Original Article

Comparison of Nested Polymerase Chain Reaction and Microscopy as Diagnostic Tools in Congenital Malaria: a Study at Tjark Corneile Hillers Hospital Maumere, Indonesia

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Abstract

Background: Microscopic detection is the conventional method for detecting malaria parasites. Although it is efficient and inexpensive, it has its limitations. In recent years, polymeras chain reaction (PCR) has been considered superior to microscopy in detecting mixed *Plasmodium* infections or infections with low parasite density. To determine whether microscopic or nested PCR (nPCR) is better at detecting congenital malaria (CM).

Methods: Blood smear examination and nPCR were performed with blood samples taken from mothers and their newborns, who were likely to be suffering from CM and in whom one of the symptoms was low birth weight (LBW). The sensitivity and specificity of each method were then compared.

Results: During one year of study, the prevalence of CM among 92 LBW newborns was determined to be 6.8% using microscopy and 7.8% using nPCR. Among the 92 mother–infant paired subjects, CM was detected in 34 subjects (37%) by microscopy and in 39 subjects (42.4%) by nPCR. nPCR was more sensitive (76.5% vs 66.7%) but less specific (77.6% vs 84.9%) than microscopy. When the two methods were compared, nPCR gave significantly better results in diagnosing CM (AUC = 0.770; $P < 0.001$).

Conclusion: Although microscopy remains the most appropriate method for the diagnosis of CM in remote areas, nPCR can be considered a complementary test.

Keywords: congenital, malaria, newborns, nested PCR, microscopy

Introduction

Infection with *Plasmodium* species during pregnancy has been associated with low birth weight (LBW), which increases the risk of perinatal and infant mortality (1). Another adverse outcome that has received less attention is the placental passage of *Plasmodium* parasites and the subsequent risk of malaria in the newborn, known as congenital malaria (CM). Estimating the prevalence of CM is complicated because several factors make diagnosis difficult. It is difficult to distinguish cases of CM from autochthonous malaria in endemic regions because case definitions differ, such as presence of parasites in the placenta at delivery or clinical

diagnosis of malaria in newborns. Diagnosis is further complicated by low density of the parasites in peripheral blood of newborns. These complications have led to estimates that have ranged from 0.03% to 46.70% in endemic areas (2−4).

The traditional microscopic examination to detect malaria parasites has been the standard method for diagnosis because of its efficacy and lower cost compared with other diagnostic tools (5). However, microscopic examination has some limitations. The species may be incorrectly identified, especially when the slides are not properly prepared or the infecting species are present at low density or mixed species are present. It also requires considerable expertise (5,6). Therefore, molecular tests such as the polymerase chain reaction (PCR) has been used as a complementary tool to achieve more accurate diagnoses. In recent years, PCR has been developed as a diagnostic tool for malaria, and most studies have found it to be superior to microscopy in detecting mixed infections or infections with low density, due to its higher sensitivity (5).

In this study, the utility of microscopy and PCR in diagnosing CM infection was compared using mother−infant paired samples at Tjark Corneile (TC) Hillers Hospital, Maumere, which serves a remote area in Indonesia in which malaria is endemic.

Materials and methods

Definitions used in this study

CM was defined as an infection resulting from the transmission of live parasites from an infected pregnant woman to her foetus that occurs prenatally or during delivery and persists after birth (9). In addition, only asexual malaria parasites are present in the peripheral blood smear of the newborns during the first seven days of life, irrespective of the clinical symptoms (10,11). LBW was diagnosed if the birth weight of the newborns was less than 2500 grams, irrespective of the duration of gestation (12). Pre-term infant was defined as birth before 37 weeks of gestation, based on the Ballard score (13).

Study design and study population

A preliminary study conducted at TC Hillers Hospital showed that the species causing CM were *Plasmodium falciparum, P. vivax* or a mixture of both (8). This cross-sectional study of the utility of microscopy and PCR as diagnostic tools in CM included 92 mother−infant pairs and was conducted from December 2012 through December 2013. Samples were taken from newborns at TC Hillers Hospital, Maumere. Aliquots were then shipped in containers equipped with dry ice to the Faculty of Medicine University of Brawijaya (FMUB), Malang-East Java, Indonesia, which is approximately 1060 km away. Thin and thick blood smear examinations were performed at the FMUB Department of Parasitology and then confirmed by nested PCR (nPCR) examination at the FMUB Department of Biomedical. Blood samples from each mother−newborn pair that fulfilled inclusion criteria were aseptically obtained.

