

Molecular evidence of mixed *P. vivax* and *P. falciparum* infections in northern Islamic Republic of Iran

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البيانات الجزيئية على العدوى بالمتصورات النشيطة والمنجلية في شمال جمهورية إيران الإسلامية
صديقة ذكري، شادي ممقاني، زينب شاهسافاري، أحمد ريسي، شهنام أرشي، نفيد دينباراست جديدي

الخلاصة: تواجه المناطق الشمالية من جمهورية إيران الإسلامية عودة دخول الملاريا إليها. وتقارن هذه الدراسة الكشف الجزيئي لأنواع المتصورات بالدراسة المجهرية الأساسية. فعند استعمال الدراسة المجهرية للأفلام الشخينة فإن 100% من الحالات المدروسة في محافظتي بارس - عباد وأردبيل، والبالغ عددها 142 حالة قد أصيبت بعدوى بنوع وحيد هو المتصورة النشيطة. إلا أن التفاعل السلسلي للبوليمراز قد كشف العدوى المختلطة بنوعين معاً هما المتصورة النشيطة والمتصورة المنجلية في 7% من هذه الحالات. أما في محافظة مازندران فإن اثنين من الأفلام الدموية العشرين التي فحصت قد شخصا الإصابة المقتصرة على المتصورة المنجلية، فيما شخصت الإصابة المقتصرة على المتصورة النشيطة بالأفلام الثمانية عشرة المتبقية. كما أن التفاعل السلسلي للبوليمراز قد شخص 17 حالة على أنها مصابة بالمتصورات النشيطة، وحالتين على أنهما مصابتان بالمتصورات المنجلية، وحالة واحدة على أنها مصابة بالمتصورات المنجلية والنشيطة معاً، وذلك من بين الحالات العشرين مجتمعة. إن الكشف غير المتوقع للمتصورات المنجلية يحث على إجراء استقصاء عاجل ومعالجة فورية لحالات الملاريا في المنطقة.

ABSTRACT This study compared basic microscopy with molecular detection of *Plasmodium* species. According to thick-film microscopy, 100% of 142 malaria cases in Pars-Abad, Ardebil province, were infected with a single species, *P. vivax*. However, nested polymerase chain reaction (PCR) detected mixed species infections of both *P. vivax* and *P. falciparum* in 7.0%. In Mazandaran province, 2/20 blood films were diagnosed with only *P. falciparum* and 18/20 with only *P. vivax*. However, nested PCR detected 17/20, 2/20 and 1/20 with *P. vivax* only, *P. falciparum* only and mixed species respectively. The unexpected presence of *P. falciparum* urges prompt investigation and immediate treatment of malaria cases in this region.

Preuve moléculaire d'infection mixte par *Plasmodium vivax* et *P. falciparum* dans le nord de la République islamique d'Iran

RESUME Cette étude a comparé la microscopie de base avec la détection moléculaire des micro-organismes des espèces de *Plasmodium*. L'examen de goutte épaisse du sang a montré que 100 % des 142 cas de paludisme à Pars-Abad (province d'Ardebil) étaient infectés par une seule espèce, *P. vivax*. Toutefois, la PCR (amplification en chaîne par polymérase) nichée a permis de détecter une infection mixte par *P. vivax* et *P. falciparum* dans 7,0 % des cas. Dans la province de Mazandaran, on a trouvé *P. falciparum* seul dans 2 frottis sanguins sur 20 et *P. vivax* seul dans 18 frottis sur 20. Toutefois, la PCR nichée a permis de détecter *P. vivax* seul dans 17 frottis sur 20, *P. falciparum* seul dans 2 frottis sur 20 et les deux espèces dans 1 frottis sur 20. La présence inattendue de *P. falciparum* impose une investigation rapide et le traitement immédiat des cas de paludisme dans cette région.

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Introduction

Malaria is the world's worst health problem. Control measures are becoming less effective, and the threat of epidemic malaria is increasing in many tropical areas, mainly in relation to population movements and climatic changes. Population movements within countries or across international borders are linked to political instability and economic problems [1].

In the past, malaria was highly endemic in most parts of the Islamic Republic of Iran. In 1924, it was estimated that out of a population of 13 million, 4–5 million people had contracted malaria [2]. Since 1958, malaria infection was eradicated from most of the country; however, there is continued transmission in the south-east region and also small pockets of transmission in different parts of the country [3].

