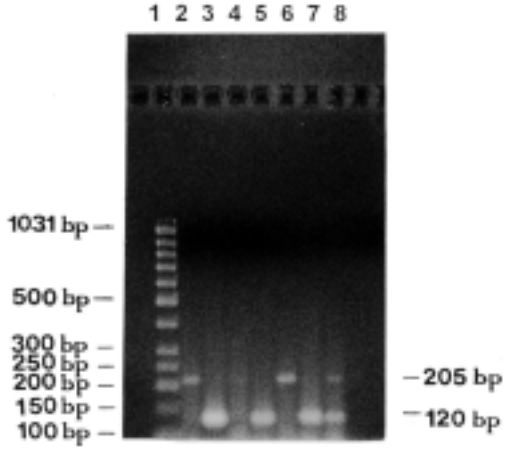

Fig 2- Duplex PCR product obtained from malaria patients by including species-specific primer pairs of *P. falciparum* and *P. vivax* in one reaction. lane 1: marker is 50 bp ladder; lane 6: *P. falciparum* - positive control; lane 7: *P. vivax*-positive control; lane 8: mixed infection of *P. vivax* and *P. falciparum*.

to identify mixed infection in one PCR assay tube (Fig2).

DISCUSSION

The results obtained by nested PCR assay were equivalent or superior to those obtained by microscopy. All microscopy-positive samples were positive by nested PCR, but nested PCR could reveal mixed infection that was not detected by microscopy. It may arise from vivax species being dominant over falciparum species. A diagnosis in which *P. falciparum* is missed is potentially of grave consequence to the patient, especially in Thailand, where *P.vivax* infections are usually treated with chloroquine, an anti-malarial drug to which the local *P.falciparum* parasites are highly resistant (Snounou *et al*, 1993b). The detection of mixed infection is important not only for successful medical treatment, but also for ascertaining the true incidence of each species and consequently its transmission potential. Many studies have demonstrated the greater sensitivity and specificity of PCR compared with thick blood films. The detection of low *P.vivax* and *P. falciparum* parasitemia by PCR at levels undetectable by microscopy, has been reported by Snounou *et al* (1993a) and Zakeri *et al* (2002). So, nested PCR using the 18 SSrRNA gene of human plasmodium is useful for the diagnosis of low parasitemia. The PCR is a superlative tool for obtaining accurate diagnosis, which is essential for correct treatment.

Moreover, the common mixed infection of *P. falciparum* and *P.vivax* found in Thailand can be easily detected using duplex PCR, and it accelerates the molecular diagnosis of malaria.

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