

## Diagnostic Value of Plasmotec® Malaria-3 Antigen Detection on Gold Standard Microscopy

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### ABSTRACT

Plasmotec® Malaria-3 is a rapid malaria diagnostic test that uses four-line tests and targets three malaria proteins, namely *Plasmodium falciparum* specific protein (HRP-2), *Plasmodium vivax*-specific LDH (Pv-LDH) and non-specific Plasmodium LDH (pLDH). Microscopy as a gold standard has many disadvantages and the availability of malaria Rapid Diagnostic Tests (RDTs) in detecting three proteins is still very limited. This study aimed to determine the diagnostic value of Plasmotec® Malaria-3 against gold standard microscopy, comparing the Plasmotec® Malaria-3 and microscopy antigen species detection, determining the Parasitemia Index (PI) cut-off using Plasmotec® Malaria-3. This study was a cross-sectional study with 105 whole blood samples obtained from the Merauke Papua General Hospital which fulfilled the inclusion and exclusion criteria. Samples were examined by thick and thin drops and then examined with Plasmotec® Malaria-3. Diagnostic values of Plasmotec® Malaria-3 against the microscopy were Sn 100%, Sp 98.04%, PPV 98.18%, NPV 100%, LR + 51, LR-0, diagnostic accuracy of 99.05%. Comparison of Plasmodium species between Plasmotec® Malaria-3 and microscopy was not significantly different, p-value = 0.172. The cut-off of PI in *P.falciparum* and *P.vivax* in Plasmotec® Malaria-3 based on the Receiver Operating Characteristic (ROC) curve could not be determined with AUC=0.577, p-value=0.385 and AUC=0.423, p-value=0.385, respectively. This study concluded that the comparison of Plasmodium species between Plasmotec® Malaria-3, and microscopy was not significantly different. This study suggested that further research is needed to find the diagnostic value of non-*falciparum* and non-*vivax Plasmodium* against Plasmotec® Malaria-3.

**Keywords:** Malaria, microscopy, Plasmotec® Malaria-3, HRP-2, Pv-LDH, pLDH

### INTRODUCTION

Malaria is a vector-borne disease caused by the protozoan parasite of the genus Plasmodium, which is transmitted by the bite of female Anopheles mosquitoes (main vectors), blood transfusions, use of shared needles and transplants.<sup>1,2</sup> Malaria is still one of the world's public health problems, an important cause of morbidity and mortality, especially in high-risk groups such as infants, toddlers, and pregnant females, as well as in adults generally.<sup>1</sup> Malaria causes around one million deaths annually throughout the world.<sup>3</sup>

Positive cases of malaria in Indonesia amounted to 465,764 cases in 2010, and this number decreased in 2015 to 209,413 cases. The National Annual Parasites Incidence (API) trend noted that in 2011 - 2015 malaria positive cases decreased from 1.75% (in 2011) to 0.85% (in 2015), but malaria cases still remained high in some endemic areas, such as Papua, West Papua, Maluku, North Maluku, and East Nusa Tenggara provinces.<sup>4</sup>

Papua Province has the highest malaria burden in Indonesia, with an API of 45.85% in 2016. Merauke Regency in Papua province is the most eastern district in Indonesia with 255,022 inhabitants inhabiting 20 regional districts, 11 sub-districts, and 179 villages. Merauke Regency experienced a decline in Annual Parasites Incidence (API) from 2010 by 19/1000 population to 15.28/1000 population in 2016, but malaria still remains a health problem in Merauke district.<sup>5</sup>

One effort to reduce malaria mortality and morbidity, namely by increasing the accuracy in diagnosing malaria, is followed by accuracy in treatment. The clinical symptoms of malaria that are not specific and the difficulties in distinguishing malaria from other tropical infections are often an obstacle in establishing a diagnosis of malaria. This condition encourages a high demand and challenges a practical and effective laboratory examination method without ignoring the value of sensitivity, specificity, accuracy, and economics in controlling malaria globally.<sup>6</sup>

