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Author(s)	Toma, Hiromu; Kobayashi, Jun; Vannachone, Bouakham; Arakawa, Takeshi; Sato, Yoshiya; Nambanya, Simone; Manivong, Khemphavanh; Inthakone, Souliya
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A FIELD STUDY ON MALARIA PREVALENCE IN SOUTHEASTERN LAOS BY POLYMERASE CHAIN REACTION ASSAY

HIROMU TOMA, JUN KOBAYASHI, BOUAKHAM VANNACHONE, TAKESHI ARAKAWA, YOSHIYA SATO, SIMONE NAMBANYA, KHEMPHAVANH MANIVONG, AND SOULIYA INTHAKONE

Department of Parasitology, Faculty of Medicine, University of the Ryukyus, Nishihara, Okinawa, Japan; Institute of Malariology, Parasitology and Entomology (IMPE), Vientiane, Lao People's Democratic Republic; Malaria Station, Khammouane Provincial Health Office, Thakhek, Khammouane, Lao People's Democratic Republic

Abstract. A detection survey for malaria infection by routine microscopy and polymerase chain reaction (PCR) assay was conducted on 336 inhabitants of two villages in Khammouane Province, Lao People's Democratic Republic (Lao PDR), in July 1997. Malaria infection was demonstrated in 58 (17.3%) subjects by microscopy and in 117 (34.8%) by PCR assay. Specimens positive by both methods were frequent in young villagers, suggesting the presence of many subclinical infections in older persons. The most common species of malaria parasite was *Plasmodium falciparum* (82.9%). Polymerase chain reaction assay detected mixed infections with 2–4 species in 27 specimens (23.1%). The results demonstrate that there are many subclinical malaria infections with low parasite level and infection with all four human malaria species in Lao PDR.

INTRODUCTION

Lao People's Democratic Republic (Lao PDR) is bordered by five countries in Southeast Asia. In this country, malaria is a major public health problem as it causes high morbidity and mortality in children and severe losses in socioeconomic development.[1] The Lao government has organized a nationwide anti-malaria network consisting of a malaria station in each province and a malaria center in each district, and has started anti-malaria activities including DDT residual spraying, mass distribution of chloroquine, improvement of diagnostic facilities, proper treatment of malaria cases, and delivery of insecticide impregnated bed nets. The exact prevalence of malaria in Laos, however, is not well known because the recorded cases were detected cases according to hospital admission with other clinical findings. Additionally, diagnosis of malaria is sometimes inadequate because of a lack of equipment and the low skill of microscopists.

In order to determine the true prevalence of malaria, it is essential to conduct case detection surveys. The authors have carried out active surveys of case detection on the inhabitants of three villages in a southeastern province in 1995 and 1996, when 2.2–7.6% of persons were positive by microscopic examination of blood smear.[2] The detection of parasites by microscopy, however, is difficult in subclinical infections, and may therefore lead to an underestimate of the value.[3–5] The current study was undertaken to assess the prevalence of malaria in Laos by the nested PCR method.

MATERIALS AND METHODS

Study area and subjects. The study was carried out in two villages (Phavang and Sisomsouen Villages) in Khammouane Province, a southeastern province 350 km from Vientiane, the capital of Laos. Phavang Village is located 55 km north of Thakhek, capital of the province. Sisomsouen Village is located 5 km southeast of Phavang. Socioeconomic situations in both villages were poor, i.e. houses were made of wood and bamboo, and there were no toilet facilities in the majority of them. The main occupations of the villagers are farming, fishing, and hunting.

The total estimated population in Phavang Village was 230 and in Sisomsouen Village, 430. Blood samples were collected from 143 and 193 of residents of each village, respectively. Samples were collected after informed consent was obtained for the survey of malaria infection. The ages of the subjects ranged from 2–68 years old (mean 22.9 $\pm$ 19.3) in Phavang and 0–75 years old (mean 21.8 $\pm$ 19.1) in Sisomsouen. Children $<$ 15 years old constituted $>$ 50% of the residents examined. The population of male : female participants in Phavang and Sisomsouen was 1.03 and 1.38, respectively.

Ethical approval for the study was granted by Institute of Malariology, Parasitology, and Entomology (IMPE) and the Ministry of Health (MOH), Vientiane, Lao PDR.

Blood smear examination. Microscopic diagnosis for malaria was carried out by examination of thick and thin blood smears stained with Giemsa. Blood was collected from a single finger prick. Thick smears were considered negative if no parasite was seen after examination of approximately 100 oil immersion fields. Blood parasitemia was calculated on the basis of positive smears collected in Phavang. It was based on the number of parasites per μl of blood in a thick smear assuming a leukocyte count of 8,000/μl.

