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Comparison of Microscopy and Polymerase Chain Reaction Examination Results in the Detection of Malaria Parasites

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Abstract

Background: Microscopy remains the mainstay method for malaria diagnosis worldwide, although species misidentifications have been detected in practices due to various limitations, such as hypnozoites detection and lower parasitemia in asymptomatic malaria. Polymerase Chain Reaction (PCR) is a molecular diagnostic method with high accuracy in detecting species of organisms. This study was aimed to evaluate the diagnostic performance of microscopy compared to nested PCR in detecting malaria parasites.

Methods: A cross-sectional study was conducted with previous data on malaria assessment in East Nusa Tenggara. More than 500 asymptomatic respondents were included by the systematic random sampling method from 5 sub-districts area in the region based on API. Microscopic assessment by thick and thin blood smears was made following protocols from the Ministry of Health, while DNA isolation was done using 200 µl fresh blood sample and nested PCR amplification protocol with specific primers of the malaria parasites species Plasmodium sp.

Results: A total of 555 specimens were collected, and 1.6% (9/555) of those were microscopy-positive and 32.6% (181/555) were detected positive by nested PCR. Of microscopy-positive samples, 33.3% (3/9) were P. falciparum and 66.7% (6/9)
were P. vivax, whereas among PCR-positive samples, 31.5% (57/181) were P. falciparum, 52.5% (95/181) were P. vivax, and 16.0% (29/181) were mixed infection of both species. From this study, microscopy was found to had a slight measure of agreement (κ = 0.055) compared to nested PCR.

Conclusion: In lower parasitemia and asymptomatic malaria, the microscopic assessment may not be sensitive. Thus, this increases the need of using PCR assessment to confirm the identification of malaria parasites.

Keywords: Malaria, Microscopy, Plasmodium, PCR

Introduction

Malaria is a life-threatening disease caused by *Plasmodium sp.* parasites that are transmitted to people through the bites of infected female *Anopheles* mosquitoes.[1] In 2018, there were 228 million malaria cases recorded globally, with the Southeast Asia region became a region with the second-highest prevalence of malaria cases after Africa.[2] Indonesia as a tropical country has a highly varied malaria endemicity level. Until 2018, a high level of malaria endemicity is still concentrated in the eastern region

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of Indonesia, with East Nusa Tenggara province occupied the third-highest API in the country (API 3.42).[3] To support the malaria-free Asia Pacific by 2030, the Ministry of Health of the Republic of Indonesia has set a target of phased elimination at the provincial level with Indonesia's target of achieving national malaria elimination by 2030.[4], [5] This is stated in the Decree of the Minister of Health of the Republic of Indonesia No. 293/MENKES/SK/IV/2009 concerning the Elimination of Malaria in
Indonesia, which also states that an area can be declared a malaria-eliminated area if there are no more cases of indigenous transmission for three consecutive years and guaranteed with the ability to carry out good surveillance.[5]

In malaria-endemic areas, there is a tendency in the form of asymptomatic malaria, which is malaria with minimal clinical manifestations associated with low-density *Plasmodium sp.* infection that can only be detected by molecular methods.[6], [7] Until recently, microscopic examination is the gold standard used method for the diagnosis of malaria.[8] However, the microscopic examination has several problems that need to be considered properly so it can be an ideal method for malaria diagnosis. When examined by trained microscopists, microscopic examination can detect parasites up to a limit of 10-50 parasites/µl of blood.[8] But in fact, the average microscopists can only detect 50-100 parasites/µl of blood.[9] For those examiners who are less skilled, the detection limit can only be 100-500 parasites/µl of blood.[8] This makes microscopic examination difficult to detect low parasitemia *Plasmodium sp.* infection and therefore difficult to detect asymptomatic malaria.[10]–[12] This is a challenge for malaria elimination efforts in a region, considering that if asymptomatic malaria infection is not detected, it can become a reservoir for malaria parasites that contribute to the transmission of malaria.[13] Besides, the examiner's subjectivity especially in the diagnosis of mixed infections is also one of the drawbacks of microscopic examination.[12] While in fact, precise identification of all malaria cases is one of the most important things for a proper diagnosis to achieve proper treatment and subsequently, reach the goal of malaria elimination.[14]

