Available online at www.sciencedirect.com
SCIENCE DIRECT
Acta Tropica 97 (2006) 174–180
ACTA TROPICA
www.elsevier.com/locate/actatropica

Plasmodium vivax dihydrofolate reductase point mutations from the Indian subcontinent

Suminder Kaur [a], Surendra K. Prajapati [b], Kavitha Kalyanaraman [a], Asif Mohmmed [a], Hema Joshi [b], Virander S. Chauhan [a,*]

[a] *International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India*
[b] *Genetics Division, Malaria Research Centre, 22, Sham Nath Marg, New Delhi 110054, India*

Received 18 March 2005; received in revised form 24 October 2005; accepted 24 October 2005
Available online 23 November 2005

Abstract

Mutations in *Dihydrofolate Reductase* (*dhfr*) gene of *Plasmodium vivax* are known to be associated with resistance to antifolate drugs. To analyze the extent of these mutations in *P. vivax* population in India, *dhfr* gene was isolated and sequenced for 121 *P. vivax* isolates originating from different geographical regions of Indian subcontinent. These sequences were compared with the gene sequence that represent wild type sequence (accession no. X98123). *P. vivax dhfr* (*Pvdhfr*) sequences showed limited polymorphism and about 70% isolates showed wild type *dhfr* sequence. A total of 36 mutations were found at 11 positions in 121 isolates. A majority of mutant isolates showed double mutations at residues 58 (S → R) and 117 (S → N), known to be associated with pyrimethamine resistance, but only 19% showed double mutations at residues 57 (F → L) and 58 (S → R). *Pvdhfr* alleles showing quadruple mutation (F57L, S58R, T61M andS117T) were found in two isolates. Three other mutations reported earlier at residue 13, 33 and 173 were not found in any of the Isolates. Six novel mutations at residues 38 (R → G), 93 (S → C), 109 (S → H), 131 (R → G), 159 (V → A) and 188 (I → V) were observed in seven isolates. Whether these novel mutations are linked to pyrimethamine resistance remains to be established.

Keywords: Malaria; *Plasmodium vivax*; Dihydrofolate reductase; Antifolate; Pyrimethamine; Point mutations; Drug resistance

1. Introduction

Development of resistance against antimalarial drugs is a major hurdle to combat malaria in most parts of the world. Due to the global spread of chloroquine resistance, the combination of sulfadoxine–pyrimethamine (S–P) becomes the first line therapy for complicated *Plasmodium falciparum* malaria in areas of endemicity. Pyrimethamine, an effective drug, interacts with the folate synthesis pathway of the parasite where dihydrofolate reductase (DHFR) enzyme reduces dihydrofolate to tetrahydrofolate. Pyrimethamine competitively inhibits parasite DHFR thereby blocking thymidylate synthesis (Hyde, 2005). In *P. falciparum*, resistance to pyrimethamine is attributed to one or more point mutations in parasite's *dhfr* gene (Anderson and Roper, 2005). Epidemiological studies, underpinned by biochemical analysis, strongly suggest that the drug pressure leads to the sequential appearance of point mutations and progressive selection of *P. vivax* parasites with increasing

* Corresponding author at: International Centre for Genetic Engineering and Biotechnology, PO Box 10504, Aruna Asaf Ali Marg, New Delhi 110067, India. Tel.: +91 11 2618 9358; fax: +91 11 2616 2316.
E-mail address: virander@icgeb.res.in (V.S. Chauhan).

0001-706X/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.actatropica.2005.10.003

levels of resistance (Imwong et al., 2003; Hastings et al., 2004).

In malaria endemic areas, prevalence of *P. vivax* often exceeds *P. falciparum*. Polymorphism in *Pvdhfr* gene is also known to be wide spread in different *P. vivax* endemic areas. Since *P. vivax* cannot be maintained in culture, therefore, the in vivo and in vitro evaluation of pyrimethamine resistance could not be established for this species of malaria. However, recent studies have shown that mutations at amino acid residues 57 (F → L), 58 (S → R) and 117 (S → N) confer resistance to pyrimethamine (Hastings et al., 2005). It has been suggested that these mutations emerge in the field populations of *P. vivax* in areas where S–P is used extensively for the treatment.

