Detection of mixed *Plasmodium falciparum* & *P. vivax* infections by nested-PCR in Pakistan, Iran & Afghanistan

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**Background & objectives**: Species identification and information on transmission pattern of malaria parasite in any malaria endemic area is key to success for a malaria control programme. In this investigation, malaria diagnosis using molecular method was used to assess the transmission pattern of malaria parasite in three malaria endemic regions: Afghanistan, Iran and Pakistan.

**Methods**: Blood samples were collected from the patients presenting with vivax malaria from Afghanistan (n = 108), Iran (n = 200) and Pakistan (n = 199). Malaria parasite detection was made by the gold standard (microscopy) and also nested-PCR assay, using 18S small sub-unit ribosomal RNA (ssrRNA) gene.

**Results**: Based on microscopy method, the level of mixed infection was zero to 2.5 per cent; however, nested-PCR assay detected 6.5, 22 and 23.5 per cent mixed infections in samples collected from Afghanistan, Iran and Pakistan, respectively. The present results showed that the co-infection of *P. vivax* with *P. falciparum* was frequent in malaria endemic regions of Iran and Pakistan.

**Interpretation & conclusion**: The present data suggest the need for improving microscopy diagnosis method and the clinician should also have careful clinical observation, along with the reports on Giemsa-stained thick blood films, particularly in summer time when *P. vivax* is predominant. Also sharing information on transmission pattern of mixed infection among these countries may help in designing better control strategies for malaria.

**Key words** Afghanistan - diagnosis - Iran - mixed infections - Pakistan - *Plasmodium falciparum* - *Plasmodium vivax*

The objective of the global Roll Back Malaria initiative was to reduce the malaria burden by half in 2010 compared with 2000¹. Epidemiology of malaria varies considerably and depends on the biology of the vectors, the parasites and the human hosts and also socio-economic factors. On the other hand, the
main technical interventions for control of malaria used in a specific region are based on prompt and effective treatment of malaria cases, prevention of malaria by reducing exposure to infective mosquito bites, prevention of malaria through chemotherapeutic measures, and prevention and control of malaria epidemics. Prompt and accurate diagnosis of malaria is the important key for disease management and also reduction of unnecessary use of anti-malarial medicines. In addition, drug selection for treatment of malaria depends on species of malaria parasite present in suspected patients.

The standard method for detecting Plasmodium infection is the microscopic examination of Giemsa-stained thick blood smear. Although effective and inexpensive, this method is laborious and time-consuming, and its sensitivity drops with the decrease of parasitaemia and mixed infections. Further, most microscopists on finding of one species might not look for a rare second species in the smear. However, molecular techniques have been able to detect and identify malaria parasites in mixed and low level parasitaemia. Different studies conducted in Africa, South-East Asia, Venezuela and Iran have revealed high prevalence of mixed parasites infections by PCR assay, that could not be detected by blood thick smear indicating the usefulness of molecular techniques for decision making in disease control and treatment. We designed this investigation to re-assess malaria diagnosis using sensitive nested-PCR techniques and also measure the frequency of P. vivax and P. falciparum mixed infections in three malaria endemic countries in the Middle East: Afghanistan, Iran and Pakistan.

Material & Methods

Study sites: Samples were collected from patients infected with P. vivax mono-infection, reporting to the health facilities located in Herat province in Afghanistan, in the Northwest of Afghanistan on border with Iran and Turkmenistan. Risk of malaria transmission in Herat is moderate to high. In 2007, 5 and 412 confirmed cases with P. falciparum and P. vivax were reported from public health facilities, respectively.

In Iran, samples were collected on admission from patients diagnosed with vivax malaria in southeast malaria endemic regions. In these regions, transmission is year-round with two peaks, the first from May to August with P. vivax as the predominant species and the second from October to November when both P. falciparum and P. vivax infections are recorded. In this study, Chabahar district in Baluchistan province with continuous introduction of immigrants was selected. In 2007, approximately 16,489 malaria cases were reported in Iran, 90 per cent of whom were microscopically diagnosed as P. vivax, and remaining as P. falciparum. Most of the cases (94%) were recorded in the southeastern provinces (the Ministry of Health, unpublished data).

