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Molecular monitoring of *Plasmodium falciparum* resistance to antimalarial drugs after adoption of sulfadoxine–pyrimethamine plus artesunate as the first line treatment in Iran

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ABSTRACT

The main objective of this investigation was whether the combination therapy of sulfadoxine pyrimethamine (SP) plus artesunate (AS) protects against the spread of resistance to SP in malaria-endemic south-eastern Iran. Infected blood samples of *Plasmodium falciparum* ($n = 170$) were collected during 2008–2010 after the adoption of SP–AS as the first line treatment in Iran. Four different genes of *P. falciparum* [dihydropteroate synthetase (*pfdhps*), dihydrofolate reductase (*pfdhfr*), chloroquine (CQ) resistance transporter (*pfcrt* K76T) and multidrug resistance1 (*pfmdr*1 N86Y)], associated with SP and CQ resistance were analyzed using PCR–RFLP methods. The result showed 4.1, 95.9 and 100% prevalence of *pfdhfr* 51I, 59R and 108N, respectively and the majority of patients (95.9%) were found to carry both 59R and 108N. The prevalence of single mutation at *pfdhps* 437G gene was 26.9% before the adoption of SP–AS, but as SP was used as the first line treatment; this mutation started to increase and reached a high level of 55.5% in 2008 (χ^2 test, $P < 0.05$). However, three years after the introduction of SP–AS, this prevalence was reduced from 55.5% (in 2008) to 39.1% (in 2009) and then 40.5% (in 2010). The frequency of parasites carrying *pfdhfr*/*pfdhps* mutations ($N_{51}R_{59}N_{108}/G_{437}$) decreased from 53.3% in 2008 to 39.1% in 2009 and 38% in 2010. In addition, no significant reduction was seen in the frequency of mutant alleles of *pfcrt* 76T and *pfmdr*1 86Y after CQ was discontinued from study areas as a treatment for *P. falciparum*. This is explained by the fixation of *pfcrt* 76T in the falciparum populations that need more time to recover from CQ sensitivity in the absence of drug pressure in this region. In conclusion, the present findings suggest that in Iran, SP is still effective for the treatment of uncomplicated falciparum malaria as a partner drug of Artemisinin Combination Therapy (ACT) in this region.

1. Introduction

Since 2007 malaria elimination has become one of the goals of the World Health Organization (WHO) in countries with unstable or low malaria transmission (Guerra et al., 2008). Antimalarial drugs play a central role in all stages of malaria elimination, including the early control and the later stages of maintaining interruption of transmission and also prevention of malaria reinfection. However, drug resistance is a big challenge in any control and elimination programs (Snow et al., 2004; Olliaro, 2005) and in this situation effective control and elimination will not be possible.

The problem of drug-resistant malaria is worldwide and as countries move towards elimination (WHO, 2010), assessing the efficacy of the first and the second line drugs is very important. In several parts of the world, sulfadoxine–pyrimethamine (SP) combination was being used as a first line drug after the development of resistance to chloroquine (CQ). However, widespread use of SP combination had resulted in a rapid loss of sensitivity and emerging multidrug-resistant parasites (Raman et al., 2010; Enosse et al., 2008; Mita et al., 2006).

One of the key components to prevent antimalarial resistance is routine monitoring of therapeutic efficacy of the first and the second line antimalarial drugs (WHO, 2009, 2005). In fact, routine monitoring makes it possible to detect drug resistance at early stages of development, hence, to avoid the further selection and spread of multidrug resistance. It also allows proper case management and early detection of changing patterns of drug efficacy in order to revise national malaria treatment policies. Therefore, providing information on drug resistance to the current classes of effective anti-malarial drugs such as chloroquine (CQ), sulfadoxine–pyrimethamine (SP) and also artemisinin is important for an early warning of the anti-malarial resistance development.

In Iran, the burden of malaria declined gradually over the last few years from 15,712 total cases in 2007 to 6122 in 2009 (Center

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for Diseases Management and Control, Tehran, Iran, unpublished data). Most of the patients are adults and may experience several infections by *Plasmodium falciparum* and/or *Plasmodium vivax* with clinical symptoms; however, there is no record of severe malaria or death due to malaria in this region. The predominant species is *P. vivax* with only 5–10% of *P. falciparum* cases. A combination of CQ and primaquine had been used as a first line antimalarial drug for treatment of uncomplicated *P. falciparum* in Iran up to 2005. Due to the prevalence of CQ-resistant parasites in this region (Raeisi et al., 2006; Zakeri et al., 2008), SP plus CQ was officially introduced as the first line treatment for confirmed *P. falciparum* cases in 2006. *In vivo* (Raeisi et al., 2006) and subsequent molecular studies (Zakeri et al., 2008; Ursing et al., 2006) indicated that resistance to CQ is highly prevalent among the Iranian *P. falciparum* population in this region; that is why, CQ usage for treating uncomplicated falciparum malaria was terminated in the therapeutic regime. Therefore, SP plus artesunate (AS) and artemether–lumefantrine (Coartem®) were adopted in the national guideline for treating malaria cases as the first and the second line treatment in 2007, respectively.

