Malaria Journal

Research

Detection of malaria parasites by nested PCR in south-eastern, Iran: Evidence of highly mixed infections in Chahbahar district

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Published: 8 February 2002

Malaria Journal 2002, **1**:2

Received: 3 February 2002
Accepted: 8 February 2002

This article is available from: http://www.malariajournal.com/content/1/1/2

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Keywords: Malaria, P. falciparum , P. vivax , diagnosis, mixed infection, Polymerase Chain Reaction (PCR)

Abstract

Background: Rapid diagnosis and correct treatment of cases are the main objectives of control programs in malaria-endemic areas.

Methods and results: To evaluate these criteria and in a comparative study, blood specimens were collected from 120 volunteers seeking care at the Malaria Health Center in Chahbahar district. One hundred and seven out of 120 Giemsa-stained slides were positive for malaria parasites by microscopy. Eighty-four (70%) and 20 (16.7%) were identified as having only *Plasmodium vivax* and *Plasmodium falciparum* infections, respectively, while only 3 (2.5%) were interpreted as having mixed *P. vivax-P. falciparum* infections.

The target DNA sequence of the 18S small sub-unit ribosomal RNA (ssrRNA) gene was amplified by Polymerase Chain Reaction (PCR) and used for the diagnosis of malaria in south-eastern Iran. One hundred twenty blood samples were submitted and the results were compared to those of routine microscopy. The sensitivity of PCR for detection of *P. vivax* and *P. falciparum* malaria was higher than that of microscopy: nested PCR detected 31 more mixed infections than microscopy and parasite positive reactions in 9 out of the 13 microscopically negative samples. The results also confirmed the presence of *P. vivax* and *P. falciparum*.

Conclusions: These results suggest that, in places where transmission of both *P. vivax* and *P. falciparum* occurs, nested PCR detection of malaria parasites can be a very useful complement to microscopical diagnosis.

Background

More than half of the world's population in approximately 100 countries is exposed to malaria. Iran is situated in the Eastern Mediterranean region, where about 45% of the population live with the risk of both falciparum and vivax malaria. Countries of this region are situated in ei-

ther Afrotropical (such as Somalia, Sudan), Oriental (such as Pakistan, south-eastern Iran, part of Afghanistan) or Palearctic (such as Turkmenistan, Uzbekistan, Tajikistan) eco-epidemiological zones regarding malaria [1].

The malaria endemic areas of Iran are located in the south-eastern part of the country, bordered in the south by the Persian Gulf and the Gulf of Oman, and to the east by Afghanistan and Pakistan. The south-eastern corner of Iran consists of the Sistan and Bluchistan province, the Hormozgan province and the tropical part of Kerman province with a combined population of approximately 3 million and is considered to be "refractory malaria region". Annual Parasite Incidence (API) was reported to be 8.74 per 1,000 population [1]. In this part of the country, malaria is of the oriental type, hence more difficult to control than elsewhere in Iran. Inherent problems are the drug resistance of *P. falciparum*[2,3] and the vector resistance to insecticides [4], complicated by an increasing rate of imported malaria, mostly *P. falciparum*, from Afghanistan and, to a lesser extent, from Pakistan. Parasite and insecticide resistance, aggravated by war and socio-economic factors have limited the possibilities for disease control. A large proportion of the malaria cases diagnosed in the south-eastern part of Iran, and an increased risk of local transmission has been observed in these regions.

Drug selection for the treatment of malaria depends on species of malaria present. Delayed or missed diagnosis of falciparum malaria increases the risk of complicated or severe disease, which may be fatal especially in non-immunes. *P. falciparum* from much of the world, including Iran, is largely chloroquine resistant and thus would not be eradicated by the standard treatment used for *P. vivax*. When parasite levels are very low, the information obtained by microscopy is limited, and in some cases biased, by the inability to devote the necessary amount of time to the examination of blood smears.

Although malaria is one of the major public health problems in Iran, with more than 32,616 cases in 1998 [5], no study has been done to date using the polymerase chain reaction (PCR). As part of a project aimed at improving the diagnosis and control of malaria in Iran, we evaluated a nested polymerase chain reaction (PCR) assay for the detection of malaria parasite species in blood samples obtained from individuals in Chahbahar district of Sistan and Bluchistan province.

