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PERFORMANCE OF MULTIPLEX REAL TIME PCR FOR DIAGNOSIS MALARIA IN FOREST ENDEMIC AREAS IN TANAH BUMBU REGENCY SOUTH KALIMANTAN PROVINCE INDONESIA

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ABSTRACT

Malaria incidence has declined in Indonesia for years, but it is still found a foci malaria in forest areas. Rapid Diagnostic Test (RDT) has been used as a diagnostic tool in remote forest areas because other tests are difficult to carry out due to poor infrastructure. Microscopic examination requires expert personnel to determine the right interpretation. The disadvantage of both methods of diagnosis is that they cannot detect the low density of parasites, causing the diagnosis to be missed. For this reason, it is necessary to examine the molecular diagnosis to determine the right species. This cross-sectional study was conducted on 105 people aged 15-64 years in forest malaria endemic areas who were randomly selected. The diagnosis of malaria is established through rapid diagnostic test, microscopic, real-time PCR examination for identification of genus, and multiplex real time PCR for species identification. The performance of multiplex real time PCR, microscopic and RDT was compared and evaluated each other. From 105 blood samples, RDT identified 38 malaria cases, consist of 9 as *Plasmodium falciparum* infection, 9 as *Plasmodium vivax* and 20 as mixed infection. Light microscopy identified 9 as *P. falciparum* infection, 5 as *P. vivax* infection and 5 as mixed infection. Multiplex real time PCR detected 7 as *P. falciparum* infection, 6 as *P. malariae* infection, and 4 as mixed *P. vivax* and *P. malariae* infection. Using microscopy as a reference test, real time PCR showed an overall sensitivity of 100% and specificity of 91,9%. Multiplex real time PCR for species *P. falciparum* had 77,8% sensitivity and specificity of 91,83%. RDTs sensitivity of 94,7% and specificity of 76,4%. Using multiplex real time PCR as a reference, microscopy had shown better performance than RDT with sensitivity 73,7% and specificity 100% but RDT had low sensitivity with 14,3% (95% CI 11,45 – 26,87) and specificity of 70,5% (95% CI 65,78-79,67). All three tests performed well to diagnose malaria in this area, but multiplex PCR performed better for detecting Plasmodium species, especially species that produce submicroscopic infections or subpaten infections. Multiplex real-time PCR is the most sensitive malaria

diagnostic method that can be used in malaria elimination programs. It has better performance in species identification, species detection with low parasitic density and mixed infection.

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1. Introduction

Malaria is still a problem in the world. More than 200 thousand cases of malaria occur annually in the world and 95% occur in sub-saharan africa. Malaria cases have increased from 227 milion in 2019 to 241 million in 2020 in the world. its occurred due to the Covid-19 Pandemic which caused all health resources to be deployed to overcome this pandemic [1]. Indonesia has the second most malaria cases after India in Asia. Although most areas in Indonesia are malaria-free, there are still focus areas of malaria, most of which are located in remote forests and referred to as forest malaria [2], [3].

An accurate diagnosis followed by effective treatment is essential to eliminate malaria. The methods used for malaria diagnosis are microscopy which is the gold standard, rapid diagnostic test (RDT), and nucleic acid-based amplification assays such as polymerase chain reactions (PCR) and loop-mediated isothermal amplification [4]. Microscopes are still the main method of malaria diagnosis in many countries including Indonesia because it can identify species through thick or thin peripheral blood smears. However, the sensitivity and specificity of microscopy depends on the quality of the stained slides and the microscopic competence [5]. In addition, microscopy cannot detect sub-microscopic malaria (infections with a very low density of parasites) and mixed infections [5- 7].

Rapid diagnostic tests are easy to use, fast, cost-effective, and a tool that can be used in the field for the diagnosis of malaria [8]. Three antigens are typically targeted for commercially available RDTs, namely *P. falciparum* histidine-rich protein 2 (Pf-HRP2), lactate dehydrogenase (LDH), which can be either pan-specific (i.e., genus-specific) or species-specific (P.f.-specific or P.v.-specific), and genus-specific aldolase which detects all Plasmodium species [9]. However, RDT has limitations when detecting asymptomatic or low-density infections as well as from parasitic strains that have removal in genes encoding HRP2 or HRP3, their structural homologs [10], [11].