In the north and the Caspian Sea region, we are facing the re-emergence and reintroduction of malaria, especially in Ardebil and to a lesser extent in Gilan and Mazandaran provinces, through the former Soviet Union states. Here, malaria transmission was interrupted in all northern parts of Iran by 1977, but it reappeared in 1994 following large population displacements in the border areas of Azerbaijan in 1991 [4]. From Azerbaijan, malaria was also introduced to the north-west of the Islamic Republic of Iran, which was affected by a serious epidemic of *Plasmodium vivax* malaria. Malaria has been seen on the Iranian side of the border, in the most receptive areas along the lowland Aras River basin, which is at an altitude of 150 m. Along this particular valley, the villages on both sides of the border are within the flying range of *Anopheles* spp. The truck drivers who travel along the Aras valley to supply the Azerbaijan enclave of Nakhichevan may be carriers of the malaria parasite and facilitate

the reintroduction of the disease in the north-west of the country [2].

The main malaria vector in this area is the *An. maculipennis* species complex (especially *An. sacharovi*) and the problem is resistance of this vector to dichloro-diphenyl-trichloroethane (DDT) and dieldrin. Malaria control in this area is based on case detection and treatment together with selective indoor residual spraying [5].

The diagnosis of malaria still relies on identification of the malaria parasite by light microscopy of Giemsa-stained thick and thin blood smears. This procedure is cheap and simple, but it is labour-intensive and requires personnel who are well trained in the morphological differentiation of the *Plasmodium* spp. [6] in order for successful diagnosis and treatment. Rapid non-microscopic tests for the detection of *Plasmodium* spp. infection, based on molecular biological techniques, have been introduced to overcome problems associated with the low sensitivity in diagnosing malaria infections with a low level of parasitaemia by microscopy.

The aim of this study was to determine the prevalence of *Plasmodium* spp. in the northern part of the Islamic Republic of Iran and to carry out correct species identification using polymerase chain reaction (PCR) methods for the first time in these areas. We also aimed to assess the accuracy of basic microscopy and to compare PCR-based diagnosis with microscopy results, in order to determine the frequency of mixed malaria infections and, ultimately, to improve the diagnosis and control of malaria in this area of the country.

Methods

Population and study area

The main study was conducted in the city of Pars-Abad, Ardebil province, located in a

westward extension of the Mughan steppe, most of which lies within Azerbaijan. Malaria transmission is markedly seasonal, with cases peaking in June–September. According to the annual report of the Iranian Centre for Disease Management and Control (CDMC) in 2002, the annual parasite incidence (API) was reported to be 0.27 per 1000 population [unpublished data]. A total of 142 malaria patients aged from 1.5 to over 70 years attending the Malaria Diagnostic Laboratory in Pars-Abad were invited to participate in this study during June to October 2002.

A smaller study was conducted in Mazandaran province in the northern part of Iran near the Caspian Sea. Here, malaria transmission is seasonal with a peak in June–September and the API was reported to be 0.05 per 1000 population. Most cases are Afghan refugees, who are migrating to the north of the Islamic Republic of Iran. A total of 20 samples were collected from malaria patients aged 16–61 years from different parts of Mazandaran province during June–September 2002.

Data collection

On arrival of the patients at the clinic, thick and thin blood smears were made from finger-prick samples on separate slides. Before treatment, and after obtaining informed consent, 2 mL venous blood was collected by the clinic staff from patients with slide-confirmed Plasmodium infection. Samples were stored at -20°C and sent to the Malaria Research Group at the Pasteur Institute in Tehran for further analysis.

Microscopy tests

For malaria parasite detection by microscopy, thick and thin blood smears were fixed and stained with 10% Giemsa and examined under oil-immersion ($100\times$) for 100

fields by expert microscopists at the local malaria diagnostic laboratory in the study areas. To confirm the results, all smears were sent to the Pasteur Institute in Tehran and examined by staff at the biotechnology department of the Malaria Research Group.

Parasite species were identified using both thick and thin film preparations. The percentage of the parasitaemia was calculated from a total count of 1000 red blood cells (RBC) in a Giemsa-stained thin blood film [7]:

$$\% \text{ parasitaemia} = \frac{\text{total no. infected RBC}}{\text{total no. RBC}} \times 100$$

PCR analysis

Extraction of parasite deoxyribonucleic acid (DNA) was carried out as described by Snounou et al. [8]. Briefly, 500 μL of blood samples were thawed on ice before being mixed with cold phosphate-buffered saline. The parasite DNA was extracted after the lysed erythrocytes had been treated with saponin followed by proteinase K, using a phenol:chloroform mixture. The positive control DNAs were as follows: *P. falciparum*-infected blood was obtained from *in vitro* cultured parasites routinely maintained at the Pasteur Institute of Iran. DNA samples of *P. vivax* were obtained from patients in the southern part of Iran [7] and *P. malariae* was kindly provided by the Pasteur Institute of France.

