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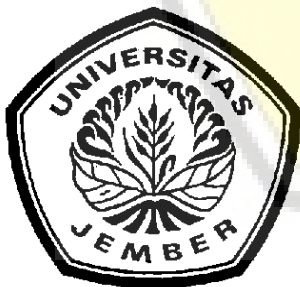
**Sensitivity and Specificity of Nested PCR for Diagnosing Malaria:
Cases in Several Areas of Indonesia**

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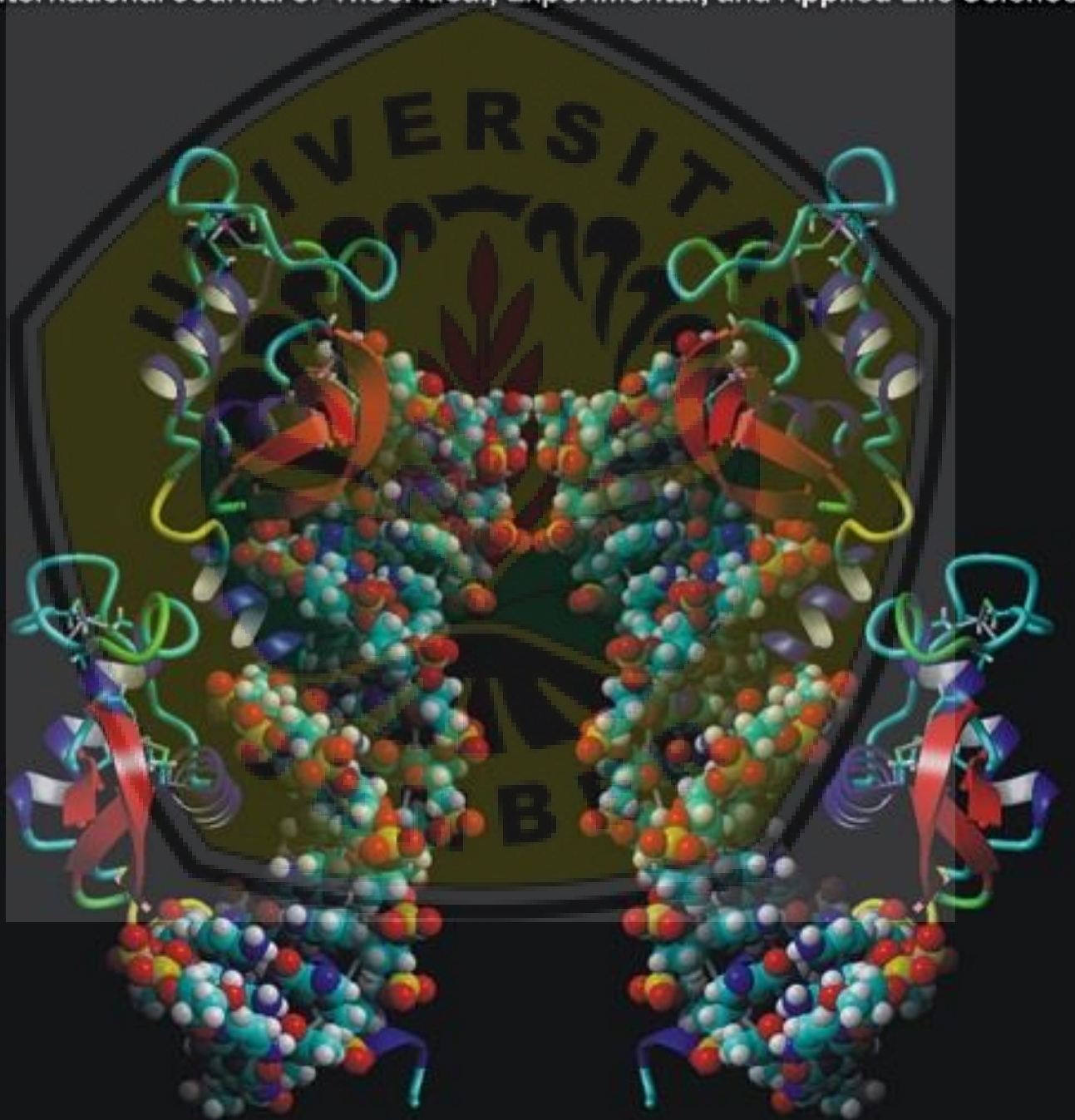
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Sensitivity and Specificity of Nested PCR for Diagnosing Malaria: Cases in Several Areas of Indonesia