PCR is very sensitive and has the ability to detect 2-5 parasites/µL compared to RDTs that can detect if the number is more than 100 parasites/µL and a microscope with a limit of detection of 50-500 parasites/µL [12], [13]. Therefore, PCR is increasingly used for malaria diagnosis and is a useful tool for epidemiological studies that map sub-microscopic malaria [14]. However, PCR is expensive, requires qualified personnel, and has a long turn-around time; thus, they are impractical for use in the field or clinical setting [15].

To evaluate efforts to implement targeted malaria elimination requires highly sensitive malaria diagnostic tools, especially for screening and mass treatment strategies [16]. This effort can be made with the use of molecular tests for the diagnosis of malaria. Currently molecular tests for the diagnosis of malaria have developed. One of them is the development of real time PCR and multiplex real time PCR. This method can increase the processing time, which is faster than conventional PCR. This method is quantitative and closed which reduces time, labor, reagents, costs, and the risk of contamination [17]. Multiplex real-time PCR has improved the ability to detect mixed Plasmodium infections and detection of Plasmodium species

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in cases of low parasitemia, with a detection limit of less than 2 parasites per ml and has the advantage of simultaneous detection of multiple targets in one go to improve sensitivity and specificity compared to microscopy and RDTs [18].

2. MATERIALS AND METHODS

2.1 Study area and study design

Teluk Kepayang sub-district in Tanah Bumbu Region still has a forest malaria endemic village. Some areas in that sub-district are still in the form of primary rain forest (20o 57' 0" LS – 30o 38' 24" LS and 115o 24' 0" BT – 116o49' 12" BT) [19]. Based on data from the Central Bureau of Statistic For Tanah Bumbu Regency, the population in that district was 8.154 in 2020 [20]. Data were collected in September 2020 until July 2021. All participants in this study were resided in forest villages (Batu Bulan and Batu Paha), with annual parasite index in 2017 was 80,8 [19]. Sampling was done using simple random sampling. The study was conducted on 105 male and female participants between the ages of 15 and 60 years, are not pregnant, are not experiencing severe pain and are willing to be research subjects. The research involved the health office, the malaria program manager at the local public health center, and the village authorities.

2.2 Data collection procedure

Researchers and village officials will visit every house to conduct interviews, collect venous blood, and observe the surrounding environment. Blood samples were taken through the median cubital vein and put into an EDTA anticoagulant tube. We used a standardized questionnaire to collect data from participants. For validity, it is based on the calculation of corrected item total correlation. The statement is valid if the R value > 0.483. Reliability test using Crohnbach's alpha with p=0.806 The questionnaire was administered by trained interviewers to collect data on patient age, gender, the residence of villages, education, occupation, and ethnicity. Using venous blood taken from each patient, a thick and thin smear is prepared for a microscope. RDT testing was performed on-site and blood in EDTA tubes was collected and transported by cold chain to Eijkman Laboratory for molecular tests.

3. MALARIAL DIAGNOSIS

3.1 Rapid Diagnostic Test

RDTs testing was performed as per manufacturer instruction using SD Bioline Malaria Ag Pf/Pv (Standard Diagnostic Inc, Abbot, US). This kit will detect HRP2 Ag specific to *P. falciparum* and pLDH specific to *P. vivax* and differentiate mono infection and mix infection using whole blood. Using a capillary tube (5 µL), the whole blood sample is collected until it reaches the black line and transferred to the cassette sample well. Four drops of diluent are added vertically for testing in the well. The result is read after 15–30 min. A result is considered negative if a color band is observed in line "C;" it is *P. falciparum*- positive if 2 color bands are observed inline "P.f" and C, or 3 color bands in lines P.f, "Pv" and C. The tests were performed in the field laboratory as a routine malaria testing in mass blood survey (MBS).

