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Detection and Species Identification of Plasmodium using Nested PCR and Diagnosis of *P.falciparum*, *P.vivax*, *P.knowlesi* and mixed infection in South Nias Regency, North Sumatera, Indonesia.

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ABSTRACT

The aim of this study was to determine the sensitivity and specificity of diagnostic test to infection of *P.falciparum*, *P.vivax*, P.*malariae*, P.*ovale* and P.*knowlesi* in endemic areas in South Nias regency using Nested-2 PCR method. The number of subject in this study were 98 people, consist of 50 female subjects (51%) and 48 male subjects (49%). Majority of the samples were 15 years old to 59 years old (41,8%) and 5 years old to 14 years old (36,7%) and the least were over 60 years old (6,1%). Most symptoms were fever (74,5%), headache (72,4%) and splenomegaly (30,6%). The result of the study with Nested-2 PCR on parasite *P.falciparum* and *P.vivax* showed sensitivity 100%, specificity 100%, NPP 95.83%, NPN 100%, positive likelihood ratio 5,17%, negative likelihood ratio 0,31% and the prevalence of *P.knowlesi* 0,97%. It concluded that Nested-2 PCR method has a high sensitivity and specificity so it can be used as diagnostic support for malaria cases.
Keywords: Malaria, Nested 2 PCR, *Plasmodium knowlesi*, sensitivity, specificity

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INTRODUCTION

Malaria is a parasitic disease with high morbidity and mortality rates in Indonesia and other tropical and sub tropical countries even through these days [1]. In Indonesia, almost half of the country's population of 250 milliom live in malaria-endemic areas. In Java and Bali, where approximately 70% of the country's population live, malaria is hypoendemic and vivax malaria predominates. In the outer island groups, the incidence of malaria is much higher with the prevalence of *Plasmodium falciparum* and *Plasmodium vivax* infection almost equal [2]. Prompt and accurate diagnosis of malaria is the important key for disease management and also reduction of unnecessary use of anti-malaria medicines. The selection of the drug for treatment of malaria also depends on species of malaria parasites present in suspected patients [3]. The standard method for detecting Plasmodium infection is the microscopic examination of Giemsa-stained thick blood smears (TBS). this method is effective and inexpensive, but time consuming, and the sensitivity drops with the decrease of parasitemia [4]. Rapid immunochromatographic tests were developed to aid better conduct of diagnosis. The basis of these tests is the detection of antigens in the blood of malaria patients. But in cases of low parasitemia, this method is insensitive, and it is also given possibility of false positive results due to the persistence of antigenemia weeks beyond the actual infection [5]. However, molecular techniques have been able to detect and identify malaria parasites in mixed and low level parasitaemia [6]. In relation to Plasmodium, the advantages of PCR in malaria detection is the significant improvement in the identification of species in mixed infections [7]. The study conducted by Costa et al [8] on malaria infection in Brazil showed that mixed infections in the Amazon region may be underestimated as a consequence of a poorly performed thick smear techniques.

South Nias regency is the western part of North Sumatera Province, approximately 92 miles from town of Sibolga or North Tapanuli district. South Nias regency is one of Indonesia region which has high incidence of malaria. The aim of the study was to determine the pattern of spread of the plasmodium species in South Nias regency and determine the sensitivity, specificity and prevalence using nested PCR technique compared with RDT (Rapid Diagnostic Test) and microscopy methods as gold standard.

MATERIAL AND METHODS

DNA Extraction

The samples that used in this study was dry blot samples of suspected patients of malaria in South Nias regency. DNA was extracted using Instagene^{TM} Matrix (BioRad) and Chelex 5% with established procedures. The resulting DNA template then stored in refrigerator at the temperature -20^{o}C

Nested 1 PCR

Nested 1 PCR conducted in master mix room. Primers used in this step were : rPLU1 : 5′-TCA AAG ATT AAG CCA TGC AAG TGA-3' and rPLU5 : 5'-CCT GTT GTT GCC TTA AAC TTC-3'. Mastermix was prepared by mixing primers first the buffer, dNTP's dan H2O. The mixture was homogenized by pipetting, then Taq polymerase added into the mixture. The PCR was carried out with total cycles 25 cycles under conditions:

Denaturation : 94^{o}C, 1 minutes
Annealing : 58^{o}C, 2 minutes
Extension : 72^{o}C, 5 minutes
Final extension : 72^{o}C, 5 minutes