Many malaria diagnostic methods have been developed since the WHO stated the importance of new, fast, easy, accurate, and inexpensive examination in detecting *Plasmodium malariae*. Moreover, in overcoming various shortcomings of microscopy examination as the gold standard for malaria examination established by the WHO.<sup>6-8</sup> Lack of microscopy examination includes the inability to detect very low parasitemia (low titer), so it is not useful in non-endemic areas of malaria, often does not identify a mixed infection (mixed infection), requires time and experts in reading microscopy preparations.<sup>6,9</sup>

Another inspection method developed to diagnose malaria is an examination by detecting proteins or enzymes produced by Plasmodium, in the form of a specific protein *Plasmodium falciparum* namely Histidine-Rich Protein 2 (HRP-2) or *Plasmodium vivax* specific Plasmodium Lactate Dehydrogenase (Pv-pLDH) and the non-specific Plasmodium Lactate Dehydrogenase (pLDH). This method has been widely used and is considered alternative malaria examination.<sup>5</sup>

Commercial enzyme examinations generally use serological techniques by detecting antigens, such as Rapid Diagnostic Tests (RDT) or Immunochromatography Test (ICT) and Enzyme-Linked Immunosorbent Assay (ELISA). Availability of RDTs with detection of three proteins at the same time is still very limited in the market, and currently available are Plasmotec<sup>®</sup> Malaria-3 (PT Indec Diagnostics, Jakarta, Indonesia) and Palutop<sup>®</sup> +4 Optima (All. Diag, Strasbourg, France). Plasmotec<sup>®</sup> Malaria-3 is quite widely used in Indonesia, but research on this RDT is still very little. They are the reason for researchers to examine the Plasmotec<sup>®</sup> Malaria-3.

This study aimed to determine the diagnostic value and compare the results of Plasmodium species antigen detection using Plasmotec<sup>®</sup> Malaria-3 RDT against gold-standard microscopy and determine the cut-off index of the Plasmodium species parasitemia antigen using Plasmotec<sup>®</sup> Malaria-3.

## METHODS

This study had received ethical approval from the Health Research Ethics Committee of the Faculty of Medicine, Airlangga University Surabaya with number 22/EC/KEPK/FKUA/2019. This was a cross-sectional analytical study conducted at the Merauke Papua Hospital and Clinical Pathology

Laboratory Faculty of Medicine, Airlangga University/Dr. Soetomo Hospital Surabaya and took place from November 2018 - June 2019. A total of 105 whole blood samples were obtained from Merauke Hospital and accommodated using Ethylene Diamine Tetraacetic Acid (EDTA) tubes. Furthermore, the examination of EDTA samples was carried out at the Merauke Regional Hospital Laboratory microscopically by thick and thin drops of blood for the identification of Plasmodium species and calculation of the Parasitemia Index (PI), and then the samples were examined using Plasmotec<sup>®</sup> Malaria-3 RDT.

The positivity of the Plasmodium species and the PI on microscopy examination were obtained from the reading of thick and thin drops prepared with Giemsa staining, with the calculation formula for the IP using thick drops of blood preparations, namely:<sup>10</sup>

$$\text{Parasites}/\mu\text{L} = \frac{\text{The number of parasites calculated} \times \text{number of leukocyte cells}^*}{200 \text{ leukocyte cells}}$$

\* If the number of leukocyte cells is unknown, the patient's leukocyte cells are assumed to amount to 8,000/ $\mu\text{L}$ .

Calculation of the index of parasitemia using thin drops of blood preparations, namely:<sup>10</sup>

$$\text{Parasites}/\mu\text{L} = \frac{\text{The number of parasites calculated} \times \text{number of erythrocyte cells}^*}{1000 \text{ erythrocyte cells}}$$

\* If there is no known number of erythrocyte cells, it is assumed that the patient's erythrocytes are 5,000,000/ $\mu\text{L}$  (male) or 4,500,000/ $\mu\text{L}$  (female).