3.2 Malaria Microscopy

After made a thick and thin blood films in the field, slides were allowed to air-dry at room temperature. The thin smear fixed with absolute methanol and stored at 2-8oC until being transported to Parasitology Laboratory Faculty of Medicine, Universitas Lambung Mangkurat. All slides were stained with 10% Giemsa solution for 10 minutes. Both thick and thin smear were examined by an experienced laboratory analist. An expert microscopist re-checked all positive slides and 10% negative slides. The microscope used 100 high power fields (HPFs) for parasite detection [21].

3.3 DNA Extraction

Genomic DNA extraction was performed using QiagenQIAamp ® 96 DNA Blood Kit (QIAGEN Inc.) from whole blood. Briefly, 200 µl whole blood add into a 1.5-ml tube for processing as per manufacturer instructions. Finally, with 200 µl volume of elution buffer the DNA was eluted and stored at − 20 °C until assayed [22].

3.4 Real time PCR

The real-time PCR method according to the steps carried out by [23]. DNA was extracted from whole blood with EDTA anticoagulants using the QIAamp DNA Blood Mini Kit (Qiagent, Valencia CA). The primary and probe set is based on the 18S rRNA sequence with u *primary forward* detention center is 5-GCTCTTTTGATTTTGGATG-3, and *the primary reverse* is 5-AGCAGGTTAAGATCTCGTTCG-3. The probe sequence is 5-ATGGCCGTTTTTTTTCGTG-3 and is labeled 5 FAM (6-carboxyfluorescein) as reporters and 3 TAMRA (6 carboxytetramethyl-rhodamin) as quenchers. Real time amplification and measurements were performed using PCR Applied Biosystems 7500 with thermal profile for qPCR: 10 min at 95°C, 40 cycles 15 seconds at 95°C, and 1 minute at 60°C. For qRT-PCR, a 30-minute cycle at 50°C is added as a first step for the reverse transcription process. For reactions, 1 µl of template was added at 9 µl of the master mix mixture containing 1 QuantiTect Probe RT-PCR Master Mix (Qiagen), 0.4 M primer, 0.2 M probe, and 4 mM MgCl2 respectively. For qRT-PCR examination, QuantiTect RT Mix (a mixture of Omniscript and Sensiscrip Reverse Enzyme) was added to the master mix mix as recommended by the manufacturer at a rate of 1µl per 100 µl of reaction parent mixture [23].

3.5 Multiplex real time PCR

Multiplex real-time PCR was performed under universal conditions (95°C for 15s, 60°C for 1 min) with the ABI TaqMan 7500. The reaction was performed with a final volume of 25 µl containing 5 µl of DNA, 12.5 µl of TaqMan universal master mix (Applied Biosystems), primers, and probes. The primers and probes used in this study are listed in Table 1 with the respective concentrations for each reaction. The Plasprobe, Ovaprobe, and Malaprobe were synthesized by Applied Biosystems, and the Vivprobe, Falcprobe, and β2 M probe were synthesized by Biosearch Technologies. All probes were purified by high-performance liquid chromatography. Samples were confirmed *Plasmodium* positive with genus-specific primers Plasmo1 and Plasmo2 and the 6-carboxyfluorescein (FAM)-labeled Plasprobe to detect a region of the *Plasmodium* 18S gene that is conserved across all five species [24].

Table 1. Primers and probes used for identification species Plasmodium.

Species	Primers or probes	Sequence (5'-3')
P. falciparum	FAL-F Primer Falc Probe	CCG ACT AGG TGT TGG ATG AAA GTG TTA A Quasar 670-AGC AAT CTA AAA GTC ACC TCG AAA GAT GAC T-BHQ-2
P. vivax	Viv-F Primer Vivprobe	CCG ACT AGG CTT TGG ATG AAA GAT TTT A Tamra-AGC AAT CTA AGA ATA AAC TCC GAA GAG AAA ATT CT-BHQ-2
P. malariae	Mal-F primer Malaprobe	CCG ACT AGG TGT TGG ATG ATA GAG TAA A FAM-CTA TCT AAA AGA AAC ACT CAT-MGBNFQ
P. ovale	Ova-F primer Ovaprobe	CCG ACT AGG TTT TGG ATG AAA GAT TTT T VIC-CGA AAG GAA TTT TCT TAT T-MGBNFQ

3.6 Data quality assurance

Data was collected by researchers and team who is skillfull. All blood films and RDT testing were

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performed based on standard operating procedures. The quality of each reagen was checked before laboratory analysis was performed. Samples and reagens were stored at appropriate temperature as indicated on manufacturer's belonging. Internal and external quality were run as required during analysis. All samples stored at -20°C.