Materials and methods

Study area

The malaria endemic areas of Iran are located in the south-eastern part of the country. These areas include the provinces of Sistan and Bluchistan, Hormozgan and Kerman. The Weather in this part of Iran is generally warm and humid. These areas incorporated less than 5% of Iran's total population, but contain more than 85% of the total incidence of malaria cases in the whole country with *P. vivax* and *P. falciparum* are both present. The Sistan and Bluchistan province is the most important area with more than 60% of all cases. The study was conducted in the Chahbahar district of Sistan and Bluchistan Province in south-eastern of Iran. In Chahbahar district, malaria transmission occurs year-round with two peaks in May-June and October-November. *P. vivax* is dominant during first peak; but *P. falciparum* is responsible for about 45% of cases and is the dominant species in second peak.

Subjects and blood sample collection

A total of 120 patients aged from 4 to over 50 years, who sought treatment at the Malaria Health Center in Chahbahar City Public Health Department in Sistan and Bluchistan province, were invited to participate in the study. All blood samples were collected during April-September of 2001. The patients were selected randomly from different ethnic and racial groups (Bluch, Fars, Afghan, Pakistani, etc.). Finger-prick blood samples were collected and thick and thin blood smears were prepared for microscopical observation.

Two millilitres blood samples for PCR assay were obtained by informed consent before treatment from patients with either slide-confirmed *P. falciparum* and *P. vivax* infection or from slide-negative patients who had been referred to the clinic because of fever and headache. A history of fever, symptoms, and drugs taken, if any, was recorded for each subject.

Examination of parasitaemia

Giemsa-stained thick blood films were routinely used for the detection of parasites without any quantitative estimation of parasitaemia. In this study, both thin and thick blood films were prepared and sent with blood samples to the Biotechnology Dept., Pasteur Institute of Iran, in order to determine the parasitaemia and to confirm the parasite species by PCR analysis. Thick and thin films were interpreted as negative only after examination with an oil immersion lens at × 1,000 magnification for at least 100 oil immersion fields by an expert microscopist. All the samples were air-dried, fixed in methanol and then stained for 15–30 minutes in Giemsa (BDH Ltd); a 1:10 diluted Giemsa (pH 7.2) was used. The stain was washed off with tap water and the smear was examined by × 1,000 magnification. The percentage of the parasitaemia was calculated from a total count of 1,000 red blood cells (RBCs) counted in a Giemsa stained thin blood film.

% Parasitaemia = Total no. of infected RBCs × 100/Total no. RBCs

Table 1: Comparison of nested PCR assay with Giemsa staining for detection of *Plasmodium* infection in Iranian malaria patients

	Microscopical diagnosis				
PCR diagnosis	*P. v*	*P. f*	*P. v* + *P. f*	Negative	Total
*P. v**	61	0	1	8	70 (58.3%)
*P. f***	0	12	0	0	12 (10%)
P. v + *P. f*	23	8	2	1	34 (28.4%)
Negative	0	0	0	4	4 (3.3%)
Total	84 (70%)	20 (16.7%)	3(2.5%)	13(10.8%)	120

P. v: P. vivax**P. f: P. falciparum*

DNA template preparation and Nested PCR assay

Extraction of parasite deoxyribonucleic acid (DNA) and PCR assay were carried out as described by Snounou *et al.*[6]. Briefly, blood samples were thawed and the parasite DNA was extracted after the lysed erythrocytes had been treated with proteinase K, using a phenol-chloroform mixture (1:1). DNA samples were processed by PCR to amplify species-specific sequences of the small sub-unit ribosomal ribonucleic acid (ssrRNA) genes of *P. vivax*, *P. falciparum*, and *P. malariae*[6]. Genomic DNA prepared from healthy individuals with no history of malaria, living in none-malarious areas of the country have been included as negative controls in all PCR diagnostic assays. The amplified products were resolved by 2.5% agarose gel electrophoresis for *P. vivax*, *P. falciparum* and *P. malariae* and stained with ethidium bromide for visual detection by ultraviolet transillumination.

In order to prevent cross-contamination, different sets of pipettes and different work areas were used for template preparation, preparation of master mix for PCR, addition of template to first and second 'nests' and PCR assays; one uninfected blood sample was included for every ten samples processed.

Results

90 (75%) males and 30 (25%) females participated in this study, aged between 4 to over 50 years old. 68% of cases were local inhabitants, 32% were immigrants from Afghanistan or Pakistan.

