Detection of Asymptomatic Carriers of *Plasmodium vivax* among Treated Patients by Nested PCR Method in Minab, Rudan and Bashagard, Iran

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**Abstract**

**Background:** *Plasmodium vivax* is the most widespread species of *Plasmodium* in humans and causing about 80 million clinical cases annually. This study was undertaken to detect *P. vivax* in asymptomatic treated *vivax* malaria patients to trace latent/sub-patent malaria infection.

**Method:** The venous blood of all detected cases with *P. vivax* in Bashagard, Minab and Roodan Districts in Hormozgan Province from 2009 to 2010 was examined by microscopic and nested PCR methods for presence of the parasite.

**Results:** In microscopic examination of peripheral blood smears, all samples were negative for the presence of the parasites. But, we detected two *P. vivax* related bands in the electrophoresis of the nested PCR products (120 bp).

**Conclusion:** Following up the malaria cases after treatment by a combination of methods, or new diagnostics such as RDTs can be included in the priorities of malaria elimination program in Iran.

**Introduction**

Malaria is the most important parasitic disease and half of the world’s population is under the risk of being infected with the diseases (1). The infection is still the most important mosquito-borne disease in Iran and one of the ma-
Molecular techniques such as nested PCR had been developed for malaria diagnosis. Polymerase chain reaction (PCR) as a DNA-based molecular detection method is more sensitive than microscopy, and is now being used widely in the field of malaria diagnosis (10, 14, 15). The real-time PCR produces fast results with very little contamination risks, a high sensitivity and specificity, and the possibility of quantification, but is relatively expensive and needs more competent employees (16-18).

Nowadays many studies have shown the important role of asymptomatic carriers of malaria as a major reservoir of parasites and maintenance of high levels of transmission (19, 20, 21). Even a few number of carriers are able to infect the anopheles and maintenance of the disease (19).

In order to better understanding and evaluating the epidemiology of malaria in Hormozgan Province this study was undertaken to detect *P. vivax* in asymptomatic treated *vivax* malaria patients to trace latent/sub-patent malaria infection. In the study both microscopic examining of blood films and nested-PCR method are used.

**Materials and Methods**

In this study, the venous blood of all detected cases with *P. vivax* in Bashagard, Minab and Roodan districts in Hormozgan Province from 2009 to 2010 was examined. Totally, 240 blood specimens from asymptomatic treated individuals during 2009 to 2010 were collected. Thick and thin blood smears were prepared in field laboratories and the thin film was fixed using methanol. All slides were stained with 10% Giemsa and examined with oil immersion (x1000) for detecting malaria parasites by a trained microscopist and rechecked by experienced microscopists at the malaria central laboratory of Minab District. Approximately 1000 μl of venous blood was collected in EDTA, stored at -20 °C and then transported to the molecular laboratory of parasitology department of Tabriz medical college for PCR method (10).

DNA was extracted by Q1Amp® DNA blood mini kit 50 (Qiagen, City Name, Germany) according to the instructions. All samples were assessed using *Plasmodium* genus specific (primary PCR) and *p. vivax/P. falciparum* species-specific primers for the *ssrRNA* gene by nested PCR method (10). Primers for *Plasmodium* genus:

**P. vivax**:

fVIV: 5′-CTT GTT GCC TTA AAC TTC-3′
rVIV: 5′-TAA AAA TTG TTG CAG TTA CG-3′

Primers for *P. falciparum* (as *P. falciparum* positive control) (205bp):

fFAL: 5′-TTA ACC TGG TTT GGG AAA ACC AAA TAT ATT-3′
rFAL: 5′-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3′

Primers for *P. vivax* (120bp):

fVIV: 5′-CGC TTC TAG CTT AAT CCA CAT AAA TGA TAC-3′
The thermo cycling condition were initial denaturation of DNA at 94 °C for 3 min, followed by 30 cycles of 94 °C for 60 s, 56 °C for 2 min, and 68 °C for 2.5 min. These steps were followed by an additional primer extension step of 7 min at 72 °C.