PCR detection. Approximately 100 μl of blood was collected in a tube containing heparin and frozen before transport to Japan. The extraction and purification of DNA were performed using a kit (GFX Genomic Blood DNA Purification Kit; Pharmacia Biotech). PCR was carried out as described by Kimura and others.[6] A small region of the *Plasmodium* 18S rRNA genes was amplified in the primary PCR. Five μl of template DNA were added to 20 μl of *Taq* buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$) and mixed with 0.4 μM each of P1 (forward 5′-ACGATCAGATACCGTCGTAATCTT-3′) and P2 (reverse 5′-GAACCCAAAGACTTTGATTTCTCAT-3′) genus-specific primers, 125 μM dNTPs, and 0.75 units of *Taq* polymerase (Takara Shuzo, Japan) on ice. Amplification was performed at 92°C for 2 min; 40 cycles at 92°C for 30 sec, and at 60°C for 90 seconds; then at 60°C for 5 min for a final extension in a thermal cycler (PTC-100, MJ Research). The primary PCR product was diluted 1:50 in TE buffer (10 mM Tris-

TABLE 1

The positive rate of malaria parasite and gametocytes among age groups in two villages in Khammouane Province, Lao People's Democratic Republic

Age group	No. examined	No. positive for malaria infection				Gametocytes	
		Giemsa	%	PCR assay	%	No. positive	%
Phavang Village							
<11	59	24	40.7	32	54.2	7	29.2
11–20	21	11	52.4	18	85.7	0	0
21–30	14	4	28.6	9	64.3	0	0
31–40	16	4	25.0	10	62.5	0	0
41–50	14	1	7.1	6	42.9	0	0
>50	19	5	26.3	12	63.2	0	0
Total	143	49	34.3	87	60.8	7	14.3
Sisomsouen Village							
<11	80	3	3.8	10	12.5	2	66.7
11–20	33	4	12.1	9	27.3	0	0
21–30	24	0	0	3	12.5	0	0
31–40	17	0	0	3	17.6	0	0
41–50	17	2	11.8	2	11.8	1	50.0
>50	22	0	0	3	13.6	0	0
Total	193	9	4.7	30	15.5	3	33.3

HCl, 1.0 mM EDTA, pH 8.0) and used as template DNA in the nested PCR. The nested PCR was performed with species-specific reverse primers corresponding to each of the four human malaria parasites (*P. falciparum,* 5′-CA-ATCTAAAAGTCACCTCGAAAGATG-3′; *P. vivax,* 5′-CA-ATCTAAGAATAAACTCCGAAGAGAAA-3′; *P. malariae,* 5′-GGAAGCTATCTAAAAGAAACACTCATAT-3′; and *P. ovale,* 5′-ACTGAAGGA-AGCAATCTAAGAAATTT-3′) in combination with P1 genus-specific primer (the same forward primer in the primary amplification). Four reaction tubes were prepared for each primary PCR product. The template DNA (2 μl) was mixed in 20 μl of *Taq* buffer with 0.4 μM each of P1 and the above species-specific primer, 125 μM each of dNTPs, and 0.75 units of *Taq* polymerase on ice. The nested PCR was performed at 92°C for 2 min, 18 cycles at 92°C for 30 sec and 60°C for 60 sec, then at 60°C for 5 min for a final extension. A negative control of human DNA was run with the samples. Ten μl of the nested PCR product was electrophoresed in 2% agarose gel (SeaKem, FMC Bioproducts). The agarose gel was stained with ethidium bromide and examined under UV light. Positive signals were observed at 110 base pairs (bp).

TABLE 2

Comparison of results by Giemsa staining and polymerase chain reaction (PCR) assay for detection of malaria parasite among the inhabitants of two villages in Khammouane Province, Lao People's Democratic Republic

Giemsa staining	PCR assay		Total (%)
	Positive (%)	Negative (%)	
Phavang Village			
Positive	49 (34.3)	0 (0)	49 (34.3)
Negative	38 (26.6)	56 (39.4)	94 (65.7)
Total	87 (60.8)	56 (39.4)	143 (100)
Sisomsouen Village			
Positive	9 (4.7)	0 (0)	9 (4.6)
Negative	21 (10.9)	163 (84.5)	184 (95.3)
Total	30 (15.5)	163 (84.5)	193 (100)

Statistics. The statistical significance of the difference in parasitemias was analyzed by the Mann-Whitney U test. $P < 0.05$ was considered significant.

RESULTS

Malaria infection rates by blood smear and PCR assay are summarized in Table 1. The rate obtained by microscopic examination of blood smears was 34.3% in Phavang and 4.7% in Sisomsouen. When the same samples were subjected to PCR assay, 60.8% and 15.5% were positive. The rate for both was highest in residents aged 11–20 years. More than 50% of the positive samples were from children < 15 years old.