The positivity and antigen detection of the Plasmodium species in Plasmotec<sup>®</sup> Malaria-3 were obtained through lines or bands that arise in the line test. Plasmotec<sup>®</sup> Malaria-3 uses the ICT method, with nitrocellulose membranes coated with non-specific anti-pLDH antibodies from *Plasmodium spp.* (*P.falciparum*, *P.vivax*, *P.malariae*, *P.ovale*) on the Pan test line, *P.vivax* specific anti-pLDH antibody on the Pv test line and *P.falciparum*-specific anti-Histidine Rich Protein II (HRP II) antibodies on the Pf test line.

Confirmation of the reading results comprising the thick drops of blood preparation and thin drops was carried out in the Clinical Pathology Laboratory Faculty of Medicine, Airlangga University/Dr. Soetomo Hospital Surabaya. The criteria for sample acceptance were patients of all ages, males, and females, clinical examinations showing symptoms of fever (specific or non-specific malaria) and who were

willing to take part in the study by signing an informed consent form. The exclusion criteria were patients receiving malaria treatment and fever patients with negative results of malaria microscopy examination.

Interpretation of microscopy and Plasmotec® Malaria-3 results was carried out by two blind and independent observers. Based on the Mc Nemar test between two observers, the results were not significantly different with a p-value <0.05.

**RESULTS AND DISCUSSION**

A total of 105 samples, each obtained positive results of the Plasmodium microscopy test as many as 54 (51.42%) samples and negative Plasmodium as many as 51 (48.58%) samples. Fifty-four positive samples of Plasmodium microscopically, obtained 54 (100%) positive Plasmodium in Plasmotec® Malaria or true positive, whereas from 51 negative samples Plasmodium microscopically, 50 (98.04%) were negative Plasmodium of Plasmotec® Malaria or true negative and 1 (0.96%) positive Plasmodium in Plasmotec® Malaria or false positive.

Plasmodium species on true positive results based on microscopy examination were dominated by *P.vivax*, *P.falciparum*, mixed Plasmodium with 34 of 54 (63%) samples, 17 of 54 (31.5%) samples and 3 of 54 (5.5%) samples, respectively. Based on the identification of parasitic density, the highest PI in *P.vivax* was obtained in the range 1,000-10,000/µL, *P.falciparum* 1,000-10,000/µL, and 10,001-200,000/µL, while the mixed results obtained evenly distributed PI, namely PI <1000, 1,000- 10,000 and 10,001-200,000/µL. The average age of patients involved in this study was 31.19 years, with the highest sex were males (63.8%). Median and long IQR fever were obtained from 28 malaria patients who had a history of fever for five days (2-60) (Table 1).

Fifty negative Plasmodium based on RDT examination consisted of 25 (50%) samples with dengue fever, 4 (8%) samples with urinary tract infections, 3 (6%) samples with pneumonia, and 4 (8%) samples with local infection, 3 (6%) samples with sepsis and 11 (22%) samples with hepatitis B.

The diagnostic value of Plasmotec® Malaria-3 against microscopy consisted of true positive = 54,

**Table 1.** Basic characteristics of subjects and study samples

Variable	Total (n)	Total %
<b>Total malaria</b>	105	52.4
Positive microscopy test	54/105	51.42
Negative microscopy test	51/105	48.58
Positive Plasmotec® Malaria-3test	55/105	52.38
Negative Plasmotec® Malaria-3test	50/105	47.62
<b>PI <i>P.vivax</i> per µL blood *</b>	34/54	63
<1.000	2/34	5.9
1.000-10.000	18/34	52.9
10.001-200.000	14/34	41.2
>200.000	0/34	0
<b>PI <i>P.falciparum</i> per µL blood *</b>	17/54	31.5
<1.000	1/17	5.9
1.000-10.000	7/17	41.2
10.001-200.000	7/17	41.2
>200.000	2/17	11.7
<b>PI mixed (<i>P.falciparum</i> and <i>P.vivax</i>) per µL blood *</b>	3/54	5.5
<1.000	1/3	33.3
1.000-10.000	1/3	33.3
10.001-200.000	1/3	33.3
>200.000	0/3	0
Age (Mean±SD) years	31.19 ± 17.97	-
<b>Gender</b>		
Male	67/105	63.8
Female	38/105	36.2
Day of fever (Median (IQR)) days (n=28)	5 (2-60)	-

