

Molecular screening of *Plasmodium* infections among migrant workers in Thailand

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ABSTRACT

Background & objective: A cross-sectional study was conducted to determine the prevalence of *Plasmodium* infections among migrant workers in Thailand.

Methods: A total of 241 migrants at Kanchanaburi, Pathumthani and Nakornpathom provinces of Thailand were recruited in our surveillance. Blood samples were examined for human malaria parasites by using microscopy and semi-nested multiplex PCR (SnM-PCR).

Results: Laboratory diagnosis revealed 6.2% total positive rate. As compared to microscopy (26.7%), SnM-PCR was more sensitive (93.3%) for malaria. *Plasmodium falciparum* was predominant than *P. vivax* (53% : 40%, respectively). The majority of positive cases were from Myanmar workers who had low parasitaemia and without symptoms. The highest prevalence (13.7%) was found among migrant workers from Kanchanaburi province in western Thailand.

Conclusion: These findings indicate risk of malaria transmission from migrant workers. Malaria surveillance should be included in the health-screening program for migrants in Thailand to manage this health risk.

Key words Malaria; migrant workers; semi-nested multiplex PCR; Thailand

INTRODUCTION

Human malaria mainly caused by four species of *Plasmodium* (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*) affects approximately 350–500 million people worldwide annually¹. It is a major public health problem in tropical and subtropical areas, particularly in developing countries in Southeast Asia region². High rate of malaria transmission with low parasitaemia in these areas is relevant to asymptomatic malaria carriers³. In Thailand, the increase of immigration from Thailand-Myanmar and Thailand-Cambodia borders where malaria is endemic has been a significant health risk in migrant workers and Thai people⁴. Lack of epidemiological data, especially the prevalence of malaria among foreign migrant workers, the impact of the situation on public health could not be determined. Until now, laboratory screening for *Plasmodium* infections has not been included in the health-screening program for migrants in Thailand^{5,6}.

Although the most economic, preferred, and reliable diagnosis of malaria is microscopic examination of thick and thin blood films, but this method is labour-intensive, time consuming, and depends on the expertise of the microscopists^{7,8}. Due to these limitations, several polymerase chain reaction (PCR)-based methods, including the semi-nested multiplex PCR (SnM-PCR), have been used for

malaria detection^{9–11}. Various reports have shown that PCR was more sensitive and specific than microscopy, particularly in cases with low parasitemia¹² but false negative results detected by PCR have occasionally been reported¹³. Two primers, which are specific to the small subunit of human ribosomal gene, were used as a positive amplification control in each sample to prevent false negatives in the SnM-PCR¹¹. Therefore, both microscopy and SnM-PCR based malaria prevalence rate may provide helpful information for risk management.

The objective of this study is to determine the prevalence of *Plasmodium* infection among migrant workers in Thailand by two laboratory diagnostic methods; the semi-nested multiplex PCR (SnM-PCR) and microscopy. Performance characteristics of the SnM-PCR for the detection and identification of *Plasmodium* parasites as compared to microscopy are also evaluated in this study.

MATERIAL & METHODS

Study areas and blood sample collection

A cross-sectional survey was conducted in three provinces of Thailand, namely Kanchanaburi, (14° 01' 09" North, 99° 31' 47" East) a province in western Thailand and Pathumthani (14° 1' 0" North, 100° 32' 0" East) and Nakornpathom (13°48' 58" North, 100° 50" East) lo-

cated in the central part of Thailand. The study was approved by the Ethics Committee of the Faculty of Medicine Technology at Rangsit University, Thailand. Blood samples were collected in EDTA anticoagulant tubes from 241 foreign migrant workers who attended the health-screening program for migrants in Thailand between June and July 2009. Thin and thick blood films were prepared for Giemsa staining and 3–5 drops of blood was spot on filter paper and kept in zip-lock plastic bags for PCR amplification.

Microscopy

Two thin and two thick blood films were stained with 3% Giemsa solution (Merck) for 30 min and microscopically examined at $\times 1000$ for species identification and the parasite density by two well-trained microscopists. The parasite density (parasites/ μ l) was done by counting 500 white blood cells from each positive thick blood film and the number of leukocyte density (WBCs/ μ l) was estimated as 8000⁷. Each blood film required approximately 20 min to read.

$$\text{Parasitaemia} = \frac{\text{No. of parasites}}{\text{No. of leucocytes}} \times 8000$$

Extraction of parasite DNA and PCR amplification

DNA was extracted by the Chelex method with minor modification¹⁴. The DNA extract was stored at -20°C until

used as the PCR template. The semi-nested multiplex PCR for malaria diagnosis was performed in a TECHNE TC-3000 Thermalcycler (Chemoscience, USA) as described previously¹¹. The amplification involved two rounds of PCR that started with initial denaturation at 94°C for 5 min, followed by PCR cycle conditions as summarized in Table 1, and ended with extension at 72°C for 10 min.