One hundred and twenty suspected malaria patients were examined by thick blood film analysis by an experienced technician in field station. By light microscopy, 84 (70%) were identified as having only *P. vivax* and 20 (16.7%) as only *P. falciparum* infections, while 3 (2.5%) were interpreted as having mixed *P. vivax-P. falciparum* infections; 13 (10.8%) were slide-negative (Table 1). The thin films showed that parasitaemia ranged from 0.001% to 5.8%, with one case of *P. falciparum* with a parasitaemia over 17%.

PCR analysis for detection of the *Plasmodium* genus and species determination is also shown in Table 1. The percentages of *P. vivax* mono infection, *P. falciparum* mono infection, and mixed infections were 70 (58.3%), 12 (10%) and 34 (28.4%), respectively. Twenty-three of the 34 mixed infections diagnosed microscopically as *P. vivax* only and 8 out of 34 as *P. falciparum* only with three mixed infection. A typical gel is shown in Figure 1.

Eight out of 13 specimens microscopically diagnosed as negative were positive as *P. vivax* with nested-PCR. One of the samples, which had been initially misdiagnosed as negative by light microscopy was also, diagnosed as mixed infection by the PCR assay. All 9 microscopically diagnosed as negative samples, have been shown to be positive by PCR, and confirmed as positive by repeating extraction and the nested PCR assay. In order to ensure that the results are true and not due to technical errors such as cross contamination, assays were all carried out with concurrent human negative controls.

One of these patients had initially been diagnosed with *P. vivax* ten days before this study and treated with chloroquine and primaquine. At the time of blood samples collection, the technician diagnosed *P. falciparum* by thick blood film analysis. This patient may have had a mixed *P. vivax-P. falciparum* infection, where treatment only cured the *P. vivax* infection. Another patient presenting with a history of malaria symptoms for the past 14 days was diagnosed as negative by repeated examination of thick blood films, but proved to be positive for *P. falciparum* by PCR. Two other cases, initially diagnosed positive for *P. vivax* and *P. falciparum*, were still positive by microscopy and PCR 14 days after treatment with chloroquine and

