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Comparison of Malaria Microscopy and Polymerase Chain Reaction for Identification of Plasmodium in Papua, Indonesia

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ABSTRACT
To diagnose malaria, microscopic examination is still considered as the standard tool, but still there are certain reservations. This study aimed to compare the identification of *Plasmodium* species by microscopy and polymerase chain reaction (PCR). This cross-sectional investigation was carried out in June 2019 until September 2019. Blood samples were taken and examined using microscope and nested PCR. Both results were compared to evaluate the diagnostic performance of microscopy and agreement between both examinations. Only *P. vivax* and *P. falciparum* were found in current study. The sensitivity, specificity, PPV, and NPV of the microscopy examination compared to nested PCR for detecting *P. vivax*, was 91.0%, 97.5%, 96.8%, and 92.8% respectively with κ=0.889; and for *P. falciparum*, the sensitivity, specificity, PPV and NPV was 62.7%, 95.8%, 88.9%, and 82.7% respectively with κ=0.628. Among all mixed infections, only 12% were identified correctly by microscopy. Microscopy is reliable way for routine diagnostic purposes, to detect single malaria infection. However, it is unreliable to detect mixed malaria infections.

Keywords: Malaria, microscopy, polymerase chain reaction.

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INTRODUCTION

Malaria is major threat to common people through the world. It is one of main cause of death in number of under developed countries.(1) The cause of malaria is the *Plasmodium* parasite and infected female Anopheles mosquitos is responsible for its transmission.(2,3) Potential risk factors of this disease are poor sanitation, life styles, housing situation, living conditions and personal hygiene, similiar as other infections such as *Helicobacter pylori*.(4) According to WHO's annual Malaria Report published in 2019 disclosed that, almost 228 million malaria cases reported through the world in 2018 with around 405.000 deaths. Most vulnerable group to be affected by malaria are children under 5 year age.(5) In 2018, 67% of all malaria deaths are accounted of this group.(6) Although surveillance has been carried out since April 2000 malaria is still a tropical health problem in Indonesia.(7) According to the Indonesia Ministry of Health, malaria cases are more prevalent in eastern Indonesia. Papua as the easternmost province, has the most annual parasite index among all provinces in Indonesia.(8,9)

WHO recommends first diagnosis of malaria before the start of its treatment, in order to avoid the drug resistance. It is also important for identifying malaria-negative patients, which need further investigations for suitable treatment.(10) For management and effective control of malaria, its accurate diagnosis and use of appropriate anti-malarial drug is very important. (11)

The examination of blood film under microscope is a standard laboratory technique for malaria diagnosis in endemic countries.(12) It is not very much expensive and can be used for finding *Plasmodium* species and its density.(13–15) However, microscopic examination in endemic areas has several limitations, such as shortage of well-trained competent microscopist, labor-intensive, time-consuming, inadequate quality control, and poor diagnosis.(16–20) On the other hand, polymerase chain reaction (PCR) is more sensitive, highly specific and can detect very low density of parasitemia. It has features of repeatability and reproducibility even with low parasite densities.(3,21) However, costs and infrastructure required to operate and maintain PCR methods are not feasible in most field settings.(13,22) Huge time span is required between sample collection, their transportation, then processing and finally sending the results to the physician. These drawbacks limits the usefulness of PCR technique in routine clinical practice.(20)

The definition of a "gold standard" for the purpose of malaria diagnosis is an important issue that should be addressed.(21) Microscopic examination of blood films has long been used for making assessment regarding the results of trials of vaccine and drug and for the purpose of using them as standard reference in evaluating new method for malaria diagnosis.(3) Although microscopy has its limitations and molecular diagnostic techniques have improvements, which pose the question of whether PCR should become the reference standard for finding the parasites of malaria.(20) Current study aimed to compare the identification of *Plasmodium* parasite by microscopy and PCR and to determine the reliability of microscopy to be used as a gold standard for clinical trial and routine examinations in Papua, Indonesia.

METHODOLOGY

Current cross-sectional investigation was carried out in June 2019 until September 2019. The population of this study was all patients in the emergency department, inpatient department, and outpatient clinic of Merauke General Hospital, Papua, Indonesia. Adult and child patients with clinical signs of fever were included in this study.

5 mL EDTA containing tubes were used to collect blood samples. Thick and thin blood film was made using Giemsa stain and interpreted on a light microscope at 1000 times magnification by 2 certified microscopists. Microscopy interpretation was done before other examinations.

Remaining blood samples were spotted on a Whatman™ paper (GE Healthcare Companies) for further molecular studies. These blood samples Whatman paer were air-dried, and double zip-lock plastic bags with silica gel were used to store these samples at 4 °C, and subsequently transported to the Department of Parasitology of Brawijaya University, Malang, Indonesia for diagnostic confirmation by PCR.