DNA samples were processed by PCR to amplify species-specific sequences of the small subunit ribosomal ribonucleic acid (ssrRNA) genes of *P. vivax*, *P. falciparum*, and *P. malariae* using primers and cycling parameters as described previously [8].

Two negative controls were included in each set of amplification reactions: 1 with no DNA and 1 from genomic DNA prepared from healthy individuals with no his-

tory of malaria who were living in non-malarious areas of the country. 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; 1 uninfected blood sample was included for every 10 samples processed.

Results

Of the 142 blood samples from malaria patients in Pars-Abad, Ardebil province, 77 (54.2%) were from females and 65 (45.8%) males; their ages ranged from 1.5 to 70 years old. The highest proportion was in the age group 11–20 years. All cases were local inhabitants of Iranian nationality.

Using light microscopy of thick blood films by local technicians, all 142 (100%)

Giemsa-stained slides were identified as having only *P. vivax* infection. No *P. falciparum* and mixed infections were diagnosed (Table 1). The thin films showed that parasitaemia ranged from 0.01% to 4.70%, with a mean of 0.74%. The results of the PCR assays (Table 1) were as follows. *Plasmodium* spp. were detected in all (100%) collected blood samples: 132 (93.0%) infections were due to *P. vivax* only and 10 (7.0%) due to mixed *P. vivax* and *P. falciparum* infections. No *P. falciparum* alone or *P. malariae* infections were detected.

Of the 20 malaria patients in Mazandran province, 2 (10%) were females and 18 (90%) males. Twelve (12) patients out of 20 were immigrants from Afghanistan, who came to the province for work.

In the microscopy analysis of thick blood films, 18 samples out of 20 were diagnosed as only *P. vivax* infection; however, 2 were *P. falciparum* alone. No mixed infection was diagnosed (Table 2). The results of the PCR assays showed 17 (85.0%) were due to *P. vivax* infection alone and 2 (10.0%) to *P. falciparum* alone (Table 2). One sample showed both *P.*

Table 1 Comparison of nested polymerase chain reaction (PCR) assay with Giemsa staining for detection of *Plasmodium* spp. infection in malaria patients from Pars-Abad, Ardebil province

PCR diagnosis	Microscopy diagnosis			Total No.
	<i>P. vivax</i> No.	<i>P. falciparum</i> No.	<i>P. vivax + falciparum</i> No.	
<i>P. vivax</i>	132	0	0	132
<i>P. falciparum</i>	0	0	0	0
<i>P. vivax + falciparum</i>	10	0	0	10
Total	142	0	0	142

Table 2 Comparison of nested polymerase chain reaction (PCR) assay with Giemsa staining for detection of *Plasmodium* spp. infection in malaria patients from Mazanderan province

PCR diagnosis	Microscopy diagnosis			Total No.
	<i>P. vivax</i> No.	<i>P. falciparum</i> No.	<i>P. vivax + falciparum</i> No.	
<i>P. vivax</i>	17	0	0	17
<i>P. falciparum</i>	0	2	0	2
<i>P. vivax + falciparum</i>	1	0	0	1
Total	18	2	0	20

vivax and *P. falciparum* mixed infections, but this was not detected by routine microscopy.

Discussion

In the 1990s, central Asia experienced considerable problems in preventing and controlling malaria. The wars and political instability in Afghanistan and Iraq and the difficult economic and social conditions among the former republics of the Soviet Union have resulted in human migration, and consequently a reduction in the quality of health services. The shortage of essential equipment and supplies for malaria prevention and control has also weakened malaria prevention activities. Lack of knowledge and experience in malaria prevention and control among health service staff has been another obstacle in the planning and effective implementation of these measures [4].

At present, there are 3 areas in central Asia that cause concern for countries of the Eastern Mediterranean Region. Afghanistan is a source of importation of malaria

to all its neighbours. In particular, the oriental type of *P. falciparum* is widespread in some areas of the country. In areas on the borders between the Islamic Republic of Iran, Azerbaijan, and to some extent Armenia, malaria transmission was interrupted by 1977, but reappeared in 1994 following large displacements of the population in the border areas of Azerbaijan in 1991. The malariogenic potential on the Iranian side is quite high along the Aras River at altitudes below 150 m. North Iraq also has a problem of *P. vivax* malaria that sometimes leads to epidemics.

