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Comparison of microscopy and PCR for the detection of human *Plasmodium* species and *Plasmodium knowlesi* in southern Myanmar

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ABSTRACT

Objectives: To determine the distribution of *Plasmodium* (*P*) species including *Plasmodium knowlesi* and to compare the specificity and sensitivity of microscopy with nested PCR in malaria diagnosis.

Methods: The study was conducted in Kawthaung, southern Myanmar. Ninety clinically suspected malaria patients were screened for malaria by Giemsa stained microscopy and confirmed by nested PCR.

Results: Among the participants, 57 (63.3%) were positive and 33 (36.7%) were negative by microscopy. Of positive samples, 39 (68.4%) were *Plasmodium falciparum*, 17 (29.8%) *Plasmodium vivax* and 1 (1.8%) *Plasmodium malariae*, whereas 59-amplified by PCR were 40 (67.8%), 18 (30.5%) and 1 (1.7%) respectively. PCR amplified 2 microscopy negative samples. Two samples of *P. falciparum* detected by microscopy were amplified as *P. vivax* and vice versa. All samples were negative for *Plasmodium ovale*, *P. knowlesi* and mixed infections. Microscopy had a very good measure of agreement ($\kappa = 0.95$) compared to nested PCR. Sensitivity and specificity of microscopy for diagnosis of *P. falciparum* were 92.5% (95% CI: 79.6–98.4) and 96.0% (95% CI: 86.3–99.5) respectively, whereas for *P. vivax* were 83.3% (95% CI: 58.6–96.4) and 97.2% (95% CI: 90.3–99.7).

Conclusions: *P. knowlesi* was not detected by both microscopy and PCR. Giemsa stained microscopy can still be applied as primary method for malaria diagnosis and is considered as gold standard. As to the lower sensitivity of microscopy for vivax malaria, those with previous history of malaria and relapse cases should be diagnosed by RDT or PCR combined with microscopy. Inaccuracy of species diagnosis highlighted the requirement of training and refresher courses for microscopists.

1. Introduction

Malaria continues to be a public health problem in many countries, although most are working towards malaria elimination. In Southeast Asia, *Plasmodium knowlesi*, a simian malaria parasite is infecting humans. All countries in Southeast Asia have reported *P. knowlesi* cases with the exception of Lao PDR and Timor Lesti [1].

Malaria is one of the priority diseases to be controlled in Myanmar. An estimated 57% of population lives in malaria risk area. In 2012, malaria morbidity and mortality rates were 8.08 per 1 000 population and 0.83 per 100 000 populations, respectively [2]. The highest morbidity rate (34.01) and second highest mortality rate (1.88) were found in Myanmar-Thai border area [2]. Myanmar had 20% of rapid diagnostic test (RDT) - confirmed malaria cases in 2014 [3] and by far the greatest malaria burden among Southeast Asian countries [4]. Given an extensive migrant population, the

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widespread use of oral artemisinin-based drugs, and its geographical proximity to India, Myanmar is critical to the success of efforts to prevent the spread of artemisinin resistance globally [4].

Human malarial parasites of four *Plasmodium* species are prevalent in Myanmar. *Plasmodium falciparum* was the predominant species accounting for 62.1% of malaria infection while *Plasmodium vivax* was 34.5% and others were 3.3%. The proportion of mixed infection was low (3.3%). *Plasmodium malariae* and *Plasmodium ovale* were of very low proportion of 0.14% in 2012 [2]. Cases of Myanmar patients infected by *P. knowlesi* were reported from neighboring countries of Myanmar. In a study conducted at China–Myanmar border area, prevalence of *P. knowlesi* infection was more than 20%, mostly co-infected with other *Plasmodium* species [5], and in another study conducted at Thailand–Myanmar border area, two cases were infected with *P. knowlesi* [6].

Detection of malaria parasites by light microscopy of Giemsa stained blood film remains the primary method for the diagnosis of malaria in health clinics and hospitals throughout the world [7]. The quality of microscopy based diagnosis is frequently inadequate for ensuring good health outcomes. An acceptable microscopy service is one that is cost-effective, provides results that are consistently accurate and timely enough to have a direct impact on treatment. The effectiveness of malaria microscopy depends on maintaining a high level of staff competency and performance at all levels [7]. Due to the morphological similarities in trophozoites, schizonts & gametocytes between *P. knowlesi* and *P. malariae* as well as ring stage between *P. knowlesi* and *P. falciparum*, *P. knowlesi* malaria is often misdiagnosed by conventional microscopy [8,9].

