International Journal of Antimicrobial Agents 41 (2013) 447–451

Contents lists available at SciVerse ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Malaria treatment failure with novel mutation in the *Plasmodium falciparum* dihydrofolate reductase (*pfdhfr*) gene in Kolkata, West Bengal, India

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ARTICLE INFO

Article history:
Received 2 February 2012
Accepted 3 January 2013

Keywords:
Plasmodium falciparum
Early treatment failure
dhfr
dhps
Single nucleotide polymorphism

ABSTRACT

The aim of this work was to define the cause of sulfadoxine/pyrimethamine (SP) treatment failure in *Plasmodium falciparum* infections in a malaria-endemic zone of India. Samples were collected from 176 patients in Kolkata from November 2008 to July 2009. In vitro susceptibility testing was performed on all isolates. Parasite DNA was extracted, and PCR and restriction fragment length polymorphism (RFLP) analysis of different codons of the *dhfr* gene (16, 51, 59, 108 and 164) and *dhps* gene (436, 437, 540, 581 and 613) were performed. Finally, sequencing of the products was performed to confirm the mutations. The in vivo treatment response to SP among the 176 patients was determined. A novel mutation of isoleucine was observed at codon 108 of the *dhfr* gene, which was highly correlated with in vitro SP resistance as well as early treatment failure. A double *dhfr* mutation (108I+51I) was observed in 77.3% of isolates, and triple mutation of the *dhps* gene was observed in 18.2% of isolates. In this endemic zone, SP treatment failure is due to a novel *dhfr* mutation (108I+51I) and any one of the *dhps* mutations (S436A, A437G, A581G or A613T/S). An increase in these mutations was highly correlated with SP resistance ($P < 0.0001$).

1. Introduction

Malaria is a major public health problem in India [1]. Sulfadoxine/pyrimethamine (SP) combination was used as a second-line treatment against chloroquine-resistant *Plasmodium falciparum* before the introduction of artemisinin-based combination therapy (ACT) [1]. Extensive use of SP has led to the rapid emergence and spread of SP-resistant parasites [2]. Pyrimethamine inhibits the dihydrofolate reductase (DHFR) that is present in *Plasmodium* as a bifunctional enzyme with thymidylate synthase (DHFR-TS). The target of sulfadoxine is dihydropteroate synthase (DHPS), which is part of the bifunctional enzyme 7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase–DHPS (PPPK-DHPS). In vitro resistance to pyrimethamine is known to be associated with the key *dhfr* mutation S108N. Additional mutations in *dhfr* N51I, C59R and I164L confer higher levels of resistance [3]. Mutations in the *dhps* gene (S436A, A437G, K540E, A581G and A613T/S) have been previously reported as increasing in vitro resistance to sulfadoxine [3].

Kolkata is the capital of West Bengal, located on the lower delta of the River Ganga, in eastern India. Kolkata is a malaria-endemic zone where SP combination was used for more than two decades; as a result, SP drug pressure in this population increased. The present investigation aimed to determine the cause of in vivo SP clinical failure and in vitro SP resistance in Kolkata.

2. Materials and methods

2.1. Collection of Plasmodium falciparum isolates

This study was conducted from November 2008 to July 2009 just before the launch of ACT by the National Vector Borne Disease Control Programme (NVBDCP). Initially, 226 suspected patients with high fever for the past 24 h were enrolled for this study. Patients with *P. falciparum* monoinfection (confirmed by microscopic examination of Giemsa-stained thin and thick blood films), a parasite density of 1000–200 000 asexual parasites/μL of blood and no recent history of self-medication with antimalarial drugs were finally included in the study [4]. Informed consent was obtained from the respective patients (adult) or the guardians of child patients.

2.2. In vivo drug testing

In this study, 28-day therapeutic efficacy was determined according to the World Health Organization (WHO) standard [5].

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http://dx.doi.org/10.1016/j.ijantimicag.2013.01.005

SP combination was administered under the supervision of doctors. Adults were given a single oral dose of three tablets, each containing 1000 mg of sulfadoxine and 50 mg of pyrimethamine, whereas children were given 20 mg/kg body weight of sulfadoxine and 1 mg/kg body weight of pyrimethamine as a single dose. Clinical conditions and parasite density were monitored on Days 0, 1, 2, 7, 14 and 28 [6]. The therapeutic efficacy of SP was classified as adequate clinical and parasitological response (ACPR), early treatment failure (ETF) or late treatment failure (LTF) as described by the WHO [5]. Patients who did not respond to SP treatment were treated with ACT (artesunate + SP).

2.3. In vitro drug sensitivity assay

An in vitro drug sensitivity assay was performed on the clinical isolates with prior adaptation to the in vitro culture conditions as described previously [7]. If the blood sample had a parasitaemia >1.0%, then fresh uninfected O^+ erythrocytes were added to adjust the parasitaemia to 0.6–1%. Ethanol and cell culture-grade dimethyl sulphoxide (DMSO) were used to prepare stock solutions and dilutions of pyrimethamine and sulfadoxine, respectively. Briefly, 200 μL/well of the parasitised erythrocyte suspension was distributed in microculture plates (WHO plate) and 25 μL of each drug concentration (ranging from 50 nM to 25 600 nM of pyrimethamine and sulfadoxine) was distributed in the wells. Three wells (without drug) were used as a control for the experiment and each concentration was studied in duplicate or triplicate. Plates were incubated for 48 h at 37 °C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 with a relative humidity of 95%. Plates were used for the hypoxanthine incorporation assay, and the 50% inhibitory concentration (IC_{50}) was determined as stated previously [6]. The calculation was based on probit/logit regression analysis. Three culture-adapted cloned strains of *P. falciparum* (SP-sensitive strain 3D7, and SP-resistant strains W2 and FCR3-D7) were used for quality control.

