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## The Risk of Re-Emergence of *Plasmodium malariae* in South-East of Iran as Detected by Nested Polymerase Chain Reaction\*

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**Abstract:** For the exact detection of parasite species, nested polymerase chain reaction based on small subunit ribosomal RNA gene was conducted, using 140 blood samples from symptomatic malaria cases. Microscopic examination of blood smears detected *P. vivax*, *P. falciparum* and a number of mixed infections with these two species and no detection of *P. malariae*. With the molecular based identification technique, in addition to diagnosis of a higher number of mixed infections, two cases of *P. malariae* was also identified after 22 years. These results point to the need of the development or utilization of a more accurate diagnostic method to distinguish between *P. vivax* and *P. malariae*. It is concluded that *P. malariae* seems to be re-emerged in South-East of Iran and its co-existence with the other two plasmodium species, *P. vivax* and *P. falciparum*, needs consideration during human malaria diagnosis to avoid ignorance and misdiagnosis of this parasite species. This is particularly important in view of the fact that the choice of drug for the anti-malarial therapy depends on the parasite species.

**Key words:** *Plasmodium malariae*, nested PCR, diagnosis

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### INTRODUCTION

In 2005 more than three billion people were at risk of malaria infection (Guerra *et al.*, 2006). In addition to *Plasmodium falciparum* there are four other malaria species known to infect humans; *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi* (Vythilingam *et al.*, 2006). Previous studies about malaria endemic areas in Iran showed that Sistan va Baluchestan province, located in the South-East of the country, is considered as the most prevalent province for human malaria, containing 42-60% of total malaria cases in Iran (Sadrizadeh, 2001). It should be noted that a relatively high rate of mix infections with *Plasmodium vivax* and *P. falciparum* has been reported by Zakeri *et al.* (2002) and Ebrahimzadeh *et al.* (2006, 2007). A single case of *P. malariae* has not been reported in this region for a long time, since 1983; a number of 233 cases were detected in blood donors by either microscopy or fluorescent antibody test during a period of 10 years between 1973 to 1983 (Edrissian, 1985). It was not known whether this species has been really disappeared in this endemic area after 1983 or has been possibly misdiagnosed due to the inaccurate routine diagnostic methods.

The gold standard method for malaria diagnosis is known to be the microscopic examination of thin and thick blood smears stained with Giemsa (Anonymous, 2000; Boonma *et al.*, 2007). Although this method is highly sensitive, the hemolysis of erythrocytes during the preparation of slides impairs the correct identification of malaria parasites. The non-assessed morphology of infected red blood cells and altered shapes of parasite interfere with the recognition of the species, often leading to

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misdiagnosis between *P. vivax* and *P. malariae* and underestimating the latter species. Lately, alternative diagnostic methods based on parasite DNA detection, such as Polymerase Chain Reaction (PCR), have been used for identification of malaria species (Boonma *et al.*, 2007). Detection of possible infections with *P. malariae* in the study area, where this species was considerably reported in the past, using two methods of direct smear microscopy and ssrRNA nested PCR, was of the principle aims in the present study.

## MATERIALS AND METHODS

This survey was carried out on the population seeking care at the malaria health centers of Sistan va Baluchestan province on October-May 2004-2005, covering two different peaks of malaria. The research proposal was approved by the ethics committee at Zahedan University of Medical Sciences. They included 140 patients referred to health centers all over the province.

In this survey, Giemsa-stained slides, microscopic method and nested PCR assay based on the sequence of the Plasmodium small subunit ribosomal genes (ssrRNA) were used. This nested PCR technique has been described enable to detect all four human *Plasmodium* spp. (Snounou *et al.*, 1993). Fingerprick blood samples were collected and thin-thick blood smears were prepared for microscopic observation. The blood films were dried and stained according to the standard protocols (Wilcox, 1960). These blood films were scanned for about 5 min (~100 microscopic fields with x1000 magnification) before considering the sample as negative.

A blood sample was collected by venous sampling from each patient to use in PCR assay by informed consent before treatment. DNA was extracted using the method of Foley *et al.* (1992) and nested PCR amplification was done according to Snounou *et al.* (1993), using Biometra Thermocycler (England). In an initial amplification reaction, the oligonucleotide primer pair, rPLU5 and rPLU6 were used (Snounou *et al.*, 1993; Ebrahimzadeh *et al.*, 2007). The size of DNA target, amplified by these outer primers, is about 1200 bp. The second reaction was performed for the specific detection of all human *Plasmodium* spp. using four primer pairs, each specific to one species (Snounou *et al.*, 1993; Ebrahimzadeh *et al.*, 2007). For *P. malariae* specific detection, the forward and reverse oligonucleotide primers (generating 144 bp fragment), is as follows: PmF, 5'- ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC -3'; PmR, 5'- AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA -3'. Positive control samples were also used; they were provided from Pasture Institute, Paris, France. A negative control reaction was also established. Electrophoresis of the PCR products was performed using 2% agarose gel followed by ethidium bromide staining and visualizing under UV light.

## RESULTS AND DISCUSSION

One hundred and forty cases of symptomatically considered malaria studied here, consisted of 92 (65.7%) males and 48 (34.3%) females, aged between 4 to 55 years old. The results of microscopy demonstrated 85 cases (60.7%) as *P. vivax* infection, 29 cases (20.7%) *P. falciparum*, 4 cases (2.9%) mixed infections (*P. vivax*+*P. falciparum*) and 22 cases (15.7%) as negative (Table 1).