Providing the data regarding resistance to antimalarial drugs is a critical factor in drug policy decision-making and also controls programs. As monitoring the efficacy of antimalarial drugs through *in vivo* and also *in vitro* tests are not sensitive enough to detect emerging resistance of any anti-malarial drug and are also costly (Bickii et al., 1998; Fidock et al., 2004), available molecular markers can be used to predict resistance to SP (Kublin et al., 2002) and CQ (Djimdé et al., 2001) very easily and with reasonable reliability. Therefore in the present investigation, the distribution of dihydropteroate synthetase (*dhps*), dihydrofolate reductase (*dhfr*), CQ resistance transporter (*crt* K76T) and multidrug resistance (*mdr*1 N86Y) mutations associated with SP and CQ resistance, respectively, was studied in *P. falciparum* isolates over 5 years after withdrawal of CQ and the use of SP mono-therapy in the first line treatment of uncomplicated falciparum malaria in the south-eastern part of Iran. In fact, this study was also a follow-up to our previous investigation (Zakeri et al., 2010), performed three years after the introduction of SP as the first line treatment in the same area, which was used to evaluate the prevalence of mutations associated with resistance to SP when combined with artesunate (SP–AS).

2. Materials and methods

2.1. Study area, sampling, and in vivo study

In this study, blood samples were collected from Chabahar districts in Sistan and Baluchistan province in the south-eastern part of Iran. In this area malaria transmission is year-round with two peaks. The first peak is 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. Blood samples ($n = 170$) were collected on day 0 (D0) from uncomplicated *P. falciparum*-infected patients aged from 6 to 70 years old during 2008–2010. The inclusion criteria were as follows: presence of fever for the preceding 48 h (axillary temperature $\geq 37.5\,^{\circ}C$), mono-infection with *P. falciparum*, no intake of anti-malarial drugs in the preceding 4 weeks, no signs of complications, no history of allergic reactions to SP and an informed consent from patients or their parents.

In this investigation, all patients with non-severe malaria attack presenting at the outpatients clinic were enrolled for a 28-day follow-up according to the WHO protocol (WHO, 2002). We excluded subjects meeting WHO criteria for severe or complicated malaria, pregnant women, children less than five years of age, those suffering from malnutrition, severe allergic reaction to the study drugs, presence of a non-malarial febrile illness (e.g. measles, acute lower respiratory tract infection, severe diarrhea with dehydration) or other known underlying chronic or severe diseases (e.g. cardiac, renal and hepatic diseases, HIV/AIDS), persistent vomiting, self-medication with antimalarials during follow-up and withdrawal of consent. For all eligible patients, a medical history was taken and clinical examination was made. A finger-prick blood sample was obtained to measure parasite density. Approximately 1 ml venous blood was obtained in a tube containing EDTA from the patients who were confirmed to be positive for the presence of *P. falciparum* parasites. Then, the patients were orally given SP–AS based on the national guideline for treating malaria. Consumption of each dose was observed by one of the primary health care staff as a member of the study team under supervision of a qualified physician. The patients were followed up on days D7, D14, D21, and D28 following treatment (post treatment). Treatment outcomes were classified according to the WHO guideline as early treatment failure (ETF), late clinical failure (LCF), late parasitologic failure (LPF), or adequate clinical and parasitological response (ACPR). The study was reviewed and received ethical clearance from Pasteur Institute of Iran. All blood samples were collected in a tube containing EDTA, stored at $4\,^{\circ}C$, and then transported to the main laboratory in Tehran.

2.2. DNA extraction and molecular diagnosis

The blood samples were stored at $-20\,^{\circ}C$ before genomic DNA extraction. Parasite DNA was extracted from the infected red blood cells using phenol/phenol–chloroform followed by ethanol precipitation from 250 μl of the whole blood as described previously (Snounou et al., 1993). The DNA was resuspended in a TE buffer (10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA) and kept at $-20\,^{\circ}C$ until use. Detection of *Plasmodium* species in all samples was performed by nested-PCR amplification as described previously (Snounou et al., 1993).

2.3. Detection of pfcrt K76T and pfmdr1 N86Y mutations by restriction digestion of PCR products (PCR/RFLP)

Nested PCRs were performed for *pfcrt* and *pfmdr*1 genes on all examined samples. The PCR primers and conditions for both genes were based on those published previously (Zakeri et al., 2008). For *pfcrt*, the PCR products were digested with *Apo*I (Fermentas, Vilnius, Lithuania) to determine the polymorphisms at codon 76 and for *pfmdr*1, *Apo*I and *Nsi*I (Fermentas, Vilnius, Lithuania; New England Biolab, Beverly, MA, USA, respectively) were used to identify at codon 86 according to the manufacturer's instruction. Digestions were done in 20 μl reactions containing 10 μl of PCR products according to the manufacturer's instruction. Digested products were subjected to electrophoresis on 2–2.5% agarose gels, and visualized by ultraviolate (UV) transillumination.

2.4. pfdhfr and pfdhps genotyping

Genotyping was done on blood samples collected before treatment at day 0. The PCR primers and conditions for both *pfdhfr* and *pfdhps* genes were based on our previously published data (Zakeri et al., 2010, 2007, 2003). For *pfdhfr*, the PCR products were digested with *Tas*I and *Taq*I (Fermentas, Vilnius, Lithuania; New England Biolab, Beverly, MA, USA, respectively) to determine the polymorphisms at codons 51 and 59, respectively. Three enzymes: *Alu*I, *Bsr*I and *Mva*I (all from Fermentas, Vilnius, Lithuania) were used to identify wild and mutant *pfdhfr* allele at codon 108 and *Dra*I (New England Biolab, Beverly, MA, USA) was used to detect mutation at position I164L. For *pfdhps*, the PCR products were digested