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Performance comparison between Conventional Polymerase Chain Reaction and AbTes Real-time polymerase chain reaction with the gold standard of microscopy imaging detection method

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ABSTRACT

Background: Microscopy imaging-based detection is the main component of the malaria diagnostic test. The healthcare technician's skill and experience will influence the results of the malaria microscopy examination. The polymerase chain reaction (PCR) is another sensitive detection technique that can be used as a diagnostic tool. PCR methods can be based on the conventional method or Real-time PCR. Real-time PCR is said to be more sensitive than the conventional one. This study aimed to compare the performance of Malaria microscopic imaging, Conventional PCR, and Real-time PCR (abTESTMMalaria qPCRII) for detecting Plasmodium in human blood.

Methods: This research was a cross-sectional analytical study that successfully generated 150 specimens from November 2018 to June 2019. Blood samples were examined using microscopy imaging detection, RT-PCR, and conventional PCR. Mann-Whitney test was used to analyze the performance of each test, which was significant if $p < 0.05$.

Results: A total of 150 participants were recruited. Ninety-eight subjects were detected positive by microscopic method and then compared to RT-PCR and conventional PCR. *Plasmodium vivax* dominated the features of the subject in all three tests. The tests show several multiple infections, like double and triple infections, especially when done by RT-PCR (28.6%). Mann-Whitney analysis revealed the significant difference between microscopic detection and Real-Time PCR AbTes in detecting *P falciparum* (Pf), *P vivax*, and *P malariae* (Pm) ($p<0.05$). PCR Conventional and Real-time AbTes also differed significantly while evaluating Pm.

Conclusion: Real-time PCR (abTEST Malaria qPCRII) positivity was beyond microscopic imaging and conventional PCR. It also shows significant differences with another test detecting *P falciparum*, *Plasmodium vivax*, and *P malariae*.

Keywords: Conventional PCR, microscopic imaging, plasmodium, Real-time PCR (abTESTMMalaria qPCRII).
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INTRODUCTION

Malaria is a *vector-borne disease* caused by 5 species of *Plasmodium* that live and multiply in human red blood cells. The five species of *Plasmodium* that cause malaria found in Indonesia today are *Plasmodium falciparum* (*Pf*), *Plasmodium malariae* (*P.*), *Plasmodium vivax* (Pv), *Plasmodium ovale* (Po), *Plasmodium knowlesi* (*Pki*).[1] Malaria cases remained high in several endemic areas, causing several thousand infections and 2000 deaths in Indonesia. Malaria infection persists in the Papua area, with around 74 % of cases reported annually.[2]

An accurate diagnosis will make successful treatment and lower the complications. The need for practical and efficient diagnostics is increasing for the global control of malaria And malarial mortality. Clinical diagnosis may be challenging to differentiate Malaria from other tropical infections based on patient signs and symptoms or medical findings. Therefore, the necessity for laboratory-based confirming diagnosis is critical.[3]

The gold standard of malaria diagnosis is microscopic examination. The advantages of microscopic imaging are the simple technique with low cost. The ability to assess the appearance of the parasite, type of Plasmodium species, and count parasite density had become microscopic imaging excellency. Microscopic examination can be cumbersome because the staining and interpretation processes are labor-intensive and time-consuming. The examiner must be an expert and trained before identifying the species accurately, especially at low parasitemia or in mixed malarial infections. Microscopy imaging has limitations in differentiating Plasmodium species that are similar, especially Pk with Pm, Pf, and Pv.[4,5]

Molecular assay for Malaria has grown rapidly nowadays. PCR-based techniques are a recent development in the molecular diagnosis of malaria. They have proven to be one of the most specific and sensitive diagnostic methods, particularly for malaria cases with low parasitemia or mixed infection. New laboratory diagnostic techniques that display high sensitivity and

specificity without subjective variation are urgently needed. The molecular method is needed to confirm malaria diagnosis in low parasite density.[6-8] Several studies were performed to evaluate the diagnostic performance of conventional or Real-time PCR. AbTES Malaria qPCRII was used to validate inconclusive results by Quantifast Real-time PCR. The AbTes demonstrated a lower Limit of detection (LOD) compared to Quantifast (≤ 0,125 parasite/μL vs. 20 parasite/ μL).[9] Objective of the study was to have a field study in Merauke, Papua, to evaluate the performance of three diagnostic methods based on microscopic imaging, conventional PCR and AbTES Malaria qPCRII.