Currently, malaria detection using molecular-based technology Polymerase Chain Reaction (PCR) technique has been said to have advantages and even overcomes challenges in microscopic examination.[15] PCR is considered to be able to detect malaria more sensitive and more accurately because it can detect very low levels of parasitemia, up to 1-3 parasites/µl of blood.[15] Besides, it can also identify mixed infections more easily than microscopic examination.[16], [17] Although PCR requires skilled human resources, high costs, and standard laboratory equipment which is usually quite difficult in some areas, PCR is said to be superior to microscopy because it can be used to measure malaria transmission and is more sensitive so that it can find cases which are not detected by microscopic examination.[15], [18] A comparative evaluation of microscopy and RT-PCR in Kenya on 500 suspected malaria subjects concluded that microscopy showed a 75.2% agreement with PCR ($\kappa = 0.51$).[15] On the other hand, a comparative study in Myanmar with 90 samples showed that microscopy had a very high agreement value with PCR, as indicated by its κ value which was at 0.95.[19] This shows that there are variations in the results from the comparison of microscopy with PCR examinations in detecting malaria parasites. Therefore, to accelerate the elimination of malaria in Indonesia, this study sought to evaluate and analyze the results of microscopy and PCR examination in detecting malaria parasites in a malaria-endemic area of East Nusa Tenggara, Indonesia.

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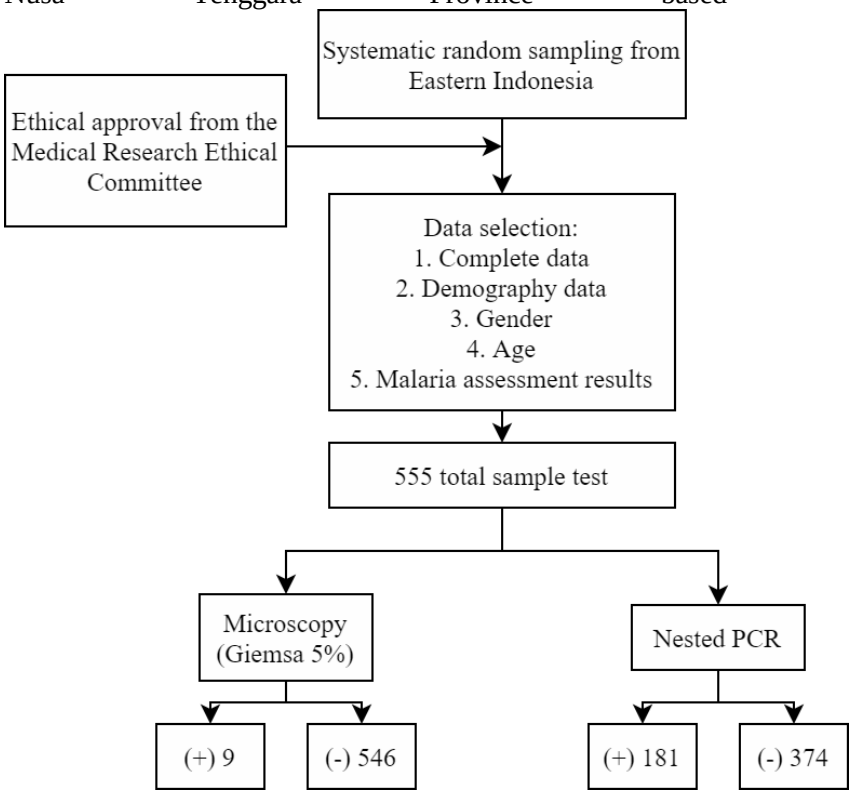
Methods

This is a cross-sectional study from previous data of malaria assessment in East Nusa Tenggara with an API ≤5‰ population. The data was collected from August 2013 until September 2014 from 5 sub-districts of South-Central Timor Regency, East Nusa Tenggara Province based on API.



Picture 1. Research Flowchart

Research subjects were selected by the inclusion criteria that include complete data consist of demography data, gender, age, and malaria assessment results. There was no incomplete data, thus no respondent was excluded and total sample analysis was undertaken. Variables that were selected in this study include microscopy and PCR examination results and were analyzed by conformity assessment with the Kappa value method. This study has been approved by the

Medical Research Ethical Committee, Faculty of Medicine, Universitas Padjadjaran, with ethic license number 1035/UN6.KEP/EC/2020.