A total of 50 μ l of the first reaction mixture contained (1 \times) PCR buffer with 1.5 mM MgCl_2 (Invitrogen, USA), 200 μ M of dNTP (Invitrogen, USA) containing each of dATP, dCTP, dGTP, dTTP the PCR primers, 2.5 U of *Taq* DNA Polymerase (Intron, Korea), and 1 μ l of DNA template. The amounts of three primers used in Nest 1 were 25 pmol for UNR, 25 pmol for PLF, and 1.25 pmol for HUF.

The second reaction consisted of (1 \times) PCR buffer with 1.5 mM MgCl_2 , 200 μ M of dNTP containing each of dATP, dCTP, dGTP, and dTTP, 1 U of *Taq* DNA Polymerase, the PCR primers and 3 μ l of 1:10 diluted first reaction DNA. The concentrations of five primers used in Nest 2 were 100 pmoles for PLF, 60 pmoles for FAR, 12.48 pmoles for MAR, 25 pmoles for OVR, and 10 pmoles for VIR. The final volume was adjusted to 25 μ l with triple distilled water.

The PCR products were visualized on an UV transilluminator after 2% agarose gel electrophoresis and staining with ethidium bromide. The sizes of the PCR products, in terms of number of base pairs (bp) were estimated

Table 1. Primers and cycle conditions of semi-nested PCR for amplification of the small subunit of the human ribosomal gene (Human ssrDNA) and the small subunit of the *Plasmodium* ribosomal gene (*Plasmodium* ssrDNA)

Primers	Cycle conditions	PCR product size (bp)
Nest 1: UNR-PLF (<i>Plasmodium</i> ssrDNA) PLF (5'-AGTGTGTATCAATCGAGTTT-3') UNR (5'-GACGGTATCTGATCGTCTT-3')	$94^{\circ}\text{C}/45 \text{ sec}; 62^{\circ}\text{C}/45 \text{ sec}; 72^{\circ}\text{C}/60 \text{ sec}$ (40 cycles)	794 to 825
Nest 1: UNR-HUF (Human ssrDNA) HUF (5'-GAGCCGCCTGGATACCG-3') UNR (5'-GACGGTATCTGATCGTCTT-3')		231
Nest 2: PLF-MAR (<i>P. malariae</i>) PLF (5'-AGTGTGTATCAATCGAGTTT-3') MAR (5'-GCCCTCCAATTGCCTTCT-3')	$94^{\circ}\text{C}/20 \text{ sec}; 62^{\circ}\text{C}/20 \text{ sec}; 72^{\circ}\text{C}/30 \text{ sec}$ (35 cycles)	269
Nest 2: PLF-FAR (<i>P. falciparum</i>) PLF (5'-AGTGTGTATCAATCGAGTTT-3') FAR (5'-AGTTCCCTAGAAATAGTTACA-3')		395
Nest 2: PLF-OVR (<i>P. ovale</i>) PLF (5'-AGTGTGTATCAATCGAGTTT-3') OVR (5'-GCATAAGGAATGCAAGAACAG-3')		436
Nest 2: PLF-VIR (<i>P. vivax</i>) PLF (5'-AGTGTGTATCAATCGAGTTT-3') VIR (5'-AGGACTTCCAAGCCGAAG-3')		499