**Table 2.** Diagnostic value of Plasmotec® Malaria-3 RDT against microscopy gold standard

Plasmotec® Malaria-3	Microscopy	
	Positive	Negative
Positive	54	1
Negative	0	50
Sn (%) (CI 95%)	100 (93.4-100)	
Sp (%) (CI 95%)	98.04 (89.55-99.95)	
PPV (%) (CI 95%)	98.18 (88.58-99.73)	
NPV (%) (CI 95%)	100 (-)	
LR+ (CI 95%)	51 (73.2-355.14)	
LR- (CI 95%)	0.00 (-)	
Diagnostic accuracy (CI 95%)	99.05 (94.81-99.98)	

false-positive = 1, true negative = 50 and false-negative = 0, then Sn = 100% and Sp = 98.04%, PPV = 98, 18%, NPV = 100%, LR + = 51, LR- = 0 and diagnostic accuracy = 99.05% (Table 2). The results of the sensitivity and specificity of Plasmotec® Malaria-3 on gold standard microscopy in this study obtained a high value, namely 100% and 98.04%. The results showed good compatibility between Plasmotec® Malaria-3 and microscopy. RDT sensitivity was influenced by various factors as explained by previous researchers, which included location and population sampling, antigenemia protein in the sample itself and optimal temperature stability of the reagent kit.<sup>9,11</sup>

The location and sampling population in this study were in Merauke, one of the districts in the Papua Province. Riskesdas in 2013 stated that Papua Province was one of the provinces with the highest malaria burden in Indonesia with an API of 45.85% in 2016. High malaria burden (endemic areas) will affect the background of malaria exposure in the population, thus providing higher parasitic densities compared to non-endemic regional populations.<sup>4</sup> Parasitic density will produce a high antigenemia protein resulting in a high positivity in the detection of RDT protein antigens, as well as the positivity of Plasmodium detection by microscopy. In this study, a high PI was obtained with each dominance of 1,000-10,000 parasites/ $\mu$ L was 41.2% in *P.falciparum* and 52.9% in *P.vivax*, while the PI < 1,000 parasites/ $\mu$ L was 5.9% in *P.falciparum* and *P.vivax*.

Rapid diagnostic test sensitivity is also influenced by optimal temperature stability of the reagent kit (4 - 30°C). If the optimal temperature is maintained, both during transportation of reagent kit delivery or during the inspection, the RDT performance will run well and provide valid results.<sup>12,13</sup> In this study, the temperature stability of the reagent kit was maintained at 4 - 30°C with the storage of reagent

kits carried out in the refrigerator before being used with controlled temperatures. RDT sensitivity was also influenced by the suitability between observers in reading RDT results.<sup>12</sup> Relevance between observers in this study was obtained by the Mc Nemmar test with a p-value < 0.05 with results as not significantly different.

The results of the diagnostic values in this study were supported by previous studies Arum *et al.* who obtained the diagnostic value of RDT against microscopy with Sn 100%, Sp 96.99%, PPV 83.2%, and NPV 100%.<sup>14</sup> However, it was not supported by Fransisca *et al.* with a sensitivity of Plasmotec® Malaria-3 to the microscopy of 73%.<sup>15</sup> This difference can be related to the low density of parasites in the Fransisca study with PI < 1,000 parasites/ $\mu$ L of 26.2% in *P.falciparum* and 45.9% in *P.vivax*. This condition could be influenced by the extrapolation of the search for samples outside the area that had been determined in the Fransisca *et al.* study, which produced low parasitic density. Low parasitic densities more often provided low sensitivity too.<sup>16,17</sup> RDT has been shown to produce lower sensitivity in areas with low parasitic density.<sup>18,19</sup>

One false-positive result of *P.falciparum* in Plasmotec® Malaria-3 in this study could be caused by protein antigenemia HRP-2 malaria parasite which could still be detected in the patient's blood for up to 30 days after antimalarial therapy. The long duration of HRP-2 was caused by the slow clearance of HRP-2 in the blood. These factors were the reason for not recommending the use of RDT as a therapeutic monitoring examination, other than the factor of the presence of positive gametocytes in the blood after antimalarial therapy. The presence of gametocytes in the blood would still produce all three antigenemia proteins (HRP-2, p-LDH, and aldolase). Unlike the case with antigenemia p-LDH and aldolase proteins that had fast clearance from

