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Validity of p-LDH/HRP2-Based Rapid Diagnostic Test for the Diagnosis of Malaria on Pregnant Women in Maluku

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ABSTRACT

Introduction: Pregnant women are one of the groups at risk for infection by the malaria parasites in endemic areas. The dangerous impacts of malaria in pregnancy are anemia and severe malaria that can cause death for mother, fetus and newborn. Clinical symptoms that are likely to be not typical until asymptomatic in pregnancy are one of the obstacles on diagnosing malaria in pregnancy in endemic areas. p-LDH/HRP2-RDT (Pf/Pan) is one of the WHO recommended RDT product on round 1-4 and has been used in Maluku. This tool is able to detect antigens of the Plasmodium metabolism results in peripheral blood so that it is regarded to be more sensitive than microscopic examination. The use of p-LDH and HRP2-RDT (Pf/Pan) for the detection of P. falciparum HRP-2 antigen and P. vivax, P. malariae, P. ovale p-LDH antigen have not been previously evaluated in the Province of Maluku.

Objectives: To evaluate the validity of p-LDH/HRP2-RDT (Pf/Pan) compared with microscopic examination and nested Polymerase Chain Reaction (PCR) as the gold standard for the diagnosis of malaria in pregnancy in Maluku.

Methods: This was a cross-sectional study using a diagnostic test of malaria in pregnant women. The study was conducted in Ambon City health center, Savana Jaya Buru Island health center and Haulussy Ambon Local Hospital. Sample data, the data of pregnancy, RDT results and microscopic results on the field were recorded in the questionnaire. Nested PCR examination was conducted at the Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada as well as second reading for microscopic examination.

Results: The results showed that p-LDH/HRP2-RDT (Pf/Pan) had the same sensitivity with microscopic of 11%, a specificity of 100% higher than microscopic 96% compared with nested PCR as the gold standard, p-LDH/HRP2-RDT (Pf/Pan) had PPV and NPV of 100% and 98% compared with nested PCR as the gold standard. p-LDH/HRP2-RDT (Pf/Pan) sensitivity was 80% compared to the microscopic examination.

Conclusion: diagnostic malaria in pregnancy in Maluku with p-LDH/HRP2-RDT (Pf/Pan) was less sensitive than nested PCR and microscopic.

Keywords: Malaria, pregnant woman, diagnostic test, validity, p-LDH/HRP2 Rapid Diagnostic Test (RDT) (Pf/Pan)
INTISARI


Tujuan: penelitian ini adalah untuk mengevaluasi validitas p-LDH/HRP2 Pf/Pan dibandingkan dengan pemeriksaan mikroskopis dan nested Polymerase Chain Reaction (PCR) sebagai standar baku untuk diagnosis malaria dalam kehamilan di Maluku.


Hasil: Validitas RDT p-LDH/HRP2 Pf/Pan memiliki sensitivitas sama dengan mikroskopik yaitu 11%, spesifisitas 100% lebih tinggi dibandingkan spesifisitas mikroskopik yaitu 96% dengan nested PCR sebagai standar baku emas. p-LDH/HRP2-RDT (Pf/Pan) memiliki nilai PPV dan NPV yaitu 100% dan 98% dibandingkan nested PCR sebagai standar baku emas. Sensitivitas p-LDH/HRP2-RDT (Pf/Pan) adalah 80% dibandingkan dengan pemeriksaan malaria dengan mikroskopis.

Simpulan: diagnosis malaria menggunakan p-LDH/HRP2-RDT (Pf/Pan) pada ibu hamil di Provinsi Maluku sebagai daerah endemis malaria, memiliki nilai sensitivitas yang lebih rendah dibandingkan dengan pemeriksaan nested PCR dan mikroskopis.

Kata kunci: Malaria, wanita hamil, tes diagnostik, validitas, RDT p-LDH/HRP2 (Pf/Pan)

INTRODUCTION

Until currently, malaria has been one of the infectious diseases that causes death in the world after tuberculosis1. One of the risk groups infected with malaria is pregnant women. The impact of malaria in pregnant women is anemia, low birth weight and abortion; therefore, this is a threat to malaria endemic areas. The number of pregnant women and infants who die due to malaria and severe anemia due to malaria is 10,000 pregnant women and 200,000 babies each year1,2. Cases of malaria especially in pregnant women are still a problem in Eastern Indonesia, including Maluku. Maluku is one of provinces with the highest API (Annual Parasite Incidence) in Eastern Indonesia3.