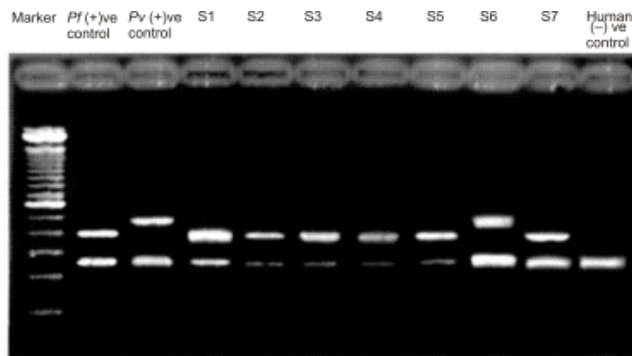


Fig. 1: Agarose gel electrophoresis showing amplicon of semi-nested multiplex PCR (SnM-PCR) for amplification of the small subunit of human ribosomal gene (*Human ssrDNA*) and the small subunit of *Plasmodium* ribosomal gene (*Plasmodium ssrDNA*). Lane 1: Marker, molecular weight marker (100 bp); Lane 2: *P. falciparum* 18S rRNA amplicon from DNA of *P. falciparum* (laboratory strain K1); Lane 3: *P. vivax* 18S rRNA amplicon from *P. vivax* infected patient blood; S1-S5 and S7: *P. falciparum* 18S rRNA amplicon from *P. falciparum* infected patient blood; S6: *P. vivax* 18S rRNA amplicon from *P. vivax* infected patient blood; and Lane 11: Human ssrDNA amplicon from non-infected blood.

according to their mobility relative to the molecular size standard (100 bp weight marker) (Invitrogen, USA). The Nest 1 reaction amplified approximately 794 to 825 bp of the small subunit of the *Plasmodium* ribosomal gene (depending on the *Plasmodium* species; *P. falciparum*–825 bp; *P. vivax*–798 bp; *P. malariae*–822 bp; *P. ovale*–794 bp; and 231 bp of the small subunit of the human ribosomal gene. In Nest 2 reaction, each *Plasmodium* species would show the specific band with different size (Fig. 1). The appropriated bands of 269, 395, 436, and 499 bp, were indicated as *P. malariae*, *P. falciparum*, *P. ovale*, and *P. vivax* infections respectively.

All the PCR reactions were done in duplicate and the UNR-HUF fragment is the control for each individual sample, and it must be present in every sample (Fig. 1). The absence of this amplification product shows that the PCR was inhibited and the results of the second PCR must be ignored in order to avoid false negatives.

Data analysis

Prevalence of *Plasmodium* infections was analyzed by using SPSS software package (SPSS 11 for Windows). Chi-square or Fisher's Exact test was used to analyze differences of the data and association between techniques. The *p*-values < 0.05 were considered statistically significant.

RESULTS

A total of 241 migrant workers were recruited in the study; 120 from Pathum Thani, 48 from Nakhon Pathom,

and 73 from Kanchanaburi. Of the participants, 203 (84.23%) and 38 (15.77%) were from Myanmar and Cambodia, respectively. There were 118 (49%) males and 123 (51%) females. Each participant attended and passed the health-screening programs including physical examination and clinical interviews. The results demonstrated that these migrant workers had no clinical symptom of *Plasmodium* infections.

Table 2 presents the prevalence of *Plasmodium* infections among migrant workers related to demographic data. Total positive rate detected by microscopy and/or semi-nested multiplex PCR was 6.2% (15 of 241). In Kanchanaburi and Pathum Thani, the respective infection rate was 13.7% (10 of 73) and 4.2% (5 of 120), but no case report was found in Nakhon Pathom. This difference was statistically significant ($p = 0.004$). Out of 203, 14 Myanmar workers were infected with malaria whereas only one positive case out of 38 was found in Cambodian workers. A higher prevalence of *Plasmodium* infections was found in males (6.5%; 8 of 123) than in females (5.9%; 7 of 118). No statistically significant difference between the prevalence of *Plasmodium* infections was found among each nationality ($p = 0.281$) and both genders ($p = 0.854$).

The migrant workers, who had erythrocytic stages of *Plasmodium* spp or parasite DNA in their blood samples but no signs and symptoms of malaria, seem to be asymptomatic. Among these asymptomatic *Plasmodium* infections, the most prevalent species was *P. falciparum* (8 of 15), followed by *P. vivax* (6 of 15) and *P. malariae* (1 of 15) (Table 3). There was no positive case for mixed infections. Furthermore, our data noted that semi-nested PCR (14 of 15 positive cases) were approximately 3.5 fold more sensitive than microscopy (4 of 15 positive cases). Classified by laboratory methods, the prevalence of *Plasmodium*

Table 2. Prevalence of *Plasmodium* infections among migrant workers related to demographic data

Demographic data	No. examined (Total = 241)	No. positive* (%)	<i>P</i> -value
<i>Residence provinces</i>			
Pathum Thani	120	5 (4.2)	0.004
Nakhon Pathom	48	0	
Kanchanaburi	73	10 (13.7)	
<i>Nationality</i>			
Myanmar	203	14 (6.9)	0.281
Cambodian	38	1 (2.6)	
<i>Sex</i>			
Female	118	7 (5.9)	0.854
Male	123	8 (6.5)	

*Detected by microscopy and/or PCR.