Malaria in India contributes around 2–2.5 million cases every year. According to statistical data published by National Malaria Eradication Program (NMEP) in the year 1997, in India, the incidence of *P. vivax* malaria is 60–70%. For treatment of *P. vivax* malaria worldwide, the first choice of treatment still remains the combination of chloroquine–primaquine. In recent years chloroquine resistance *P. vivax* parasites have been reported in several locations with high-level resistance confirmed in parts of Indonesia, New Guinea and India (Bombay) (Imwong et al., 2003). However, there are very limited reports on extent of drug resistant genotypes among *P. vivax* population in Indian Subcontinent.

In the present study, we have analyzed sequences of *Pvdhfr* genes from 121 *P. vivax* field isolates from different geographical regions of India. A small fraction of samples showed one to four mutations at different residues including mutations known to be responsible for pyrimethamine resistance. In addition, a number of unique and novel mutations were also identified in the present study.

2. Materials and methods

2.1. Geographical regions for sample collection

Blood samples from patients having acute *P. vivax* infections were collected from various clinics/hospitals in different geographical locations in India (Fig. 1): New Delhi (39), Mohan Nagar (49), Mirzapur (8), Cuttack (3), Navi Mumbai (5), Goa (8), Chennai (8) and Car Nicobar (1). The diagnosis was made by microscopic examination of giemsa-stained thin and thick blood smears. All the patients were found to be only infected with *P. vivax*. These samples were collected during July–September 2004. All the blood samples were collected with the consent of each patient and approval of the protocol

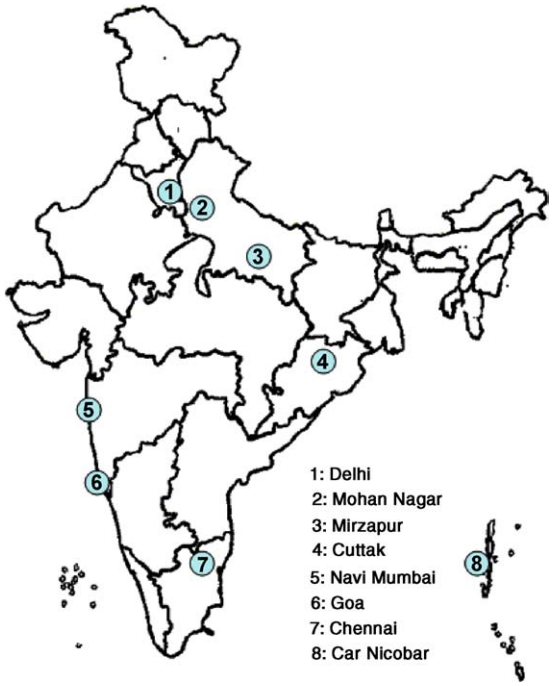

Fig. 1. Map of India showing *P. vivax* sampling sites.

was obtained from Human volunteer Research Ethical Committee of the International Center for Genetic Engineering and Biotechnology and Ethical Committee of Malaria Research Centre, New Delhi, prior to the study.

2.2. Parasite DNA extraction and amplification

Template DNA was isolated from the blood spots of infected patients using QIAamp DNA extraction mini-kit (QIAGEN) following manufacturers' protocol. The PvDHFR gene sequences were amplified from these DNA samples by primary and nested PCR following Imwong et al. (2001). Oligonucleotides were designed using a sequence of *dhfr-ts* gene of *Plasmodium vivax* (GenBank accession no. X98123). The primary amplification was performed using the external primers, VDT-OF (5′-ATGGAGGACCTTTCAGATGTATTTGACATT-3′) and VDT-OR (5′-GGCGGCCATCTCCATGGTTATTTTATCGTG-3′), wherein the entire *P. vivax dwhfr-ts* gene (1.8 kb) was amplified. This primary amplification product was then used for performing nested PCR, to amplify PvDHFR domain (711 bp). The oligonucleotide pair used were VDT-OF (5′-ATGGAGGACCTTTCAGATGTATTTGACATT-3′) and VDF-NR (5′-TCACACGGGTAGGCGCCGTTGATCCTCGTG-3′).

The PCR cycling parameters were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles