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Molecular Evidence for *Plasmodium falciparum* Resistance to Sulfadoxine–Pyrimethamine but Absence of *K13* Mutations in Mangaluru, Southwestern India

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Abstract. In most of India, sulfadoxine–pyrimethamine (SP) *plus* artesunate serves as first-line treatment for uncomplicated falciparum malaria. In 112 clinical *Plasmodium falciparum* isolates from Mangaluru, southwestern India, we sequenced molecular markers associated with resistance to SP, lumefantrine, and artemisinin (*pfdhfr*, *pfdhps*, *pfmdr1*, and *K13*). The *pfdhfr* double mutation 59R-108N combined with the *dhps* 437G mutation occurred in 39.3% and the *pfdhfr* double mutation *plus* the *pfdhps* double mutation 437G-540E in additional 24.1%. As for *pfmdr1*, the allele combination N86-184F-D1246 dominated (98.2%). *K13* variants were absent. No evidence for artemisinin resistance was seen. However, the antifolate resistance alleles compromise the current first-line antimalarial sulfadoxine–pyrimethamine *plus* artesunate, which may facilitate the emergence of artemisinin resistance. Artemether–lumefantrine, introduced in northeastern parts of the country, in the study area faces the predominant *pfmdr1* NFD genotype, known to impair lumefantrine efficacy. Further monitoring of resistance alleles and treatment trials on alternative artemisinin-based combination therapies are required.

Emerging artemisinin resistance of *Plasmodium falciparum* in Southeast Asia threatens global malaria control.[1] In India, the countrywide first-line antimalarial drug for uncomplicated falciparum malaria is artesunate *plus* sulfadoxine-pyrimethamine (SP) (*plus* single dose primaquine) except for the northeastern states, where artemether–lumefantrine is recommended because of intense SP resistance.[2] Sulfadoxine–pyrimethamine treatment failure rates vary greatly across India,[3] as do the frequencies of associated mutations in the parasite's *dihydrofolate reductase* (*pfdhfr*) and *dihyropteroate synthase* (*pfdhps*) genes.[4–8] Cumulative *pfdhfr* and *pfdhps* mutations render *P. falciparum* resistant to pyrimethamine and sulfadoxine, respectively.[9] Work on Asian strains suggests a predominant sequential accumulation of mutations, in that two initial mutations preferentially occur in *pfdhfr* (108N, 59R), followed by two in *pfdhps* (437G, 540E) and a third in each of *pfdhfr* and *pfdhps*.[10] Artemisinin resistance, which so far largely means delayed parasite clearance, in vitro findings, and/or associated mutations in the Kelch 13 (*K13*) propeller domain of *P. falciparum*, is spreading in mainland Southeast Asia including neighboring Myanmar,[1,11] but has not been confirmed in India so far.[12] Here, we assessed molecular markers of resistance to artesunate–SP some 2,500 km away from India's hotspot of SP resistance (and potential gateway of artemisinin resistance) in the northeastern states, namely, in the city of Mangaluru, coastal southwestern Karnataka. In addition, we assessed *P. falciparum multidrug resistance-1* (*pfmdr1*) alleles to appraise the potential of, for example, lumefantrine as an alternative to SP as partner drug.

Malaria outpatients were recruited between June and December 2015 at the malaria diagnostic unit of Wenlock Hospital, the largest governmental hospital in Mangaluru, southwestern India. All study participants provided written informed consent (of parent/guardian in case of children < 18 years of age), and the study protocol was reviewed and approved by the Institutional Ethics Committee of Kasturba Medical College, Mangaluru, Manipal University (IEC KMC MLR 05-1598). Permission to conduct the study was given by the Directorate of Health and Family Welfare Services, Government of Karnataka. Study details and clinical manifestation are presented elsewhere.[13] Patients confirmed to have falciparum malaria were treated by hospital staff according to standard guidelines on an outpatient basis, that is, artesunate–SP for 3 days *plus* single dose primaquine on the second day. Genomic DNA of patients infected with *P. falciparum* was extracted from full blood aliquots (QIAamp DNA Blood Mini Kit; Qiagen, Hilden, Germany). *Plasmodium* species was ascertained by nested polymerase chain reaction (PCR) assays.[14] In addition, following amplification, PCR products were bidirectionally sequenced (Source BioScience, Berlin, Germany), and multiple sequence alignment was performed to detect polymorphisms in *pfdhfr*, *pfdhps*, *pfmdr1*, and *K13* using BioEdit v.7.2.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and SnapGene v.3.1 (GSL Biotech, Chicago, IL) software. *Plasmodium falciparum* 3D7 (PF3D7_1343700) retrieved from PlasmoDB was used as reference for the *K13* alignments, and for *pfdhfr* and *pfdhps*, references were NCBI XM_001351443.1 and GenBank Z30654.1, respectively. We specifically analyzed the mutations *pfdhfr* N51I, C59R, S108N, I164L; *pfdhps* S436A/F, A437G, K540E, A581G, A613S/T; and *pfmdr1* N86Y, Y184F, and D1246Y. Sequencing plots did not suggest the presence of polyclonal infections.