To overcome some limitations of microscopy, PCR based assays have been developed. PCR is more sensitive and specific than microscopy, particularly in cases with low parasitemia or mixed infections and the nested PCR has been considered the molecular gold standard for malaria detection [10–12]. PCR assay is the sensitive technique for collecting accurate malaria epidemiological data and is useful especially in low endemic areas and where the parasitemia is a very low level and also in submicroscopic cases [13].

Although there were reported cases of Myanmar patients infected by *P. knowlesi* at the Thai-Myanmar and China–Myanmar borders, the presence of *P. knowlesi* infection has never been clearly specified and officially recorded in Myanmar. Thus the confirmation of the infection in the region is getting more important. This study was conducted to determine the distribution of *Plasmodium* species including *P. knowlesi* and to compare the specificity and sensitivity of microscopy with nested PCR in malaria diagnosis.

2. Materials and methods

2.1. Study site

The study was conducted from 2013 to 2015 in Kawthaung township (10°N and 98.30°E) located at the mouth of Kyan river on the Malay Peninsula and Thai border town of Ranong to the East (Figure 1). Kawthaung is one of three districts in Tanintharyi Division where majority of the residents live along the coastal plains and river valleys. Malaria in this region is mainly caused by *P. falciparum* and *P. vivax*. In 2011, slide positivity rate and RDT malaria positivity rate among clinically suspected malaria were 39.26% and 43.16% respectively [2].

2.2. Patients and sample size

Assuming that prevalence of *P. knowlesi* infection being 5% among malaria patients [2] at 95% confidence interval, 5% precision and 20% refusal rate, the required sample size was 90. Adult male and female febrile patients suspected to be positive for malaria who signed informed consent form were recruited into this study.

2.3. Procedures

On obtaining written informed consent from each patient, 6–8 drops (0.3–0.4 mL) of capillary blood sample from finger prick were collected under aseptic condition. Two to three drops were used for preparation of thick and thin blood films for Giemsa staining. The rest was spotted on a 3 MM Whatman filter paper, air-dried at room temperature, kept individually in zip lock bag and stored at − 20 °C until required for molecular analysis.

2.3.1. Microscopic examination

Three blood slides, two thick and a thin blood smears were prepared from each patient. The registration number of the patient and the date were recorded on the glass slide with a permanent glass pen. One thick blood film was stained rapidly (10% Giemsa for 10–15 min) for initial screening, while another thick and thin blood slides were stained with 2% Giemsa for 45–60 min and were used to determine the parasite density and species identification, respectively.

Parasite density was calculated as described in [14]. A blood slide was considered negative when examination of 100 microscopic fields revealed no asexual parasites. A further 100 fields of thick film was examined to exclude mixed infections; the thin film was examined for confirmation for any doubtful case [14]. Two trained and experienced microscopists examined all the slides independently and parasite densities were calculated by averaging the two counts. Blood smears with discordant results (differences between the two microscopists in species diagnosis, in parasite density of > 50%) were re-examined by a third, independent microscopist and parasite density was calculated by averaging the two closest counts [7].

2.3.2. DNA extraction and nested PCR

Parasite DNA was extracted from dried blood collected on 3 MM filter paper by using QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. The extracted DNA was stored at − 20 °C until required for molecular analysis. Nested PCR was performed as described [13,15]. Briefly, primary amplification with genus specific primers was followed by secondary PCR using specific primers for all five species (*Plasmodium* – *falciparum*, *vivax*, *ovale*, *malariae* and *knowlesi*). Standard control samples of both positive and negative were included in every PCR reaction. PCR products of nest 2 were subjected to electrophoresis in 2% agarose gel, stained with ethidium bromide, visualized under UV light. Interpretation of the results was done based on band sizes.

