

## Research Article

## Malaria Detection using Polymerase Chain Reaction (PCR) Method in Pregnant Women's Saliva on Several Hospitals in North Sulawesi Province

### *Deteksi Malaria dengan Metode PCR pada Saliva Perempuan Hamil di Beberapa Rumah Sakit di Provinsi Sulawesi Utara*

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#### Abstract

**Objective:** To detect malaria by PCR examination of saliva in pregnant women and to obtain the incidence of malaria and the type of plasmodium causing malaria in pregnant women at various hospital in North Sulawesi.

**Method:** A descriptive cross sectional study in pregnant women during antenatal care at the Department of Obstetrics and Gynecology Faculty of Medicine University of Sam Ratulangi/Prof. Dr. R.D. Kandou General Hospital in Manado, R.W. Monginsidi Hospital in Manado, Bethesda Hospital in Tomohon, and Datoe Binangkang Hospital in Kotamobagu, from 1 April until 31 May 2008.

**Result:** There were 43 pregnant women clinically diagnosed with malaria, 23 (53.49%) by PCR examination of saliva and 20 (46.59%) by blood smears. From 23 cases of malaria in pregnancy detected by PCR, there were 18 diagnosed as tropical malaria, 3 tertian malaria, and 2 mixed malaria.

**Conclusions:** The incidence of malaria in pregnancy at various hospital in North Sulawesi using PCR methods for saliva examination from April 1 - May 31 2008 is 53.49%. In this study malaria are mostly caused by Plasmodium falciparum with the largest incidence in primigravida, in the first trimester.

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**Keywords:** malaria, PCR, pregnancy, saliva

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#### Abstrak

**Tujuan:** Untuk mendeteksi malaria dengan PCR melalui saliva perempuan hamil dan mengetahui angka kejadian malaria pada perempuan hamil serta jenis plasmodium penyebab malaria pada perempuan hamil di beberapa rumah sakit di Sulawesi Utara.

**Metode:** Uji potong lintang pada perempuan hamil yang melakukan pemeriksaan antenatal di Bagian/SMF Obstetri dan Ginekologi Fakultas Kedokteran Unsrat/RSUP Prof. Dr. R.D. Kandou di Manado, RS R.W. Monginsidi di Manado, RS Bethesda di Tomohon dan RS Datoe Binangkang di Kotamobagu sejak 1 April - 31 Mei 2008.

**Hasil:** Sejak 1 April - 31 Mei 2008 didapatkan 43 perempuan hamil yang diduga malaria secara klinis, 23 (53,49%) dinyatakan positif malaria dengan pemeriksaan PCR terhadap spesimen saliva dan 20 (46,59%) dengan apusan darah. Dari 23 perempuan yg terdeteksi positif malaria melalui PCR, 18 pasien didiagnosis sebagai malaria tropika, 3 pasien malaria tertiana, dan 2 pasien mixed malaria.

**Kesimpulan:** Kejadian malaria pada perempuan hamil tersangka klinis malaria pada beberapa RS di Sulawesi Utara menggunakan metode PCR melalui spesimen saliva periode 1 April - 31 Mei 2008 adalah sebesar 53,49%. Jenis plasmodium terbanyak pada penelitian ini adalah Plasmodium falciparum dan angka kejadian malaria tertinggi ditemukan pada primigravida, pada trimester pertama.

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**Kata kunci:** kehamilan, malaria, PCR, saliva

#### INTRODUCTION

In Indonesia, malaria ranks on the eight place in the ten deadliest diseases, with 0.7% mortality rate in the urban area and 1% in the suburban area (SKRT 2001). In North Sulawesi, based on the data from the outpatient clinic at public health care, malaria is still in the ten most common diseases. In 1989, malaria was recorded to be the second most common disease (17%) after common cold and holds 9% of inpatient rate at various hospitals in

North Sulawesi. Malaria infection in North Sulawesi were caused by plasmodium falciparum, vivax and the mixed of both plasmodium.<sup>1-3</sup>

Pregnant women are very susceptible to malaria infection because of various causes. A pregnant women underwent a decline on T cell and LMI (Leukocyte Migration Inhibition Index) percentage and if a pregnant women are infected with malaria, the decrease will be more radical.<sup>2,4</sup>

Clinical manifestation of malaria in pregnant women depended on the immunity level of the women. The immunity depended on the transmission rate at the place where the women live. In an endemic malaria area like North Sulawesi, where women are always exposed to infected mosquitoes every months, the malaria immunity developed significantly. The most susceptible group to be infected with malaria in these areas is pregnant women.<sup>3,4</sup>