Table 3. *Plasmodium* infections among 241 migrant workers diagnosed by microscopy and semi-nested multiplex PCR (SnM-PCR)

Species	No. of positives		
	Total	SnM-PCR	Microscopy
<i>P. falciparum</i>	8 (53.3)	7 (87.5)	2 (25)
<i>P. vivax</i>	6 (40)	6 (100)	2 (33.3)
<i>P. malariae</i>	1 (6.7)	1 (100)	0
<i>P. ovale</i>	0	0	0
Total	15	14 (93.3)	4 (26.7)

Figures in parentheses indicate percentages.

infections tested by semi-nested PCR (5.8%; 14 of 241) was higher than that of microscopy (1.7%; 4 of 241). There was highly significant difference in the prevalence between the two laboratory methods ($p < 0.001$). As shown in Fig. 2, the SnM-PCR and microscopy showed concordant results in 229 blood samples including 3 positive cases and 226 negative cases. However, 12 non-concordant results between microscopy and SnM-PCR were that one microscopy positive sample was not detected by semi-nested PCR and microscopy could not detect 11 SnM-PCR positive samples.

DISCUSSION

Our study revealed that 6.2% of foreign migrant workers infected with malaria parasites. Higher prevalence was found in these workers as compared to 0.055% of Thai people². It is likely due to low socioeconomic status in migrant workers. Poor economy in migrants has resulted in the difficulty to access healthcare services and

treatment facilities. In agreement with our previous report¹⁵, the present study showed lower prevalence of *Plasmodium* infections in the central part of Thailand (Pathum Thani and Nakorn Pathom provinces). The highest prevalence in Kanchanaburi province confirmed a major risk of spreading *Plasmodium* infections in the western part of Thailand, especially on Thailand-Myanmar border¹⁶.

The species of *Plasmodium* commonly identified among migrants were the same as those seen in Thai people. The most prevalent species in this study was *P. falciparum*, followed by *P. vivax* and *P. malariae*. These parasites are also frequently found in other countries of the Southeast Asia region^{2,17}. Malaria cases reported in our study were mainly single infections with low parasitaemia. The failure of malaria detection by microscopy and inadequate initial treatment in these migrants seem to increase malaria carriers, that may result in an upward trend in the prevalence of malaria in Thailand.

The SnM-PCR is more sensitive in malaria detection than microscopy. In accordance with the fact that microscopy could not detect malaria parasites when the density is $< 5\text{--}50$ parasites/ μl ¹⁸. Our data suggest that microscopy is insufficiently sensitive to be used alone for screening of malaria, particularly in low parasitaemia. However, one positive sample by microscopy was negative for malaria by the SnM-PCR. It may be due to PCR inhibitors in blood samples^{19–23} and/or the competitive DNA targets²⁴. The false negative results should be of concern, especially *P. falciparum*, the most virulent human malaria.

Approximately, 70% of the total population in the South-east Asia region including Myanmar, Cambodia, Lao People's Democratic Republic, and Thailand are at risk of malaria^{2,18}. The abundance of drug resistant malaria and the increase of immigration from the foreign migrant workers in this region appear to represent the added risk of spreading multidrug resistant malaria in Thailand^{25–26}. According to the significant public health problem, laboratory diagnosis in the health-screening program for foreign migrant workers is urgently needed for active malaria surveillance. Considering the highest achievement and cost-effectiveness, the DNA extracts from pooled blood samples should be used as templates in the amplification reaction of the Nest 1 of SnM-PCR for detection of *Plasmodium* infections with low parasitaemia²⁷. Quality assurance program is also essential for microscopy.

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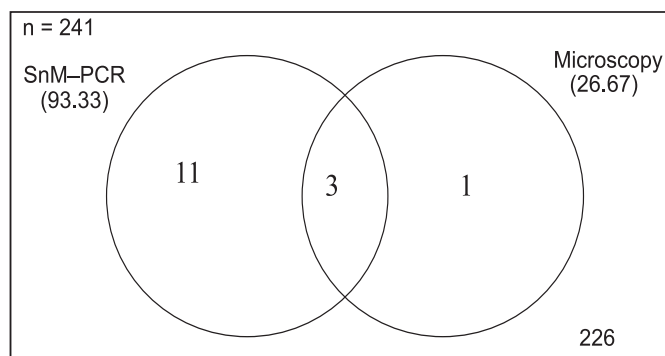


Fig. 2: Malaria detection by semi-nested PCR (SnM-PCR) and microscopy. The numbers of positive cases were shown in circles and the sensitivity was expressed as percentage in parentheses. (n = Total number of the samples examined).

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