1. Data Collection

Blood sampling was done for making thick and thin blood smears (Giemsa 5%) using the protocols from the Ministry of Health. Thick and thin blood smears were read using immersion oil with 100 fields of view. 3 ml of fresh blood samples were taken using an EDTA tube BD vacutainer 3 ml and stored at -20°C for molecular examination. DNA isolation was done using a 200 µl fresh blood sample and nested PCR amplification protocol following the commercial PCR kit. Five primers of the *Plasmodium* species (*P. falciparum*, *P. vivax*, *P.*

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ovale, *P. malariae*, and *P. knowlesi*) were detected using the nPCR method. The amplified samples were visualized using 1-1.5
% Agarose gel containing 2 µl ethidium bromide (EtBr). Visualization of the nPCR results using ultraviolet illumination with a DNA ladder.[20]

2. Data Analysis

All collected data were proceeded using the IBM® SPSS® 22nd version software and analyzed using descriptive statistics in the form of conformity assessment with the Kappa value method.

Results

1. Characteristics of Respondents

A total of 555 respondents were collected for this research based on the inclusion and exclusion criteria. Table 1 shows the distribution of both

microscopy and PCR positive-malaria parasite assessment results based on the characteristics of the respondents. In positive-microscopic results, the number of males is superior to females. The most prevalent age is the 31-40- year-old group and >51-year-old-group. From 5 sub-districts research location, the highest positive-microscopic result is located at Batu Putih, with all negative results are detected at Oinlasi, Oe'ekam, and Panite.

In contrast with microscopy, the number of females with positive results are superior to males. The most prevalent age is the >51-year-old group and the lowest group is the <15-year-old group. From 5 sub-districts research locations, the highest number of positive results is located at Oe'ekam, with Oinlasi and Panite as the lowest.

Table 1. The Distribution of Microscopy and PCR Positive-Malaria Parasite Assessment Results

Characteristics	Positive-Malaria Parasite Assessment Results	
	Microscopy n=9 (%)	PCR n=181 (%)
Gender		

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Male	6 (66.7)	78 (43.1)
Female	3 (33.3)	103 (56.9)
Age (years old)		
<15	0 (0.0)	3 (1.7)
16-20	1 (11.1)	4 (2.2)
21-30	2 (22.2)	18 (9.9)
31-40	3 (33.3)	47 (26.0)
41-50	0 (0.0)	44 (24.3)
>51	3 (33.3)	65 (35.9)
Sub-district		
Oinlasi	0 (0.0)	25 (13.8)
Oe'ekam	0 (0.0)	45 (24.9)
Panite	0 (0.0)	25 (13.8)
Batu Putih	5 (55.6)	43 (23.8)
Oenino	4 (44.4)	43 (23.8)

2. Detection of malaria parasites by microscopy

555 subjects were included in this study and screened for malaria by microscopy. Among these participants, 1.6% (9/555) were positive and 98.4% (546/555) were negative. (Table 2) Of positive samples, 33.3% (3/9) were detected as *P. falciparum*, and 66.7% (6/9) were detected as *P. vivax*. Monoinfection was detected in all microscopy positive cases. Neither *P. ovale* nor *P. malariae* infections were detected. (Table 3)

Table 2. Result of Microscopy and PCR Examination

		Nested PCR examination		Total
		Positive	**Negative**	
Microscopy examination	**Positive**	8	1	9
	Negative	173	373	546
Total		181	374	555

Table 3. Species Identification by Microscopy and nPCR Examination

		Nested PCR examination				Total
		P. falciparum	***P. vivax***	**Mix infection (*P. falciparum* & *P. vivax*)**	**Negative**	
Microscopy examination	***P. falciparum***	3	0	0	0	
	P. vivax	1	2	2	1	
	Negative	53	93	27	373	
Total		57	95	29	374	

	3
	6
	546
	555

3. Detection of malaria parasites by nested PCR

After screened for microscopy, all 555 samples were also examined by nested PCR examination. Among these 555 samples, 32.6% (181/555) were positive and 67.4% (374/555) were negative. Of positive samples, 31.5% (57/181) were detected as *P. falciparum*, 52.5% (95/181) were detected as *P. vivax*, and 16.0% (29/181) were detected as mixed infection (*P. falciparum* and *P. vivax*). Neither *P. ovale* nor *P. malariae* infections were detected.