Laboratory diagnosis on malaria is established by various methods such as microscopic examination, QBC examination, immunoserological examination and biomolecular examination. In a situation where the level of parasites in the blood are low (low parasitemia) and the symptoms are uncommon or even asymptomatic, microscopic findings are less sensitive compared to QBC methods and other dipstick examinations. PCR has a higher sensitivity compared to other examinations.<sup>5-8</sup>

PCR examinations in malaria can be used in cases when a patient highly suspected for malaria but the microscopic examinations are negative, to confirm and identified the type of plasmodium and to monitor the responds to medication especially in potentially resistance area.<sup>5-8</sup>

With new information of the parasite life cycle, detection and screening for malaria infection through blood sampling (finger tip or vein) are needed. But this brings difficulties in some communities, where they have beliefs that blood sampling are taboo and can cause some limitations. Needle utilization needs skills and more budgets, but in this study we used saliva to detect *P. falciparum* with PCR. Thus, detection of malaria infection underwent improvement in sampling method. Screening of parasites in large scales and for epidemiology surveys can be accomplished without blood sampling and needle utilization.<sup>5-8</sup>

## METHOD

This is a descriptive cross sectional study in pregnant women during antenatal care at the Department of Obstetrics and Gynecology Faculty of Medicine University of Sam Ratulangi/Prof. Dr. R.D. Kandou General Hospital in Manado, R.W. Monginsidi Hospital in Manado, Bethesda Hospital in Tomohon, and Datoe Binangkang Hospital in Kotamobagu from 1 April until 31 May 2008.

## SAMPLING CRITERIA

### Inclusion Criteria

Every women that was confirmed as pregnant, suspected clinically of having malaria, and agreed to participate in this study.

### Exclusion Criteria

Pregnant women diagnosed with malaria, or already on malaria therapy.

## OPERATIONAL FRAMEWORK

The pregnancy is confirmed if definite pregnancy signs are positive.

Pregnant women was diagnosed with malaria by doctor based on the previous laboratory examination.

Pregnant women on malaria therapy is a pregnant women diagnosed with malaria and is on anti malaria medication.

The diagnosis of malaria is clinically suspected if there were signs and symptoms such as fever, shivering, headache, decreased appetite, nausea and vomiting.

Malaria PCR is a genotype and biomolecular examination to detect plasmodium DNA, in this case specific parasite nucleotides.

Positive Nested-PCR means that the DNA of malaria parasite was found using PCR technique. Negative Nested-PCR means that the DNA of malaria parasite was not found using PCR technique.

## INSTRUMENTS

Basic data questioner, Instruments and PCR reagents, Primer nested PCR, Instruments and electroforesis reagents.

## PROCEDURE

### Research approval

The study is carried out after the patients met the study criteria, receive good explanation of the study and signed a research consent. Basic data questioner was filled by research officer. Data was recorded and included in the research status and

registration. One to five ml of saliva was taken by sputtering in a dish given and was stored under -20° C temperature.

### **DNA extraction**

300 µl saliva was added with 900 µl buffer lysis cell, mixed throughly for 10 minutes in 4° C temperature, then centrifuged for 10 minutes. The supernatant in the centrifuge was disposed and 300 µl nuclear lysis solution on pellet was added and mixed by vortex and incubated for 2 hours or through the night in 55° C temperature or then 200 µl protein precipitation solution was added. It is stirred for 20 seconds, then centrifuged for 10 minutes with maximum speed. Tube filled with 1 µl reagent was prepared, the supernatant was taken and placed in a new container, 2 propanolol was added, mixed throughly for 15 minutes in maximum speed. The supernatant was disposed afterwards, then it was rinsed with 70% ethyl alcohol, dried for 3.5 minutes, and 50 µl dehydration solution was added. Then DNA and TrisEDTA was mixed. Sampel was stored in - 20° C before used.

### **PCR reaction**

Primer that was used in this nested PCR study can amplify a DNA segment and gene found in plasmodium. The first primary pair will produce a DNA segment of 262 pair. The second base primer is specific for Plasmodium sp.