3.7 Data analysis

The data was analyzed by using SPSS version 28.0 for window. The descriptive analysis will be carried out in the form of percentage and frequency to describe patient sociodemographic. The sensitivity, specificity, predictive value and Kappa coefficient were estimated by comparing results from three assays and 95% confidence interval was computed.

3.8 Ethical Consideration

This study was reviewed and approved by the ethical commission research at the Faculty of Medicine, Lambung Mangkurat University Banjarmasin, Indonesia with number 603/KEPK- FK ULM/EC/V/2021.

4. FINDING AND DISCUSSION

4.1 Sociodemographic characteristic of study participants

A total of 105 participants in forest malaria endemic areas were recruited into the study. Most of the inhabitants work in the forest as forest workers. The lowest age was 16 years old, the highest was 60 years old and mean age was 32 years. The mean of length of stay in the village was 83.81 month ranging from 1 month - 30 years. The lowest body temperature was 36.1°C, the highest was 37.8°C and average temperature was 36.6 °C. The complete data can be seen in table 2 below.

Table 2 Demographic and risk factors of malaria infection

Variables	Total (N=105)
Age (years) (median, range)	32 (16-60)
15 – 45	90 (85. 7%)
more than 45	15 (14. 3%)
Sex	
Male	67 (63. 8%)
Female	38 (36. 2%)
Ethnicity	
Banjar	100 (95. 2%)
Dayak	5 (4. 8%)
Villages	
Batu Bulan	67 (64. 5%)
Batu Paha	38 (35. 5%)
Education	
No school	46 (43. 8%)
Elementary	44 (41. 9%)
Middle school	10 (9.5%)
High school	5 (4. 8%)
Occupation	
Forest workers	79 (75. 2%)

Non-Forest workers	26 (24. 8%)
Temperature (ºC) (mean, range)	36.66 (36. 1 – 37.8)
History of malaria	
Yes	70 (66. 7)
Not	35 (33. 3)
Interval of previous malaria infection (month) (total; mean, range)	(70; 6; 3-14)

4.2 Laboratory result of study participants for diagnosis malaria by RDTs, microscopy, real time PCR, multiplex real time PCR.

Among 105 participants, malaria positive cases by RDTs were 38 (36,19%), by microscopy were 19 (18,1%), by real time PCR (for genus) were 26 (24,8%). Malaria positive cases by multiplex real time PCR was 17 (16,1%). Malaria positivity rate was highest for RDTs. The positivity rate was increased among the age groups 15-45 years old, in male participants, and in forest workers. Malaria positivity rate for RDTs was higher in participants with history or previous malaria, but in microscopy, real time PCR and multiplex real time were higher in participants with no previous malaria infection. The complete data can be seen in table 3.

Table 3. Malaria positivity rate by RDTs, microscopy, real time PCR, Multiplex real time PCR in forest malaria areas

Characteristic	Malaria positivity			
	RDTs (n=38) %	Microscopy (n=19) %	Real time PCR (n=26) %	Multiplex real time PCR and AbTes real time PCR (n=17) %
Age Group				
15-45 (n=90)	33 (86,8)	17 (89,5)	22 (84,6)	15 (88,2)
> 45 (n=15)	5 (13,2)	2 (10,5)	4 (15,4)	2 (11,8)
Sex				
Male (n=67)	25 (65,8)	12 (63,2)	19 (73,1)	10 (58,8)
Female (n=38)	13 (34,2)	7 (36,8)	7 (26,9)	7 (41,2)
Occupancy				
Forest Worker (n=79)	31 (81,6)	19 (100)	26 (100)	17 (100)
Non-Forest (n=26)	7 (18,4)	0	0	0
History Of Malaria				
Yes (n=70)	27 (71,1)	5 (26,3)	10 (38,5)	6 (35,3)
Not (n=35)	11 (28,9)	14 (73,7)	16 (61,5)	11 (64,7)