2.3.3. Statistical analysis

Laboratory data was recorded on a daily basis and entered into a database. With nested PCR as the reference standard,

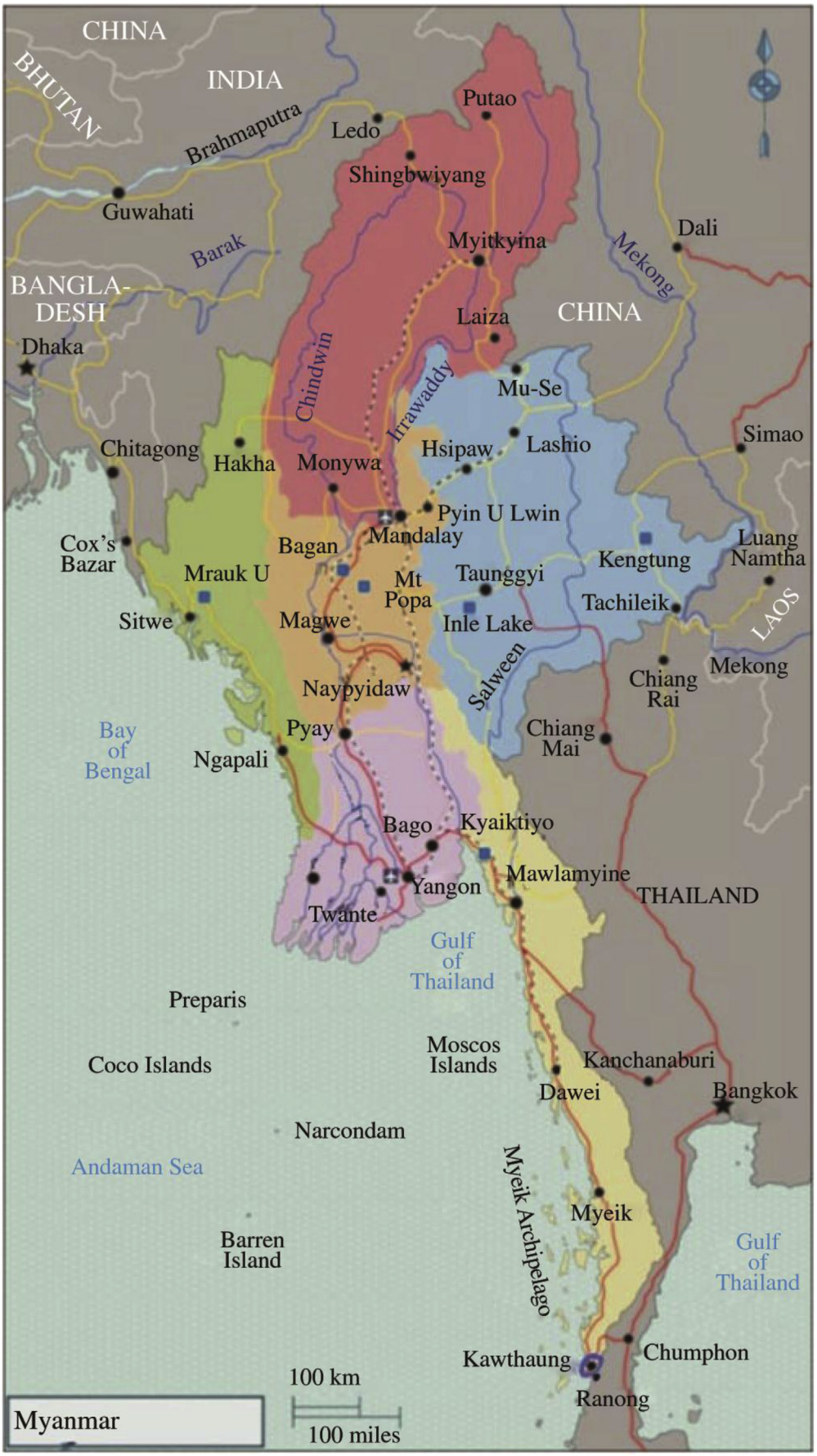

Figure 1. Map of Myanmar – the blue circle indicates the study site.

microscopy results were considered true positive, true negative, false positive and false negative. Sensitivity, specificity and kappa coefficient of tests were determined by STATA version 13 (StataCorp LP, Texas, USA).

