Residual Antimalarial Levels in *Plasmodium Falciparum* Malaria Patients from Selected Sites in India: An indication of Drug Pressure

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\textbf{Abstract:} Irrational treatment practices and self-medication of antimalarials are common in malaria-endemic areas. Such types of practices may affect the treatment outcome as well as could promote the spread of drug resistance in the community. The aim of the study was to monitor residual antimalarials level in *Plasmodium falciparum* infected patients belonging to Bilaspur and Betul districts in India. A total of 139 patients were enrolled for the treatment of antimalarials as per national treatment policy, and their clinical follow-up was done as per WHO guidelines (2009). Heparinised blood was taken on 31ET filter paper for monitoring residual antimalarials on day 0 and post-treatment sulphadoxine levels on day 7 using High Performance Liquid Chromatography. 27.3% of the patients showed residual antimalarials on day 0. Surprisingly, the residual levels did not correlate well with the information collected on case record forms. A significantly lower parasite density/µl of blood was observed in samples having residual antimalarials than the samples without residual antimalarials (\(P<0.05\)). The residual antimalarials detected in the blood might be due to previous drug episodes, self drug intake, and irrational treatment practices. Awareness towards rational treatment practices are the need of hour.

\textbf{Introduction}
Malaria imposes a substantial socio-economic challenge and together with six other diseases, it accounts for 85% of global infectious disease burden [1]. Annually, approximately 3.4 billion people worldwide expose to malaria, 1.2 billion are at high risk amongst them[2]. India alone contributes 50% of the 2 million reported cases in the South-East Asia region; majority of which are reported from Odisha, Chhattisgarh, Jharkhand, Madhya Pradesh, Rajasthan and North-Eastern states in India[2, 3].

Unfortunately, the control of malaria is hampered due to development of antimalarial drugresistance emergence of antimalarial drug resistance in South East Asia region to commonly used antimalarial drugs particularly for *Plasmodium falciparum* (*Pf*). The resistance to anti-malarial drugs has also increased the global cost of controlling the disease, including the cost of new drug development. The north-eastern region of India has been the epicentre of antimalarial drug resistance in the past and the history of resistance against antimalarials in this region include chloroquine (CQ) resistance in *Pf* in Karbi Anglong district in Assam in the 1973 followed by sulphadoxine-pyrimethamine (SP) resistance in the same region in the1979[4,5]. Ineffective treatment regimen including the use of monotherapies with artemisinin, lack of compliance to the prescribed medications and intake of counterfeit medicines are associated contributing factors in promoting drug resistance[6,7]. Before 2005, CQ was first line therapy for *Pf* malaria in India. Despite the fact that ACT (AS+SP) became the first line treatment for uncomplicated *Pf* malaria cases in India[8]; SP, quinine (QN), mefloquine (MQ) and CQ were still found to be prescribed as well as circulating widely in the private health sectors [9]. A study conducted in six states of India revealed 14.8% prescription of artesunate (AS) monotherapy in Jharkhand, one of the malaria endemic site in the country [10].

The standard WHO protocol to monitor the efficacy of antimalarial medicines does not exclude patients with history of antimalaria intake [11], however, the intake of antimalarial drugs prior to inclusion in an \textit{in-vivo} study may interfere with the estimation of treatment outcome because of accumulation of residual or sub-therapeutic levels of antimalarials. Standard case record forms were filled to ascertain the use of antimalarials prior to \textit{in-vivo} efficacy.
studies. However, with a recall period of a week or fortnight, this information was unreliable and needs further confirmation. Thus, this information might not give an accurate and complete picture of drug use in that area. Therefore, it is important to measure the antimalarial drugs level using field adapted methods. Globally, this information has been captured by a number of studies, including quantification of residual antimalarials such as CQ or SP in urine or blood in the general population or patients [12-14]. But to the best of our knowledge this is the first study report from highly endemic sites of malaria where simultaneous quantification of five residual antimalarials namely CQ, sulphadoxine (SDX), pyrimethamine (PYR), QN and MQ in human blood samples was done. In addition, association of residual antimalarial levels with parasite densities of Pf was also analysed. This work will highlight the use of over the counter residual antimalarial levels in the patients participating in in-vivo therapeutic efficacy studies in highly endemic regions of malaria.