4.3 Diagnostic performance of RDTs, microscopy, real time PCR and multiplex real time PCR

Using microscopy as a reference test, real time PCR showed an overall sensitivity of 100% (95% CI 96-100), specificity of 91,9% (95% CI 93,54-96,87). Multiplex real time PCR for species *P. falciparum* had 77,8% sensitivity (95% CI 71,47-81,67) and specificity of 91,83% (95% CI 86,57-95,87). RDTs sensitivity of 94,7% (95% CI 92,57-99,67). Using multiplex real time PCR as a reference, microscopy had shown better performance than RDT with sensitivity 73,7% and specificity 100% but RDT had low sensitivity with 14,3% (95% CI 11,45 – 26,87) and specificity of 70,5% (95% CI 65,78-79,67). Real time PCR had shown good sensitivity and specificity for detection genus Plasmodium but Multiplex real time PCR had good performance to identified other species non falciparum. RDT showed low sensitivity and specificity to detect *P. falciparum* (33,3% and 76,7%). Other species had identified by RDT like *P. vivax* and mixed *P.*

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falciparum and *P. vivax* infection. Microscopy had identified *P. vivax* and mixed infection but multiplex real time PCR had not found *P. vivax* monoinfection, but in mixed infection with P. *malariae*. Interestingly, multiplex real time PCR had identified *P. malariae* monoinfection in this study, which was not found in that areas in previous study. This species was not computed performace because it was not found in others assay. Kappa value for microscopy and multiplex real time PCR was 0,78, RDT and multiplex PCR was 0,68. Kappa value for RDT and microscopy was 0,74.

Table 4. Performance of multiplex real time PCR, RDTs and microscopy

Assay	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
RDT vs microscopy				
overall	94,7(92,57-99,67)	76,4 (71,57-79.46)	47,4 (43,6-54,76)	98,5(94.3-100)
P. falciparum	33.3(31,46-43,67)	76,7(70,34-78.54)	33,3 (29,76-41,45)	95,2(93,8=99,2)
Real time PCR vs microscopy				
overall	100(96-100)	91,9(93,54-96,87)	73,07 (70,45-84,23)	100 (93,0-100,0)
Multiplex real time PCR vs microscopy				
P. falciparum	77,8(69,8-88,45)	91,83(89,78-100)	100(94,45-100,0)	98,4(93,24-100,0)
RDT vs multiplex real time PCR				
overall	96,2 (93,0-100,0)	83,5(76,78-85,43)	65,8(61,78-72,87)	98,5(94,23-100,0)
P. falciparum	14,3 (11,45-26,87)	70,5 (65,78-79,67)	11,1 (9,87-21,56)	92,5 (89,57-98,23)
Microscopy vs multiplex real time PCR				
overall	73,7 (70,97-87,67)	100 (93,7-100)	100 (94,3-100)	91,86 (89,67-96,23)
P. falciparum	100 (93-100)	97,7(91,77-100)	77,8 (71,67-86,45)	100 (92,78-100)

5. DISCUSSION

An accurate and fast diagnosis is essential to control malaria infection. This study evaluated the performance of RDT, microscopes, and real-time PCR in diagnosing malaria in forest malaria areas that are low transmission. This study obtained the prevalence of forest malaria based on microscopic examinations of 18.1%, based on RDT of 36.19%, real time PCR of 24.8% and based on multiplex real time PCR and AbTes real time PCR of 16.1%. Positivity rates reported in this study were highest for RDTs followed by real time PCR, microscopy and multiplex real-time PCR. Real-time PCR detected all positive tests in a microscope and 10,36% on a positive RDT. The level of positivity by all diagnostic methods increases among the younger age group and decreases or becomes zero in the older age group. These results are in line with research conducted in the Western Arsi Zone, Ethiopia that positivity rates among younger age groups are higher when determined by microscopes, RDTs and molecular tests [13].