To make it visible and weigh the amplified DNA, 10 µl of PCR product was applied on 1.7% agarose gel under 100V electric field for 45 minutes. The gel then was immersed in Ethidium Bromide 0.5 µg/ml for 10 minutes and after being washed by deionized distilled water, was observed and analyzed by using Transilluminator (450 nm UV).

Positive cases with at least 400 parasites in µl blood (from Malaria Laboratory of Minab district and Malaria Laboratory in Tehran University of Medical Sciences - School of Public Health) were used as positive control, and the samples of non-infected individuals and those with P. falciparum were used as negative control.

Result

Totally 240 individuals with a history of vivax malaria treated a year earlier, were involved in this study, including 194, 31 and 15 individuals from Bashagard, Minab and Roodan districts, respectively.

In microscopic examination of peripheral blood smears, all samples were negative for the presence of the parasites. But, we detected two Plasmodium vivax related bands in the electrophoresis of the nested PCR products (120 bp). One of the cases was from Bashagard and another was from Minab district. The positive controls were all positive and the negative control was negative. The result was shown in Fig.1.

Discussion

In recent years, the economy of 4 malaria endemic provinces in Iran (Sistan and Baluchestan, Kerman, Bushehr and Hormozgan) has suffered a substantial loss during malaria epidemics, and malaria control activities imposed heavy expenses to the socio-economic developmental programs (22).

In this situation one of the major challenges is detecting of asymptomatic infection which is not detectable by routine tests due to the lack of malaria symptoms and having low levels of parasitaemia. So, these patients become gametocyte carriers and have a key role in continuing disease transmission that might combat the malaria elimination program (23-25).

Considering the challenge of asymptomatic malaria in the elimination program, the aim of this study was to detect P. vivax in asymptomatic treated patients to trace latent/sub-patient malaria infections. In this study both microscopic examining of blood films and nested-PCR method were used.
Many studies have recently shown the importance of asymptomatic malaria carriers as the parasite reservoir and the transition of the disease. In a study, conducted in 2009 on 38 individuals with a history of *vivax* malaria in a year before, no infection was detected among studied individuals by microscopic examination of thick and thin blood smears but one of the samples was identified as *P. vivax* infection by nested-PCR method (26). In an investigation on 500 symptomless individuals randomly selected in Hormozgan Province to evaluate the presence of *Plasmodium* infection using ELISA method, 1% of the studied population (5 of 500) was positive and had high IgG antibody titer, although no cases of infection with *Plasmodium* were found by parasitological and molecular methods (25). Among 120 volunteer patients in Chabahar, Iran, that were investigated by microscopic examination and PCR methods, 107 cases were positive for malaria by both methods and 13 out of 120 were negative by microscopy of which 9 cases reported positive using PCR. The result also showed 3 cases as having mix infection of *P. vivax* and *P. falciparum* by microscopy and 34 mix positive cases by nested PCR method (27).

The present study was performed in Hormozgan Province which is one of the main malaria endemic regions in Iran. To increase the accuracy and reliability of the results, microscopic and molecular techniques were simultaneously used to detect *Plasmodium* parasite among studied cases as asymptomatic carrier.

Microscopic technique was used as a gold standard and nested-PCR as a sensitive and specific method for asymptomatic malaria diagnosis which can detect low parasite densities (28). Detection of malaria species and correct treatment can reduce the number of malaria-infected individuals who carry the parasites and may thus reduce the risk of re-introducing of malaria into other parts of the country, outside the province, where an interruption of transmission had been earlier achieved. Nested PCR detection of malaria can be a helpful complement to microscopic test, as a gold standard method, to obtain the real prevalence of each species and also for the follow-up of the patients after specific treatment. (27). Zulma Milena Cucunubá et al., 2008, carried out a study on 212 symptomatic patients by microscopic examination of blood smears on day 0, 14 and 28 and nested PCR technique. Nested PCR detected 50% more infections than the thick smears examination (29). The PCR method has had also remarkable advantages in detection of mix infections of malaria. In Iran, Afghanistan and Pakistan, both nested PCR and microscopic examination applied for detection of mix infection of *P. vivax* and *P. falciparum*. The result showed 0%-2.5% mix infection by microscopy and 22%, 65% and 23% in Iran, Afghanistan and Pakistan, respectively, using nested PCR (30).