Materials and methods

Study sites
The study was carried out during the year 2011-2012 at Gaurella Community Health Centre at Bilaspur district, Chhattisgarh and Ghodadongri Primary Health Centre (PHC) at Betul district, Madhya Pradesh (Figure 1). In these areas, malaria transmission takes place round the year due to favourable environmental conditions supporting vector survival. Pf infections are predominantly recorded from October to December. Amongst the two study states, Chhattisgarh contributes 13.3% and Madhya Pradesh contributes 8.7% of annually reported malaria cases in the country with 2.2% cases from Bilaspur district and 2.6% of cases from Betul district respectively [3]. At the time of the study, ACT (AS+SP) was the first line treatment for uncomplicated Pf malaria in these areas and was available with Auxiliary Nurse Midwives (ANMs), Accredited Social Health Activists (ASHAs) and other community health volunteers. All the study procedures were conducted in accordance with the Institutional Ethics Committee of the National Institute of Malaria Research (NIMR), Ministry of Health and Family Welfare, Govt of India.

Population screening and sample collection
Finger prick blood samples of 139 patients (70 from Chhattisgarh and 69 from Madhya Pradesh) in the age group of >6 months to ≤65yrs and infected with uncomplicated Pf malaria were collected on day 0 (D0). The inclusion/exclusion criteria for selection of patients was according to WHO protocol (2009) [11]. A written informed consent was obtained from all participating patients. From all eligible patients, a medical history was taken and clinical examination was made. Finger prick blood samples were used for counting parasite density on day 0 until 28 days of follow-up. Also, hundred microliters (100 µl) of heparinised blood was taken on 31ET filter papers (Whatman GE Health Care, UK) for monitoring residual drug on day 0 and SDX drug level on day 7. The filter papers were then allowed to dry at room temperature and stored in a zipper pouch with desiccant at 4°C in a refrigerator until analyzed. Treatment with standard first line therapy of AS+SP (ACT) was initiated in appropriate dosages according to age.

Treatment and follow-up
In this study, all eligible patients with non-severe malaria presenting at the outpatients clinic were enrolled for a 28-day follow up according to the WHO protocol [11]. All of them received oral doses of artemesunate (AS) + sulphadoxine-pyrimethamine (SP) (age wise combi-blister pack) on day 0 and primaquine (PQ) (0.75 mg/kg bwt.) on day 2, as per the national guidelines[8]. Subjects were observed for 30 minutes after ingesting the drugs to ascertain
its retention. Those who vomited the first dose were retreated with an identical dose. Subjects who vomited twice were dropped from the study. Patients were asked to return for follow-up visits on days 1, 2, 3, 7, 14, 21 and 28 days. Parasitological assessment and temperature measurement was done during each follow-up visit.

Parasites identification and quantification
Thick and thin blood films were prepared and stained with 10% Giemsa solution. While thin film were studied for species identification, the parasite density was determined by counting the number of parasites in thick film per 200 white blood cells (WBCs), based on a mean WBC count of 8,000/μL. A slide was considered negative when no parasite was observed against count of 1000 white blood cell. Each of these slides were examined under a light microscope on days 0, 1, 2, 3, 7, 14, 21, 28 by experienced microscopist (s) at the site and were cross-checked for quality control at National Institute of Malaria Research, New Delhi by third experienced microscopist.

HPLC analysis
Baseline blood samples (day 0) collected from patients reporting no antimalarial intake prior to the study were screened for the presence of five antimalarial drugs such as CQ, SDX, PYR, QN and MQ using a modified HPLC method [15]. The level of partner drug (SDX) of AS+SP was also determined on day 7. Extraction of the standard drugs (CQ, QN, SDX, PYR and MQ; Sigma Aldrich, USA), blank whole blood spot (control sample) and each of the collected samples were carried out according to the protocol of Blessborn et al., 2010, with slight modification[15]. This involved the use of multi-mode solid phase extraction column (M-M SPE, Biotage, USA) and elution of the samples by methanol:triethylamine (97.3 v/v) mixture. Eluates were dried under a gentle stream of air at 70°C and were then dissolved in 100 μl of methanol:HCl (0.01 M) 10:90 v/v. Twenty microliter (20 μl) of each of these standards and samples were injected into the HPLC system. HPLC was performed on a Hitachi gradient system equipped with binary pump (Model L-2100/2130) and multi wavelength UV detector (Model L-2420 UV-VIS). Analytes extracted from the M-M SPE column were analyzed using two different mobile phases (A) acetonitrile:ammonium formate (20 mM in 1% formic acid) (5:95 v/v) and (B) acetonitrile:ammonium formate (10 mM in 1% formic acid) (80:20 v/v) and were run according to previously described gradient program[15]. The compounds were analyzed on a Tosoh ® 5 μm C18 (150 mm × 2 mm) column protected by a precolumn security guard C4 (8mm ×2 mm) (Tosoh Bioscience, PA). The UV detector was monitored at 280nm. Data acquisition and quantification were performed using Hystar™ and Data Analysis™ (Bruker, Bremen, Germany).

Estimation of dose intake time for sulphadoxine
To estimate the probable timing of drug intake, we compared the whole blood concentrations of SDX at baseline (C0) and on Day 7 (C7) after a complete treatment with AS+SP for the same patients. Assuming a terminal elimination half-life (t½) of 7.2 days for SDX, an inter-individual variability of 30%[12] and a similar dosage on pre-study exposure and during the study, a back-calculation was done to estimate the intake time of the drug before baseline sampling:

\[ \text{Intake time} = \ln(C_7/C_0) \times \frac{t_1/2}{\ln(2)} + 7\text{[days]} \]

The variability on t½ was used to estimate a 90% confidence interval around this intake time, considering plausible inter-individual variations in elimination rate [16]. Similar calculation was not attempted for pyrimethamine because of its short half-life period [12].

Statistical analysis
All statistical analyses were done using the SPSS software version 14. Geometric mean of parasite densities at 95% confidence interval (CI) was calculated. Frequencies were compared using the X² test. The differences were considered statistically significant at an error probability less than 0.05 (p<0.05).

Results
Baseline demographic data
A total of 139 (70 patients from Bilaspur and 69 from Betul) eligible patients with microscopy-confirmed \textit{Pf} malaria and who satisfied the inclusion/exclusion criteria were enrolled in the study. Common symptoms of malaria such as fever, headache, chills/rigor and vomiting were reported by the patients at the time of recruitment. The total number of males and females included in the population study were 53.2% and 46.8% respectively. The patients were categorised on the basis of age as: Infants; < 5yrs (3.6%), 5-15 yrs (38.8%) and adults (57.6%). 62(88.6%) patients from Bilaspur reported to have taken any prior course of antimalarial drug before the initiation of our study while 8(11.4%) patients from Bilaspur and 69 (100%) from Betul did not aware about the intake of any antimalarials. The clinical and demographic characteristics of these patients have been tabulated in table 1.
Detected levels of antimalarial drugs in population

The presence of antimalarial drugs above the detection limit (50 ng/ml) was observed in the blood of 38 (27.34%) patients: 25 (18.0%) had SDX, 17 (12.2%) had CQ, 5 (3.5%) PYR while 1 (0.7%) each had QN and MQ (Table 2). On day 0, the median (range) blood concentration was 5300 ng/ml (100-54100 ng/ml) for SDX, 180 ng/ml (51-263 ng/ml) for CQ, 900 ng/ml (100-1600 ng/ml) for PYR. QN and MQ residual level was observed to be 279 ng/ml and 367 ng/ml in one patient each (Fig. 2 & Table 2). Residual levels of SDX above the minimum inhibitory concentration (MIC) i.e. 20µg/ml were detected in six patients [17]. In addition, fourteen patients had residual levels of CQ more than the MIC (MIC: 90 ng/ml) [18].

