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A Field Study on Malaria Prevalence along the Myanmar Thailand Border by Rapid Diagnostic Test (RDT) and Polymerase Chain Reaction Assay (PCR)

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Authors' contributions

This work was carried out in collaboration between all authors. Author SC designed the study, wrote the protocol and wrote the first draft of the manuscript. Author YP managed the literature searches, analyses of the study performed the statistic analysis and author WN managed the experimental process and identified the species of Malaria parasite. All authors read and approved the final manuscript.

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ABSTRACT

Background: Thailand has a national goal to eliminate malaria from 80 percent of the country by 2020. An accurate detection and prevalence are critical to effective management of malaria. Rapid diagnostic tests (RDTs) detecting parasite lactate dehydrogenase (pLDH) antigen are used to identify individuals with *Plasmodium falciparum* infection even in low transmission settings seeking to achieve elimination.
Aims: The aim of this study was to evaluate the exact prevalence of malaria in the Thai border area where malaria is endemic by RDT compared with PCR.
Methodology: One thousand one hundred thirty blood samples were obtained from study subjects who live along the Myanmar Thailand Border. RTD was performed with the parasite lactate

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dehydrogenase (pLDH) antigen-based lateral flow test and the primer set used for PCR was designed on the species-specific nucleotide sequence of 18S rRNA plasmodium gene.
Results: Malaria infection was demonstrated in 70 (6.2%) subjects and 97 (8.6%) subjects by RDT and PCR respectively. PCR detected a significantly higher number of malaria infection than RDT (*P<0.05*). Comparison of RDT negative and PCR positive samples suggested that RDT negatives resulted from low parasitaemia. Moreover, PCR was able to identify the species of Plasmodium parasite. Three species, *Plasmodium falciparum, Plasmodium vivax* and *Plasmodium malariae* were detected. No *Plasmodium ovalae* was detected from any of the study location. *P. falciparum* was predominant along border with a percentage of 31.9 of positive suspected patients. Mixed infections with two or three malaria species were detected in 54 specimens (55.7%).
Conclusion: The result demonstrates that PCR should be undertaken to assess the prevalence of malaria in border areas to progress towards malaria elimination in Thailand.

Keywords: Malaria; prevalence; rapid diagnostic test; polymerase chain reaction assay.

1. INTRODUCTION

Malaria is a life threatening parasitic disease transmitted by *Anopheles* mosquitoes. It is the most highly prevalent tropical disease, with economic and social impact. In Southeast Asia, the number of malaria cases has been grossly underestimated [1,2]. The largest focus of *falciparum* malaria in this region is situated in Myanmar, with a reported annual caseload of 70,941 in 2010 [3]. In that same time period, Thailand had about 70 percent decreased in reported malaria cases between 2000 and 2011, from 78,561 cases to 24,897 cases. According to Thai government increased funding for malaria control, overall incidence declined [4,5]. Central Thailand has been malaria-free for more than three decades. However, malaria endemic areas are still located along the forested border. Malaria in Thailand is forest related, with high prevalence along the densely forested border areas [6,7]. The border between Thailand and Myanmar is 2,107 km long and is mostly forested and mountainous. It is inhabited by a mosaic of ethnic groups and is characterized by intense migration fluxes between the two countries. Malaria control in this border area is particularly challenging, because of a reservoir of malaria in Myanmar, where the disease burden is higher than in Thailand and differences in adequate control measures on the two sides of the border. In addition, decades of internal conflicts and economic impact in Myanmar have resulted in massive population displacement, and over 150,000 refugees have allowed continued malaria transmission in Thailand [8]. The Thai government has organized a nationwide anti-malaria network consisting of malaria centers in each district, proper treatment of malaria cases and improvement of diagnostic facilities [6]. Current malaria control activities in Thailand are also supported by the Mekong Malaria program (MMP) and Global Funds [9,10]. These grants aim at eliminating and combating artemisinin resistance in the Mekong region [11]. Thailand is pursuing spatially progressive elimination and has a national goal to eliminate malaria from 80 percent of the country by 2020 [6].

