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Emergence of sulfadoxine–pyrimethamine resistance in Indian isolates of *Plasmodium falciparum* in the last two decades

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ABSTRACT

Genotyping the sulfadoxine–pyrimethamine (SP) genes will help in identifying the genes under drug selection and the emergence of resistance in *dhfr* and *dhps* genes. India is an important hotspot for studying malaria due to the immense climatic diversity prevalent in the country. The central and eastern parts of the country are most vulnerable sites where malaria cases are reported throughout the year. From different regions of the country 173 field isolates were genotyped at various loci in *dhfr* and *dhps* genes collected between 1994 and 2013. This encompasses the period before antimalarial resistance emerged and the period after the use of combination therapy was made mandatory in the country. We observed the rise of resistant SP alleles from very low frequencies (in the year 1994) to steadily rising (in the year 2000) and maintaining this increasing trend subsequently (in the year 2013) as shown by the sequence analysis of *dhfr* and *dhps* genes. This study assessed the prevalence of mutations in *dhfr* and *dhps* genes associated with SP resistance in samples indicative of increase in resistance levels of *Plasmodium falciparum* to SP even after the change in malaria treatment policy in the country.

1. Introduction

India with one of the largest population endemic to malaria is at a huge risk of malaria infection and the disease burden gets multiplied due to drug resistance (Sharma, 1999). From South East Asia India itself contributes to about 77% of the total malaria cases (World Malaria Report, 2012). After the first reports of CQ resistance in 1973 and of SP resistance emerged in 1979; a national drug-policy was introduced in 1982 with SP as the antimalarial treatment for CQ-resistant regions (Misra, 1996; National Anti Malaria Programme, 2013). In the year 2005 artesunate + SP became the second line drug for treating CQ resistant cases and in 2007 this combination therapy became the first line of treatment in high endemic regions. In the year 2010 this combination therapy became the first line treatment throughout the country (National drug policy on malaria, 2010). The overlap of treatment lines during the years 2005, 2007 and 2010 leads to emerging mutations due to drug pressure associated with drug resistance genes *viz. crt*, *mdr-1*, *dhfr* and *dhps* (Sharma, 2012). Currently the malaria treatment in India uses artesunate with SP hence the drug pressure due to SP continues to exist. In India, not much data is available on the prevalence of drug resistance genes in parasite before and after the change in malaria treatment policy. The continuous increase in the SP resistance needs to be assessed at regular intervals in parasite population to reveal the SP sensitivity in our country. Knowledge of the resistant antifolate genes in the field regions is essential to detect the spread of SP resistance over wide areas and for effective implementation of the drug policy in India. Thus it is of relevance that SP genes in the parasite population be analyzed to understand the emergence of resistance pattern during this time. In our lab we had collected field samples of asymptomatic and uncomplicated patients from different regions during the period 1994 to currently 2013. The present study was carried out to evaluate the pattern of mutations in the *dhfr* and *dhps* genes in Indian *Plasmodium falciparum* field isolates collected at different time intervals during the last 20 years from various geographical regions.

2. Materials and methods

2.1. Sample collection

Blood samples of the *P. falciparum* patients were collected from six different regions of the country during the years 1994 through 2013. The sample collection was part of a previous study and the blood samples were stored as dried blood spots at −20 °C sealed in air tight bags. The

Abbreviations: AS + SP, artesunate + sulfadoxine–pyrimethamine; *P. falciparum*, *Plasmodium falciparum*; CQ, chloroquine; *dhfr*, dihydrofolate reductase; *dhps*, dihydropteroate synthetase; *crt*, chloroquine resistance transporter; *mdr-1*, multi drug resistance 1; SNPs, single nucleotide polymorphisms.

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previous study had undertaken the collection of field samples after microscopic examination of the parasite (Biswas et al., 1996; Gupta et al., 2014). The study was approved by the institutional ethics committee (IEC) (reference no. ECR/NIMR/EC/2) and the ethical guidelines of the institute were followed for sample collection. A total of 185 samples from both sexes having uncomplicated malaria were analyzed in this study (Fig. 1). The samples were collected from patients with no prior medication/treatment history. No *in vivo* studies were undertaken by us.