Table 1: The results of microscopic and PCR methods in the study area

PCR/microscopic results	Pv	Pf	Mixed-infection Pf+Pv	Negatives	Total results of Microscopic method
Pv	2+59*	0	24	0	85
Pf	2	16	11	0	29
Mixed infection					
Pf+Pv	0	0	4	0	4
Negatives	11	3	2	6	22
Total results of PCR method	2+72*	19	41	6	140

\*Two cases of *P. malariae* have been detected by PCR which initially demonstrated *P. vivax* by microscopic method



Fig. 1: Agarose gel electrophoresis of nested PCR products of malaria ssrRNA. Lane 1, 100 bp ladder marker; lane 2, positive control; lanes 3-4, negative control and lanes 5-6, *Plasmodium malariae* clinical isolates

The PCR analysis for detection of the malaria parasite spp, identified 2 cases of *P. malariae* parasite (Fig. 1). They were identified in blood samples belonging to two Iranian settlers in Iranshahr district which were initially diagnosed as *P. vivax* by microscopy. The PCR technique found *P. vivax*, *P. falciparum*, mixed infections (*P. vivax* + *P. falciparum*) and negative, in 72 cases (51.4%), 19 cases (13.6%), 41 cases (29.3%) and 6 cases (4.28%), respectively (Table 1).

The results of the present survey confirms further the higher sensitivity and accuracy of the PCR method for Plasmodium detection (Padley *et al.*, 2003; Hunag *et al.*, 2005) comparing to the microscopic examination that is believed to be considered as a gold standard method (Anonymous, 2000; Moody, 2002; Hanscheid, 2003; Coleman *et al.*, 2006). Using the ssrRNA PCR in this study, we could detect and identify two cases of *P. malariae* after a period of two decades.

Microscopic analysis is currently used for identification of *Plasmodium* spp. This technique is appropriate for routine clinical diagnosis (Anonymous, 2000). However, its sensitivity is limited and its diagnostic accuracy depends greatly on the expertise of microscopists in the field. Further, information based on microscopy is restricted, particularly at low levels of parasitaemia and in cases of mixed infections. The detection limit of malaria parasites by microscopy is 20-30 parasites  $\mu\text{L}^{-1}$  of blood (Gilles, 1993), but often, due to the pressure of specimen load, inadequate staining, poorly maintained microscopes, even this limit may be decreased in both quality and quantity.

The accurate detection and the correct identification of malaria parasites are crucial, since the drug of choice for a successful treatment of malaria depends on the Plasmodium species. Moreover, the correct identification of parasites is of fundamental importance to develop adequate strategies for malaria control in endemic areas. The PCR method for detection and identification of the species of Plasmodium proved to be far more sensitive and accurate in the malaria diagnosis particularly when more than one species are infecting the same individual. However, factors that limit the PCR use as routine such as high cost, the need of adequate equipments and trained technical personnel may be considered, particularly in developing countries where malaria is a major health problem (Hanscheid and Grobusch, 2002). Furthermore, in the endemic areas, which lack the above conditions, the PCR do not provide a rapid diagnosis, which is essential for the management of clinical cases in endemic areas. A number of studies showed that PCR is more reliable than the microscopy in detection of malaria in low parasitaemic areas (Roper *et al.*, 1996; Mens *et al.*, 2007). The nested PCR utilized in the present study, also was more sensitive than the traditional microscopy of blood films for diagnosis

of malaria infections. This method is reproducible and has several other advantages over other diagnostic methods. However, the PCR technique requires equipments that are not easily available in the field.

In this study, there was a double slide reading by two different microscopists over the subjected blood smears. However, no case of *P. malariae* was detected by microscopy, whereas two cases of this species were detected and identified by PCR (Fig. 1, Table 1). They were initially demonstrated by microscopy as *P. vivax*.

This is the first documented molecular data for detection of *P. malariae* species in the field study in South-East of Iran. A similar study based on molecular technique that was performed previously (Zakeri *et al.*, 2002), reported single infections and highly mixed infections of *P. vivax* and *P. falciparum* in a part of study area, Chahbahar district, however, no *P. malariae* was found. During 1973 to 1983, a number of 233 cases of *P. malariae* were detected from blood donors in Iran, by light microscopy and direct immunofluorescent test (Edrissian, 1985). But, there was not a documented report of *P. malariae* in Iran since 1983.

A consequent repeated microscopic examination of newly prepared slides from the two blood smears confirmed the presence of *P. malariae*-specific morphological stages. This indicates further the misdiagnosis of *P. malariae* relying on microscopy only, as the none-specific blood stages of parasite may cause confusion in differentiation between *P. malariae* and *P. vivax*. This can be the source of underestimation of the number of *P. malariae* cases in the endemic areas. Comparison between two methods confirmed the value of PCR as a tool for obtaining reliable data on malaria infection; it proved especially useful in the identification of single-species *P. malariae* infection.

It is not known whether the appearance of *P. malariae* after about two decades has been due to the vector transmission in the region or due to the recrudescence originated from hidden old infections.

Infection caused by *P. malariae* can be easily treated with chloroquine alone, except possibly in Indonesia, where resistant strains have recently been reported (Maguire, 2002). Chloroquine resistance against *P. falciparum* in the study area has been reported (Edrissian *et al.*, 1999); however, this drug is still used as the first choice for treatment of *P. vivax* alone as well as *P. falciparum* malaria accompanying with fansidar and primaquine. As there is no evidence of drug resistant *P. malariae* in this region, the regimen of chloroquine therapy is probably suitable for the cases of *P. malariae* infections.

It is concluded that *P. malariae* seems to be re-emerged in South-East of Iran and its co-existence with the other two plasmodium species, *P. vivax* and *P. falciparum*, needs consideration during human malaria diagnosis to avoid ignorance and misdiagnosis of this parasite species.

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