**Table 3.** Comparison of Plasmodium detection results between Plasmotec® Malaria-3 and microscopy gold standard

Plasmotec Malaria-3	Microscopy				p-value	RT PCR				P-value
	P.f n (%)	P.v n (%)	Mixed n (%)	Total n (%)		P.f n (%)	P.v n (%)	Mixed n (%)	Total n (%)	
P.f	15 (100)	0 (0)	0 (0)	15 (100)	<b>0.172</b>	6 (40)	0 (0)	9 (60)	15 (100)	<b>&lt;0.001</b>
P.v	1 (2.6)	34 (89.5)	3 (7.9)	38 (100)		0 (0)	29 (76.3)	9 (23.7)	38 (100)	
Mixed	1 (100)	0 (0)	0 (0)	1 (100)		0 (0)	0 (0)	2 (100)	2 (100)	
Total	17 (31.5)	34 (63)	3 (5.5)	54 (100)		6 (10.9)	29 (52.7)	20 (36.4)	55 (100)	

the blood after antimalarial therapy.<sup>9</sup>

In addition, the cross-reaction with rheumatoid factor and heterophilic antibodies in the patient's blood was one of the causes of false positivity in the RDT.<sup>13</sup>

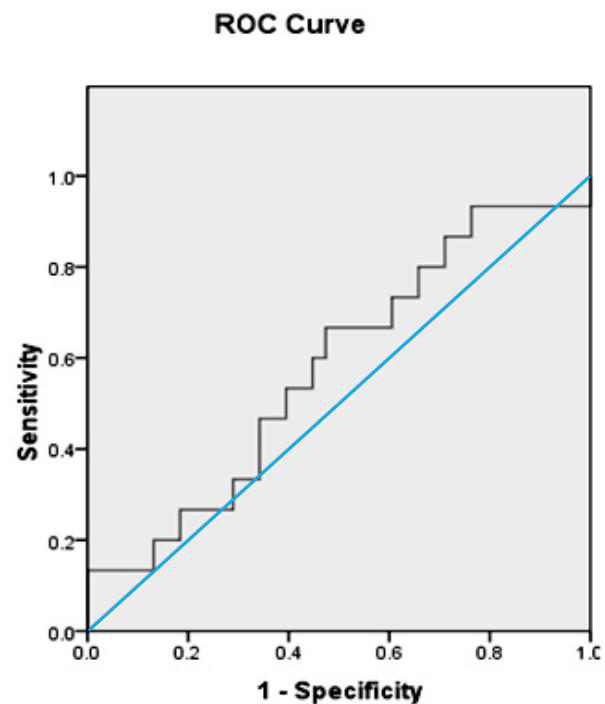
The comparison of Plasmodium species antigen detection results between Plasmotec® Malaria-3 and gold standard microscopy were not statistically significantly different with a p-value = 0.172. The detection results of 15 samples of *P.falciparum* in Plasmotec® Malaria-3 were 15 (100%) detected as *P.falciparum* also on microscopy, 38 samples detected as *P.vivax* in Plasmotec® Malaria-3 obtained 34 (89.5%) samples detected as *P.vivax* on microscopy, 1 sample identified as mixed (*P.falciparum* and *P.vivax*) in Plasmotec® Malaria-3 obtained 0 (0%) samples detected as mixed on microscopy, but detected as *P.falciparum* (Table 3).