2.2. Sample sites

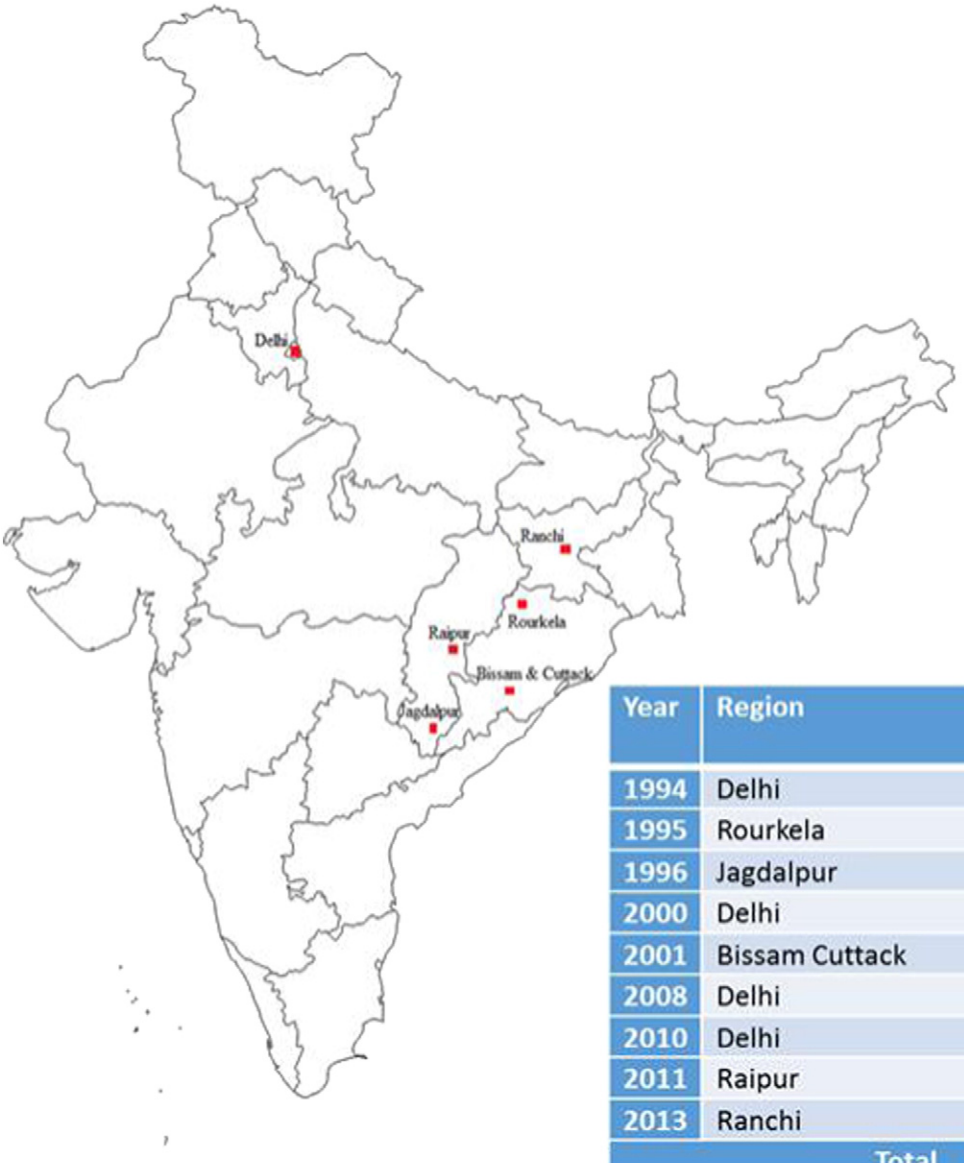
Samples from six sites were analyzed in this study collected from three states and one union territory of the country (Fig. 1). The sites from Orissa state located in the southeast of the country included; Rourkela and Bissam Cuttack and from Jharkhand state, samples were collected from Ranchi. The samples from central part of the country were from Raipur and Jagdalpur situated in the Chattisgarh state. Apart from Delhi all the other sites are malaria endemic regions varying from meso to hyper endemic. The samples from Delhi were collected during the peak malaria transmission season after the rainy season, *i.e.*, August to October in different time intervals (Fig. 1). The samples from Rourkela and Bissam Cuttack were collected during the month of October, from Jagdalpur during April–May, from Raipur in the months of July–August and from Ranchi in the month of November.

2.3. DNA extraction

The parasite genomic DNA from filter paper was extracted using QIAamp DNA mini kit (QIAGEN), according to the manufacturer's instructions with slight modifications. As archived samples were used in this study, at least four punches of same dimensions (4 mm) were taken from individual dried blood spots and incubated overnight in PBS at 4 °C for comparable results. DNA samples were then kept at −20 °C until further processing.

2.4. Species identification

Nested PCR targeting 18S rRNA genes was performed with extracted DNA from confirmed samples to differentiate the *P. falciparum* species from mixed infections. The primers used for 18S rRNA PCR have been previously described (Johnston et al., 2006).



Year	Region	No. of sample
1994	Delhi	15
1995	Rourkela	22
1996	Jagdalpur	13
2000	Delhi	17
2001	Bissam Cuttack	21
2008	Delhi	18
2010	Delhi	19
2011	Raipur	29
2013	Ranchi	31
	Total	185

Fig. 1. Map of India depicting the different regions of sample collection and the table specifying the number of samples collected from different regions.