In Pakistan, samples were collected on admission from patients diagnosed with vivax malaria in the health facility (Tehsil Head Quarter Hospital Saddah) located in Kurram Agency in Federally Administered Tehsil Areas (FATA) of Pakistan. Transmission in the area is seasonal with P. vivax as the predominant species during July to August, while from October to November both P. falciparum and P. vivax infections are recorded. In 2007, approximately 23,234 cases were reported from FATA, mostly P. vivax (84%) and Kurram Agency reported 2,757 cases. In FATA, P. vivax is the predominant species, but P. falciparum is also on rise in recent years.

Sample collection: Patients of different age groups, presenting with the signs and symptoms of malaria who sought treatment at the health centers, were screened for P. vivax mono-infection, using thick and thin Giemsa stained films. Informed consent was taken from all the enrolled adult patients and the parents or guardians of the children. One ml of whole blood sample was obtained from consented patients aged from 4 to 70 yr during April to October 2008. The isolates were collected from P. vivax-infected patients from Afghanistan (n = 108), Iran (n = 200) and Pakistan (n = 199) during April to October 2008. This study was approved by the Ethical Review Committee of Research in Pasteur Institute of Iran and the Ministry of Health in Afghanistan and Pakistan.

Malaria parasite detection: Thick and thin blood smears were made from finger-prick samples, stained with 10 per cent Giemsa and examined under oil-immersion (100 ×) by expert microscopists at Malaria Clinic in each study areas. The percentage of the parasitaemia was calculated in the main laboratory as described previously.

Preparation of Plasmodium species DNA: Parasite genomic DNA was prepared by phenol/phenol-chloroform extraction and ethanol precipitation as described previously. The DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA) and kept at -20°C until use.
Detection of parasites by nested-PCR assay: Plasmodium species DNA (P. vivax, P. falciparum and P. malariae) were detected by nested-PCR amplification of the small sub-unit ribosomal ribonucleic acid (ssrRNA) genes using the primers and cycling parameters as described previously. This assay could detect the presence of one to ten parasites/µl of blood. The positive controls were genomic DNA prepared from an Iranian patient infected with P. vivax confirmed with microscopy with 3 per cent parasitaemia as well as DNA from the cultured strain K1 of P. falciparum and P. malariae species, respectively. The P. malariae genomic DNA used as positive control in this study was kindly provided by G. Snounou (Universite Pierre & Marie Curie, Paris, France). Two negative controls were included in each set of amplification reactions: first had no DNA and the second was from genomic DNA prepared from healthy individuals with no history of malaria, living in non malarious areas of the country. 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 and addition of template to first and second nests and PCR assays. The amplified products were resolved by 2-2.5 per cent agarose gel electrophoresis and stained with ethidium bromide for visual detection by ultraviolet transillumination.

Results

In samples collected from Heart, Afghanistan (n = 108), 62 per cent were males and 38 per cent females. The highest proportion of samples was in the age group of 1 to 10 yr (36%). All patients were Afghani nationals (Table). Only 1.8 per cent (n = 2) of Herati patients had travelled to Iran but not to Pakistan, 2 to 4 wk prior to attending the clinic (Table). By light microscopy, all samples collected from Herat were found to be infected with only P. vivax (100%) (Fig. A). Thin films showed that overall parasitaemia ranged from 0.01 to 4 per cent.

In Iranian samples (n = 200), 78 per cent (n = 156) were male and 22 per cent (n = 44) were female. Sixty nine per cent (n = 138), 20 per cent (n = 40) and 11 per cent (n = 22) of the patients recruited in southeast were Iranian, Pakistani and Afghani nationals, respectively. In addition, 1 per cent (n = 2) and 18 per cent (n = 36) of the patients had travelled to Afghanistan and Pakistan, respectively, 2 to 4 wk prior to attending the clinic (Table). Using light microscopy of thick blood films by local technicians, 95.5 per cent (n = 191) Giemsa-stained slides were identified as having only P. vivax, while 2 per cent (n = 4) were interpreted as having mixed P. vivax-P. falciparum infections, with 2.5 per cent (n = 5) negative results (Fig. A). Thin films showed that parasitaemia ranged from 0.001 to 5 per cent.

All patients recruited from Sadda region were Pakistani nationals with no history of travel to the neighbouring provinces or countries. Of these patients, 55.8 per cent (n = 111) were male and 44.2 per cent (n = 88) were females. The highest proportion of samples was in the age group of 1 to 10 yr (52%). By light microscopy, all 199 (100%) Giemsa-stained slides were identified as having only P. vivax infection, with no record of P. falciparum and/or mixed infections of both species (Fig.).

