Infection, Genetics and Evolution 31 (2015) 183–189

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

High prevalence of *pfdhfr–pfdhps* triple mutations associated with anti-malarial drugs resistance in *Plasmodium falciparum* isolates seven years after the adoption of sulfadoxine–pyrimethamine in combination with artesunate as first-line treatment in Iran

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ARTICLE INFO

Article history:
Received 23 September 2014
Received in revised form 15 November 2014
Accepted 3 January 2015
Available online 31 January 2015

Keywords:
Plasmodium falciparum
Drug resistance
Molecular analysis
Sulfadoxine–pyrimethamine
pfdhfr
pfdhps

ABSTRACT

The spread of anti-malarial drug resistance will challenge any malaria control and elimination strategies, and routine monitoring of resistance-associated molecular markers of commonly used anti-malarial drugs is very important. Therefore, in the present investigation, the extent of mutations/haplotypes in *dhfr* and *dhps* genes of *Plasmodium falciparum* isolates ($n = 72$) was analyzed seven years after the introduction of sulfadoxine–pyrimethamine (SP) plus artesunate (AS) as first-line anti-malarial treatment in Iran using PCR–RFLP methods. The results showed that the majority of the patients (97.2%) carried both 59**R** and 108**N** mutations in pure form with wild-type genotype at positions N51 and I164. Additionally, a significant increase ($P < 0.05$) was observed in the frequency of $\mathbf{R}_{59}\mathbf{N}_{108}/\mathbf{G}_{437}$ haplotype (79.2%) during 2012–2014. This raise was because of the significant increase ($P < 0.05$) in the frequency of 437**G** mutation (81.9%), which more likely was due to more availability of SP as anti-malarial drug for treatment of falciparum patients in these malaria-endemic areas of Iran. However, no quintuple mutations associated with treatment failure were detected. In conclusion, the present results along with *in vivo* assays suggest that seven years after the adoption of SP–AS as the first-line treatment in Iran, this drug remains efficacious for treatment of uncomplicated falciparum malaria, as a partner drug with AS in these malaria-endemic areas.

1. Introduction

Malaria is a fatal and an acute febrile disease. The most severe form of and death from this disease are caused by *Plasmodium falciparum*. Nowadays, due to the lack of an effective vaccine, chemotherapy remains one of the mainstays for the control and management of falciparum malaria. However, the spread of drug-resistant *P. falciparum* parasites (to commonly used anti-malarial drugs) seriously threaten the global malaria control and elimination programs (Snow et al., 2004; Olliaro, 2005). Therefore, continuous monitoring of the efficaciousness of anti-malarial drugs in disease-endemic areas is crucial for early detection of parasites with reduced susceptibilities to the drugs (WHO, 2010).

To overcome and combat the spread of drug-resistant *P. falciparum* parasite from malaria-endemic to other freed malaria areas, molecular surveillance of drug resistance is necessary. This surveillance is very important for early detection of resistance to anti-malarial drugs such as chloroquine (CQ) and sulfadoxine–pyrimethamine (SP) and also for avoiding the further selection and extension of multidrug resistance (Maïga et al., 2007; Lynch et al., 2008; Certain et al., 2008; Harris et al., 2010). Such molecular study also provides a baseline data for the health authorities responsible for ensuring access to efficient anti-malarial drugs that could be fundamental to accelerate elimination program nationwide.