There are many reports on the use of molecular techniques for confirmation of diagnosis, epidemiological studies and drug efficacy assessment of malaria (2,31-33). Application of PCR methods for the detection of low density of malaria parasites and mixed infections has been demonstrated repeatedly (2). Whenever malaria elimination program is implemented it is critical to identify reservoirs of drug-resistant parasites and asymptomatic carriers. Furthermore, the relapsing cases caused by the activation of hypnozoites in the liver 2-3 years after the initial infection eventually could threaten the progress of the program. Each of these items can establish transmission of the disease and returning malaria outbreaks in population with relatively low level of acquired immunity (2).

We could detect two asymptomatic carriers in individuals with a history of *vivax* malaria treatment at least one year before participation in the study. This finding can have a significant impact on the adoption of best practices and removing of asymptomatic carriers of malaria parasites in elimination program of the country.

In a study conducted in Afghani immigrants without malaria symptoms and signs in south-eastern Iran by conventional light microscopy,
1.6% of subjects demonstrated *P. vivax* in their peripheral blood thick smear (34). Asymptomatic carriers could be due to reasons such as drug resistance, latent forms of the parasite or re-infection. Determination of each of these items requires detailed epidemiological and molecular follow-up of the cases in the time of onset of the disease. Unfortunately, due to the long time elapsed from the onset of the disease there was not any possibility for us to further follow up the cases. But, regardless of the causes, this phenomenon has a decisive role in the success of malaria elimination program in Iran and can be a potential risk for development of outbreaks of malaria. Molecular detection of malaria can be a helpful complementary test to microscopic examination for correct diagnosis of the disease and for the follow-up of patients after specific treatment (2).

**Conclusion**

It seems that detection of malaria reservoirs by molecular methods should be included in the list of major priorities of the malaria elimination program in Iran. Following up the malaria cases after treatment by a combination of methods, or new diagnostics such as RDTs (Rapid Diagnostic Tests), can be included in the priorities of malaria elimination program in Iran.