### **PCR amplification**

PCR mixture was prepared with volume reaction as many as 25 µl for every PCR tube: 17 µl sterile aquabidest, 10XPCR buffer + MgCl<sub>2</sub> 2.5 µl, 10 mMol dNTPs 2 µl, 0.25 µl enzyme, each 0.5 µl primer and 3 µl DNA template. The first PCR stage used Primer P<sub>1</sub>F-Up and P<sub>1</sub>R, and for the second stage used P<sub>1</sub>F and each specific primer. The first stage PCR product was then diluted to a final concentration of 50 times and was used as templates for the second PCR stage.

The PCR machine (Perkin Elmer) was programmed for 30 amplification cycle in phase I and 35 cycles in phase II. The parameters used for the PCR cycle Phase I includes the initial denaturation for 7 minutes at a temperature of 95° C, denaturation for 30 seconds at a temperature of 95° C, annealing for 30 seconds at a temperature of 55° C,

polymerization for 30 seconds at a temperature of 72° C and extension for 7 min at 72° C.

The parameters used for the PCR cycle phase II includes the early denaturation for 7 minutes at a temperature of 95° C, denaturation for 30 seconds at a temperature of 95° C, annealing for 30 seconds at a temperature of 55° C, polymerization for 30 seconds at a temperature of 72° C polymerization and extension for 7 min at 72° C, then the temperature lowered to 4° C.

### **Reading of the PCR**

Agar material was prepared by dissolving 1.5 g agarose in 100 ml of 1x TBE buffer, and then added to 5 µl ethidium bromide that is mutagenic, heated until boiling. It was then printed using a comb as a shaper of the wells. A total of 8 µl sample of PCR was then mixed with 2 µl loading dye and then poured into 1.5% agar wells. As the marker 10 µl 100 bp DNA ladder was used. Electrophoresis was carried out for 20 minutes at a voltage 100 volts. Visualization of the results of electrophoresis can be performed by trans-illuminator using ultraviolet light and photographed with a Polaroid camera as documentation.

## **RESEARCH ETHICS**

All pregnant women who participated in this study had undergone counseling and had received explanation of the purpose and objectives of this study. If the woman agreed, then she must sign a letter of informed consent. When a woman was found positive for malaria in this study, then a proper step of treatment was carried out.

## **DATA ANALYSIS**

In this study, data was collected, assessed, tabulated and presented in tables and percentages.

## **RESULTS**

This research was conducted from April 1<sup>st</sup> to May 31, 2008. During the study, there were 43 pregnant women clinically suspected with malaria who underwent in-patient and ambulatory care in Map research hospitals, namely: 6 patients at the Prof. Dr. R.D. Kandou General Hospital in Manado, 24 pa-

tients at the Wolter Monginsidi Hospital, 7 patients at Datoe Binangkang General Hospital in Kotamobagu, 6 patients at Bethesda Hospital in Tomohon.

Of the 43 pregnant women clinically suspected malaria, there were 23 positive pregnant women with malaria confirmed with PCR through saliva specimens. Among them, there were 18 women diagnosed with tropical malaria (*Plasmodium falciparum*), 3 pregnant women with tertian malaria (*Plasmodium vivax*), and 2 pregnant women with mixed malaria (*Plasmodium falciparum* and *Plasmodium vivax*).

In this study, there were 23 (53.49%) pregnant women diagnosed with malaria infection by PCR and 20 (46.59%) by blood smear. The results were quite high; this could be due to the time of sampling which occurred in April to May which is the rainy season. Based on the literature it was said that the rainy season associated with 5 fold the risk of clinical malaria and 2 fold the risk of parasitemia compared to the dry season.<sup>6,9</sup>

These results are similar to those found in Ghana where Mockenhaupt et al found that of 839 pregnant women examined with suspected malaria, PCR methods diagnosed malaria infections by 52.9%, while blood smear only diagnosed 19% of malaria infection. Tjitra research in eastern Indonesia also found that 50% of clinically suspected malaria infected were malaria positive.<sup>10,11</sup>

Pregnant women are highly vulnerable to malaria infection because of several things. There were a decreased percentage of T cells and LMI (Leucocytes Migration Inhibition Index) and if the woman is infected with malaria, they will further decrease.<sup>2,4,9</sup>

**Table 1.** Characteristics of Samples

Characteristics	n = 43	%
Age		
≤ 20	12	27.9
21 - 25	7	16.3
26 - 30	16	37.2
> 30	8	18.6
Parity		
Prim gravidity	19	44.2
Secondary gravidity	9	20.9
Multi gravidity	15	34.9
Gestational Age		
< 13 weeks	8	18.6
13 - 28 weeks	16	37.2
> 28 weeks	19	44.2