Despite SP treatment failure in most parts of the world, this drug remains a key tool for malaria control and elimination, particularly when it is administered as a partner drug with artemisinin (Aponte et al., 2009). These drugs target the inhibition of the activity of dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*), both coding for essential enzymes in the folate biosynthesis pathway. The level of *P. falciparum* susceptibility to SP depends on the frequency of mutations identified in the *dhfr–dhps* genes that encode these enzymes (Peterson et al., 1988, 1990; Plowe et al.,

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http://dx.doi.org/10.1016/j.meegid.2015.01.020
1567-1348/

1997; Wang et al., 1997a; Wongsrichanalai et al., 2002; Le Bras and Durand, 2003; Bwijo et al., 2003) and they are located on chromosomes 4 and 8, respectively. Therefore, increasing the number of mutations in *dhfr* and *dhps* genes of *P. falciparum* (*pfdhfr* and *pfdhps)* contributes to an increased risk of treatment failure (Happi et al., 2005). In this regard, point mutations in codons N51I, C59R, and S108N (*pfdhfr* triple mutant) have been associated with pyrimethamine resistance *in vitro* (Cowman et al., 1988; Peterson et al., 1988, 1990; Plowe et al., 1995, 1998) and with treatment failure *in vivo* (Peterson et al., 1988, 1990; Kublin et al., 2002). Codons S436A/F, A437G, and K540E of *dhps* have been linked to sulfadoxine resistance (Brooks et al., 1994; Triglia et al., 1997; Wang et al., 1997b; Plowe et al., 1998; Kublin et al., 2002). Infections carrying the *pfdhfr–pfdhps* quintuple mutant (*pfdhfr* 51**I**/59**R**/108**N** + *pfdhps* 437**G**/540**E**), synergistically confer *in vivo* SP treatment failure (Kublin et al., 2002; Mockenhaupt et al., 2005) and are highly predictive of clinical failure (Staedke et al., 2001; Kublin et al., 2002; Happi et al., 2005). Additional mutations in *pfdhfr* I164L and *pfdhps* A581G have been associated with high level of SP resistance and failure (Gesase et al., 2009).

In 2009, with the implementation of the "Malaria Elimination Program", which is in agreement with the global vision of malaria elimination and eradication program, the Iranian Center for Diseases Management and Control (CDMC) moved from control to pre-elimination (WHO, 2009) and then to elimination phases (WHO, 2011). In Iran, the burden of malaria has declined gradually from 96, 340 in 1991 to 15,712 total cases in 2007, and further reduction was reported in 2013, with total cases of 1,373 due to the scaling-up of different interventions (CDMC, Tehran, Iran, unpublished data). Resistance to CQ was reported in Sistan and Baluchistan Province in 1983 (Edrissian et al., 1986), and this resistance was increased to more than 78.5% of treatment failures in this area (Raeisi et al., 2006). As the intensity of CQ resistance increased, in 2005, the country changed the CQ, as first-line antimalarial treatment, to a combination of SP–CQ and artemether–lumefantrine (CoArtem®), as the second-line drug (Zakeri et al., 2008). Due to the high rates of treatment failures with CQ (Zakeri et al., 2008) and inadequate efficacy of treatment with SP–CQ combination, in 2007, the Iranian CDMC decided to replace SP–CQ with SP–AS (artesunate) (ACT) as the first-line recommendation for uncomplicated falciparum malaria as recommended by WHO in many malaria-endemic countries (WHO, 2006).

SP is still used as a partner drug in combination with AS in the first-line treatment of uncomplicated *P. falciparum* in Iran; therefore, the main aim of the present study was to investigate the distribution of mutations in *pfdhfr* and *pfdhps* genes that are associated with SP resistance seven years after its implementation. This investigation is a follow-up study to our previous works in the same area (Zakeri et al., 2010; Afsharpad et al., 2012) that provides data to evaluate the efficaciousness of SP as a partner drug in combination with AS. Furthermore, the second aim of the present work was to compare the trend in the distribution and the prevalence of *pfdhfr* and *pfdhps* mutations among *P. falciparum* samples in various periods of time with different levels of SP pressure in the study areas.

2. Materials and methods

2.1. Study area and sample collection

This study was performed in Sistan and Baluchistan Province, an area located in Southeastern Iran, where malaria transmission is seasonal, and most of the infected individuals are symptomatic. In this investigation, samples ($n = 93$) were collected from patients 4–60 years of age presenting at Primary Health Care Centers in Chabahar district in Sistan and Baluchistan Province, Iran with uncomplicated falciparum malaria from 2012 to 2014. The criteria for participation in the study were: presence of fever for the preceding 48 h (axillary temperature $\geqslant$37.5 °C), mono-infection with *P. falciparum*, no intake of anti-malarial drugs in the preceding 4 weeks, no signs of complications, and no history of allergic reactions to SP–AS. Finger-prick blood samples were collected on Whatman 3-mm filter papers (Whatman International, Ltd., Maidstone, England) from febrile patients who were confirmed to be positive for the presence of *P. falciparum* parasites before treatment with SP–AS. All filter papers were dried, individually stored in sealed plastic bags containing silica gel and kept in the dark at room temperature. After collection, the coded filter papers were subsequently transported to Malaria and Vector Research Group at Pasteur Institute of Iran for molecular analysis. A written informed consent was obtained from all patients (adults or parents or legal guardians of children), and an ethics approval was obtained from Committee of Research at Pasteur Institute of Iran.

2.2. DNA extraction and molecular diagnosis

Genomic DNA was extracted from dried blood spots by using Tris–EDTA buffer-based extraction (Bereczky et al., 2005). DNA samples were processed by nested PCR method to amplify species-specific sequences of the small-subunit ribosomal ribonucleic acid (18ssrRNA) genes of *Plasmodium vivax*, *P. falciparum*, and *Plasmodium malariae* (Snounou et al., 1993). The PCR products were electrophoresed on 2–2.5% agarose gel (Invitrogen, Carlsbad, CA).

2.3. Amplification of pfdhfr and pfdhps genes by nested PCR

Nested PCRs were carried out for *pfdhfr* and *pfdhps* genes, and all amplifications were performed in a reaction mixture with final volume of 25 μl, including 2 mM $MgCl_2$, 200 μM dNTP mixture (Invitrogen, Carlsbad, CA, USA), 1 unit Taq polymerase (Invitrogen, Carlsbad, CA, USA), and a pair of primers (10 pmol each). In addition, 2–5 μl DNA of the first reaction product was used as template in the first and second PCR. Negative controls (reaction mixtures without DNA) were used in all PCRs. All PCRs were performed at 95 °C for 5 min as first denaturation, 30 cycles at 94 °C for 1 min as denaturation, at 72 °C for 1 min as extension, and at 72 °C for 5 min as final extension. Annealing temperature was carried out as shown in Table 1. The secondary PCR products were analyzed by electrophoresis on 2% agarose gel (Invitrogen, Carlsbad, CA, USA) and visualized by an ultraviolet light.

2.4. Detection of pfdhfr and pfdhps mutations by restriction fragment length polymorphism (RFLP)

RFLP was performed to detect single nucleotide polymorphisms (SNPs) in *pfdhfr* at positions N51I, C59R, S108N/T, and I164L, and in *pfdhps* at positions S436A/F, A437G, K540E, A581G, and A613S/T. In the case of *pfdhfr* gene, two enzymes, *Tas*I and *Taq*I (both from Fermentas, Vilnius, Lithuania; New England Biolab, Beverly, MA, USA, respectively), were used for digestion of the secondary PCR products to define wild and mutant types at positions N51I and C59R, respectively. *BsrI* (Fermentas, Vilnius, Lithuania) was used to identify mutations at codon 108, and *Dra*I (New England Biolab, Beverly, MA, USA) was used for identification of mutations at codon 164. For *pfdhps*, the PCR products were digested with five enzymes, including *Ava*II, *Fok*I (both from New England Biolab, Beverly, MA, USA), *Mnl*I, *BstU*I, and *Mwo*I (all from Fermentas, Vilnius, Lithuania) in order to analyze the mutations at codons 437, 540, 436, 581, and 613, respectively. RFLP was carried out in 20-μl reaction, including 10 μl PCR products and 10 μl master mix containing ddH_2O, buffer, and enzyme according to the manufacturer's instructions. Digested