Table 2 shows the relationship between the results of microscopy and PCR. In Phavang, a total of 87 specimens were positive by either microscopy or PCR. Forty-nine (56.3%) of the positive specimens were positive by both methods, and 38 were positive only by PCR. False-negatives were not observed with the PCR assay, and all infections diagnosed by microscopy were confirmed by PCR. In Sisomsouen, 9 (29.0%) specimens were positive by both microscopy and PCR, and the remaining 21 specimens were positive only by the PCR assay.

Results stratified according to age are presented in Figure 1. Specimens positive by both methods were frequently observed in subjects < 20 years old in both Phavang and Sisomsouen. However, the proportion of positives by PCR only was relatively greater in the older age groups, suggesting that there were many cases with low parasitemia among this group. The intensity of parasitemia was higher for subjects < 20 years old in Phavang, although a significant difference was shown only for those 21–30 years old (Figure 2).

Malaria species detected in the present survey are summarized in Table 3. In Phavang, > 88% of the parasites were *P. falciparum* by both methods. *Plasmodium vivax* was identified in only 4 specimens by microscopy but as many as 30

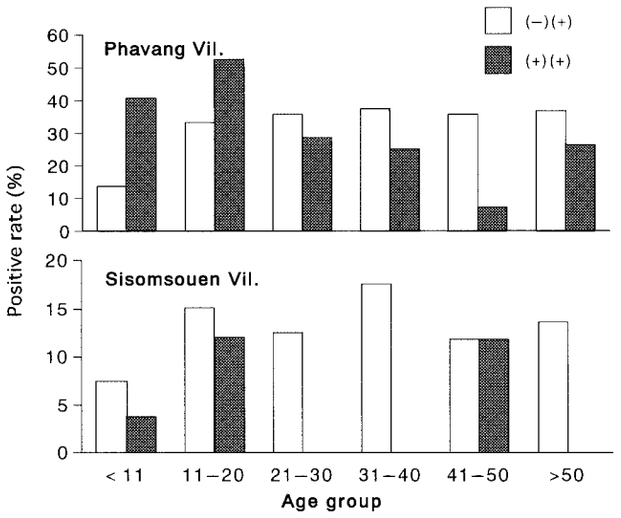

FIGURE 1. Relation of the results from microscopical examination and polymerase chain reaction (PCR) assay for malaria infection by age group. (−)(+) indicates that the specimen was negative by miroscopic examination and positive by PCR assay. (+)(+) indicates the specimen was positive by both methods.

specimens were positive by PCR. Three specimens were positive for *P. malariae* by PCR, although this species could not be demonstrated by microscopy. On the other hand, *P. ovale* were detected in five specimens by PCR assay, and only one of the five was confirmed by microscopy. The PCR assay detected 24 specimens with mixed infection while only two were detected by microscopy. Twelve cases (27.3%) of mixed infection were revealed by PCR among cases that were microscopically diagnosed as *P. falciparum* infection alone. Mixed infections were also confirmed by PCR assay in 9 of 43 cases negative by microscopy. Among the 24 specimens with mixed infection, 19 were *P. falciparum* and *P. vivax*. Mixed infections with three or four malaria species were confirmed in three specimens. In Sisomsouen, the results were similar. More than 66% of parasites were *P. falciparum* by both methods. *Plasmodium malariae* and *P. ovale* infections were also demonstrated in five and one specimens, respectively. These were not detected by microscopy.

DISCUSSION

The true measurement of parasite incidence is of fundamental significance in the design of malaria control measures. Although almost all data on the prevalence of malaria in Laos are based on the number of passively detected cases, the official record of malaria cases by the national referral institute for malaria in Laos indicated the occurrence of 370,529 cases, including 566 fatal cases, in 1997. Khammouane Province surveyed in the present study is also known as a high endemic area; a total of 1,630 cases including 24 fatalities were reported in the same year. On the other hand, there have been few active case detection surveys to estimate subclinical infections in Laos. In a past survey in Keodom District of Vientiane Province, a total of 1,105 villagers, accounting for 7% of the total population, were examined for malaria infection. Positive smears were reported to be 2.4%.[7] Recently, we carried out active surveys in three villages in Khammouane Province, and positive rates of 2.2–7.6% were obtained by microscopy.[2] In the pre-