Sample size

To be considered for the study, a mother−newborn pair had to meet the following inclusion criteria: 1) pre-term infant delivery; 2) LBW infant; 3) infant illness during the first seven days of life with symptoms such as anaemia, poor feeding, jaundice, seizures, fever, or hepatomegaly; and 4) informed consent obtained from each newborn's mother or guardian for participation in this study. However, to alleviate study bias, exclusion criteria were implemented. Infants who suffered from asphyxia or multiple congenital anomalies or were suspected to suffer from bacterial sepsis were excluded. Mother−infant pairs who experienced rupture of the membrane more than 18 hours before delivery or a traumatic delivery process were also excluded. After informed consent was obtained, blood was obtained from mother and from the newborn shortly after delivery. The appropriate sample size was calculated on the basis of a comparative statistical analysis formula (7), with an estimated CM prevalence of 14.7% and the species identified as *P. falciparum* and *P. vivax* (8). On the basis of a calculated sample size of 64 mother−infant pairs, subjects were consecutively enrolled during a one-year period. A total of 92 mother−infant pairs were enrolled in the study.

Blood smear

Peripheral blood samples of 0.5 mL and 3 mL were aseptically taken from the infant and mother, respectively. Thick and thin smears, which required about 50 µL and 10 µL blood, respectively, were stained with Giemsa solution. Identification of *Plasmodium* species using thin smear was performed by a trained medical analyst and confirmed by a parasitology consultant. Parasite densities were determined by calculating the number of infected erythrocytes per 1000 erythrocytes and are expressed as percentages.

Nested PCR

DNA samples were extracted from whole blood using the absorption method described in the blood lysate and purification protocol of the PureLink™ Genomic DNA Kit (Invitrogen, USA). After purification, all samples were stored at −20 °C until further processing for nPCR. A pair of outer primers (rPLU1 and rPLU5) was used to amplify a section of the *Plasmodium genome*. Two pairs of inner primers, one for *P. falciparum* (rFal1–rFal2) and one for *P. vivax* (rVIV1–rVIV2), were used for species amplification. A DNA template of 1 µL was used in 25 µL master mix (Go Tag®Green Master Mix, Promega) under the following conditions: 1 µL of each primer (10 µM), 12.5 µL of PCR master mix and 9.5 µL of double-distilled H_2O. The nest 2 reaction was identical to the nest 1 reaction (14). Sterile aqua dest was used as a negative control.

Statistical analysis

Statistical analyses were performed using SPSS software version 17. A descriptive analysis was used to calculate the prevalence of the outcome variables, each diagnosed by microscopy and nPCR methods. The prevalence of CM infection was expressed as a percentage. The sensitivity and specificity of each method were compared using microscopy and nPCR alternately as the gold standard. Thus, when the sensitivity and specificity of the microscopic method were examined, nPCR was used as the gold standard, and vice versa. Receiver Operating Characteristic (ROC) curves of both methods were analysed using a significance level of 5% (7).

Ethical considerations

This study was approved by the Ethical Committee for Medical Research of the Medical Faculty University of Brawijaya (Ethical Clearance no. 368A/EC/KEPK/12/2012). Informed consent was obtained from mothers or legal guardians of all newborns.

Results

Comparison of CM diagnosis by microscopy and nPCR

CM was diagnosed when similar *Plasmodium* species were detected in both the mother's and infant's peripheral blood, either by microscopy and/or nPCR. During the one year study period, the prevalence of CM diagnosed by microscopy and PCR among all LBW newborns was 6.79% and 7.78% respectively. Table 1 provides a matched-sample description of how microscopy and PCR

performed in relation to each other, using the alternate method as the gold standard.

In this study, discrepancies were found in 21 (22.8%) samples. The *Plasmodium* species identified by each method are specified in Table 2. An example variation assessment by each diagnostic tool is shown in Figure 1.