2.3.4. Ethical consideration

The study protocol was performed according to the Helsinki declaration and had been approved by the Ethics Review Committee on Medical Research involving Human Subjects, Department of Medical Research (Lower Myanmar). Written informed consent was obtained from all patients. Those with microscopy-positive malaria were treated by local health staff according to the National Treatment Guidelines.

3. Results

3.1. Detection of malaria by microscopy

Ninety clinically suspected adult malaria cases were included in this study and screened for malaria by microscopy. Among these participants, 57 (63.3%) were positive and 33 (36.7%) were negative. Of positive samples, 39 (68.4%), 17 (29.8%) and 1 (1.8%) were diagnosed as *P. falciparum*, *P. vivax* and *P. malariae*, respectively. Monoinfection only was detected in all microscopy positive cases. Neither *P. ovale* nor *P. knowlesi* infections were detected.

3.2. Determination of parasite density

Level of parasitemia was determined for 57 microscopy-positive cases. 43 patients had parasite density of 1 000 and above while 13 patients had less than 1 000 parasites/μL. Patients with *P. falciparum* had high level of parasitemia [mean density 8 874 parasites/μL (SEM = 2127) with the range from 50 to 74 320 parasites/μL] than those with *P. vivax* [mean density 4 029 (SEM = 868), range 200–10 110 parasites/μL)].

3.3. Detection of malaria by nested PCR

As shown in Table 1, PCR amplified 59 samples, of which 40 (67.8%), 18 (30.5%) and 1 (1.7%) were *P. falciparum*, *P. vivax* and *P. malariae*, respectively. Mixed infections were not detected. All samples were negative for *P. ovale* and *P. knowlesi*. PCR amplified 2 microscopy negative samples (one each as *P. falciparum* and *P. vivax*). Two samples of microscopy positive *P. falciparum* were amplified as *P. vivax* and vice versa.

Table 1
Results of microscopy and nested PCR.

			Nested PCR				Total	
			Positive			Negative		
			PF	PV	PM			
Microscopy	Positive	PF	37	2	0	0	39	57
		PV	2	15	0		17	
		PM	0	0	1		1	
	Negative	PF	1	0	0	31	33	33
		PV	0	1	0			
Total			40	18	1	31	90	90

PF = *P. falciparum*; PV = *P. vivax*; PM= *P. malariae*.

3.4. Sensitivity and specificity of malaria microscopy in comparison with nested PCR

Giemsa stained microscopy detected *Plasmodium* species in 57 out of 59 PCR positive samples having sensitivity of 96.6% and specificity of 100%. Microscopy had a very good measure of agreement (κ = 0.95) compared to nested PCR. Among 40 PCR-confirmed *P. falciparum* cases, 37 cases were correctly diagnosed by microscopy while 2 cases were misdiagnosed as *P. vivax* and 1 as negative. Among 18 PCR positive *P. vivax* cases, 15 were correctly diagnosed as *P. vivax* by microscopy while 2 were misdiagnosed as *P. falciparum* and 1 as negative case. One PCR confirmed *P. malariae* case was correctly diagnosed by microscopy (Table 1). Therefore sensitivity and specificity of microscopy for diagnosis of *P. falciparum* were 92.5% (95% *CI*: 79.6–98.4) and 96.0% (95% *CI*: 86.3–99.5), respectively, while for *P. vivax* it was 83.3% (95% *CI*: 58.6–96.4) and 97.2% (95% *CI*: 90.3–99.7), respectively.

4. Discussion

Myanmar is located in South East Asia Region and bordering with Bangladesh, China, India, Laos and Thailand. There are 16 townships in Myanmar Thailand border and the study site Kawthaung is across the Thailand border town named Ranong. Kawthaung was one of the areas with highest annual incidence rates of clinical malaria 5–49 per 1 000 [16]. Early, adequate diagnosis and prompt treatment is one of the main strategies in controlling malaria. In Kawthaung, different malaria diagnosis methods are used which is mainly clinical diagnosis based on signs & symptoms and laboratory diagnosis (microscopy with or without RDT). Microscopic examination of Giemsa stained blood films is a standard laboratory method for malaria diagnosis. In areas where microscopy is not available, immediate confirmation of malaria is by RDTs. Microscopic diagnosis has many advantages such as 1) low direct costs if the infrastructure maintaining service is already available, 2) is sensitive if the quality of microscopist is high and able to differentiate between malaria species, 3) can determine parasite densities and 4) use to diagnose other diseases [7].