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ABSTRACT

Indonesia is still included in high endemic area of malaria infection. Early detection as well as appropriate and quick treatment is needed to be able to prevent and treat malaria in Indonesia. Laboratory examination using a microscopic method is still used as the gold standard to diagnose malaria cases. However, the morphology similarity of some *Plasmodium* species and the number of parasites that can be seen under microscopy causes malaria diagnosis become difficult if only relying on microscopy diagnostic method. The purpose of this study is to analyze the sensitivity and specificity of nested PCR compared to microscopic examination in diagnosing malaria cases. A cross-sectional study has been carried out in some areas of Indonesia and the microscopic analysis as well as nest PCR was done in Laboratory of Parasitology and Laboratory of Central Biomedical Faculty of Medicine, Universitas Brawijaya, Malang East Java Indonesia. A total of 149 blood samples from patients with clinical symptoms of malaria had been obtained from Sumatra, Sulawesi and East Java during December 2011 to December 2013. From 149 sample, 81.9% samples were diagnosed malaria positive by microscopy examination, whereas the PCR results showed that 90.6% of samples were positive. Nested PCR sensitivity is 97.5%, and microscopy 88.2%. Nested PCR specificity is 40.7%, whereas microscopy 78.5%. PPV and NPV for nested PCR are 88,2% and 78.5% respectively, and for microscopy are 97.5% and 40.7% respectively. Nested PCR has a higher sensitivity than microscopy in diagnosing malaria and is able to detect mixed infection better than microscopic examination. However, it is statistically less specific than microscopy examination.

Keywords: *Nested PCR, microscopy, sensitivity, specificity, Malaria, Indonesia*

INTRODUCTION

Malaria is one of infectious diseases that remains a global public health problem around the world and concerned by the World Health Organization (WHO) for eradication. Most regions in Indonesia are still malaria endemic areas such as Papua, Maluku, Nusa Tenggara, Sulawesi, Borneo, and some regions such as

Lampung in Sumatra, Bengkulu and Riau. Even though the endemicity is already very low, outbreaks of malaria in Java and Bali are still often seen. This is due to ease of transportation for the mobilization of the population, causing increased cases of imported malaria. *Plasmodium* that causes malaria are consist of 5 species, which are *Plasmodium falciparum*, *Plasmodium vivax*,

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Plasmodium ovale, *Plasmodium malariae*, and *Plasmodium knowlesi* [1, 2].

Laboratory diagnosis by examination of blood smear using a microscope is still the gold standard in malaria diagnosis. This examination is able to identify some species of *Plasmodium* precisely and at the same time it can count the number of parasites so that the degree of parasitemia can be known. However, the morphological changes of malaria parasite, the *P. knowlesi* species morphology which is difficult to distinguish from *P. falcifarum* and *P. malariae* makes malaria diagnosis become difficult if only relying on microscopy diagnosis technique. Effect of endemicity of malaria, rapid migration, as well as travel from endemic areas, indirectly also enhance the problem in laboratory diagnosis and therapy in malaria [2].

Microscopic examination has limitations in detecting Plasmodium, especially when a blood smear is not made properly or the low degree parasitemia or mixed infection occurs [3,4]. Therefore, the biomolecular examination like polymerase chain reaction (PCR) is considered as an alternative examination to obtain more accurate results. In the last few years, PCR has been developed as a malaria diagnostic method, most of the research that has been done reporting PCR advantages compared to microscopic examination techniques, especially in the case of a mixed infection and malaria infections with a low degree parasitemia [5].

The purpose of this study is to analyze the sensitivity and specificity of nested PCR compared to microscopic examination in diagnosing malaria cases and in determining the accuracy of each method in endemic area of Indonesia.

MATERIALS AND METHODS

Study design

This study was a cross-sectional study, comparing the species that cause malaria by microscopic examination and nested PCR. This study is conducted at the Parasitology Laboratory, Faculty of Medicine, Universitas Brawijaya for microscopy examination of the blood smear and Biomedical Laboratory, Faculty of Medicine, Universitas Brawijaya for nested PCR examination.

Subjects

Samples were collected for 24 months from December 2011 to December 2013. The samples were obtained from Sumatra, Sulawesi and Malang East Java, Indonesia. The participants of the study were obtained

by convenience sampling, and 149 patients with clinical symptoms of malaria were involved in this study.

Samples collection

Fingerpick blood samples were taken from every patient from Sumatera and Sulawesi and placed on oil-free and clean microscopy glass slides. Two blood spots were taken from each patient on filter paper (Whatman #903, GE Healthcare) labelled with the participant's code/ number. Each filter paper was dried separately to avoid contamination. The samples were then stored in small plastic bags and transported to the Medical Faculty – Universitas Brawijaya for molecular analysis. From each patient from Malang, five (5) mL peripheral venous blood (medial cubital vein) was collected in an EDTA vacutainer sample collection. Blood samples were processed for thick and thin blood-smears for microscopic examination and DNA isolation for nested PCR.

Microscopic examination

Both thick and thin blood smears were prepared on a single slide. Thin blood smears were fixed with methanol after air-drying and then were stained in a 30% Giemsa solution for 10 minutes. Thin and thick blood films were examined at laboratory of parasitology by experienced medical laboratory analyst and negative result was considered if no *Plasmodium* parasites were seen in 100 high power (1000 ×) fields.

Polymerase Chain Reaction (PCR)

The extraction and purification of Plasmodium DNA from the blood spots on filter paper were used as a modified chelex-based DNA extraction method by the InstaGene Whole Blood Kit (Bio-Rad Laboratories, Hercules, CA, USA). Identification and classification of species by nested PCR was performed for all samples as described before [2, 6]. Individuals who interpreted PCR results were blinded to the results of microscopic examination

Data analysis

To determine the sensitivity and specificity of nested PCR compared with microscopy examination, we used a diagnostic test with 2 × 2 tables (crosstabs) with SPSS ver.16.0. Sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) and accuracy of each method of examination were calculated based on the crosstabs results.

Ethical clearance

This study protocol was performed according to the Helsinki Declaration and approved by the Ethical Committee of Medical Research, Faculty of Medicine, Universitas Brawijaya. Written informed consent was obtained from Bioethical Division of Medical Research, Faculty of Medicine, Universitas Brawijaya.

RESULTS AND DISCUSSION

Microscopic examination results

From 149 samples that have been examined by microscopic examination 81.9% (122/149) were found malaria positive, in which, *P. falciparum* was found in 40.3% (60/149) of samples, *P. vivax* 27.5% (41 /149) and mixed infections of *P. falciparum* and *P. vivax* was 14.1% (21/149). No *P. ovale*, *P. knowlesi*, and *P. malariae* were found in all the samples.

PCR results

By nested PCR, 90.6% (135/149) of samples were malaria positive, in which, *P. falciparum* was found in 2% (3/149) of all samples, *P. vivax* 65.1% (97/149), *P. malariae* 0.7% (1/149), mixed of *P. falciparum* and *P. vivax* 18.8% (28/149) and a mixed of *P. vivax* and *P. knowlesi* 4% (6 /149). Complete data comparison of the results of microscopy examination and nested PCR test are presented in Table 1 and Table 2.

Tests sensitivity and specificity

In this study, the diagnosis of malaria obtained from PCR method has a higher sensitivity (97.5%) compared to the microscopic examination (88.2%) method, but the microscopic examination method has a higher specificity (78.5%) compared to nested PCR (40.7%) method. The positive predictive value (PPV) and negative predictive value (NPV) from the nested PCR

Table 1. Comparison of microscopy examination and nested PCR

Test results	Microscopy		Nested PCR	
	Amount	Percentage (%)	Amount	Percentage (%)
Negative	27	18.1	14	9.4
<i>Plasmodium falciparum</i>	60	40.3	3	2
<i>Plasmodium vivax</i>	41	27.5	97	65.1
<i>Plasmodium knowlesi</i>	0	0	0	0
<i>Plasmodium malariae</i>	0	0	1	0.7
<i>Plasmodium ovale</i>	0	0	0	0
Mixed <i>Plasmodium falciparum</i> and <i>Plasmodium vivax</i>	21	14.1	28	18.8
Mixed <i>Plasmodium vivax</i> and <i>Plasmodium knowlesi</i>	0	0	6	4
Total	149	100	149	100

Table 2. Conversion result of microscopic examination and nested PCR

Microscopic	PCR							
	Negative	<i>P. falciparum</i>	<i>P. vivax</i>	<i>P. knowlesi</i>	<i>P. malariae</i>	<i>P. ovale</i>	<i>P. falciparum</i> and <i>P. vivax</i>	<i>P. vivax</i> and <i>P. knowlesi</i>
Negative (27)	11 40,7%	0 0,0%	13 48,1%	0 0,0%	0 0,0%	0 0,0%	2 7,4%	1 3,7%
<i>P. falciparum</i> (60)	1 1,7%	3 5,0%	33 55,0%	0 0,0%	0 0,0%	0 0,0%	22 36,7%	1 1,7%
<i>P. vivax</i> (41)	2 4,9%	0 0,0%	34 82,9%	0 0,0%	0 0,0%	0 0,0%	2 4,9%	3 7,3%
Mixed <i>P. falciparum</i> and <i>P. vivax</i> (21)	0 0,0%	0 0,0%	17 81,0%	0 0,0%	1 4,8%	0 0,0%	2 9,5%	1 4,8%

method were 88.2% and 78.5% respectively. While the PPV and NPV obtained for microscopic examination method were 97.5% and of 40.7% respectively.

In this study, most of the samples for nested PCR used dried blood drops on filter paper. Nested PCR identifies the *Plasmodium* using genus and species-specific marker. Nested PCR examination showed greater positive results (90.6%) compared with the results of a microscopy examination (81.9%). In this study there were 16 samples (10.7%) reported negative by microscopy examination, turned positive by nested PCR examination. These results indicate that nested PCR method has a higher sensitivity compared to microscopic examination, in line with the results of previous studies in Africa, Asia and Latin America that reported the PCR method had a better ability to detect malaria on the low density of parasites or on subclinical malaria [7].

Microscopic examination can only detect the presence of the *Plasmodium* in the level of 20 parasites/mL of blood, the results also depend on the expertise and interpretation of the examiner. Whereas nested PCR can detect the presence of the *Plasmodium* in level 6 parasites/ml of blood when the blood sample uses dried blood drops. This allows the PCR method to detect the presence of malaria parasites in a low level of parasitaemia and can also detect the presence of mixed infections which is often not detected by microscopic examination [6, 8, 9]. Although PCR has a good sensitivity and specificity, the routine use of nested PCR for the diagnosis of malaria cannot be done because of the complexity of the method, the high cost and it requires trained technicians and experts to conduct nested PCR method. Quality control and maintenance tools are also important parts in the nested PCR process, so the method is difficult to do for a routine inspection in malaria [10].

In this study, nested PCR has a higher sensitivity (97.5%) compared to microscopic examination (88.2%), but it has lower specificity (40.7%) compared to microscopic examination (78.5%). These results are consistent with several previous studies reported in Brazil, Angola, and Indonesia (Maumere Region) [11, 12, 13]. Studies in several countries found microscopic method sensitivity varies between 34% to 54% and the specificity varies between 86% to 95%. When microscopic method used as the gold standard to assess the specificity of the nested PCR method, false-negative results on microscopic examination with positive nested PCR would be considered as false positive. Thus,

making the microscopic method became more specific than nested PCR method [13, 14].

It is known that blood components, such as hemoglobin and immunoglobulin-G (IgG) or immunoglobulin-M (IgM) can influence the outcome of PCR examination. Although errors in the PCR process cannot be eliminated, but with the PCR-trained technicians and good process control, the error in the PCR process can be minimized. One way to reduce the error process is to implement a policy of 3 space (three-room rule), one room for DNA isolation, one room for preparation of reagents, and one room for amplification (PCR). This policy will be able to minimize false positive results due to contamination. Discrepancy results of microscopy examination and blood smear is difficult to describe and verify except for re-examination on each sample by using the two methods and performed by the same technician (expert in the examination of microscopy blood smear and nested PCR) and carried out with strict control in process [15].

Differences in outcome is also possible because of an error in the interpretation of the results of the microscopic examination and the possibility of errors can also occurs in the nested PCR process, from DNA extraction process, mixing with the mastermix, nesting process, and the reading of electrophoresis gel. Poor quality blood smear will cause artifacts, bacteria, fungi, sludge dye, and dirt or cells debris that resemble the malaria parasite. The possibility of false negatives found in microscopic examination becomes higher with smaller number of parasites. In this study, microscopic examination ability to identify *P. falciparum* was dominant (43%) while the PCR result only obtained a few positive samples of *P. falciparum* (2%). An explanation of the differences in these results was very striking which was a theory that stated if a species of *Plasmodium* was at a high level of parasitemia, this condition would preclude the identification of other species in PCR, for example, high levels of the *P. vivax* would hinder the amplification and identification of *P. falciparum* in the PCR [13, 16]. In this study, with nested PCR, a high prevalence of *P. vivax* was obtained (65.1%) although not all of them were single *P. vivax* infection, rather than a mixed of *P. vivax* and *P. falciparum*.

CONCLUSION

Nested PCR has a higher sensitivity than microscopic examination in diagnosing malaria and is able to detect mixed infection better than microscopic

examination. However, it is statistically less specific than microscopy examination.

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