Accuracy of microscopy and nPCR method in diagnosing CM

In order to determine which method showed better accuracy, the sensitivity, and specificity of microscopy and nPCR, using the alternative method as the gold standard, were evaluated. Table 3 provides a description of the accuracy of
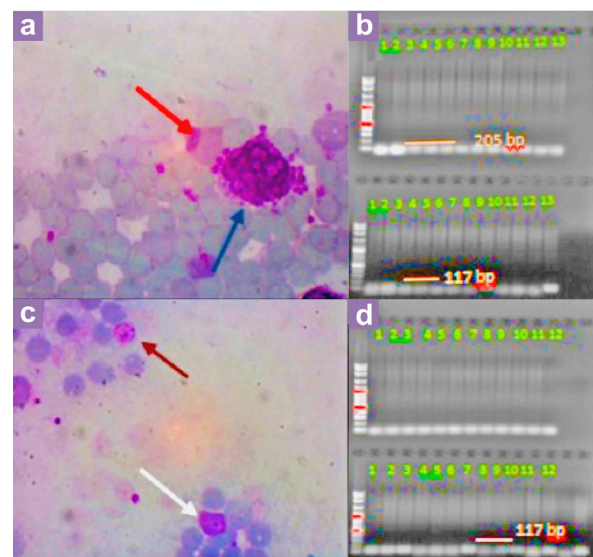

Figure 1: Example of comparative identification of *Plasmodium* species using microscopy and nPCR. a) Identification by microscopy showed trophozoites/ring form on enlarged red blood cell (red arrow). Active macrophage ingesting *Plasmodium* is showed by blue arrow; b) Confirmation by PCR showed mixed infection of *P. falciparum* (upper gel, 1st column, 205 bp) and *P. vivax* (lower gel, 1st column, 117 bp). c) Identification by microscopy showed trophozoites/ring form on enlarged red blood cell (white arrow) but there was an image resembling schizont of *P. falciparum* on upper field (brown arrow); d) Confirmation by PCR showed single infection of *P. vivax* (lower gel, 12th column, 117 bp).

Table 1: Identification of congenital malaria: comparison between microscopic and nPCR

		PCR		Total
		Negative (%)	Positive (%)	
Microscopy	Negative	45 (48.9)	13 (14.1)	58 (63)
	Positive	8 (8.7)	26 (28.3)	34 (37)
Total		53 (57.6)	39 (42.4)	92 (100)

Table 2: Identification of *Plasmodium* species: comparison between microscopic and nPCR

Species	Diagnostic Tools	
	Microscopy (%)	Nested PCR (%)
P. falciparum	3 (8.8)	9 (23.1)
P. vivax	29 (85.3)	29 (74.4)
Mixed Infection P.falciparum & P.vivax	2 (5.9)	1 (2.5)
Total	34 (100)	39 (100)

Table 3: Accuracy of congenital malaria diagnostic tools: comparison between microscopic and nPCR

Diagnostic Tools	Sensitivity (%)	Specificity (%)	AUC	95% CI	*P* value
Microscopy	66.7	84.9	0.758	0.653 – 0.863	< 0.001*
Nested PCR	76.5	77.6	0.770	0.667 – 0.874	< 0.001*