Misidentification of Microscopy and False-Positive (*P. vivax*) Microscopy

One sample of microscopy positive *P. vivax* was amplified as *P. falciparum*, two samples of microscopy *P. vivax* were amplified as mixed infection consisted of *P. falciparum* and *P. vivax*, and one sample of microscopy positive *P. vivax* was amplified as a negative case by nested PCR. **False-Negative Microscopy**

Among 546 samples that were detected as negative by microscopy, 31.7% (173/546) were detected as positive by nested PCR. Among those 173 false-negative microscopies, 30.6% (53/173) were amplified as *P. falciparum*, 53.8% (93/173) were amplified as *P. vivax*, and 15.6% (27/173) were amplified as mixed infection consisted of *P. falciparum* and *P. vivax*.

4. Performance of microscopy against nested PCR as the reference method

Using nested PCR as the reference standard, 181 samples were positive for malaria while 374 samples were negative for malaria. Microscopy correctly identified 8 samples as positive, but misidentification of species was detected. Microscopy showed a low sensitivity (4.4%) and high specificity (99.7%) with positive and negative predictive values were 88.9% and 68.3%, respectively.

Table 4. Test performance of microscopy with nested PCR as the reference method

Test characteristic	Microscopy
TP (PCR = 181)	8
FP (PCR negative)	1
TN (PCR = 374)	373

FN (PCR positive)	173
Sensitivity [95% CI]	4.4% [2.07 – 8.83]
Specificity [95% CI]	99.7% [98.3 – 99.9]
PPV [95% CI]	88.9% [50.7 – 99.4]
NPP [95% CI]	68.3% [64.2 – 72.2]
Kappa value [95% CI]	0.055 [0.015 – 0.095]

TP true positive, *FP* false positive, *TN* true negative, *FN* false negative, *PPV*
positive predictive value, *NPV* negative predictive value

In this study, microscopy had a slight measure of agreement (κ = 0.055) compared to nested PCR. Among 57 PCR-confirmed *P. falciparum* cases, 3 cases were correctly identified by microscopy while 1 case was misidentified as *P. vivax* and 53 were misidentified as negative. Among 95 PCR-confirmed
P. vivax cases, 2 cases were correctly identified as *P. vivax* by microscopy, while 93 were misidentified as negative. Among 29 PCR-confirmed mixed infections (*P. falciparum* and *P. vivax*), 2 cases were misidentified as *P. vivax* infection only by microscopy, and 27 cases were misidentified as negative by microscopy. And among 374 PCR-confirmed negative cases, 373 cases were correctly negatively-detected by microscopy, while 1 case was misidentified as *P. vivax* infection by microscopy.

Discussion

Indonesia is located off the coast of mainland Southeast Asia in the Indian and Pacific oceans, and it is the most populous country in the Southeast Asia region.[21] East Nusa Tenggara province of Indonesia is comprised of islands in the Lesser Sunda Islands group and it is located in the southeast portion of the country.[22] In 2018, the province had an Annual Parasite Incidence (API) at 3.42, the third-highest in the country of Indonesia.[3] South Central Timor Regency was one of the areas with the highest annual incidence rates of malaria in

East Nusa Tenggara.[23] Early-adequate diagnosis and prompt treatment are some of the principal strategies in controlling malaria. Until recently, the microscopic examination of Giemsa-stained blood films is the gold standard used laboratory method for malaria diagnosis. In areas where microscopy is not available, immediate confirmation of malaria is done by RDTs.[19] Microscopic diagnosis has many advantages such as 1) cost-effective if the infrastructure maintaining service is already available, 2) is sensitive enough if the microscopist can differentiate between malaria species, and 3) allows for the identification of parasitemia percentage, parasitic morphology, and speciation.[19], [24]