One of the WHO strategies for malaria control in pregnant women is the early diagnosis of malaria in pregnant women with microscopic examination and the use of RDTs, thus appropriate malaria therapy could prevent the effects of malaria in pregnancy for both mother
and fetus\(^4\). Although malaria examination with the thick blood preparation can detect the number of parasites 50-100 parasites/µl, the sensitivity is dependent on several factors e.g. microscopic examination of slide-making skills, microscopic reading accuracy or availability of proper equipment\(^5\). RDT offers some conveniences such as easier to use in the field since it does not require special skills and provides faster results. Regarding the sensitivity, RDT could detect >100 parasites/ul in the peripheral blood\(^6\). However, RDT sensitivity is also influenced by various factors, namely: parasitic factors (species and degree of parasitemia, parasite antigen structure variability and antigen ability); factor of RDT condition, technical factors of the use of RDT, and its interpretation\(^7\).

A low number of parasites in the peripheral blood is due to sequestration process in the placenta by *P. falciparum* infection because the attachment of VAR2CSA protein antigen on the surface of erythrocytes with Chondroitin Sulfate A (CSA) and Hyaluronic acid (HA) receptors from the placenta is produced in the second trimester\(^8\). This causes microscopic examination to be less sensitive than RDT which is able to detect antigens as the metabolism results of *Plasmodium* in blood. On infection by *P. vivax*, the sequestration process in the placenta does not happen, but *P. vivax* infection during pregnancy is likely to lead to babies born with low birth weight\(^8\).

Degree of parasitemia is also influenced by the immunity system. Pregnant women who are infected with malaria and living in endemic areas will have the ability to suppress parasitemia and develop protective specific immunoglobulins (IgG) and cell mediated immunity (anti-parasite immunity) so that the symptoms of malaria in pregnant women become oligosymptomatis to asymptomatis on very low parasitic conditions in the blood due to antitoxic immunity\(^11\).\(^12\).

p-LDH/HRP2-RDT Pf/Pan is one of RDT products recommended by the WHO in round 1 to round 4 for use in zone 2, like Asia that has a mixed *P. falciparum* infection of *P. vivax* and *P. malariae* as well as *P. ovale*\(^13\). This tool has the form of cassette containing membrane strip covered by a specific monoclonal antibody against HRP-2 at the Pf line for the detection of *P. falciparum* and specific polyclonal antibodies against p-LDH at the Pan lines for detection of *P. vivax*, *P. ovale* and *P. malariae*\(^13\).\(^14\). Antigen of p-LDH on HRP2-RDTPf/Pan has the lower sensitivity than HRP-2 antigen. The sensitivity for the detection of *Pvivax* is only 50% compared to the microscopic as the standard, if the number of parasites is 1-50 parasites/µl of blood, and the sensitivity for the detection of *P. falciparum* from 93.8 % up to 100 % compared to the microscopic as the standard, if the number of parasites is 1-50 parasites/ µl of blood\(^14\).

PCR is a method to amplify DNA fragments in a short period of time\(^15\). This test is very specific and sensitive (approaching 100 %). This test can detect at least 2 parasites, even 1 parasite/µl blood\(^16\).\(^17\), but this PCR test has disadvantage: a) the provision of DNA and RNA primer is very complicated, b) the tools used for hybridization are complex, c) tools for PCR amplification and detection of amplification product is very sophisticated and expensive, d) it takes a long time (24 hours) so that it is only suitable for epidemiological and experimental studies\(^16\).
This study is to evaluate the validity of p-LDH/HRP2-RDT (Pf/Pan) compared with microscopic examination and nested Polymerase Chain Reaction (PCR) as the gold standard for the diagnosis of malaria in pregnancy in Maluku.

MATERIALS AND METHODS

In this cross sectional study, the population was pregnant women in Ambon City Public Health Center (PHC), Haulussy Ambon Local Hospital and Savana Jaya P. Buru Health Center. The inclusion criteria included pregnant women who lived and settled in the Maluku; pregnant women with symptoms of fever (temperature > 37.4 °C) in the past 2 weeks; pregnant women without symptoms of fever, but had any of the following sign: anemic conjunctiva, weakness, fatigue, lethargy, muscle pain, headache, vaginal bleeding, nausea and vomiting, and diarrhea. The exclusion criteria included pregnant women who were taking antimalarial drugs or taking malaria drugs within the last 2 weeks and pregnant women who were not willing to sign the informed consent of research. Fifty nine pregnant women in this study who met the inclusion and exclusion criteria were recruited.