In this study, the results of species identification between RDT, microscopic and multiplex real time were different. In RDT and microscopic no *species of P. malariae* nor mixed infections of *P. vivax* and *P.malariae were* found. This suggests multiplex real time PCR is able to detect infections with low parasite levels as often found in *P. malariae* infections. Although this PCR detection capability is very good in detecting submicroscopic infections, the discrepancy between RDT, microscopic and multiplex real time PCR can be caused due to the limit of detection of each different examination [25- 27].

Unlike previous studies which showed that multiplex PCR has the best sensitivity and specificity [28] but in this study the results were not satisfactory. This is because the quality of DNA for PCR examination is not

enough good and the amount of DNA is small due to dilution with anticoagulants that are not appropriate so as to affect the results of the examination [30], [31]. In real time PCR obtained 26 samples that were positive for the genus Plasmodium, and then forwarded with multiplex assay for species identification. Due to dubious results such as due to the thresshold cycle of more than 36 and low Tm peak, this examination is continued with multiplex AbTes real time PCR examination. The final results were obtained *P. falciparum* infection as much as 41.2%, *P. malariae* as much as 35.3% and mixed infections as much as 23.5%.

Although the performance of multiplex real time PCR in this study is different from previous studies, the molecular diagnosis method remains the best choice for determining the diagnosis of malaria, especially in low-endemic areas with low parasitic density [32]. Multiplex real-time PCR can be an alternative diagnostic tool for epidemiological studies and verification of elimination in malaria elimination settings. Multiplex real-time PCR has the advantage of simultaneous detection of several species of Plasmodium in a single reaction [33- 35]. In addition, this method can also identify mixed infections that are often undetected and poorly reported by RDTs and microscopes. Mixed infection detection provides accurate information for the treatment of patients, and for epidemiological studies regarding the transmission of malaria. In an Israel study, of the 10 mixed infections identified by real-time PCR, only one was identified with a microscope and RDT testing [36]. In another study conducted in Switzerland to evaluate microscopy and real-time multiplex PCR, the multiplex qPCR test correctly identified mixed species and infections with low-level parasitemia [37]. A study to determine the prevalence of mixed infections using real-time PCR in northern Ethiopia from 168 samples, found the prevalence of mixed infections was 1.8% with a microscope and 12.5% with real-time PCR [17].

The study found the negative predicted value of multiplex real-time PCR was 98.4% using a microscope as a reference test. This means multiplex real-time PCR can used for diagnosis of malaria in both of genus and species. With the performance and quality of multiplex real time PCR in this study, this method becomes an ideal diagnostic tool for use in malaria elimination. In this study, RDTs and microscopes have the possibility of errors in diagnosis, especially in the identification of species that will negatively affect the elimination program, and potentially contribute to ongoing transmission. Multiplex real-time PCR have identified 17 cases of malaria in this study, consist of 7 infections of P. *falciparum*, 6 infections of *P. malariae* and 4 as a mixed infection *of P. vivax* and *P. malariae* from a total of 105 individuals living in forest malaria endemic areas in this study. Of these malaria cases, *P. malariae* was not detected by the RDT nor light microscopy tests. However, microscopy and multiplex real time PCR methods showed a good performance to identification of *the species P. falciparum*.

6. CONCLUSION

Multiplex real-time PCR is the most sensitive malaria diagnostic method that can be used in malaria elimination programs. It has better performance in species identification, species detection with low parasitic density and mixed infection detection than microscopes and RDTs especially in low malaria transmission settings. The test can be used for epidemiological and community-based prevalence studies and for verification of elimination in areas where malaria elimination is launched. However, microscopes and RDTs still have acceptable performance to be used as malaria diagnostic tools in health facilities due to affordability and easier performance diagnostic methods.

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AbTes real time PCR examination, and the Parasitology Laboratory for microscopic examination.

AUTHOR'S CONTRIBUTIONS
II: conceptualization of work, analysis of data, drafting and revising, final report:
UH: drafting and revising, final approval of the version to be published
HA: analysis of data, revising draft

DATA AVAILABILITY
All relevant data are within the paper

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COMPETING INTEREST
The authors have declared that no competing interests exist exist

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