Improving diagnostic accuracy in malaria control and elimination must be technically challenging. Since the WHO recognized the diagnostic tool for simple, quick and cost-effective tests for determining the presence of malaria parasites, numerous rapid diagnostic tests (RDT) have been developed. The use of RDTs provides the most feasible means of rapidly expanding diagnostic testing, especially in peripheral health facilities. The test kit is simple to use. Following WHO recommendation of using RDT in all suspected malaria cases, they are widely applied in regional clinics in endemic areas. They can be performed by a health worker [12,13]. In Thailand, the rapid diagnostic tests (RDTs) such as Immunochromatographic test (ICT), that are based on the recognition of Plasmodium antigen in the blood circulation of patient, have been used in diagnosing and determining malaria prevalence. RDTs were implemented in the management of febrile illnesses in remote malaria endemic areas. It was supported by a global Fund [10]. Most ICT products are suitable for diagnosis of *P. falciparum* and *P. vivax* malaria [14]. Recently, the interpretation of malaria diagnosis and epidemiology have been changed by molecular tools, for instance by revealing grand reservoirs of asymptomatic infection and by detecting distribution of *Plasmodium* spp. infection. According to DNA amplification, all species could be identified [15]. The current study compared the performance of RDT with PCR for assessment of true malaria

prevalence along the Myanmar Thailand's border to achieve its elimination goals.

2. MATERIALS AND METHODS

2.1 Clinical Sample

The blood samples were collected from study subjects who came to malaria clinic during June to September 2014. A total of 1130 EDTA blood samples were obtained from subjects. All subjects lived in study area and had fever. EDTA blood was taken before treatement. The template DNA was extracted from 200 µl of EDTA blood using QIAamp DNA Blood Mini kits (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's protocol. The three hospitals and nine malaria centers in this study were closed to border areas where there was migration of people between the two countries. They were in the province of Chiang Mai, Mae Hong Son and Kanchanaburi near the Myanmar Thailand border. This study was performed using a protocol approved for medical research on human subjects, Department of Medical Sciences, Ministry of Public Health, Thailand.

2.2 Rapid Diagnostic Test (RDT)

The Rapid diagnostic test was performed with the parasite lactate dehydrogenase (pLDH) antigen-based lateral flow test (DMSc Malaria Pf/PAN Rapid test, Thailand) according to the manufacturer's instructions. The test is a device that detects malaria antigen in a small amount of blood, 5 µl, by immunochromatographic assay with monoclonal antibodies which detects *P. falciparum* specific and pan-specific antigens and impregnated on a test strip. The result, a colored test line, is obtained in 30 minutes. Two trained readers examined the immunochromatographic test, independently. A test was considered negative if only the internal control line was visible. The result of an immunochromatographic test was considered not valid if the internal control was not visible.

2.3 Nested Polymerase Chain Reaction (Nested PCR)

All samples were tested for *P. falciparum, P. vivax, P. malariae and P. ovale* by nested PCR. The species-specific nucleotide sequences of the 18S rRNA genes were applied as described previously with slight modifications [15]. A small region of the *Plasmodium* 18S rRNA genes was amplified in the primary PCR. One µl of template DNA were added to 21 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, 200 µM dNTPs, and 0.05 units of *Taq* polymerase. Amplification was performed at 94°C for 10 min; 35 cycles at 92°C for 30 sec, 60°C for 90 sec and at 72°C for 60 sec; then at 72°C for 5 min for a final extension in a thermal cycler. The primary PCR product was diluted 1:40 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'-CAATCTAAAAGTCACCTCGAAAGATG-3'; *P. vivax,* 5'-CAATCTAAGAATAAACTCCGAAGAGAAA-3'; *P. malariae,* 5'-GGAAGCTATCTAAAAGAAACACTCATAT-3' and *P. ovale,* 5'-ACGATCAGATACCGTCGTAATCTT-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 (1 µl) was mixed in 21 µl of *Taq* buffer with 0.4 µM each of P1 and the above species-specific primer, 200 µM each of dNTPs, and 0.05 units of *Taq* polymerase on ice. The nested PCR was performed at 94°C for 10 min; 20 cycles at 92°C for 30 sec, 60°C for 1.5 min, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. Ten µl of the nested PCR product was electrophoresed in 2.5% agarose gel. The agarose gel was stained with ethidium bromide and examined under UV light. The expected band sizes were approximately 160 bp for the outer primer PCR product and approximately 110 bp for the inner primer PCR product.

2.4 Statistical Methods

The statistical analysis for significance was done using Chi-square. $P<0.05$ was considered significant.