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**References**

1. Suárez-Mutis MC, Cuervo P, Leoratti FM, Moraes-Avila SL, Ferreira AW, Fernandes O, Coura JR. Cross sectional study reveals a high percentage of asymptomatic *Plasmodium vivax* infection in the Amazon Rio Negro area, Brazil. Rev Inst Med Trop Sao Paulo. 2007; 49(3):159-64.
2. Harris I, Sharrock WW, Bain LM, Gray KA, Bobogare A, Boaz I, Lilley K, Krause D, Vallely A, Johnson ML, Gatton ML, Shanks GD, Cheng Q. A large proportion of asymptomatic *Plasmodium* infections with low and sub microscopic parasite densities in the low transmission setting of Temotu Province, Solomon Islands: challenges for malaria diagnostics in anelmination setting. Malar J. 2010; 9:254.
3. Morgan, U. M. & Thompson, R. C. A. Molecular detection of parasitic protozoa. Parasitology. 1998;117: S73-S85.
4. Zaman J, Shahbazi A, Asgharzadeh M. *Plasmodium vivax* dfr mutations in isolates from malarious areas of Iran. Korean J Parasitol. 2011; 49(2):125-131.
5. Zeyrek YF, Kurcer MA, Zeyrek D, Simsek Z. Parasite density and serum cytokine levels in *Plasmodium vivax* malaria in Turkey. Parasite Immunol. 2006; 28:201–207.
6. Sundar S, Rai M. Laboratory diagnosis of visceral leishmaniasis. Clin Diagn Lab Immunol. 2002; 9:951–958.
7. Boonma P, Christensen PR, Suwanarusk R, Price RN, Russell B, Lek-Uthai U. Comparison of three molecular methods for the detection and speciation of *Plasmodium vivax* and *Plasmodium falciparum*. Malar J. 2007; 6:124.
8. Schneider P, Wolters I, Schoone G, Schallig H, Sillekens P, Hermens R, Sauerwein R. Real-time nucleic acid sequence-based amplification is more convenient than real-time PCR for quantification of *Plasmodium falciparum*. J Clin Microbiol. 2005; 43:402–405.
9. Veron V, Simon S, Carne B. Multiplex real-time PCR detection of *Plasmodium falciparum*, *P. vivax* and *P. malariae* in human blood samples. Exp Parasitol. 2009;121:346–351.
10. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, Thaithong S, Brown KN. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol. 1993;61:315–320.
versus conventional PCR for malaria parasite detection in low-grade parasitemia. Exp Parasitol. 2007;116:427–432.
12. McKenzie FE, Sirichaisinthop J, Miller RS, Gasser RA, Jr, Wongsrithanalai C. Dependence of malaria detection and species diagnosis by microscopy on parasite density. Am J Trop Med Hyg. 2003;69:372–376.
13. Safeukui I, Millar P, Boucher S, Melinard L, Fregeville F, Receveur MC, Pistone T, Fialon P, Vincendeau P, Fleury H, Malvy D. Evaluation of FRET real-time PCR assay for rapid detection and differentiation of *Plasmodium* species in returning travelers and migrants. Malar J. 2008;7:70.
14. Babiker HA, Schneider P, Reece SE: Gametocytes: insights gained during a decade of molecular monitoring. Trends Parasitol 2008, 24:525-530.
15. Farcas GA, Zhong KJ, Mazzulli T, Kain KC. Evaluation of the Real-Art Malaria LC real-time PCR assay for malaria diagnosis. J Clin Microbiol. 2004;42:636–638.
16. Andrews L, Andersen RF, Webster D, Dunachie S, Walther RM, Bejon P, Hunt-Cooke A, Bergson G, Sanderson F, Hill AV, Gilbert SC. Quantitative real-time polymerase chain reaction for malaria diagnosis and its use in malaria vaccine clinical trials. Am J Trop Med Hyg. 2005;73:191–198.
17. Elsayed S, Plewes K, Church D, Chow B, Zhang K. Use of molecular beacon probes for real-time PCR detection of *Plasmodium falciparum* and other *Plasmodium* species in peripheral blood specimens. J Clin Microbiol. 2006;44:622–624.
18. Vo TK, Bigot P, Gazin P, Sinou V, De Pina JJ, Huynh DC, Fumoux F, Parzy D. Evaluation of a real-time PCR assay for malaria diagnosis in patients from Vietnam and in returned travelers. Trans R Soc Trop Med Hyg. 2007;101:422–428.
19. Tada MS, Marques RP, Mesquita E, Dalla Martha RC, Rodrigues JA, Costa JD, et al. Urban malaria in the Brazilian Western Amazon Region I: high prevalence of asymptomatic carriers in an urban riverside district is associated with a high level of clinical malaria. *Mem Inst Oswaldo Cruz* 2007;102(3):263-269.
20. Alves FP, Gil lh, Marrelli MT, Ribolla PE, Camargo EP, Da Silva LH. Asymptomatic carriers of *Plasmodium* spp. As infection source for malaria vector mosquitoes in the Brazilian Amazon. J Med Entomol. 2005;42(5):777–779.
21. Haghdoost AA, Mazhari S, Bahadini K. Estimating the relapse risk of *Plasmodium vivax* in Iran under national chemotherapy scheme using a novel method. J Vector Borne Dis. 2006;43(4):168-172.
22. Diseases Management Center of MOH, I.R. Iran. Annual Reports of Malaria. 2006.
23. Roper C, Elhassan IM, Hviid I, Giha H, Richardson W, Babiker H, Satti GM, Theander TG, Arnott DE. Detection of very low level *Plasmodium falciparum* infections using the nested polymerase chain reaction and a reassessment of the epidemiology of unstable malaria in Sudan. Am J Trop Med Hyg. 1996;54:325-331.
24. Alves FP, Durlacher RR, MenezesMJ, Krieger H, Silva LH, Camargo EP. High prevalence of asymptomatic *Plasmodium vivax* and *Plasmodium falciparum* infections in native Amazonian populations. Am J Trop Med Hyg. 2002;66(6):641-8.
25. Turki H, Zoghi S, Mehrizi AA, Zakeri S, Raesi A, Khazan H, Haghdoost AA. Absence of asymptomatic malaria infection in endemic area of Bashagard District, Hormozgan Province, Iran Iranian J Parasitol: Vol. 7, No.1, 2012; pp.36-44.
26. Shahbazi A, Raesi A, Asmar M, Naddaf S, Nateghpour M, Anaraki G. Detection of *Plasmodium vivax* by Nested PCR in malariaous areas of western north of Iran. Bi-monthly Journal of Hormozgan University of Medical Sciences 3. 2009; 13 (1) :7-12 (In Persian).
27. Zakeri S, Najafabadi TS, Zare A and Dinparast N D. Detection of malaria parasites by nested PCR in south-eastern, Iran: Evidence of highly mixed infections in Chahbahar district. Malaria J. 2002;1:2.
28. Coleman RE, Sattabongkot J, Promtheparmor, Manechai N, Tippayachai B, KengleechaA, Rachapaew N, Zollner G, Miller RS, Vaughan JA, Thimasam K, Khuntrir B. Comparison of PCR and microscopy for the detection of asymptomatic malaria in a *Plasmodium falciparum* endemic area in Thailand. Malar J. 2006; 5: 121.
29. Zulma Milena Cucunubá, Ángela Patricia Guerra, Sonia Judith Rahirant, Jorge Alonso Rivera, Liliana Jazmín Cortés, Rubén Santia-go Nicholls. Asymptomatic Plasmodium spp. infection in Tierralta, Colombia. Mem Ínst Oswaldo Cruz, Rio de Janeiro. November 2008; 103(7): 668-673.