However, the effectiveness of malaria microscopy depends on maintaining a high level of staff competence and accurate performance at all levels. Misdiagnosis of malaria often happens in cases with low level parasitemia, especially when antimalarial drugs are taken inappropriately. Misdiagnosis of malaria results in the unnecessary prescription of high cost drugs and the unnecessary exposure of the patient to potentially toxic drugs. This is a needless burden to both the patient and the medical services [7]. In this study, microscopically diagnosed two *P. vivax* cases were detected as *P. falciparum* by PCR. Those cases were treated with chloroquine in accordance with National Guideline for Treatment of malaria. Chloroquine resistant *P. falciparum* malaria has long been documented in Myanmar and monotherapy is unethical clinical practice in treating falciparum malaria. To reduce malaria morbidity and mortality, early diagnosis and prompt treatment are essential. As the choice of treatment usually relies on the exact diagnosis, misdiagnosis will lead to mismanagement. Failure to give prompt treatment to falciparum malaria patients may result in unnecessary

complications which may result in fatality. This finding shows the usefulness of molecular diagnostic facility in reducing malaria mortality and morbidity.

Two cases of PCR confirmed as *P. vivax* were diagnosed as *P. falciparum* by microscopy. These cases were treated with artemisinin-based combination therapy (ACT) but not with primaquine. *P. vivax* patient was unnecessarily exposed to ACT and that kind of drug pressure on parasite population is one of the causes of drug resistance. Failure to add primaquine in these cases will lead to the formation of gametocytes which are the infective stage of the parasite to the mosquitoes. This highlights the important role of molecular diagnosis to reduce transmission especially for a country like Myanmar in the pre-elimination era.

In addition, inaccuracy of species identification by microscopic examination in this study highlighted the requirement of adequate training and retraining of microscopists. Adequate training can increase the yield of accurate malaria diagnosis which helps to reduce illness, potential death, mistreatment and disease burden and also save the resources for malaria control [7].

Greater sensitivity and specificity of nested PCR over microscopy had been reported by many studies [17–20]. Compared to nested PCR, overall Giemsa stained microscopy for malaria diagnosis had sensitivity of 96.6% and specificity of 100% in our study. With regards to species diagnosis by microscopy, sensitivity for identifying *P. falciparum* was much higher than *P. vivax* (92.5% *vs*. 83.3%) whereas the specificity for both *P. falciparum* and *P. vivax* was relatively similar (97.2% *vs*. 96.0%). Lower sensitivity of microscopy in diagnosing *P. vivax* in this study was probably due to presence of artefacts, low quality of stained smears, low parasitemia in vivax infection and high prevalence rate of falciparum malaria in the study area. This finding was noticed to be consistent with a study in Ethiopia [17].

Detection of one microscopy positive *P. malariae* confirmed by PCR indicated presence of *P. malariae* infection in Kawthaung but of very low prevalence rate. It is because *P. malariae* and *P. ovale* infections in Myanmar were very low having only 0.14% in 2012 [2].

Although there were reported cases of Myanmar patients infected by *P. knowlesi* at the Thai-Myanmar and China–Myanmar borders, microscopy and nested PCR results of our study confirmed that *P. knowlesi* was not detected among 90 clinically suspected malaria patients in Kawthaung, southern Myanmar. It is recommended to study *P. knowlesi* infection in humans in larger sample of the population and also extend to other potential geographical area like China–Myanmar border.

P. knowlesi was not detected by both microscopy and nested PCR in clinically suspicious malaria patients in Kawthaung. Microscopic examination of Giemsa stained blood film can still be used as primary method for diagnosis of malaria. As to the lower sensitivity of microscopy for diagnosis of vivax malaria, those with previous history of malaria and relapse cases should be diagnosed by RDT or PCR combined with microscopy. Inaccuracy of species diagnosis by microscopy highlighted the requirement of adequate training and regular refresher training of microscopists who are involved in malaria diagnosis.

Conflict of interest statement

We declare that we have no conflict of interest.

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