The variables assessed in this study were: sensitivity, specificity, positive predictive value, negative predictive value of p-LDH/HRP2-RDT tool compared with microscopic. Nested PCR is used as the gold standard. Informed consent had been signed by all who participated in the study. Permit of the study was approved by the research ethics committee of the Faculty of Medicine, Gadjah Mada University. Tests done were:

a. Microscopic examination (developing thick and thin blood smear preparation)

Malaria thick and thin slides were prepared, stained and counted according to WHO (2011) in Basic Malaria Microscopy 2nd edition.

b. Examination with Rapid Diagnostic Test

p-LDH/HRP2-RDT was provided in the form of cassette. RDT examination was used to assess the presence of HRP-2 antigen in infections (P. falciparum) and p-LDH antigen in P. vivax, P. malariae, P. ovale infections. The RDT examination was done by two health personal (midwifes or nurses). Before examining RDT, RDT officers ensured that it was not expired. Blood specimens taken with a disposable loop were inserted into the rounded-shape sample well. The officers then put four drops of assay buffer into the box-shaped assay diluent hole next to the sample well and allowed it to stand for fifteen minutes and read. Results should not be read after 30 minutes. Result was considered as positive Pf (P. falciparum) when 2 lines (test line “Pf” and the control line “C”) were appeared in the result window; a positive Pan (P. vivax, P. malariae, P. ovale) if 2 lines (test line “Pan” and the control line “C”) were appeared on the window; mixed infections (P. falciparum and P. vivax or P. malariae, P. ovale) if 3 lines (test line “Pf”, test line “Pan” and control line “C”) were appeared on the window. Result was considered as negative if only one control line “C” was appeared in the result window. No color that appeared on the control strip means that the examination should be repeated.

c. Nested PCR examination

This examination was performed in the laboratory of Parasitology of FM-UGM, Yogyakarta. Peripheral blood samples were collected on filter paper (Whatman 3 MM chromatography paper). DNA was then extracted from the filter paper with a method and isolated using Chelex method.
Nested PCR was done through two steps, for nested one, a pair of primer was used rPLU 1 and rPLU 5. Second amplification was nested two for genus (rPLU 3 and 4) followed by nested two for species (rFAL 1 and rFAL2, rMAL1 and rMAL2, rVIV1 and rVIV2, rOVA1 and rPLU2 primers. PCR condition was set as follow: initial denaturation 94°C for 1 minute, annealing 59°C for 2 minutes, extension 72°C for 5 minutes, denaturation repetition 94°C for 1 minute annealing 59°C for 2 minutes, extension 72°C for 5 minutes and repeated 35 times. Nested PCR was done as suggested by CR product for nested two genus was positive at 240 bp, and nested species was confirmed positive when a band with specific base pairs was appeared (205 bp for \( P. falciparum \), 144 bp for \( P. malariae \), 117 bp for \( P. vivax \), and 226 bp for \( P. ovale \)).

Validity of the diagnostic tool of p-LDH/HRP2-RDT (Pf/Pan) including the sensitivity, specificity, positive predictive value, negative predictive value was then assessed by comparing the RDT data with microscopy and nested PCR as the gold standard.

![Figure 1](image.png)

**Figure 1. Example of Nested 2 PCR specific species result showed the presence of \( P. falciparum \) of blood samples taken from pregnant women at Ambon Primary Health Center, Savana Jaya Primary Health Center, Haulussy General Hospital, Ambon on September 2012 – April 2013**

**RESULTS AND DISCUSSION**

In this study, the prevalence of malaria in pregnant women with RDT Pf / Pan is 6.8% (4/59), a microscopic 8.5% (5/59), 61% (36/59) nested PCR. Maternal age with the highest prevalence of malaria using PCR is in 21-29 age group by 27 (67.5%). The prevalence of malaria in pregnant women in first pregnancy and two pregnancy as much as 23/39 (58.9%) less than multigravida malaria infection in 13/20 (65%) by PCR, in contrast to previous studies that found the prevalence of malaria is more common in the first and second pregnancies compared to multigravida.

- M: 100 bp Standard
- λ: Marker
- K(-): Negative control
- K(+): Positive control
- Lane 001 to 033: Sample DNA
- Lane 001 to 009: PCR Result
- Lane 018 to 027: Specific band at 205 bp
The risk of reinfection in pregnant women in malaria-endemic areas will cause the immune system has the ability to suppress parasitemia\textsuperscript{11,12}. A low number of parasites in the blood due to suppression of the immune system and the effects of sequestration of the malaria parasite of \textit{P. falciparum} can cause the sensitivity of the RDT is become less\textsuperscript{7,19}. The low number of parasites in the blood, requires malaria RDTs as diagnostic tool with the high sensitivity in malaria endemic areas.