Of the 276 patients infected with *P. falciparum*, 138 isolates (50%) were randomly selected, and of those, 112 (81.1%) were successfully typed for all alleles under study (including 53 mixed *Plasmodium vivax–P. falciparum* infections). Among the 112 patients (median age, 30.5 years; range, 10–65), 92.0% (103) were male; 79.5% (89) had migrated to Mangaluru a median of six months (range, 1–240) before presentation; and 20.5% (23) originated from Mangaluru city, 33.9% (38) from the local Karnataka state, 27.7% (31) from the northern/northeastern states, and 17.9% (20) from other regions of

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India. Their socioeconomic status was low (data not shown), and 69.6% (78) of patients were construction workers or daily laborers. The geometric mean parasite density was 9,572/μL (95% confidence interval, 7,516–12,190/μL). Each 4.5% (five) of patients was admitted to ward or had severe malaria, respectively. Intake of antimalarials (chloroquine) in the preceding 6 weeks was reported by 0.9% (two) of patients.

Only one-third of *pfdhfr* alleles were wild type, and this figure was slightly lower for *pfdhps* (Table 1). For *pfdhfr*, two-thirds of isolates exhibited the double mutation 59R-108N, whereas for *pfdhps*, the single mutation 437G dominated over the double mutation 437G-540E. Together, almost 40% of isolates showed *pfdhfr* 59R-108N *plus pfdhps* 437G in addition to one in four isolates with the *pfdhfr* double mutation *plus* the *pfdhps* double mutation. No *K13* polymorphisms were detected. Also, we observed only wild-type alleles at codon 86 of *pfmdr1* (N86), and almost exclusively so in the combination N86-184F-D1246 (NFD).

Isolates from native Mangalureans and from migrants did not differ in terms of isolates with *pfdhfr*/*pfdhps* double–single or double–double mutations (60.9% [14/23] versus 64.0% [57/89]; $P = 0.78$). Of note, this figure was not increased in migrants from the north/northeastern states (67.7%, 21/31; $P = 0.60$), but tended to do so in migrants from the local state of Karnataka (78.9%, 30/38; $P = 0.13$), and it was reduced in migrants from elsewhere in India (30.0%, 6/20; $P = 0.04$). The time since migration was not associated with carrying these SP-resistant parasites ($P = 0.74$).

We show that in coastal southwestern India, most of the *P. falciparum* isolates have mutations conferring SP resistance, whereas *K13* variants associated with artemisinin resistance are absent. Anticipating a further intensification of SP resistance as seen elsewhere in India,[3] the useful therapeutic lifetime of the current combination artesunate–SP appears limited. However, considering the fixation of *pfmdr1* N86 and the almost fixation of the NFD allele combination, artemether–lumefantrine might not be a promising candidate for replacing artesunate–SP in this area. Moreover, based on the limited dataset, SP resistance in Mangaluru seems to be a local rather than an imported problem.

TABLE 1

Prevalence of antimalarial drug resistance alleles and genotypes in Mangaluru, southern India

Gene	Allele or genotype	Prevalence (%, *n*/112)
pfdhfr	Wild type	33.9 (38)
	Double mutation (59R-108N)	66.1 (74)
pfdhps	Wild type	29.5 (33)
	Single mutation (437G)	45.5 (51)
	Double mutation (437G-540E)	25.0 (28)
pfdhfr/*pfdhps*	Wild type	26.8 (30)
	dhfr wild type + *dhps* single (437G)	6.3 (7)
	dhfr wild type + *dhps* double (437G-540E)	0.9 (1)
	dhfr double (59R-108N) + *dhps* wild type	2.7 (3)
	dhfr double (59R-108N) + *dhps* single (437G)	39.3 (44)
	dhfr double (59R-108N) + *dhps* double (437G-540E)	24.1 (27)
K13	Wild type	100 (112)
pfmdr1	Wild type	0 (0)
	86N-184F-1246Y	1.8 (2)
	86N-184F-1246D	98.2 (110)

Our data originate from a limited number of *P. falciparum* isolates and represent only a snapshot in a dynamic process of resistance development. As compared with recent molecular data from India, the observed *pfdhfr* double mutation 59R-108N (i.e., without 51I) is found rather in central India,[4,5] whereas the *pfdhfr* triple mutation (and also 164L) has become prevalent particularly in northeastern India.[6–8] Likewise, the *pfdhps* mutations 437G and 540E (and the respective *pfdhfr*/*pfdhps* combinations) are comparatively rare in central India but common in the Northeast.[4–8] Our data from southwestern India occupy a middle position in this regard: whereas *pfdhfr* 59R-108N occurs at a prevalence similar to central India,[4,5] the *pfdhps* mutations 437G and 540E are almost as common as in northeastern India.[6–8] We detected *pfdhfr* 59R-108N *plus pfdhps* 437G or *plus* 437G-540E in almost two in three isolates. In East Africa, the *pfdhfr* triple mutation, *pfdhps* 437G-540E, and their combination (quintuple mutant) strongly predict SP treatment failure.[15] At a lower level of SP resistance, for example, in Indonesia, SP treatment failure has been associated with *pfdhfr* 59R-108N *plus pfdhps* 437G, and the *pfdhfr* double–*pfdhps* double variant with high-grade resistance (RII/III).[16] Even when double *pfdhfr* mutations do not greatly intensify SP resistance as compared with the 108N core mutation alone,[9] the prevalence of *pfdhps* 437G and 540E suggests SP resistance in the study area to be pronounced but not yet highly intense. Given ongoing SP drug pressure, for example, on parasites transmitted to recently treated patients without detectable artesunate levels but fading SP concentrations, and the foreseeable, stepwise development of further *pfdhfr*/*pfdhps* mutations,[10] SP resistance is likely to intensify in the study area, eventually compromising artesunate–SP. Reassuringly, no molecular evidence for artemisinin resistance was seen in the present study. However, against the background of evidence for impaired and potentially further waning SP efficacy, *K13* mutations may emerge or spread after importation. In this regard, a limited number of *K13* mutations have recently been detected in the northeastern state of Arunachal Pradesh bordering Myanmar.[12]

To protect the artemisinin component, partner drugs should have the highest possible efficacy. India's National Drug Policy on Malaria recommends the use of artemether–lumefantrine in the northeastern states,[2] which bear intense SP resistance. The present study showed the predominance of *pfmdr1* N86 and of the NFD haplotype. In vitro, wild-type *pfmdr1* N86 reduces sensitivity to dihydroartemisinin and to the partner drugs lumefantrine or mefloquine (3- to 4-fold higher IC50s) but increases susceptibility to chloroquine, monodesethyl amodiaquine (active metabolite of amodiaquine), and, less pronounced, piperaquine.[17] In clinical trials, *pfmdr1* N86 predicts recrudescence in patients treated with artemether–lumefantrine.[18] Similarly, *pfmdr1* NFD parasites reinfecting after artemether–lumefantrine treatment have been shown to tolerate 15-fold higher artemether–lumefantrine blood concentrations than those with the opposite YYY haplotype,[19] although the central polymorphism appears to be *pfmdr1* N86.[18] Interestingly, artemether–lumefantrine and artesunate–amodiaquine select different *pfmdr1* alleles,[18] which suggests that artesunate–amodiaquine and dihydroartemisinin–piperaquine might be effective in parasites with reduced susceptibility to artemether–lumefantrine.[1,11,18]

The diversity of malaria in India, including geographically variable drug susceptibility and resistance alleles, impedes drug policy recommendations, which accurately fit for the whole of the subcontinent. India presently forms the western boundary of artemisinin-resistant malaria, and the term of the presently used first-line treatment artesunate–SP is limited. Against the background of spreading and intensifying SP resistance as seen in the present study, expanded monitoring of molecular makers and clinical trials on alternative first-line antimalarials are required.

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