In Walker's study, age and gravidity did not affect the prevalence of malaria infection sub microscopically in pregnant women in Cameroon. Nyunt et al (2003) conducted a study in southern Zambia and found that the average age of pregnant women with malaria was 26 years (21-39 years).<sup>12-14</sup>

In Table 2, the type of *Plasmodium* causing malaria were *Plasmodium falciparum* in 18 patients (77.3%), *Plasmodium vivax* in 3 patients (13.6%) and mixed *Plasmodium* in 2 patients (9.1%). From the literature, pregnant women are more susceptible to the infection of *P. falciparum*. This happens because only *P. falciparum* underwent sequestration, and other *Plasmodium* cycles entirely occurred in the peripheral blood vessels. Sequestration occurs in vital organs and virtually all tissues in the body.<sup>4</sup> Pregnant women have the possibility of infected with malaria *falciparum* more frequent and severe compared to non pregnant women. In endemic areas such as North Sulawesi Province, the most common cause of malaria was *Plasmodium falciparum*. In Walker's research *Plasmodium falciparum* infection to be the most common cause of malaria, found in 76.1% malaria patients. *Plasmodium falciparum* and *Plasmodium malariae* mixed infection is found in 6.9%, *Plasmodium falciparum* and *Plasmodium ovale* mixed infection in 1.8% and the mixed infection of *Plasmodium falciparum*, *Plasmodium malariae* and *Plasmodium ovale* was 0.7%.<sup>16</sup>

**Table 2.** Distribution of malaria based on parity

Malaria	P. Falciparum		P. Vivax		Mixed Malaria	
	n	%	n	%	n	%
Prim gravidity (n=19)	10	47.4	2	10.5	1	5.3
Secondary gravidity (n=9)	4	44.4	1	11.1	0	0.0
Multi gravidity (n=15)	4	26.7	0	0.0	1	6.7

Out of 43 pregnant women clinically suspected malaria, 47.4% is primigravida, 44.4% is secondary gravidity, and 26.7% is multigravida. Some studies also mention the highest prevalence of malaria infection in pregnancy was the first and second gravidity with a high parasite density. From the literature, malaria infection was also determined by the patient immune factors. Individual immunity against malaria is influenced by several factors, one of which is derived factor (active immunity) follow-

ing a recurrent infection, and it is increasing simultaneously with the increasing age and parity. This means that the younger the age of a woman in her first pregnancy, the more likely she is to be infected with malaria. The reduced risk of malaria with increasing parity has been reported in some studies, especially in women who are semi immune.<sup>9,13,15,16</sup>

The same study conducted by Coulibaly et al showed the results of 56.7% in primigravida, 43.6% in secondary gravidity and 20.2% in multi-gravida.<sup>17</sup>

The result showed that the incidence of malaria caused by *Plasmodium falciparum* infection in women of gestational age <13 weeks and 13 - 28 weeks are 50% and in women with gestational age over 28 weeks is 26.3%. *P. vivax* infection in women of gestational age of 13 - 28 weeks is 13%, and over 28 weeks is 5%. Mixed malaria at the gestational age of <13 weeks is 12.5% and over 28 weeks is 5.3%. Zhou et al also stated that the first trimester had the highest risk of malaria infection. Immunity against malaria began to decrease in pregnant women in the third trimester of pregnancy. According to Zhou, malaria infection in pregnant women are more prone to occur in the first trimester because at that period there was a decline in proliferative response of T cells resulting in decreased immunity seluler.<sup>16</sup>

Nyunt et al (2003) in their study found 40% of women suffered from malaria is in their late second trimester and third trimester of pregnancy. While Coulibaly found 55.9% pregnant women infected with malaria at their earlier pregnancy with a peak at 9-16 weeks of gestation.<sup>14</sup>

## CONCLUSION

The incidence of malaria in pregnant women clinically suspected malaria at the Prof. Dr. R.D. Kandou General Hospital, Wolter Monginsidi Hospital, Datoe Binangkang Hospital, and Bethesda Hospital in Tomohon using PCR methods through saliva specimens from 1 April to 31 May 2008 was 53.49%. The type of plasmodium causing malaria in this study was *Plasmodium falciparum* with the highest incidence of malaria was found in primi gravida and in the first trimester. PCR Method were slightly more accurate in malaria detection compared to microscopic examination.

## SUGGESTION

Further research can be done to make the PCR with saliva specimens as a standard test on suspected cases of malaria in endemic areas.

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