Plasmodium species identified between Plasmotec® Malaria-3 and microscopy were not significantly different. It could be influenced by observers involved in microscopy readings as trained observers and certified malaria training so that the opportunity to determine malaria species correctly and directly through microscopy was obtained. It could also be influenced by the level of conformity between observer readings with irrelevant results if p-value <0.05 both in thick or thin smear reading, as well as reading results on Plasmotec® Malaria-3 cassettes.<sup>15</sup>

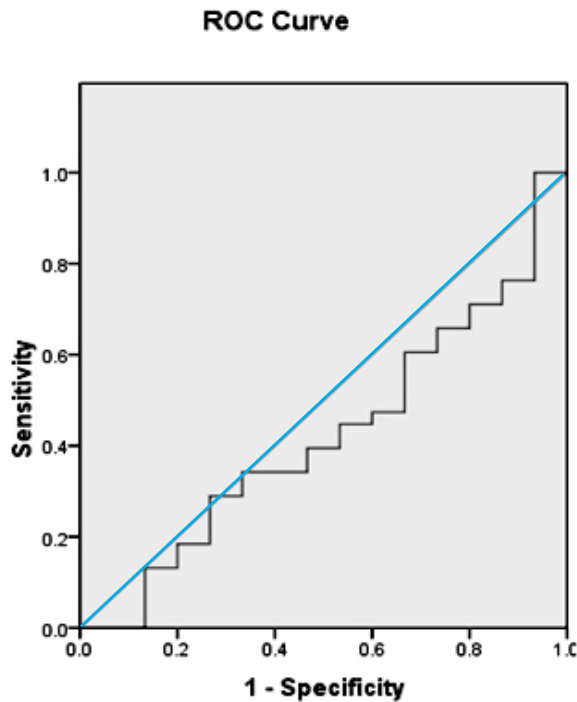
Conformity between the results of Plasmotec® Malaria-3 and microscopy was also inseparable from the density of parasitemia in this study, thus increasing the positivity and sensitivity of Plasmotec® Malaria-3. The population of malaria species in this study was dominated by *P.vivax* and then followed

by *P.falciparum*, so that although there was a high parasitemia, but because of the dominance of malaria species occupied by *P.vivax*, the chance to produce false positives in pLDH was small. It could be related to Wilson study, whose statement which revealed that patients with high *P.falciparum* parasitemia could give false-positive results in pLDH and end with high *P.vivax* findings on RDT examination.<sup>9</sup>

Based on the ROC curve analysis to determine the cut-off value of species PI in Plasmotec® Malaria-3,



**Figure 1.** ROC curve of *P.falciparum* PI in Plasmotec® Malaria-3 [AUC: 0.577 (CI 95%: 0.407-0.748, p-value = 0.385)].



**Figure 2.** ROC Curve of *P.vivax* PI in Plasmotec® Malaria-3 [AUC: 0.423 (CI 95%: 0.252-0.593, p-value= 0.385)].

a low AUC was obtained with a significance  $> 0.05$ , with *P.falciparum* obtained AUC = 0.577 with p-value was 0.38 (Figure 1) and *P. vivax* obtained AUC: 0.423 with p-value = 0.385 (Figure 2). It indicated that the cut-off value could not be determined so that calculations of sensitivity and specificity could not be continued. This could be caused by heterogeneous data variability and a wide distribution of PI values in this study. The Standard Deviation (SD) values of *P.falciparum* and *P.vivax* species in Plasmotec® Malaria-3 were found to be greater than the mean. In this study, 1 sample with  $PI \pm 1,100,000$  parasites/ $\mu$ L and 1 sample with  $PI \pm 55$  parasites/ $\mu$ L were obtained. This was different from the research conducted by Fransisca *et al.* who obtained a cut-off of each species for 100% sensitivity on *P.falciparum*  $\geq 4,800$  parasites/ $\mu$ L and *P.vivax*  $\geq 640$  parasites/ $\mu$ L.<sup>15</sup>

## CONCLUSIONS AND SUGGESTIONS

The diagnostic value of Plasmotec® Malaria-3 against gold-standard microscopy obtained Sn 100%, Sp 98.04%, PPV 98.18%, NPV 100%, LR+51, LR-0 and a diagnostic accuracy of 99.05%. The comparison of Plasmodium antigen detection results between Plasmotec® Malaria-3 and microscopy was not significantly different. Further

research is needed to find the diagnostic value of non-*falciparum* and non-*vivax* Plasmodium in Plasmotec® Malaria-3.

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