Among the 38 patients with residual drug concentrations, 9 (23.7%) had residual level for more than one drug: 1 (2.6%) patient had residual levels of SDX+PYR+CQ, 4 (10.5%) patients had SDX+PYR, 1 (2.6%) patient with SDX+QN+CQ while 3 (7.9%) patients had residual level of SDX+CQ concentration.

The SDX levels were also analysed on day 7 in blood samples of eighty six patients (38 with residual antimalarials and 48 without). The mean ±SD of SDX concentration for these patients was found to be 48.4±16.2 µg/ml (range: 15.3-99.1 µg/ml).

### Table 1: Clinical and demographic characteristics of patients (n=139) enrolled in studies

<table>
<thead>
<tr>
<th>Study Site</th>
<th>Bilaspur (Chhattisgarh)</th>
<th>Betul (Madhya Pradesh)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
<td>(N=70)</td>
<td>(N=69)</td>
</tr>
<tr>
<td>Sex [no. (%)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>44 (62.9)</td>
<td>30 (43.5)</td>
</tr>
<tr>
<td>Female</td>
<td>26 (37.1)</td>
<td>39 (56.5)</td>
</tr>
<tr>
<td>Age category [no. (%)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5 yr</td>
<td>2 (2.9)</td>
<td>3 (4.3)</td>
</tr>
<tr>
<td>5-15 yr</td>
<td>12 (17.1)</td>
<td>42 (60.9)</td>
</tr>
<tr>
<td>Adult</td>
<td>56 (80.0)</td>
<td>24 (34.8)</td>
</tr>
<tr>
<td>Temperature (˚C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>37.8 ± 1.1</td>
<td>37.8 ± 0.1</td>
</tr>
<tr>
<td>Range</td>
<td>36.1-40.6</td>
<td>37.5 - 38.1</td>
</tr>
<tr>
<td>Febrile (≥37.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes [no. (%)]</td>
<td>36 (51.4)</td>
<td>69 (100.0)</td>
</tr>
<tr>
<td>No [no. (%)]</td>
<td>34 (48.6)</td>
<td>-</td>
</tr>
<tr>
<td>Parasite count (no/µl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>233 ± 17820.4</td>
<td>1216. ± 11561.9</td>
</tr>
<tr>
<td>Gametocytes on day 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[no. (%)]</td>
<td>3 (4.3)</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td>Previous antimalarial intake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes [no. (%)]</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>No [no. (%)]</td>
<td>62 (88.6)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Unknown [no. (%)]</td>
<td></td>
<td>8 (11.4)</td>
</tr>
</tbody>
</table>

### Probable time of previous SDX intake

After comparing the whole blood concentration of SDX at baseline (day 0) and on day 7, the back estimation method indicated a median of 30 days prior to study enrolment (range 6-71 days; 90% CI), the most likely time for previous SDX intake. Majority of the patients i.e. 13 (52%) showed previous SDX intake estimated time of more than 28 days.