*significantly different ($P < 0.05$).
Abbreviation: AUC = Area Under Curve; CI = Confidence Interval.
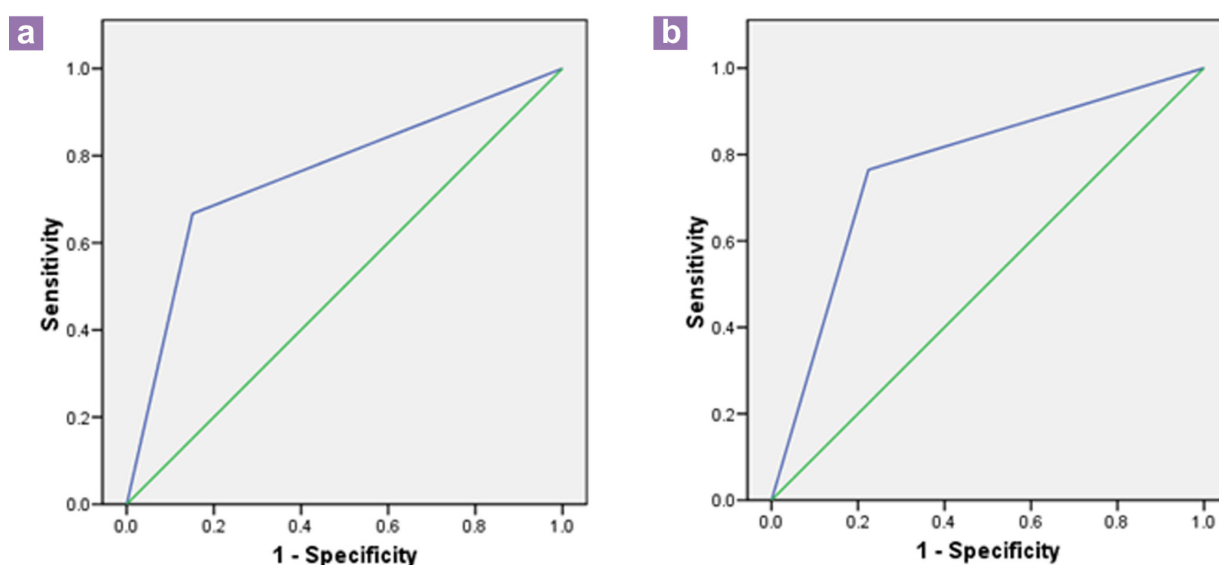
Figure 2: Summary of Receiver Operating Characteristic (ROC) plot of sensitivity and specificity of microscopy and nPCR. a) ROC plot of microscopy method (blue line) using PCR (green line) as a gold standard and AUC obtained was 0.758 ($P < 0.001$). b) ROC plot of nPCR method (blue line) using microscopy (green line) as a gold standard and AUC obtained was 0.770 ($P < 0.001$).

microscopy and nPCR in diagnosing CM, while Figure 2 shows a comparison of the ROC curves obtained using each method.

Discussion

In this study, nPCR yielded a slightly higher prevalence of CM among LBW newborns (7.78%) than microscopy (6.79%). In malaria endemic areas, CM is considered as a rare consequence of malaria infection among pregnant semi-immune women; its prevalence varies from 0 to 23%. The reasons that have been suggested for this variability include: (i) differences in the definition of CM; (ii) levels of maternal immunity; (iii) the type of blood sample examined (peripheral blood of neonates or cord blood); (iv) the expertise in blood smear examinations; (v) the method of parasite detection (Giemsa staining or PCR); or even (vi) a reflection of true environmental differences (11). In this study, blood smears were evaluated by an expert at the central education centre. However, in real situations at TC Hillers Hospitals or in other remote endemic areas, blood smears would be evaluated only by a local analyst, and the results would be dependent on available technical support and skill. This condition would probably increase the gap between CM prevalence diagnosed by both methods and would give different results from this study. However, due to the prohibition of local culture, blood samples were only taken from peripheral blood, instead of cord blood; thus, this could affect the magnitude of the actual prevalence of CM.

A previous study in Africa stated that nPCR has been used and considered a molecular gold standard for detecting human malaria parasites since 1999 (15). In recent years, PCR has been developed as a malaria diagnostic tool, and many studies have demonstrated its ability to detect mixed infections and infections with low parasite densities.5 The present study is the first analytical report comparing microscopy with nPCR for routine use in diagnosing CM at TC. Hillers Hospital, Maumere. In this study, false negative results from nPCR were found in eight samples and have been re-evaluated two times. Such cases can be confirmed by DNA sequencing; however, due to resource limitations, sequencing could not be performed. A possible explanation for the discrepant results is that the false positives observed in our study may be due to the lack of primers targeting other human malaria species, including *P. knowlesi*, a simian malaria. *P. knowlesi* has been reported to resemble *P. malariae* and *P. falciparum* when viewed with

a microscope (17). The detection threshold in the Giemsa-stained microscopic method has been estimated to be 50–100 parasites/µL (± 0.001%) under imperfect conditions. In remote areas with less skilled microscopists and poor equipment, a higher threshold is very likely (16). In this study, the parasite loads in both infants (0.962 ± 1.047%) and their mothers (0.341 ± 0.378%) were quite dense, but sample quality was not ideal. Poor blood film may cause artefacts, with bacteria, fungi, precipitated stain and dirt, or cell debris resembling malaria parasites. The chance of false negative results would increase with decreasing parasite density. The recommended number of fields on a thick blood film requiring examination before declaring a negative result varies from 100 to 400 (16). In this study, more mixed infections were identified using the microscopic method than were identified using nPCR. Another explanation for these discrepant results is that when one Plasmodium species is present at high parasite density, it may inhibit the identification of other infecting species using PCR. For example, competition for amplification at the beginning of the nPCR due to a high density of *P. vivax* may preclude detection of *P. falciparum* (17).