METHOD

This research was a cross-sectional analytical study that successfully generated 150 specimens from November 2018 to June 2019. It received ethical approval from the Health Research Ethics Committee of Airlangga University's Faculty of Medicine in Surabaya, with reference number 22 / EC / KEPK / FKUA / 2019. The sample inclusion criteria included patients of all ages, both males and females, who had a fever (specific or nonspecific malaria) during their clinical examination. Patients who had received malaria treatment were used as sample exclusion criteria.

This study was conducted at Merauke Papua Regional Hospital to collect whole blood (WB) using EDTA tubes and dried blood spots (DBS), as well as to prepare and read thick and thin drops of blood preparations. The blood film was both thick and thin. 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 made before other examinations.

Species identification and determination of parasite density

Microscopically, Plasmodium species identification and parasitemia index (PI) calculation were performed on Giemsa-stained thick and thin blood smears. The PI was calculated according to WHO guidelines.[10]

Real-Time PCR

Plasmodium DNA examination with the Rotor-Gene® Q PCR from Qiagen, Tokyo, Japan, and the abTESTMMalaria qPCR III reagent kit. The AbTESTMMalaria qPCR III (AITbiotech Pte Ltd, Singapore) can identify five Plasmodium spp. species (*P. falciparum, P. vivax, P. ovale, P. malariae, P.knowlesi*). There are two major steps to take: DNA extraction from blood samples and amplification of DNA extracts using highly specific primer pairs and probes that hydrolyze double-dye (double-dye hydrolysis probes). Double-dye hydrolysis probes are fluorescent substances that can emit light, which is then captured by optical detectors on the Rotor-Gene® Q device, resulting in an increase in signal in the wave graph. This probe binds to Plasmodium species primers compatible with the Rotor-Gene® Q optical detector channel. Each is made up of FAM (compatible with Green channels), HEX (compatible with Yellow channels), and ROX (compatible with Red channels). Quasar 705 (compatible with Crimson channel), VIC, TAMRA, TEXAS RED, and Cy5 (compatible with Orange channels). Any positive Plasmodium result occurred when the level of fluorescence exceeded the threshold value (LOD 0.48geq/L), and vice versa for any negative result.[11]

Conventional PCR

The remaining blood samples were paper (GE Healthcare Companies) for further molecular studies. These blood samples Whatman paper 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 Biotek Corporation, Canada). The 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 included positive and negative control. The primers used for amplification followed Adiatmaja research.[5]

Statistical Analysis

Mann-Whitney test was used to analyze the performance of each test, which was significant if $p < 0.05$.

RESULTS

Table 1 shows the characteristics of the research subjects. A total of 150 subjects were enrolled in the study. The average age was 28.6 vs. 31.4 in Malaria and non-malaria subjects. Male subjects were higher than females in all study groups. Ninety-eight subjects belonged to the Malaria group, and 52 were in the non-malaria group. The positivity of *P viis vax* was higher than other Plasmodium, whether based on microscopic analysis or PCR-based diagnosis, Conventional PCR, and Real-time PCR (62.2 %, 48 %, 55.1 %, respectively). P falciparum was the second rank of Malaria prevalence in this study regarding each test. We found mixed infection based on all test results, and AbTes Real-time PCR appeared to be the most number in revealing mixed infection at 25/25.5 %. All non-malaria groups were detected negative by microscopic imaging, but several numbers gave positive results when tested by conventional PCR and AbTes Real-Time PCR (2.8 % vs. 38.5 %).

We can see the different proportions of Positive Plasmodium in negative microscopic imaging in **Table 2**. As many as 32/61.5 % had negative concordance between Pv and Pf. The discordance consisted of Pf(+) in RT-PCR detected negative by Conventional PCR 3/5.8 %, Pv (+) in RT-PCR but negative in conventional PCR (10/19.3 %), Pf(+) and Pv(+) by RT-PCR but negative in conventional PCR (6/11.5 %), and Pf(+) and Pv(+) by RT-PCR but Pv(+) in conventional PCR (6/11.5 %).