DNA extraction from the filter paper was done using *Norgen's Dried Blood Spot DNA Isolation Kit* (Norgen Biotek Corporation, Canada). Nested PCR method was performed using *Bio-Rad T100 Thermal Cycler* (Bio-Rad Laboratories, Inc.) followed by electrophoresis according to the instruction manual. Electrophoresis reading was performed using *Bio-Rad Gel Doc EZ Imager* (Bio-Rad Laboratories, Inc.). Each run was included positive and negative control. The primers used for amplification are shown in table 1.

Statistical analysis was carried out using SPSS 25.0 software. The microscopy outcomes were compared to PCR results. Using PCR as the gold standard, we calculate the not only sensitivity, and specificity, but also positive predictive value (PPV), and negative predictive value (NPV) of microscopy. Interrater reliability between microscopy and PCR was analyzed using *Cohen's Kappa Coefficient* (κ).

Well briefed and well informed consent was obtained from every patient of this study. Research ethics committee of the Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia approved this study.

RESULTS

Among 146 subjects were recruited as a sample of this study. Subject characteristics were shown in Table 2. Male subjects were dominant in this study (63%). Most subjects were within the age group of 21-30 (23.3%), 11-20 (21.2%), and 31-40 (17.8%).

Identification of *Plasmodium spp.* by microscopy and PCR were categorized as *P. vivax*, *P. falciparum*, mixed *P. vivax* and *P. falciparum*, and negative. The proportion of each was shown in Table 3. *P. vivax* was the most prevalent *Plasmodium* species in current study. Other *Plasmodium spp.* were not found in this study.

Table 4 showed the cross-tabulation of *Plasmodium spp.* identification by microscopy and nested PCR. Among 25 subjects with mixed infection identified by nested PCR, there were only 3 subjects that were correctly identified as mixed infection by microscopy. The remaining subjects were identified by microscopy as *P. vivax* in 17 subjects, *P. falciparum* in 4 subjects, and negative in 1 subject.

The diagnostic test performance of microscopy was evaluated by using nested PCR as the reference standard (Table 5). The diagnostic test performance was done separately for each *Plasmodium* species identification. To identify *P. vivax*, microscopy had sensitivity (91.0%), specificity (97.5%), PPV (96.8%) and NPV (92.8%) with κ=0.889. κ value ranges between 0.80 and 0.90 showed strong agreement between the two examinations.(23) To identify *P. falciparum*, microscopy had sensitivity, specificity, PPV and NPV of 62.7%, 95.8%, 88.9%, and 82.7% respectively with κ=0.628. κ value between 0.60 and 0.79 showed moderate agreement between the two examinations.(23)

DISCUSSION AND CONCLUSIONS

Among 146 subjects were examined of *Plasmodium* species by microscopy and PCR. The sensitivity of microscopy to identify *P. falciparum* was lower than to identify *P. vivax*. The microscopy readings showed substantial discrepancy when compared to PCR, especially in mixed infection. Several of the *P. falciparum* were in subjects with mixed infection and most of them were interpreted as single *P. vivax* infection, resulting in increased false-negative readings of *P. falciparum* and thus lowered the sensitivity of microscopy to identify *P. falciparum*. The microscopists might have missed the occurance of *P. falciparum* due to the predominance of *P. vivax* in those subjects. It has been found that the co-infection of *P. vivax* and *P. falciparum* in a host (human) has interspecies suppression through competition of host red blood cell and cross-species immunity.(24) Our result was consistent with previous studies which found that microscopy had lower sensitivity to detect *Plasmodium spp.* compared to a molecular diagnostic methods like nested PCR(25), semi-nested multiplex PCR(15), and RT-PCR(26).

It is important to differentiate among various *Plasmodium* species to establish the accurate treatment regimen, and also to apply suitable strategies to control malaria in various endemic regions. Inaccurate identification of the *Plasmodium* species can create problems in public health and inappropriate treatments, can lead to produce drug resistance and malaria resurgence.(15,27–29) Drug resistance was associated with malaria resurgence because infections that were not effectively treated remained to contribute towards onwards transmission.(29) According to WHO's guidelines, high quality diagnostic techniques require to control.(15)

The application of microscopy as the gold standard for malaria diagnosis has been under scrutiny due to the reports of false-negative results even at low level of parasitemia and errors in identification of species in mixed infections.(20,28,30) The findings of this study also showed limitations of microscopy which was associated with mixed infection. Among 25 subjects with mixed infection confirmed by nested PCR, most of them were identified as single *P. vivax* infection by microscopy. Only 3 of them were identified correctly as mixed infection by microscopy.

We also observed other misidentifications by microscopy compared to nested PCR. Two subjects with negative PCR results were identified as *P. vivax* and *P. falciparum* infection respectively, two subjects with single *Plasmodium* infection were identified incorrectly, and two subjects with single *P. vivax* infection were interpreted as mixed infection by microscopy. It is known that bad quality of blood film often produce wrong information about the presence malaria parasites,(31,32) and these wrong information can cause false-positive readings of *Plasmodium* species.