### CRF information & its correlation with residual antimalarial drugs

History of previous drug intake was collected using case record form (CRF) for each patient. This information was correlated with detected residual antimalarial levels in the patient’s samples. The CRF information showed that 62 (44.6%) patients reported no antimalarial intake, while 77 (55.4%) were not sure about the previous intake. However, HPLC analysis revealed that 38 (27.3%) patients had residual antimalarials in their blood while 101 (72.7%) patients did not have residual antimalarial levels on day 0 before the onset of treatment course (Fig. 3). A weak association was observed between the information provided in CRF about the antimalarials intake and baseline residual antimalarial detected before initiation of the treatment ($\chi^2 = 1.27; P=0.17$).
Residual antimalarials: correlation with parasite densities, age and sex
Parasite density (Mean ± SD) in Pf infected patients was found to be 17804.4 ± 16005.9 asexual parasites/µl (range: 616-74040). Parasite densities were comparable between the patients with residual antimalarial concentrations (n=38) and those with no antimalarials (n=101) {15522.0±13020.1 asexual parasites/µl Vs 18203.2±16733.2 asexual parasites/µl, p<0.05} on day 0. Residual antimalarial levels was found to be negatively correlated with parasite density and statistically non-significant (r=-0.47, p=0.778). The influence of age and sex on the probability of detecting residual antimalarials at baseline showed no significant relationship (X²=23.0, P=0.10).

Table 2: Residual concentration of antimalarial drugs in blood samples of 38 patients from Bilaspur and Betul regions at Day 0, before the onset of treatment

<table>
<thead>
<tr>
<th>Antimalarials</th>
<th>No. of patients</th>
<th>Mean ± SD (ng/ml)</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphadoxine</td>
<td>25 (18)</td>
<td>1541 ± 5300</td>
<td>172.0</td>
<td>100.0</td>
<td>54100.0</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>17 (12)</td>
<td>4.8 ± 0.0</td>
<td>3.0</td>
<td>51.0</td>
<td>263.0</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>5 (3.5)</td>
<td>980 ± 900</td>
<td>100.0</td>
<td>1600.0</td>
<td></td>
</tr>
<tr>
<td>Quinine</td>
<td>1 (0.7)</td>
<td>0.0 ± 0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Mefloquine</td>
<td>1 (0.7)</td>
<td>0.0 ± 0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Parasites clearance time with presence of residual antimalarials (alone/ combinations) and without residual of antimalarial.

<table>
<thead>
<tr>
<th>Previous drug intake(Y/N)</th>
<th>Percentage of patients showing Parasites clearance time in days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Do (0 hr)</td>
</tr>
<tr>
<td>Single drug(n=29)</td>
<td></td>
</tr>
<tr>
<td>SDX(n=16)</td>
<td>0</td>
</tr>
<tr>
<td>CQ(n=12)</td>
<td>0</td>
</tr>
<tr>
<td>MQ(1)</td>
<td>0</td>
</tr>
<tr>
<td>Combination of drug (n=9)</td>
<td></td>
</tr>
<tr>
<td>SDX+PYR(n=4)</td>
<td>0</td>
</tr>
<tr>
<td>SDX+PYR+CQ(n=1)</td>
<td>0</td>
</tr>
</tbody>
</table>

Treatment outcome and follow-up
Among the 139 patients enrolled in this study, 1 patient voluntarily withdrew on day 2, and 2 patients were lost to follow-up on day 7; while, remaining 136 study participants completed the 28-day follow-up. Among them, 120(86.3%) patients cleared their parasitemia in less than 24 hrs, 18(12.9%) cleared between 24 hrs and 48 hrs, while remaining 1(0.7%) cleared between 48 hrs and 72 hrs. 100% parasite clearance was observed in all the patients by day 3. There was no reappearance of parasitemia in these patients during follow-up till day 28, indicating adequate clinical and parasitolotical response (ACPR). Interestingly, On day 2 parasite clearance was observed in the patients detected with residual level of SDX and CQ; complete parasite clearance was observed on day 1 in the patients who had combinations of residual antimalarials; whereas, parasitemia was seen up to day 2 in case of the patients with no residual levels of antimalarials. The percentage of patients showing parasite clearance on day 1 was 81.2 and 83.3 for SDX and CQ respectively (Table 3).