Ninety-eight subjects were detected as positive by microscopic imaging (table 3). All subjects were positive when tested by Real-time PCR, but three subjects (3.1 %) were detected negatively by conventional PCR. Multiple infections showed in the study. Double *Plasmodium* infection had appeared both in RT-PCR and conventional PCR. The configuration were Pf (+)Pv (+), Pv(+) Po(+), Pv(+) Pm(+) by RT-PCR. Conventional PCR only showed a combination of Pf(+) and Pv(+). Triple

infection can be seen in Table 3, only detected by Rt-PCR

A comparative analysis between the three tests is shown in table 4. Plasmodium falciparum detection had significantly different when comparing microscopic analysis and RT-PCR. Microscopic examination showed significant differences between Conventional PCR and Real-time PCR. Regarding *P malariae*, microscopic had significant differences from Real-time PCR. Both PCR tests had significant differences ($p<0.05$). In detecting *P ovale*, We found no significant difference between the three tests.

Table 1. Subject Characteristic Profile

Parameter	Malaria Subject	Non-Malaria Subject
Number of Subject (n/%)	98/65.3	52/34.7
Age (Mean/Min-Max) (years)	28.6/3-76)	31.4/5-76
Gender n/%		
Male	66/67.4	29/55.8
Female	32/32.7	23/44.2
Positive Microscopic imaging n/%	98/100	0/0
P vivax (Pv)	61/62.2	0/0
P falciparum (Pf)	34/34.7	0/0
Pv dan Pf	3/3.1	0/0
Conventional PCR n/%	98/100	52/100
P vivax (Pv)	44/44.9	1/1.9
P falciparum (Pf)	27/27.6	0/0
P malariae (Pm)	0/0	0/0
P ovale (Po)	0/0	0/0
Pv dan Pf	24/24.4	1/1.9
Pf (+) Pv (+)Pm (+)	0/0	0/0
Pf(+) Pv(+) Po(+)	0/0	0/0
Negative	3/3.1	50/96.2
AbTes Real Time PCR n/%	98/100	52/100
P vivax (Pv) (+)	54/55.1	10/19.2
P falciparum (Pf) (+)	16/16.3	3/5.8
P malariae (Pm) (+)	0/0	0
P ovale (Po) (+)	0/0	0
Pv dan Pf(+)	22/22.5	7/13.4
Pv Po (+)	1/1.0	0
PvPm (+)	1/1.0	0
Pf (+) Pv (+)Pm (+)	1/1.0	0
Pf(+) Pm(+) Po(+)	3/3.1	0
Negative	0/0	32/61.5

DISCUSSION

Microscopic imaging detected only Pf and Pv or mixed Pf/Pv in this research. It is consistent with conventional PCR in detecting Pv and Pv only. The proportion of mixed infection is higher in conventional PCR than in microscopic imaging. The limit of detection in Microscopic and Conventional PCR was different. A false-negative microscopy result is directly proportional to the level of parasite density due to its limit of detection (LOD) of about 50-100 parasites/µl of blood. LOD varies from 30–100 parasites/µL between expert and field microscopists. Excellent laboratory officer able to detect lower parasite density (5 parasite/µL). The negative microscopic result does not exclude Malaria infection exclude *Plasmodium* infection. Parasitemia lower than 500 parasites/µL can cause false-negative results.[3,6,12]

PCR can analyze as few as 1-5 parasites/µl of blood (≤ 0.0001% of infected red blood cells) compared with around 50-100 parasites/µl of blood by microscopy or RDT. Evaluation of AbTes qPCR showed that the method could detect as low as ≤ 0.125 parasites/µl. Another Real-time PCR can only detect as low as 20 parasites/µl . The advantages of Real-time PCR when compared to conventional nested PCR are the ability to detect multiple Plasmodium species in a single amplification round, higher throughput potential, and not require manual quantification of end-points using gel electrophoresis.[9]

Twenty of the 52 negative microscopy results were recognized as positive by AbTes-PCR, and only two specimens were detected as positive by conventional

Table 2. Differences profile in Negative Malaria Microscopic Imaging

Convent. PCR	AbTes RT-PCR				
	Pf (-) and Pv (-) n/%	Pf (+) n/%	Pv (+) n/%	Pf (+) and Pv (+) n/%	Total
Pf (-) Pv(-)	32/61.5	3/5.8	10/19.3	6/11.5	51/98.1
Pv (+)	0/0	0/0	0/0	1/1.9	1/1.9
Pf(+)	0/0	0/0	0/0	0/0	0/0
Pf (+) and Pv (+)	0/0	0/0	0/0	0/0	0/0
Total	32/61.5	3/5.8	10/19.3	7/13.4	52/100