When the diagnostic tools used in this study were compared, nPCR was found to be more sensitive, whereas the microscopic method was found to be more specific. These results were statistically significant ($P < 0.001$). Previous studies in Brazil and Angola revealed similar results (18,19). A recent meta-analysis reported that similar studies in many countries have demonstrated that the sensitivity of the microscopic method varies from 34–54% and its specificity varies from 86 to 95%. When using microscopy as a gold standard, a false negative result from microscopic method would consider a positive result from nPCR to be a false positive. Thus, the comparator would be bigger and make the nPCR specificity lower than that of microscopy (20). The higher sensitivity of the nPCR method showed that it has better ability to detect malaria parasites, although nPCR requires more time and is costly (6,20).

In this study, we also found a predominance of *P. vivax* infection in CM cases. Little is known about the effects of *P. vivax* during pregnancy or on infant outcomes. *P. vivax* caused markedly fewer cases of severe or fatal malaria than *P. falciparum,* which may explain the lack of severe illness or death among cohort studies, even on delay in identification and treatment. *P. vivax* generally predominates in areas where overall *Plasmodium* transmission is less intense,

and thus, protective immunity to *P. vivax* is not as commonly acquired as in areas with intense transmission (21).

Conclusion

In remote areas, microscopy remains the most appropriate diagnostic tools for CM, but nPCR should be considered as a complementary test because of its superiority, feasibility, and cost effectiveness.

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Conflicts of Interest

None.

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Authors' Contributions

Conception and design: NEJ, IRH, LEF
Analysis and interpretation of the data: NEJ, TW, LEF
Drafting of the article: NEJ
Critical revision of the article for the important intellectual content: IRH, AH, LEF
Final approval of the article and obtaining of funding: IRH, LEF
Provision of study materials or patient and collection and assembly of data: MBN
Administrative, technical or logistic support: TW, AH

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References

1. Desai M, Ter Kuile FO, Nosten F, McGready R, Asamoa K, Brabin B, et al. Epidemiology And Burden Of Malaria In Pregnancy. *Lancet Infect Dis*. 2007;**7(2)**: 93–104. doi: 10.1016/S1473-3099(07)70021-X.

2. Mukhtar MY, Lesi FE, Iroha EU, Egri-Okwaji MT, Mafe AG. Congenital malaria among inborn babies at a tertiary centre in Lagos, Nigeria. *J Trop Pediatr*. 2006;**52(1)**:19–23. doi: 10.1093/tropej/fmi044.

3. Obiajunwa PO, Owa JA, Adeodu OO. Prevalence of congenital malaria in Ile-ife, Nigeria. *J Trop Pediatr*. 2005;**51(4)**:219–222. doi: 10.1093/tropej/fmi003.

4. Falade C, Mokuolu O, Okafor H, Orogade A, Falade A, Adedoyin O, et al. Epidemiology of congenital malaria in Nigeria: a multi-centre study. *Trop Med Int Health*. 2007;**12(11)**:1279–1287. doi: 10.1111/j.1365-3156.2007.01931.x.

5. Fontecha G, Mendoza M, Banegas E, Poorak M, De Oliveira A, Mancero T, et al. Comparison Of Molecular Tests For The Diagnosis Of Malaria In Honduras. *Malar J*. 2012;**11**:119. doi: 10.1186/1475-2875-11-119.

6. Azikiwe CCA., Ifezulike CC, Siminialayi IM, Amazu LU, Enye JC, Nwakwunite OE. A Comparative Laboratory Diagnosis Of Malaria: Microscopy Versus Rapid Diagnostic Test Kits. *Asian Pac J Trop Biomed*. 2012;**2(4)**:307–310. doi: 10.1016/S2221-1691(12)60029-X.

7. Dahlan MS. Konsistensi V Menentukan Besar Sampel. In: Dahlan MS, editors. *Langkah-Langkah Membuat Proposal Penelitian Bidang Kedokteran dan Kesehatan*. 2nd edition. Jakarta: Sagung Seto; 2012. p. 79–88.

