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Original Article

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-

jor health problems especially in the south and southeast parts including Sistan and Baluchistan, Hormozgan and Kerman Provinces (2). Four species of *Plasmodium* are responsible for causing human malaria: *P. falciparum, P. vivax, P. malariae*, and *P. ovale* (3).

Plasmodium vivax is the most widespread species of *Plasmodium* in humans and causing about 80 million clinical cases annually. It remains the most widely distributed malaria parasite outside of Africa (4). *vivax* infections are rarely mortal but it can causes very debilitating illness (5). Its clinical characteristics are not enough for an accurate diagnosis, since they are shared by other occurring infections, such as typhoid, visceral leishmaniasis and tuberculosis (6). The laboratory diagnosis of *P. vivax* infections usually depends on the microscopic examination of Giemsa-stained thick and thin blood smears, immunochromatographic tests, and serological methods (7-9). These methods provide a cost effective, rapid diagnostic tool which can easily be used in the field (10). Nonetheless, these methods are subject to misdiagnosis particularly in the cases of mixed cases and low level parasitemia (7, 11-13).

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:

fPLU:5'-CTT GTT GTT GCC TTA AAC TTC-3
rPLU: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.viax* (120bp):
fVIV:5'-CGC TTC TAG CTT AAT CCA CAT AAA TGA TAC-3

rVIV:5'-ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3

The thermo cycling condition were initial denaturaation 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).

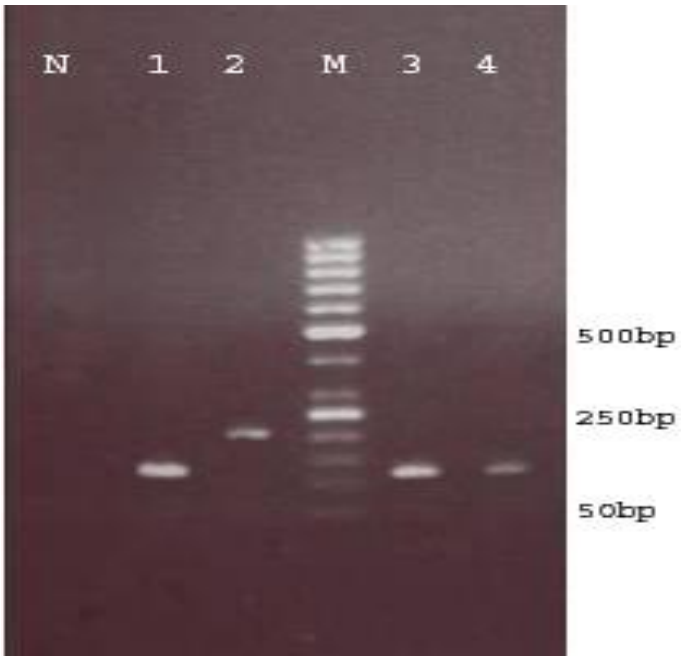

Fig.1: Agarose-gel electrophoresis of the nested PCR products of blood samples from Bashagard, Minab and Roodan districts. N: Negative control, Lane 1: *P. vivax* positive control, Lane 2: *P. falciparum* positive control, M: 50bp DNA marker ladder, Lane 3: *P. vivax* positive case from Bashagard, Lane 4: *P. vivax* positive case from Minab

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-patent 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). Wherever 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 southeastern 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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