Table 1. Comparison of the results of p-LDH/HRP2 RDT \textit{P.f/Pan}, and \textit{nested} PCR of pregnant women in September 2012 – April 2013 in Ambon Primary Health Center; Savana Jaya (P.Buru) Primary Health Center and Haulussy General Hospital in Ambon City.

<table>
<thead>
<tr>
<th>RDT \textit{P.f/Pan}</th>
<th>Nested PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positif</td>
<td>Negatif</td>
</tr>
<tr>
<td>Positif</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Negatif</td>
<td>32</td>
<td>23</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>23</td>
</tr>
</tbody>
</table>

The results of the validity of RDT Pf /Pan (samples of peripheral blood) in this study for the examination of malaria in pregnant women have a sensitivity of 9.75%, specificity 100%, PPV 100%, NPV 42% when compared with nested PCR as standard gold standard (Table 1). Meanwhile, RDT sensitivity SD Pf/Pan compared to microscopic examination was 80%, 100% specificity, 100% PPV and 98% NPV (Table 2). The sensitivity results of RDT Pf / Pan in this research is lower than the sensitivity of the RDT Pf / Pv 83.3% in Eastern Sudan. The difference of the sensitivity of the RDT tool can be influenced by differences in the criteria of the samples taken and the status of malaria in different areas\textsuperscript{18}.

Table 2. Comparison of the results of p-LDH/HRP2 RDT \textit{P.f/Pan}, and microscopic of pregnant women in September 2012 – April 2013 in Ambon Primary Health Center; Savana Jaya (P.Buru) Primary Health Center and Haulussy General Hospital in Ambon City.

<table>
<thead>
<tr>
<th>RDT \textit{P.f/Pan}</th>
<th>Microscopic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positif</td>
<td>Negatif</td>
</tr>
<tr>
<td>Positif</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Negatif</td>
<td>1</td>
<td>54</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>54</td>
</tr>
</tbody>
</table>
RDT sensitivity 80%, specificity 100%, PPV 100%, NPV 98% compared to the microscopic. In contrast to the results of research in Sei Barombang, Labuan Batu, North Sumatra found that Parascreen RDT test results had a sensitivity of 0% and a specificity of 100% compared with the gold standard microscopic. This difference may be due in Sei Barombang samples were taken from all pregnant women (with and without symptoms of malaria), whereas in this study the samples taken from pregnant women with malaria symptoms who are typical and not typical, so it can provide higher sensitivity results.

In this study, factors that could affect the validity of RDT are the condition of RDT, the use of RDT and interpretation techniques of p-LDH/HRP2-RDT Pf/Pan has been controlled by the researchers with keeping the RDT in the right temperature at 4°C-30°C and use it before the expired periods of RDT. The researchers disseminate and training on how to use the RDT tool to clinic staff and hospital personnel before the study so that officers can use RDTs correctly and can read it right interpretation of the results of the RDT.

The degree of parasitemia that can be seen by the microscope showed that the type of P. vivax malaria is highest at 1890-10800 /µl and the number of P. falciparum parasites is 5707-15613 /µl. p-LDH/HRP2-RDT Pf/Pan in this study can detect P. falciparum parasites in the blood by the number of > 5000 parasites /µl of blood, P. vivax > 1000 parasites/µl of blood.

Thirty two of the fifty-five negative RDT results, giving the positive results in the nested PCR examination (false negative RDT) in table 1, might be due to: a) low number of parasites in the blood. Previous research has found that pregnant women with microscopic examination is negative, but RT-PCR examination give the positive results, the number of parasites in the blood is very low at 2.9 parasites /µl; b) the type of p-LDH antigen produced by Plasmodium besides P. falciparum giving more lower sensitivity than HRP-2 antigen on the amount of blood parasites 1-50/µl (SD Malaria Antigen Pf/Pan®), so the majority of malaria infections in the number of parasites in the blood 1-50/µl in this study was not detected by RDT; c) for the HRP-2 antigen types produced by P. falciparum, although the sensitivity was high 93.8%, but other factors that may affect the sensitivity of HRP-2 antigen is the possible due to the variability of antigen structure of HRP-2 antigen deletion or existence HRP-2 antigen mutation. Previous research has also found a mutation of the HRP-2 antigen into HRP-3 after the sequence. Antigen HRP-2 mutations can cause the HRP-2 antigen in the sample could not be detected by RDT PHRP-2. Parasite antigens produced PHRP3 encodes amino acids alanine and histidine-rich that similar to the antigen P HRP2 but have differences in amino acid 4. This genetic variation can affect the detection of P. falciparum parasites in the amount of <1000 parasites /µl.