30. Zakeri S, Kakar Q, Ghasemi F, Raeisi A, Butt W, Safi N, Afsharpad M, Memon S M, Gholizadeh S, Salehi M, Atta H, Zamanin Gh. Dinparast DN. Detection of mixed Plasmodium falciparum & P. vivax infections by nested-PCR in Pakistan, Iran & Afghanistan. Indian J Med Res. 2010;132: 31-35.

31. Shahbazi A, Racisi A, Nateghpour M, Mirhendi H, Mohebali M, Asmar M. Polymorphism of merozoite surface protein-3α gene of Plasmodium vivax in isolates of Iran. Iranian J Parasitol. 2008;3(2):15-20.

32. Shahbazi A, Racisi A, Nateghpour M, Mohebali M, Asmar M, Mirhendi H. Partial Sequence Analysis of Merozoite Surface Protein-3α Gene in Plasmodium vivax isolates from malarious areas of Iran. Iranian J Parasitol. 2008;3(4):1-8.

33. Shahbazi A, Mirhendi H, Racisi A. Plasmodium vivax MSP-3B gene as a genetic marker for the parasite detection in comparison with ssrRNA gene. Iranian J Publ Health. 2010; 39(2):105-109.

34. Nateghpour M, Akbarzadeh K, Farivar L, Amiri A. Detection of asymptomatic malaria infection among the Afghani immigrant population in Iranshahr district of southeastern Iran. Bull Soc Pathol Exot. 2011 Oct;104(4):321-3.