Results of PCR analysis: Based on nested-PCR results, 93.5 per cent (n = 101) of Herati samples showed P. vivax mono-infection and none of the samples had P. falciparum or P. malariae mono-infections. However, 6.5 per cent (n = 7) Herati samples were found to be infected with P. vivax-P. falciparum mixed infections (Fig. B). The frequency of mixed infections in examined Herati samples was increased from April to September (data not shown).

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F, Federally Administered Tehsil Areas (FATA) of Pakistan; H, Herat province in Afghanistan; S & B, Sistan and Baluchistan
In Iranian samples, PCR analysis showed 77.5 per cent (n=155) of cases harboured *P. vivax* mono-infection and 22 per cent (n=44) had *P. vivax*-*P. falciparum* mixed infections. The number of mixed infections was increased during April to September. Only one blood sample showed no infection with *Plasmodium* species. The 3 out of 5 samples microscopically diagnosed as negative were positive as *P. vivax* only, and one had mixed *P. vivax*-*P. falciparum* infections. *P. malariae* infection was not seen in any Iranian samples (Fig.).

In Afghan samples, PCR analysis showed 75.8 per cent (n=151) were infected only with *P. vivax*; however, 23.6 per cent (n=47) were infected with both *P. vivax*-*P. falciparum* infections (Fig. B). The number of mixed infections was increased during April to September. No *P. falciparum* mono-infection as well as *P. malariae* was diagnosed among all 199 Pakistani samples by both light microscopy and nested-PCR assay.

**Discussion**

The objectives of malaria control programmes are eliminating mortality, minimizing morbidity and reducing prevalence or eradication. To achieve these goals, all malarious countries share a common need for reliable laboratory diagnostic techniques to ensure early and rational treatment. However, transmission pattern of malaria parasite could not be the same in each endemic area, rendering the need for providing precise data on transmission pattern, applicable in designing a more efficient control programme.

In Afghanistan, Iran and Pakistan, *P. vivax* is the predominant species in spring and summer with very low prevalence of *P. falciparum*\textsuperscript{15-17}. Based on microscopic method, the level of mixed infection reported from Iranian malaria endemic areas was 0.77 per cent in 2007 (the Ministry of Health, unpublished data); however, there was no report on mixed infection from Afghanistan and Pakistan (the Ministry of Health, unpublished data). The result of the present study revealed that PCR assay detected 6.5, 22 and 23.5 per cent mixed infections in Afghani, Iranian and Pakistani studied samples, respectively. Presence of mixed infections in an individual person could be the cause of important alterations in the pathogenicity. Therefore, during the first peak, when *P. vivax* is prevalent in three countries, it could have a suppressive effect on *P. falciparum*. In addition, the results of this investigation also provided some evidence on pattern of mixed infection, which could be used in designing preventive measures for accurate diagnosis of *Plasmodium* species and treatment of cases in these parts of the world. The possibility that malaria patients in our studied areas may have undetected mixed infections during first peak of transmission should be kept in mind because of the specific therapy required both for *P. falciparum* and for radical cure of *P. vivax*. This, in turn, will increase the number of individuals infected with *P. falciparum* malaria, which can carry the parasites among populations and may thus increase the risk of re-introducing this species into other parts of these countries and the world.

Interestingly, the level of mixed infections was increased from April to September in all studied areas of three countries, which was in concordance with our previous finding in malaria endemic regions in Iran\textsuperscript{12}. Currently, the roles of primary and secondary anopheles vectors in studied areas of three countries are not exactly defined and need further study. However, the presence of different *Anopheline* species in the study...
areas will support the complexity of multiplication and transmission of malaria parasites by different vectors; therefore, from control point of view, such data are highly needed.

In conclusion, the present data point to the need of improving microscopy diagnosis method in malaria endemic areas and also suggest that the clinicians should have careful clinical observation, along with the reports on Giemsa-stained thick blood films, particularly in summer time when *P. vivax* is predominant. Although molecular techniques are not practical in rural areas for diagnosis of *P. vivax* and *P. falciparum* mixed infections; these could be used to collect epidemiological facts for control of the disease in these regions. Also population movement among the three countries and also presence of similar parasite and mosquito vectors species and the transmission pattern of mixed infection suggest the establishment of a networking co-ordination for anti-malaria activities and standardization of control measures among these three countries, at least in border areas by sharing data for making decision on malaria elimination in their countries.

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References


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