However, the microscopic examination method requires well-trained microscopists, and sensitivity and specificity may vary based on the skill of microscopists. The limit of detection is also not ideal, which leads to undiagnosed and untreated cases in sub-microscopic asymptomatic individuals with low parasitemia that cause the transmission cycle to continue in the community.[24] Species misidentification also often happens in microscopically-diagnosed malaria cases.[19] This

could be due to the subjectivity of the microscopists, particularly concerning the diagnosis of mixed infections.[12] Whereas an accurate laboratory diagnosis is essential, as false-negative results can lead to untreated malaria and potentially severe consequences, and false-positive results also can lead to misuse of antimalarial drugs, exposure of parasites to sub-therapeutic blood levels of the drugs, and development of resistance.[25]

In this study, two cases of PCR-confirmed mixed infection consist of *P. vivax* and *P. falciparum* were detected only as *P. vivax* infection by microscopy. If the subsequent drug administration is given based on the microscopic examination results, these cases will be treated as *P. vivax* infection with Artemisinin-based Combination Therapy (ACT) consisted of Dihydroartemisinin-Piperaquine (DHP) + Primaquine, with primaquine given for 14 days of treatment. Fortunately, this drug regimen was similar to the recommended drug administration for mixed infection consist of *P. vivax* and *P. falciparum* based on the National Guideline for Treatment of Malaria.[26] 14 days duration of treatment for *P. vivax* infection is recommended due to the ability of *P. vivax* to form a dormant stage in the liver of the patients which can only be eradicated by administering primaquine in the recommended duration. The total treatment dose of primaquine in *P. vivax* infection corresponds with the overall efficacy of the treatment.[27] Although in this study both detections have the same drug recommendations, an opposite detection may also happen in other tests, where *P. vivax was* detected as *P. falciparum* by microscopy.[19] This kind of misidentification will lead to inadequate primaquine administration for *P. vivax*, because primaquine is only be given for the first day of treatment in *P. falciparum*-detected cases. Whereas, failure to give primaquine in the necessary duration leads to both the formation of gametocytes which are the infective stage of the parasite to the mosquitoes, and the possibility of relapsing malaria cases in the future due to the uncomplete eradication of dormant hypnozoites.[19]

Another finding in this study is one case of both PCR-confirmed negative and *P. falciparum* that was detected as *P. vivax* by microscopy. If subsequent drug administration is given based on microscopy examination, these cases will be treated as *P. vivax* with DHP + Primaquine. Primaquine in *P. vivax*, as stated above, should be given for 14 days. This may result in an excessive treatment of primaquine and thus increasing the possibility of the development of an unnecessary complication, such as hemolysis in undetected G6PD deficiency patients.[28]

Moreover, a similar thing has also happened in all false-negative cases. In this study, 53 cases PCR-confirmed as *P. falciparum*, 93 cases PCR-confirmed as *P. vivax*, and 27 cases PCR-confirmed as mixed infection (*P. falciparum* & *P. vivax*), while they were detected as negative cases by microscopy. Failure to give the appropriate treatment for both *P. falciparum* and *P. vivax* infection cases may result in unnecessary complications and thus lead to fatality and relapsing malaria cases.[19], [29] These findings show the usefulness of the molecular diagnostic method in reducing malaria mortality and morbidity and also highlight the important role of molecular diagnosis to reduce transmission especially for a country like Indonesia in the pre-elimination era.

In this study, the slight agreement of the two examination methods was recorded (κ = 0.055). This was discordant with the previous study in Kenya that showed a moderate agreement between PCR and microscopy (κ = 0.51), and a study in Myanmar that showed a very good agreement (κ = 0.95).[15], [19] Another corresponding study in Nigeria also showed a moderate agreement between PCR and microscopy (κ = 0.491).[30] This phenomenon might be happened due to the high prevalence of asymptomatic malaria in the region which

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correlates with the fact that many asymptomatic infections are submicroscopic and can only be detected by molecular methods.[20], [31]

Conclusion

In conclusion, the performance of microscopic examination in detecting and identifying malaria parasites found to be inferior compared to PCR based on the slight measure of agreement that was found in this study. Microscopy may be accurate while the parasitemia level is high. However, in lower parasitemia and asymptomatic malaria, PCR examination is the most accurate method in detecting and identifying malaria parasites. These inaccuracies of species identification by microscopic examination highlight the importance of using PCR assessment to confirm the identification of malaria parasites.

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