3. RESULTS

The positive rate obtained by RDT was 70/1130 (6.2%) whereas the same samples were subject to PCR assay was 97/1130 (8.6%). PCR detected a significantly higher number of malaria infection than RDT ($P<0.05$). Table 1 shows the

relationship between the result of RDT and PCR. A total of 70 specimens were positive by both methods, and 27 specimens were positive only by PCR. False negatives were not observed with the PCR assay, and all infections diagnosed by RDT were confirmed by PCR. Malaria species detected in this present survey are summarized in Table 2. Among PCR-positive specimens, 31/97(31.9%), 12/97 (12.4%) were detected as *P. faciparum* and *P. vivax* respectively. The PCR assay detected 54 specimens (55.7%) with mixed infection. Among the 54 specimens with mixed infection, *P. faciparum* and *P. vivax* were identified in only one specimen, but as many as 44 specimens were *P. vivax* and *P. malariae* and 9 specimens were positive for *P. faciparum*, *P. vivax* and *P. malariae*. There was no *P. ovale* in any of the 1,130 samples.

Table 1. Comparison of result by RDT and PCR assay for detection of malaria parasite among study subjects in hospitals and malaria centers near the Myanmar Thailand border

		PCR assay		
		Positive	**Negative**	**Total**
RDT	Positive	70	0	70
	Negative	27	1,033	1,060
	Total	97	1,033	1,130

Table 2. Malaria species detected by PCR assay in the Myanmar Thailand Border

Method	**Positive**	**Specimens positive for**						
		F	**V**	**M**	**O**	**FV**	**VM**	**FVM**
PCR	97	31	12	0	0	1	44	9

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

4. DISCUSSION

In order to implement an effective malaria elimination program in Thailand, accurate information on the incidence and prevalence of malaria is required. In this study, the malaria prevalence survey was conducted to provide the exact baseline parasitological information. In general, Malaria is not equally distributed in all provinces of Thailand. Central Thailand has been malaria-free for more than three decades but malaria endemic areas are still located along the forested border [6,7]. Thailand is pursuing spatially progressive elimination and has a national goal to eliminate malaria from 80 percent of the country by 2020; consequently, the exact measurement of parasite prevalence is of fundamental significance in the design of malaria control measures.

In this study, 27 specimens were positive by PCR, although these specimens were negative by RDT, suggesting that there were many subclinical cases with low level parasitemia not detected by RDT. The results indicated that the PCR assay was more effective in detecting malaria infection than RDT and the detection of parasites by RDT was difficult in subclinical infections, and may lead to an underestimate of the exact prevalence of infection. Additionally, PCR assay was effective for identification of malaria species. Three species, *P. falciparum*, *P. vivax* and *P. malariae*, were detected in these samples. No *P. ovalae* was detected from any of the study locations and *P. falciparum* was predominate [Table 2].

Even though RDT is currently the recommended the standard method of malaria diagnosis, it has the disadvantages of poor sensitivity and specificity, especially during low paresitemia. In very low transmission, asymptomatic infections remain the major reservoir of malaria parasites contributing to maintain disease transmission. In addition, most RDTs have achieved 95% sensitivity for *P. falciparum,* but not for non- *P. falciparum* and declined sensitivity at parasite densities<500/mcL blood [16].

The PCR assay applied here is extremely sensitive. The greatest advantage is the ability of PCR to detect infections with low parasitemia and endemic area where individuals are asymptomatic [17-20]. It has been estimated that PCR can detect malaria infection with parasitemia as low as 5 parasites/ µl [21]. Moreover, PCR assay was effective for the identification of malaria species and was also able to detect mixed infection [20]. In this study, 9 cases were mixed infection with three species *P. falciparum*, *P. vivax* and *P. malariae* and 45 cases were mixed infection with two species (Table 2). In mixed infections, it has been suggested that there is a tendency for one species to dominate the other [22]. Detection of mixed infection may be of clinical importance because interaction between different species simultaneously infecting the same individual could result in significant changes in the course of the infection and disease [23].

Diagnostic accuracy in malaria control and elimination must be technically reliability of the results. PCR is an essential molecular tool to

enriched capability of identifying in asymptomatic individuals in the field setting**.** This will enable a switch from passive to active malaria case detection in the field. Although RDT remains the standard method of use in all suspected malaria cases. Its sensitivity and specificity as compared to PCR is limited suggesting exploration of novel molecular technique for malaria endemic areas. These data will also be a massive help for global initiatives of malaria elimination. More studies should be conducted in the future to map malaria epidemiology along Thailand's border.

5. CONCLUSION

PCR is an effective method for assessment of exact malaria prevalence to achieve its elimination goals.

CONSENT

All authors declare that 'written informed consent was obtained from the patient for publication of this article.

The consent form used has been approved by the Department of Medical Sciences Ethics Committee.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the Department of Medical Sciences Ethics Committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki."

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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