Nested 2 PCR

Mastermix was prepared similar with nested 1, with PCR product as DNA template in nested 2 PCR. Primers that used in nested 2 PCR were :

rFal1 : 5'-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3'
rFal2 : 5'-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3'
rVIV1: 5'-CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC-3'
rVIV2: 5'-ACT TCC AAG CCG AAG CCA AGA AAG TCC TTA-3'

rMAL1:5'-ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC-3'
rMAL2:5'-AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA-3'
rOVA1:5'-ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA-3'
rOVA2:5'-GGA AAA GGA CAC ATT AAT TGT ATC CTA GTG-3'
Pmk8 : 5'-GTT AGC GAG AGC CAC AAA AAA GCG AAT-3'
Pmkr9: 5'-ACT CAA AGT AAC AAA ATC TTC CGT A-3'
If there is positive result for *P.knowlesi* (Pmk8/Pmkr9) :
PkF 1140 : 5'-GAT TCA TCT ATT AAA AAT TTG CTT C-3'
PkR1550 : 5'-GAG TTC TAA TCT CCG GAG AGA AAA GA-3'

The conditions of PCR was similar with nested 1 PCR. The electrophoresis agarose gel conducted on the condition of 80mV for 45 minutes, then visualized using gel documentation. For *P.falciparum* (rFal1, fFal2), *P.vivax* (rVIV1, rVIV2), P. *malariae* (rMAL1,rMAL2), *P.ovale* (rOVA1,rOVA2) the size of product is about 206bp, 121bp, 145 bp and 226 bp respectively.

Data Analysis

The analysis result of nested PCR and microscopy were tabulated in 2x2 tables. If malaria parasites is found, the data was added into positive category. The true positive result was added into cell a, the false positive result was added into cell b, the false negative result was added into cell c and true negative result in cell d. Then sensitivity, specificity, PPV (Positive Predictive Value), NPV (Negative Predictive Value), prevalence and likelihood ratio determined.

Ethical Clearance and Informed Consent

Ethical clearance was obtained from the research committee of health, faculty of medicine, Andalas University no 177/KEP/FK/2015. Informed consent was requested in writing form from subjects of research that states willing to participate in the study after receiving explanation of the purpose and objectives of this research.

RESULT AND DISCUSSION

Samples Characteristic

The number of samples in this study were 98 patients, consist of female 50 patients (51%), male 48 patients (49%). Most symptoms are fever as many as 73 patients (74,5%), headache 71 patients (72,4%) and splenomegaly 30 patients (30,6%). The characteristic of patients were described in Table 2.

Detection of Plasmodium species with Nested PCR

The samples of the study were the patients who met the inclusion criteria based on clinical symptoms including fever, headache, muscle pain, nausea, diarrhea, abdominal pain, decreaseing in appetite, anemia, and enlarged spleen. The samples also must have positive result based in microscopy analysis and RDT in *P.falciparum*, *P.vivax*, *P.ovale*, *P.malariae*. In this study, *P.knowlesi* also found based on nested PCR result.

Based on the result, the new types of *P.knowlesi* was found in Nias, North Sumatera. The findings come after the confirmatory test with Nested PCR with prevalence 0,97%. These findings are the achievement in the search for new species that causes the malaria, especially in North Sumatera. Perkins et al [9] reported that *P.knowlesi* is a primate parasite. Primates (non human primate) was very common in South East Asia. This causes malaria in long-tailed macaques (*Macaca fascicularis*), and also could infect humans. In general, malaria parasites tend to be host specific. For example, humans are the natural hosts for 4 species, *P.falciparum*, *P.vivax*, *P.malariae* and *P.ovale*, while long-tailed macaques are host for 5, *P.knowlesi*, *P.fieldi*, *P.coatneyi*, *P.cynomologi* and *P,inui*. Zoonotic malaria was considered to be extremely rare until a large focus of p.knowlesi infections in the Kapit Division of Sarawak, Malaysian Borneo, was described in 2004. Since then, human cases have been described in virtually all southeast sian countries and *P.knowlesi* is now considered the fifth species of Plasmodium causing malaria in humans [10].

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Profile of Sensitivity, Specificity, PPV (Positive Predictive Value), NPV (Negative Predictive Value), LR+ (Positive Likelihood Ratio), LR- (Negative Likelihood Ratio) and Prevalence of Parasites

The examination result with RDT, Microscopy and Nested PCR on *P.falciparum* and *P.vivax* are shown in Table 3. The highest sensitivity (100%) on parasite examination was between RDT (*P.vivax*) with N-PCR Pv (nested PCR *P.vivax*). the highest specificity (100%) were between RDT Pf with N-PCR Pf and Pf microscopy with N-PCR Pf. The highest PPV (95,83%) were between RDT Pf with N-PCR Pf and between microscopy Pf and N-PCR Pf. The highest NPV (100%) were between RDT Pv and N-PCR Pv. The highest LR+ (5,17%) were between RDT Pf and microscopy Pf. Whereas the highest LR- (0,31%) were between RDT Pf with N PCR Pf and microscopy Pf with N-PCR Pf.