Discussion
To our knowledge this is the first study to investigate the presence of five antimalarials (CQ, SDX, PYR, QN and MQ) in blood of patients from Bilaspur (Chhattisgarh) and Betul (Madhya Pradesh) districts with high Pf malaria before the onset of treatment. Out of these five antimalarial drugs, CQ is currently recommended as first line antimalarial for uncomplicated P. vivax (Pv) malaria cases in the country, while SP is being recommended as partner drug in the recommended ACT for the treatment of uncomplicated Pf malaria throughout the country except in the north-eastern India. In this region, artemether-lumefantrine is the first line of treatment for Pf in the north-east India, as high treatment failure to SP was observed during 2012-13[19]. Quinine is recommended as rescue for case where treatment failure cases to ACT is observed while MQ is advised for long term chemoprophylaxis. The measurement of these five antimalarials allowed a comprehensive assessment of the circulating drugs in the community under study.
Malaria is a major public health problem in India and its dynamics vary from place to place. The forest and tribal areas of Madhya Pradesh and Chhattisgarh situated in central part of India where malaria outbreaks are frequently recorded; its control is logistically difficult reason being the inadequate surveillance, poor reporting, a time-lag in reporting to decision makers and a poor geographic information system that could identify the trouble spots for a timely preventive action. Both Pf and Pv are prevalent species in these states with a preponderance of Pf. Drug resistance is a major problem for control and eradication of malaria. Besides the genetic factors, drug pressure in the community also plays a major role in emergence of drug resistant parasites [12]. To assess in the community also plays a major role in malaria. Besides the genetic factors, drug pressure in reporting to decision makers and a poor geographic information system that could identify the trouble spots for a timely preventive action. Both Pf and Pv are prevalent species in these states with a preponderance of Pf. Drug resistance is a major problem for control and eradication of malaria. Besides the genetic factors, drug pressure in the community also plays a major role in emergence of drug resistant parasites [12]. To assess drug pressure, residual level of antimalarials was monitored by HPLC in the patients enrolled at study sites under therapeutic efficacy monitored at state as well as national level.

We found that out of 139 patients enrolled in our study, 38(27.34%) carried blood residual antimalarials above the lower limit of detection (50 ng/ml) at inclusion. This could be a worrying factor and a possible indication of tolerance to residues which could act as a precursor for the development precipitation of resistance. This is a matter of concern and the issue needs to be carefully monitored at state as well as national level.

**Sulphadoxine-Pyrimethamine (SP)**

SP is currently being used as a partner drug in first line ACT (Artesunate+ Sulphadoxine-Pyrimethamine) for uncomplicated Pf malaria in India since 2010, except in the north-eastern India as mentioned elsewhere. In our study, we found residual level of SDX in blood of 25 (18%) patients on day 0 at a mean concentration of 13.4 µg/ml. This finding corroborates with the results obtained from the study in which residual plasma concentration of antimalarials was detected prior to the treatment[12]. The SDX level in ninety six patients on day 7 was found to be 48.4±16.2 µg/ml. PYR is usually given as a combination therapy with SDX. 5(3.5%) patients in our study were found to have a mean concentration of 0.98 µg/ml of PYR in their blood. PYR is usually given at a lower dose of 1.25 mg/kg in combination with SDX and its half-life is approximately 4 days; than that of SDX which has a half-life of 7.2 days[12, 20]. In present study, the mean concentration obtained for SDX and PYR was comparatively higher than the reported study; but there was no conspicuous difference in the concentration range of the residual antimalarials[12]. There are various factors which might be responsible for such differences the in mean concentration of residual antimalarials: (1) Differences in the Samples (dried blood spots) extraction procedure (2) Instrumentation technique used i.e. they have used liquid chromatography-tandem mass spectrometry which is highly sensitive as compared to HPLC used in the present study. Based on the day 0 and day 7 SDX concentration (C0 and C7), and assuming that the patients with residual SDX in their blood had taken a single dose of SP according to their body weight, we can infer by back estimation method that these patients must have taken SP approximately within one month (30 median days) prior to inclusion in our study [12]. Furthermore, it is also possible that patients might have taken a sub-therapeutic dose or counterfeit form of SP more recently[21].