Therefore, the use of PCR for diagnosis of malaria placenta remains under discussion, especially in submicroscopic condition as it is difficult to determine what have been detected by PCR. PCR is very sensitive to detected parasites nucleic acids, but it is unclear if this is a residual from a non viable sequestered parasite, or a viable parasite, or gametocyte. One positive result of microscopic examination of the P. vivax parasite number 10800 /µL gave a negative result on the results of RDT and nested PCR which might be due to miss interpretation of the slide.
CONCLUSIONS AND RECOMMENDATIONS

Rapid Diagnostic Test (RDT) p-LDH/HRP2 (Pf/ Pan) has the same sensitivity to the microscopic sensitivity that is 11% when compared with nested PCR as the gold standard and less sensitive (80%) than microscopic to diagnosis malaria in pregnant women in the Maluku province as an endemic malaria areas.

Research on the validity of the RDT for pregnant women is needed before the RDT tool is used to diagnose malaria for pregnant women, especially pregnant women who living in malaria endemic areas with oligosymptoms and the number of parasites that tend to be low in the blood. Further epidemiological research and biotechnology research to determine the presence of HRP-2 antigen variation that can affect the reliability of the RDT tool especially for the Asia Pacific region.

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b. **Abstract**: The author should provide two abstract, in Indonesian and English language. All articles should be provided with an abstract of between 200-300 words in one spacing. The abstract should be written in simple language with structured abstract style. Abstract should describe of the study using below headings: Introduction, Objectives, Methods, Results and Discussion, and Conclusion. Standard nomenclature should be used and abbreviations should be avoided.

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d. **Introduction**: The Introduction should provide the problem statement clearly, the relevant literature on the subject, and the proposed approach or solution.

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with a heading and a legend. Tables should be self-explanatory without reference to the text.

j. **Figure:** The figures should be numbered consecutively with Arabic numerals. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. The figures should be constructed in such a manner that they can be understood without reading the text. Appropriate symbols should be used on graphs and explained in the legends. Graphs should not duplicate results presented in tables. Title and comments of the figures and photographs should be provided on separate page using MS Word.

k. **References:** References should be numbered consecutively in the order in which they are first mentioned in the text (Vancouver style). Identify references by Arabic number as superscript in order of appearance. A number must be used even if the author(s) is named in the text. The original number assigned to the reference is reused each time the reference is cited in the text, regardless of its previous position in the text. For example:

```
........ it has been reported¹ ........
........ according to Sardjito² ........
........ Winstein & Swartz³ conducted ........
........ by Avon et al.⁴ ........
```

Authors are responsible for the accuracy and the completeness of their references. References should be listed numerically (Vancouver style) at the end of the text and in the same order that they have been cited in the text. For citation references with six or less authors, all authors should be listed, when seven or more authors only first three authors should be listed followed by et al. Journal names are abbreviated according to Index Medicus and Index of Indonesia Learned Periodicals (PDIN 1974). References to journal articles, books, chapters in books, theses, etc. should be listed as given in Sample References.

**Sample References**

**Scientific Journal**

1. **Standard journal article**

2. **Organization as author**

3. **No author given**

4. **Article not in English**

5. **Volume with supplement**

6. **Issue with supplement**

7. **Volume with part**

8. **Issue with part**
9. **Issue with no volume**

10. **No issue or volume**

11. **Pagination in roman numerals**

12. **Type of article indicated as needed**
    Spargo PM, Manners JM, DDAVP and open heart surgery [letter]. Anaesthesia 1989;44:363-64.

13. **Article containing retraction**

14. **Article retracted**

15. **Article containing comment**

16. **Article in comment**

17. **Article with published erratum**

**Books and Other Monographs**

18. **Personal author(s)**

19. **Editor(s) as author**

20. **Organization(s) as author**

21. **Chapter in a book**

22. **Conference proceedings**

23. **Conference paper**

24. **Scientific or technical report**
25. Dissertation

26. Patent

Other Published Material
27. Newspaper article

28. Audiovisual material

29. Computer program

30. Legal material

31. Map

32. Dictionary or Encyclopaedia

33. Classic material

34. In press

Electronic Material
35. Journal article in the internet

36. Monograph in electronic format

37. Computer program
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