2.4. DNA isolation and genotyping of the dhfr and dhps genes

Erythrocytes were separated from patient blood using a Histopaque 1077 density gradient. Parasite DNA was isolated from infected erythrocytes as described previously [6]. Extracted DNA was air-dried and re-suspended in TE buffer [10 mM Tris, 1 mM ethylene diamine tetra-acetic acid (EDTA)] and was preserved at −20 °C until use.

The primer design was based on published *pfdhfr* and *pfdhps* sequences of *P. falciparum* (GenBank accession nos. M22159 and J04643 for *dhfr* and Z30659 for *dhps*) aided by the primer designing software Primer3 (Table 1). In the nest I reaction, the *dhfr* gene was amplified using the M1 and M5 primer pair. Similarly, the *dhps* gene was amplified using R2 and R/primers. In the nest II reaction of the *dhfr* gene, 2 μL of amplified DNA from the M1–M5 primer pair was added to each of two PCR mixtures: M3 and F/to amplify the fragments containing 16-alanine, 51-asparagine, 108-asparagine and 164-leucine, or F and M4 to detect 59-arginine, 108-serine and 108-threonine [8]. Similarly for the *dhps* gene, DNA amplified with the R2–R/primer pair was added to each of two PCR mixtures: K and K/to amplify the 436, 437 and 540 codons of the *dhps* gene. An additional nest of L–L/was designed to amplify the 581 and 613 codons (Fig. 1) Polymorphisms in the *pfdhfr* and *pfdhps* genes at their specific codons were determined by enzymatic digestion with specific restriction enzymes as described previously [9]. The SP-sensitive strain 3D7 and the SP-resistant strains W2 and FCR3-D7 were used as control strains.

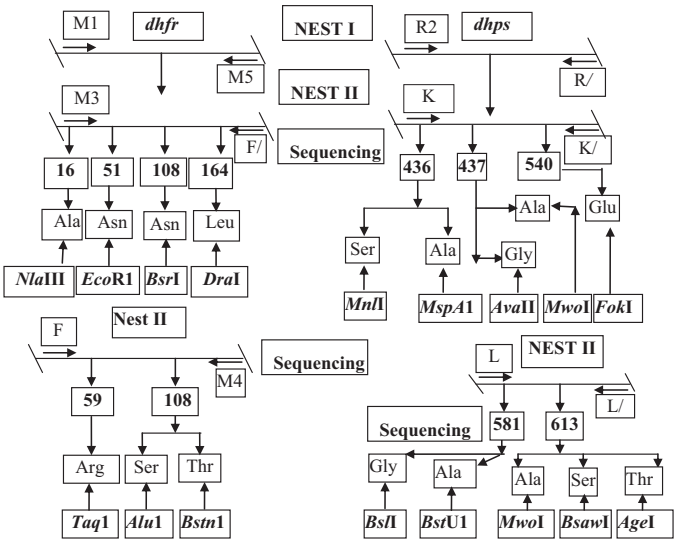

Fig. 1. Schematic representation of the nested system for the detection of polymorphisms in the *dhfr* and *dhps* genes of *Plasmodium falciparum*. Various primers and restriction enzymes were used for detection of each variant codon.

2.5. Sequencing of the dhfr and dhps genes

Sequencing reactions were carried out with an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and were run on a 3730xl Genetic Analyzer (Applied Biosystems) to confirm the mutation. Electropherograms were visualised and analysed with CEQ2000 Genetic Analysis System software (Beckman Coulter India Pvt. Ltd., Mumbai, India). Sequences were translated using the translation tool available online at the Expert Protein Analysis System proteomic server (http://www.expasy.org). Translated sequences were then aligned using the online multiple sequence alignment tool ClustalW2 (http://www.ebi.ac.uk/clustalw). Single nucleotide polymorphisms (SNPs) were confirmed by reading both the forward and reverse strands.

2.6. Assessment of isolate clonality

Multiplicity of infection (MOI) was estimated by using an allelic family-specific nested PCR (MAD20 and K1 for *pfmsp-1*, and 3D7 Africa and FC27 for *pfmsp-2*) [10]. All PCR amplifications contained a positive control (genomic DNA from W2 and 3D7 strains) and a negative control (no target DNA). Multilocus genotype analyses for drug resistance markers were performed with monoclonal isolates, i.e. isolates in which a single *pfmsp-1* and/or *pfmsp-2* allelic form was detected.

2.7. Statistical analysis

Statistical analysis was performed for the relationship between IC_{50} values of pyrimethamine and sulfadoxine by regression analysis. Fisher's exact test and Mann–Whitney *U*-test were used to study the relationship between IC_{50} values and genotypes. All analyses were performed using the statistical packages Origin 6.1 (OriginLab™ Corp., Northampton, MA) and GraphPad InStat software v.3.0 (GraphPad Software Inc., La Jolla, CA).