Alemu et al [11] in their study about comparison of Giemsa microscopy with nested PCR for diagnosis of malaria in North Gondar, north-west Ethiopia, reported that among microscopy negative samples, 13,1% (39/297) samples turned malaria-positive in nested PCR. Although Giemsa microscopy remains the gold standard for malaria diagnosis in resource-limited environment, its sensitivity and specificity as compared to nested PCR is limited suggesting exploration of novel rapid and simplified molecular techniques for malaria-endemic countries. Pembele et al [12] reported that RDT were significantly more sensitive than microscopy. The nested PCR is time and labour consuming and required expensive reagents and infrastructure, but it was confirmed the considerable sensitivity of nested PCR for detection of cases that remain undiagnosed by microscopy and RDT.

Table 1. 2x2 table of nested PCR and microscopy

		Microscopy	
		Positive	**Negative**
Nested PCR	Positive	a	b
	Negative	c	d

Calculation formula :

- Sensitivity = a: (a+c)
- Specificity = d : (b+d)
- Positive predictive value = a : (a+b)
- Negative predictive value = d : (c+d)
- Prevalence = (a+c) : (a+b+c+d)
- Positive likelihood ration = sensitivity : (1-spesificity) = a/(a+c) : b/(b+d)
- Negative likelihood ratio = (1-sensitivity) : spesicifity = c/(a+c) : d/(b+d)

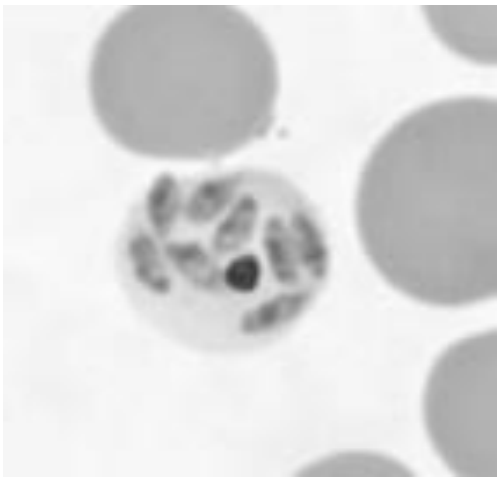

Figure 1. Morphology of *P.knowlesi* (red arrows) with Hematoxylin and Eosin staining

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Table 2. Characteristic and symptoms of the subjects

Characteristic	number (n)	Percentage (%)
Sex		
Male	48	49,0
Female	50	51,0
Age (years old)		
• 0 – 4	15	15,3
• 5- 14	36	36,7
• 15 – 59	41	41,8
• >60	6	6,1
Symptoms		
• Fever	73	74,5
• Pale	37	37,8
• Abdominal pain	27	27,6
• Diarrhea	1	1.0
• Headache	71	72.4
• Nausea	5	5.1
• Lost of appetite	6	6.1
• Splenomegaly	30	30.6

Table 3. Profile of *P.falciparum* and *P.vivax* on several parameters

No.	Parameter (%)	RDT Pf		RDT Pv		Micros Pf *vs* N-PCR Pf	Micros Pv *vs* N-PCR Pv
		Micros Pf	N-PCR Pf	Micros Pv	N-PCR Pv		
1.	Sensitivity	91,30	69,00	97,62	100,00	69,00	97,06
2.	Specificity	82,35	100,00	42,11	28,57	100,00	48,57
3.	PPV	69,23	95,83	91,11	87,18	95,83	79,52
4.	NPV	82,35	8,82	80,00	100,00	8,82	89,47
5.	LR+	5,17	∞	1,69	1,40	∞	1,89
6.	LR-	0,11	0,31	0,06	0,00	0,31	0,06

CONCLUSION

In this study conclude that Nest PCR has high sensitivity and specificity to be used as an alternative diagnostic for falciparum malaria, vivax malaria, mixed infection and knowlesi malaria. And in this study also suggested the findings of *P.knowlesi* in South Nias regency, although the prevalence is still low, but it is necessary to develop a new combination therapy for its management of the malaria.

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