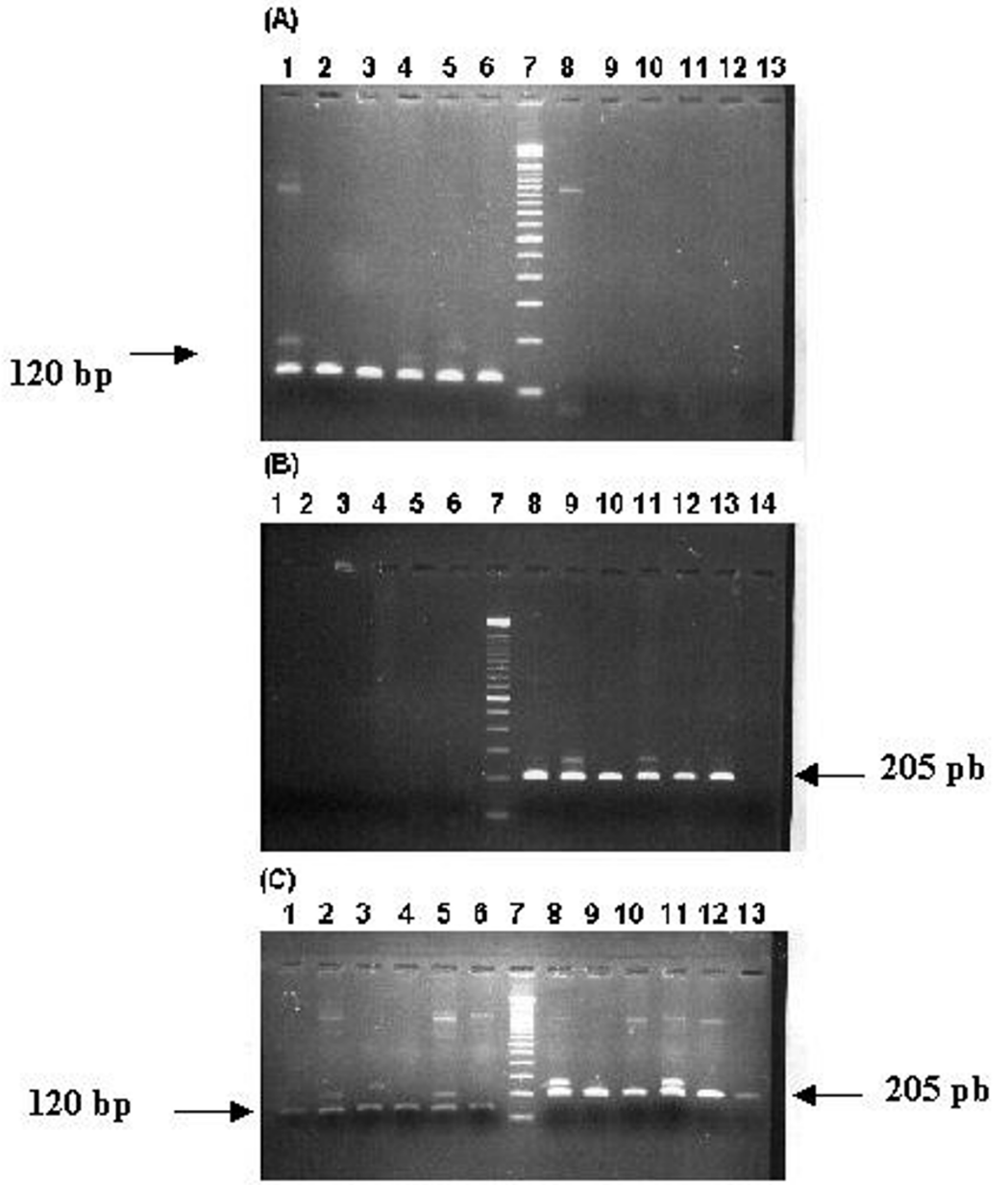

Figure 1
Schematic representation of agarose gel electrophoresis of nested PCR products from clinical specimens using species-specific oligonucleotide pairs for A (*P. vivax*), B (*P. falciparum*), C (mix *P. vivax* & *P. falciparum*). Marker is 100 bp ladder. **(A)** The representative microscopically *P. vivax* diagnosed samples, which were positive by using *P. vivax*-specific primers (left panel), and negative by *P. falciparum*-specific primers (right panel). **(B)** The representative microscopically *P. falciparum* diagnosed samples, which were positive by using *P. falciparum*-specific primers (right panel), and negative by *P. vivax*-specific primers (left panel).**(C)** Demonstrates the representative samples, diagnosed by species-specific primers as mix *P. falciparum* and *P. vivax,* and which have been diagnosed by microscopy as *P. vivax*.

primaquine; while these two cases might have been resistant strains of both species, re-infection cannot be excluded. With nested-PCR assay using malaria specific primers, no *P. malariae* was detected in any of the 120 samples tested.

Discussion

Chahbahar district has been selected as the study area for the following reasons:

1. Inhabitants are from different ethnic and racial groups (Bluch, Fars, Afghan, Pakistani and etc.), some with family members living 200–300 km away in the Bluchistan Province of Iran and the Bluchistan Province of Pakistan.

2. Movement of people from different parts of Iran, Pakistan, Afghanistan and the other side of the Oman sea to Chahbahar for holidays, job-hunting and financial activities may affect parasite populations and cause the re-introduction of malaria into areas, where an interruption of transmission had been achieved before (i.e. in the northern, central and western).

3. Presence of some *P. falciparum* cases with no response to chloroquine treatment in Chahbahar district.

To date the situation in south-eastern Iran is quite serious, because of the proximity to Afghanistan, a country with disrupted health systems.

Microscopy has historically been the mainstay of the diagnosis of malaria. A clinical diagnosis of malaria currently depends on the visualization of parasites by light microscopy of Giemsa-stained thick and thin blood smears. This procedure is cheap and simple, but it is a labour intensive procedure and requires well-trained personnel [7]. Many studies have demonstrated the greater sensitivity and specificity of PCR compared to thick blood films. The detection of low *P. vivax* and *P. falciparum* parasitaemia by PCR, at levels undetectable by microscopy, has been reported by Brown *et al.*[8], Sethabutr *et al.*[9], Snounou *et al.*[6], Wataya *et al.* [10], Khoo *et al.* [11], Black *et al.*[12], Roper *et al.*[13] and Singh *et al.*[14]. The present study between microscopy and nested PCR assay showed that the results obtained by PCR were equivalent or superior to those obtained by microscopy, in that all microscopy-positive samples were positive by PCR. In addition, the PCR test was able to detect mixed infections that were missed by microscopy. This may be due to the tendency to one species to be dominant over other species [15].