8. Jahja NE, Huwae, I.R., Nara, M. B., Enggar, L.F. Abstract. Proceedings of the 6th Annual Pediatrics Scientific Meeting. Solo, Indonesia. 2013;**53(5)**:173.

9. Carlier Y, Truyens C, Deloron P, Peyron F. Congenital parasitic infections: A review. *Acta Trop*. 2012;**121(2)**:55–70. doi: 10.1016/j.actatropica.2011.10.018.

10. Uneke CJ. Impact of placental Plasmodium falciparum malaria on pregnancy and perinatal outcome in sub-Saharan Africa: II: effects of placental malaria on perinatal outcome; malaria and HIV. *Yale J Biol Med.* 2007;**80(3)**:95–103. doi: 10.1016/j. trstmh.2010.01.013.

11. Menendez C, Mayor Alfredo. Congenital malaria: The least known consequence of malaria in pregnancy. *Semin Fetal and Neonatal Med.* 2007;**12(3)**:207–213. doi: 10.1016/j.siny.2007.01.018.

12. OECD. *Infant Health: Low Birth Weight. In: OECD, editor. Health At A Glance.* Paris (FR): OECD Publishing; 2011, p. 38–39. doi: 10.1787/health_glance-2011-11-en.

13. NICE. *Neonatal Jaundice.* United Kingdom (UK): Nice Clinical Guideline; 2010. p. 98.

14. Fuehrer HP, Fally MA, Habler VE, Starzengruber P, Swoboda P, Noedl H. Novel Nested Direct PCR Technique for Malaria Diagnosis Using Filter Paper Samples. *J Clin Microbiol.* 2011;**49(4)**:1628–1630. doi: 10.1128/JCM.01792-10.

15. Rubio J, Benito A, Roche J, Berzosa PJ, Garcia ML, Mico M et al. Semi-nested, multiplex polymerase chain reaction for detection of human malaria parasites and evidence of Plasmodium vivax infection in Equatorial Guinea. *Am J Trop Med Hyg.* 1999;**60(2)**:183–187.

16. Wongsrichanalai C, Barcus MJ, Muth S, Sutamihardja A, Wernsdorfer WH. A Review Of Malaria Diagnostic Tools: Microscopy And Rapid Diagnostic Test (RDT). *Am J Trop Med Hyg.* 2007;77**(6 Suppl)**:119–127. doi: 10.1186/1475-2875-7-118.

17. Rosanas-Urgell A, Mueller D, Betuela I, Barnadas C, Iga J, Zimmerman P, et al. Comparison Of Diagnostic Methods For The Detection And Quantification Of The Four Sympatric Plasmodium Species In Field Samples From Papua New Guinea. *Malar J.* 2010;**9**: 361. doi: 10.1186/1475-2875-9-361.

18. Andrade B, Reis-Filho A, Barros A, Souza-Neto S, Nogueira L, Fukutani K, et al. Towards A Precise Test For Malaria Diagnosis In The Brazilian Amazon: Comparison Among Field Microscopy, A Rapid Diagnostic Test, Nested PCR, And A Computational Expert System Based On Artificial Neural Networks. *Malar J.* 2010;**9**:117. doi: 10.1186/1475-2875-9-117.

19. Fancony C, Sebastiao Y, Pires J, Gamboa D, Nery S. Performance Of Microscopy And RDTs In The Context Of A Malaria Prevalence Survey In Angola: A Comparison Using PCR As The Gold Standard. *Malar J.* 2013;**12**:284. doi: 10.1186/1475-2875-12-284.

20. Kattenberg J, Ochodo E, Boer K, Schallig H, Mens P, Leeflang M. Systematic Review And Meta-Analysis: Rapid Diagnostic Tests Versus Placental Histology, Microscopy And PCR For Malaria In Pregnant Women. *Malar J.* 2011;**10**:321. doi: 10.1186/1475-2875-10-321.

21. Mendis K, Sina BJ, Marchesini P, Carter R. The neglected burden of Plasmodium vivax malaria. *Am J Trop Med Hyg.* 2001;**64(1-2 suppl)**:97–106. doi: 10.1016/S1473-3099(09)70177-X.