In the Islamic Republic of Iran, malaria is also affected by those 3 countries and the constantly increasing population movements have led to importation of malaria cases from neighbouring countries. In 1958, we started the national malaria eradication programme, which changed to malaria control in 1987 due to technical problems and administrative difficulties [3]. The main anti-malaria measures in the country are early detection and treatment of cases, and indoor residual spraying in foci of transmission.

The Islamic Republic of Iran has 3 epidemiological zones of malaria [2]. Zone 1 in the northern part of the Zagros mountain chain consists of 16 provinces inhabited by 43 million people. In 2002, there were 3060 cases in this region. The annual parasite incidence (API) was approximately 0.07 per 1000 people and 2882 (94%) of total cases were *P. vivax* malaria. Most of the provinces in this zone were clear of malaria, but after 1994, some new foci appeared in this region, particularly in the border area between the Islamic Republic of Iran and the Republic of Azerbaijan and Armenia. In Zone 2, are regions to the south of the Zagros range with a population of approximately 15 million. The API in 2002 was reported to be 0.11 per 1000 in total cases of 1726. More than 88% of the cases in this zone are imported, which led to the establishment of 2 new foci in Bushehr and Fars provinces during the year 2002. In this zone, the dominant parasite (89% of total cases) is also *P. vivax*. Zone 3 consists of 3 provinces, Hormozgan, Kerman and Sistan va Baluchistan, inhabited by approximately 3 million people. The API was 2 per 1000 in 2002. The total malaria cases in the whole country in 2002 was 15 558 with an API of 0.24 per 1000 population and about 70% (10 772) of cases were from zone 3. Also 85% of total reported *P. falciparum* cases were from zone 3.

P. falciparum infection has been eradicated from most areas of the Islamic Republic of Iran and the underlying reason for the continued transmission of this species in the south-east is probably due to the fact that *P. falciparum* belongs to the oriental ecological system. However, in the rest of the country, small pockets of transmission still exist [2]. The official records of malaria cases in Ardebil (north-west) and Mazandaran (north) provinces in 2002 showed 339 and 135 cases, respectively. All cases

were diagnosed as only *P. vivax* in Ardebil and 130 were diagnosed as only *P. vivax*, with 5 *P. falciparum*/mixed infection in Mazandaran provinces. No mixed infection was diagnosed by light microscopy methods in Ardebil.

In Pars-Abad, Ardebil, our study using nested PCR analysis found 7% of mixed *P. vivax/P. falciparum* infections among cases microscopically diagnosed as *P. vivax* only. Also 60% of Mazandaran's cases were Afghan immigrants who came from Afghanistan or the south-eastern part of the Islamic Republic of Iran. There is a risk, therefore, of introducing new isolates, including drug resistant parasites from south-east to north and this may stimulate outbreaks in this part of the country. Untreated patients with missed diagnosis of *P. vivax* and *P. falciparum* infections could be carriers of the malaria parasites within and between provinces and spread malaria to areas where malaria was interrupted before. Potentially, because of the presence of about 21 *Anopheles* species (5–6 in most provinces) in different zoogeographical areas, the Islamic Republic of Iran has a high risk for malaria transmission. Entomological and parasitological surveillance must be considered since the risk of returning malaria from in and/or outside neighbouring countries is very high.

Our results showed an unexpected presence of *P. falciparum* in the Palaearctic zoogeographic region, among the population in Ardebil province. Cases of *P. falciparum* infection outside of south-east Iran (Oriental zoogeographic region) should be given emergency status, prompt investigation and immediate treatment of malaria cases.

The presence of Afghan refugees in Mazandaran province should be considered in surveillance and monitoring of the malaria parasite, as they may harbour resistance

to *P. falciparum* parasites, which may originate from Afghanistan or south-eastern parts of the Islamic Republic of Iran and spread to the northern part of the country. A resurgence of transmission in areas where malaria was interrupted may then cause epidemics during which the entire population is vulnerable to malaria.

In this study, in addition to aiding diagnosis, we used a sensitive parasite detection system to understand the dynamics of parasite infection in the northern part of the Islamic Republic of Iran. The outcome of this study could also help to prevent spread of this disease, coordination of anti-disease activities and standardization of control methods between the 3 countries in border areas: the Islamic Republic of Iran, Azerbaijan and Armenia.

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