With the spread of parasite resistance to antimalarial drugs in Sistan and Bluchistan Province and the increasing difficulty in controlling malaria in these areas, it is important to diagnose malaria accurately and to treat it correctly. Microscopic observation of parasites stained with Giemsa in thick smears is an inexpensive and simple method that is still used in these areas with malaria transmission and where the diagnosis of malaria is part of primary health care. Several malaria infections from endemic countries are subpatent, with very low parasitaemia, and our results also showed this has occurred in our study area. The problem of population migration, together with the possibility of tourists and professionals travelling to areas at risk for malaria, has increased the number of cases in areas in which malaria transmission was low or previously eradicated. In these cases, an accurate malaria diagnosis is very important so that a possible recrudescence after an incorrect treatment of infected individuals can be avoided.

Mixed infections with asexual blood forms of *P. falciparum* and *P. vivax* are well described but relatively uncommon compared to single species infections. Shute (1951) described the frequency of mixed infections as less than 1% among the hundreds of British troops he examined in southern Italy during 1943 [8]. Traditionally, *P. falciparum* has been thought to inhibit the parasitaemia of *P. vivax*[16]. In contrast, there are several lines of evidence suggesting that *P. vivax* may have a suppressive effect on *P. falciparum*. James [17], in his classic review of studies of induced malaria was impressed that *P. vivax* was the predominant species. In their studies of induced malaria, Boyd & Kitchen [18] often used small doses of quinine which has long been known to have a greater suppressive effect on *P. vivax* than on *P. falciparum*[19]. In three instances where *P. vivax* parasitaemia rose and no drug was administered, asexual *P. falciparum* parasitaemia fell to submicroscopic levels [18]. Drug selection for the treatment of malaria depends on species of malaria present. Delayed or missed diagnosis of falciparum malaria increases the risk of complicated or severe disease, which may be fatal, especially in non-immunes, and many isolates of *P. falciparum* are chloroquine resistant and thus would not be eradicated by the standard treatment for *P. vivax*. When parasite levels are very low and in the detection of mixed species infections, the information obtained by microscopy is restricted, and in some cases biased, by the inability to devote the necessary amount of time to the examination of blood smears. A missed diagnosis of *P. vivax* concurrent with *P. falciparum* is more problematic since these species could cause relapses, thereby compounding morbidity. Because of negative microscopical diagnosis untreated patients may be carriers of the malaria parasites in these particular areas. The number of patients who had travelled to Pakistan, Afghanistan and other parts of Sistan and Bluchistan Province were high, with the risk of introducing new isolates (including drug resistant parasites) from neighbouring countries.

Conclusions

1. The nested PCR using the 18S ssrRNA gene of *P. falciparum* described by Snounou *et al.*[6] is useful as an adjunct to conventional microscopy in selected cases for the diagnosis of low-level parasitaemia. It showed high sensitivity and it could be performed relatively quickly when many samples must be tested. Also, the PCR is not affected by the subjectivity of the observer. Therefore, it is an excellent tool for obtaining accurate epidemiological data.

2. The correct diagnosis of malaria parasite species and correct treatment can reduce the number of malaria-infected individuals who carry the parasites between populations and may thus reduce the risk of re-introducing of malaria into other parts of Iran, outside this province, where an interruption of transmission had been previously achieved.

3. The results of the nested PCR also showed that the number of malaria cases, the ratio of *P. vivax* to *P. falciparum* and the level of mixed infection compared to mono-infection were increased from April to September. This may be due to the prevalence and ability of vector species to simultaneously transmit the different parasite species.

4. These results also suggest that, in malaria endemic areas where transmission of both *P. falciparum* and *P. vivax* occurs, nested PCR detection of malaria parasites can be a very useful complement to microscopical examination in order to obtain the real incidence of each species and also for the follow-up of patients after specific treatment.

Acknowledgements

We thank the Malaria Division, CDC, Iran, for their cooperation. We are grateful for the hospitality and generous collaboration of Zahedan University of Medical Sciences (Dr. M.T. Tabatabai), staff in Public Health Department, Sistan and Bluchistan province (Mr. Shahbakhsh), Chahbahar district (Dr. Zand, Dr. Bayat and Mr. Gorgij) for their assistance in collecting blood samples from the field. We are particularly indebted to Dr. S. Zeinali for his constant support. This work was supported by Pasteur Institute of Iran.

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