Still use of microscopy is quite useful diagnostic tool in malaria-hit areas, but its use as a gold standard can produce wrong outcomess in clinical trials. Expertise of technicians to make good blood film, its staining, and then reading are important requirements for better quality of microscopy .(13,14,16,26) It also is important to look into many areas to detect infection, and it requires services of at least two train microscopists.(15) Microscopy readings also produced both

false-negative readings and false-positive readings, which can greatly affect the outcomes of clinical trials. This finding was similar to previous studies.(16,26) PCR-based methods from previous studies mentioned excellent diagnostic outcomes than microscopy to detect *Plasmodium spp*.(13,25) PCR is strongly suggested for the real time diagnosis of *Plasmodium spp*. and should be used as a gold standard in clinical trials and reference laboratories with adequate infrastructure to perform molecular procedures.

The drawback of current study was a small sample size and limited finding of *Plasmodium spp*. Papua province is the endemic area of *P. vivax* and *P. falciparum*, but other *Plasmodium spp*. were very rare in this area and thus made this study unable to find other *Plasmodium spp*. Further study with other *Plasmodium spp.* and larger sample size is needed. Microscopy can be used for routine diagnostic purposes to detect single malaria infection in Papua, Indonesia and other endemic areas with limited resources. However, it is unreliable to detect mixed malaria infections. The molecular diagnostic approach is highly recommended to be used as a gold standard in clinical trials and reference laboratories.

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Table 1: Primer Sequences

Nest 1 Genus-specific primers		
Plasmodium (1600 bp)	rPLU5	5⁺-CCT GTT GTT GCC TTA AAC TTC-3⁺
	rPLU1	5⁺-TCA AAG ATT AAG CCA TGC AAG TGA-3’
Nest 2 A. Genus Specific Primers		
Plasmodium (240 bp)	rPLU3	5+-TTT TTA TAA GGA TAA CTA CGG AAA AGC TGT-3+
	rPLU4	5+-TAC CCG TCA TAG CCA TGT TAG GCC AAT ACC-3’
B. Species Specific Primers		
P. vivax (117 bp)	rVIV1	5+-CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC-3+
	rVIV2	5+-ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3+
P. falciparum (205 bp)	rFAL1	5+-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3+
	rFAL2	5+- ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3+
P. ovale (787 bp)	rOVA1	5+-ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA-3+
	rOVA2	5+-GGA AAA GGA CAC ATT AAT TGT ATC CTA GTG-3+
P. malariae (144 bp)	rMAL1	5+-ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC-3+
	rMAL2	5+-AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA-3+
P. knowlesi (153 bp)	PMK8F	5⁺-GTT AGC GAG AGC CAC AAA AAA GCG AAT-3⁺
	PMK9R	5⁺-ACT CAA AGT AAC AAA ATC TTC CGTA-3⁺

Table 2: Subject characteristics

Characteristics	Value
Gender	
Male	92 (63%)
Female	54 (37%)
Age	27 (16-41.5)*
1-10 years	17 (11.6%)
11-20 years	31 (21.2%)
21-30 years	34 (23.3%)
31-40 years	26 (17.8%)
41-50 years	16 (11%)
51-60 years	12 (8.2%)
61-70 years	7 (4.8%)
71-80 years	3 (2.1%)

*median (IQR)

Table 3: *Plasmodium spp.* identification by microscopy and nested PCR

Identification	Microscopy	Nested PCR
P. vivax	58 (39.7%)	42 (28.8%)
P. falciparum	31 (21.2%)	26 (17.8%)
Mixed *P. vivax and P. falciparum*	5 (3.4%)	25 (17.1%)
Negative	52 (35.6%)	53 (36.3%)

Table 4: *Plasmodium spp.* cross tabulation

	Nested PCR				
Microscopy	*P. vivax*	*P. falciparum*	Mixed	Negative	Total
P. vivax	39 (26.7%)	1 (0.7%)	17(11.6%)	1 (0.7%)	58 (39.7%)
P. falciparum	1 (0.7%)	25 (17.1%)	4 (2.7%)	1 (0.7%)	31 (21.2%)
Mixed	2 (1.4%)	0 (0%)	3 (2.1%)	0 (0%)	5 (3.4%)
Negative	0 (0%)	0 (0%)	1 (0.7%)	51 (34.9%)	52 (35.6%)
Total	42 (28.8%)	26 (17.8%)	25(17.1%)	53 (36.3%)	146 (100%)

Table 5: Diagnostic test performance of microscopy in *Plasmodium spp.* identification with nested PCR as reference method

Identification	Sensitivity	Specificity	PPV	NPV	κ	p
P. vivax	91.0%	97.5%	96.8%	92.8%	0.889	<0.001
P. falciparum	62.7%	95.8%	88.9%	82.7%	0.628	<0.001