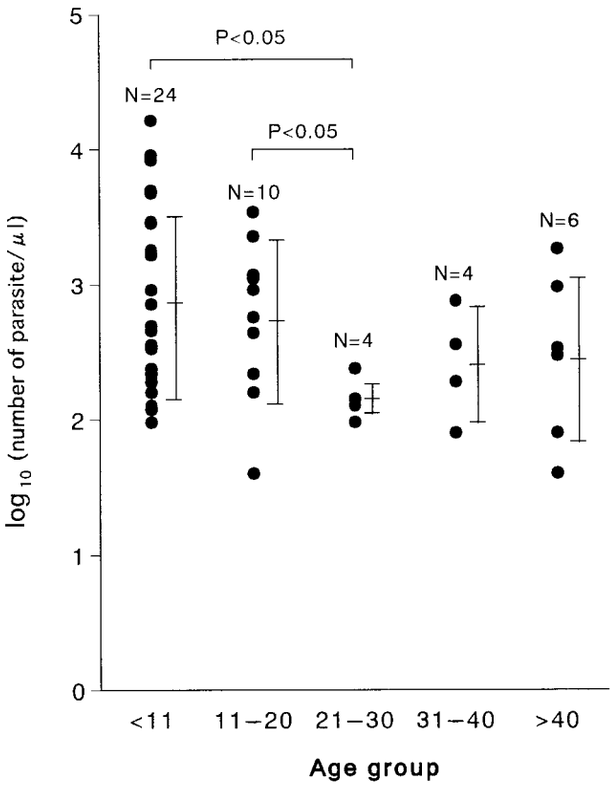

FIGURE 2. Comparison of number of malaria parasites among positive smears by age group in Phavang Village.

TABLE 3

Malaria species detected by Giemsa staining and polymerase chain reaction (PCR) assay among the inhabitants or two villages in Khammouane Province, Lao People's Democratic Republic

Method	No. positive	No. specimens positive for: F	V	M	O	FV	FM	VM	VO	FVM	FVO	FVMO
Phavang Village												
Giemsa staining	49	44	2	—	1	2	0	—	0	0	0	0
PCR assay	87	54	7	—	2	19	1	—	1	1	1	1
Sisomsouen Village												
Giemsa staining	9	7	2	0	0	—	0	0	—	—	—	—
PCR assay	30	19	5	2	1	—	1	2	—	—	—	—

F = *Plasmodium falciparum*; V = *P. vivax*; M = *P. malariae*; O = *P. ovale.*

sent study in Khammouane Province using PCR, the positive rate was 4.7% and 34.3% by microscopy and as high as 15.5% and 60.8% by PCR, suggesting that there were many subclinical cases with low level parasitemia not detected by microscopy. The positive rate was higher in younger individuals by microscopy, although subclinical infection was only detected by PCR assay and was more frequent in the older population.

The PCR assay applied here is extremely sensitive.[8–11] Ten to 100 times greater parasites can be detected as compared to microscopy.[12] Results of the present survey indicate that the PCR assay was approximately 2–3 times more effective in detecting malaria infection than microscopy. Additionally, PCR assay was effective for the identification of malaria species.[13] Species identification of malaria with low parasite numbers is problematic by microscopic examination. In the present study, > 90% of parasites identified by microscopy were *P. falciparum. Plasmodium vivax* malaria was identified in only 6 of 58 positive samples by microscopy, although as many as 37 samples with *P. vivax* infection were detected by PCR. The result may relate to the relatively low parasite numbers in the *P. vivax* infection compared to *P. falciparum.* Eight samples were found to be positive for *P. malariae* by PCR but this parasite could not be confirmed by microscopy. Similarly, six *P. ovale* infections were identified by PCR but only one of the six was microscopically confirmed. These results indicate that *P. malariae* and *P. ovale* infections are prevalent in Lao PDR. For *P. ovale* infection, there have been few reports in this country, although there are published and unpublished data on infection with this species in other Southeast Asian countries.[14–16]

Polymerase chain reaction was especially sensitive for detection of mixed infection. Twenty-seven cases were confirmed to be mixed infection with *P. falciparum* and *P. vivax* by the PCR but only two were identified by microscopy. In mixed infections, it has been suggested that there is a tendency for one species to dominate the other.[17] The result indicates that one of the infecting species is sometimes overlooked microscopically.[18] Detection of mixed infection may be of clinical importance because interactions between different species simultaneously infecting the same individual could result in significant changes in the course of the infection and disease.[19]

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Authors' addresses: Hiromu Toma, Jun Kobayashi, Takeshi Arakawa, and Yoshiya Sato, Department of Parasitology, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903–0215, Japan. Bouakham Vannachone, Simone Nambanya, and Souliya Inthakone, Institute of Malariology, Parasitology and Entomology (IMPE), Ministry of Health (MOH), Vientiane, Lao PDR. Khemphavanh Manivong, Malaria Station, Khammouane Provincial Health Office, Khammouane, Lao PDR.

Reprint requests: Hiromu Toma, Department of Parasitology, Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903–0215, Japan.

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