Table 3. **Differences profile in Positive Malaria Microscopic Imaging**

	AbTEST RT-PCR								
Convent. PCR	Pf (+) n/%	Pv (+) n/%	Pf (+) Pv (+) n/%	Pv(+) Po(+) n/%	Pv(+) Pm(+) n/%	Pf (+) Pv (+) Pm (+) n/%	Pv (+) Pm (+) Po (+) n/%	Negative n/%	Total n/%
Pf (+)	14/14.3	0/0	11/ 11.2	0/0	0/0	0/0	2/2.1	0/0	27/26.5
Pv (+)	0/0	40/40.81	1/1.0	1/1.0	1/1.0	0/0	1/1.0	0/0	44/48.0
Pf (+) and Pv (+)	2/2.1	14/14.3	7/7.1	0/0	0/0	1/1.0	0/0	0/0	24/25.5
Pf (+) Pv (+)Pm (+)	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Pf (+) Pv (+)Po (+)	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Negative	0/0	0/0	3/3.1	0/0	0/0	0/0	0/0	0/0	3/0
Total	16/16.3	54/55.1	22/22.5	1/1.0	1/1.0	1/1.0	3/3.1	0/0	8/100

PCR. One reason is that the patient has experienced an infection. There are transmission hotspots for asymptomatic infections, such as those observed for Plasmodium vivax and Plasmodium falciparum, which have been identified as contributing to transmission. It has also been observed that some submicroscopic infections never develop symptoms and may contribute to infection.[13] The quality of the blood film also determines the microscopic reading result. It often produces wrong data about the presence of malaria parasites, and this wrong information can cause false-positive or negative readings of Plasmodium species.[14]

Abtest qPCR also detected Pm and Po in mixed infection in samples. Microscopic and conventional PCR failed to find the species. This might be attributed to different detection limits also. The rare appearance of Pm Po in research can influence the experience of laboratory officers in detecting them, so they failed to determine those two malaria species. There is still a probability that Abtest will give a false positive result. A primer in real-time PCR can be unintended annealed with other gene targets resulting in a false-positive result. These findings imply that mixed Plasmodium species may require further validation.[9] The study did not perform another molecular assay to confirm the positivity of conventional PCR and AbTes Malaria Real-Time PCR. For further study, it is necessary to evaluate the correlation between PCR results with parasite density.

Table 4. **Comparison between Microscopic imaging, Conventional PCR and Real-Time PCR AbTes.**

Parameter	Statistical Significance*
Pf microscopic vs. Pf PCR Conventional	p=0.143
Pf microscopic vs. Pf Real-Time PCR AbTes	p=0.008*
Pf PCR Conventional vs Pf Real-Time PCR AbTes	p=0.230
Pv microscopic vs Pv PCR Conventional	p=0.027*
Pv microscopic vs Pv PCR Conventional	P=0.01*
Pv PCR Conventional vs Pv Real-Time PCR AbTes	p=0.238
Pm microscopic vs Pm PCR Conventional	p=1
Pm microscopic vs Pm Real-Time PCR AbTes	p=0.024*
Pm PCR Conventional vs Pm Real-Time PCR AbTes	P=0.024*
Po microscopic vs Po PCR Conventional	P=1
Po microscopic vs Po Real-Time PCR AbTes	P=0.085
Po PCR Conventional vs Po Real-Time PCR AbTes	P= 0.082

*Mann=Whitney test, significance if $p < 0.05$

CONCLUSION

Real-time PCR (abTES Malaria qPCRII) positivity was beyond microscopic imaging and conventional PCR. It also shows significant differences with another test in detecting *P falciparum*, *Plasmodium vivax*, and *P malariae*. The presence of mixed infection with discordance from other tests must be evaluated further

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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AUTHORS CONTRIBUTION

PW contributed in consepting and designing the study, literature review, data collection, data analysis, manuscript preparatioan and editing. A and NC were responsible in concepting and designing the study, data analysis, manuscript editing and supervised the research

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