**Chloroquine (CQ)**

Although, CQ has been withdrawn to be used as first-line of treatment for Pf infection [22]; it was detected in the blood of 17 (12.2%) patients in our study at a mean concentration of 0.172 µg/ml. Hodel et al., 2010 also reported the residual plasma concentration of CQ, MQ and QN before initiating the antimalarials treatment in patients[13]. This might be due to easy availability of CQ in the private clinics and pharmacy shops or due to its wide use in the treatment for a previous episode of Pv malaria. Previous study from our laboratory has reported that CQ is the most common antimalarial sold across the counter with high frequency of prescription in public as well as private health facilities as compared to other antimalarials[10]. As we employed dried blood spot method for sample collection, it was not possible to detect any artemisinin compounds. Also, short half-life of artemisinin requires sophisticated techniques as well as plasma sample collection. However, the presence of residual artemisinin cannot be ruled out as high prescription rate of this antimalarial has been previously observed in the country [10]. Both MQ and QN are not recommended for uncomplicated malaria, but their residual levels were still found to be present in the patients, although in very few samples and that too at low concentrations.

By detecting the presence of residual anti-malarial levels on day 0, it can be concluded that this be due to; (1) Self-medication due to non-availability of reachable drug to the dispensing facility or the practitioner leading to inadequate dosing and there by inability to control infection[12, 13, 23,25] (2) Irrational treatment practices by the physician at the study sites with high transmission intensity of malaria parasites, where all febrile patients were treated with a variety of available antimalarial drugs[26-27], (3) Unawareness regarding the suitable antimalarial drug to be used for treating malaria (4) Treatment of previous episode of infection with antimalarial drug after then reinfection with a new parasite resulting of previously consumed episode of drug as residue in
These factors contribute to increased drug pressure on the parasite thus encouraging resistance in Plasmodium species [30]. With these observations, it can be deduced that the entry criteria based on self-reporting of previous drug intake or information collected in case record forms are not reliable at least in this population. The influence of age and sex on the probability of residual antimalarials at entry showed no significant relationship in our study ($X^2=23.0; P=0.10$) indicating uniform antimalarial prescription or intake behaviour in the population. These estimates are approximate, as indicated by the wide confidence interval explained by the fair degree of inter-individual variability and residual error. The correlation between the parasite density and levels of antimalarial drugs was also studied. We found that 15522 asexual parasites/µl (P<0.05) were present in blood samples with residual antimalarials, while 18203.2 asexual parasites/µl (P<0.05) were present in those without residual antimalarials. This clearly showed that the residual levels of drug in blood were not enough to control parasite replication and to overcome clinical symptoms in the patients, although the parasitemia levels were little lower in the patients with residual antimalarials than without. Here it is difficult to comment whether the residual drug levels were due to the result of full or incomplete treatment and the parasites causing the current episode of infection were from the same or a new infection.

Residual levels of SDX and CQ above MIC were observed in 24% and 82.3 % patients respectively. This shows that the accessibility to CQ was more as compared to SDX and thus people could easily buy CQ for the treatment of malaria episode. Also, earlier presumptive treatment with CQ was practiced in the country to treat febrile episodes of malaria. This suggests that despite drug policy change at the national level and introduction of ACT for treatment of falciparum malaria, CQ is still being widely used for tackling this species.

One hundred and thirty six patients (136) out of a total of 139 recruited in the present study completed the 28-days follow-up after treatment with AS+SP. No microscopically visible parasitemia was observed in any patient on day 3. There was no reappearance of parasites up to 28 days of follow-up. This suggests that AS+SP was 100% efficient in treating uncomplicated Pf malaria in patients and it showed adequate clinical and parasite response (ACPR).

Prior drug intake may have an effect on the current treatment in ways that; it may lead to increased drug exposure from cumulative levels resulting either in better efficacy or more toxicity. The prevalent parasitaemia in the presence of residual antimalarials at the time of patient enrollment suggested that these clones causing the disease were already thriving in the patients and constituted less sensitive population selected by the previous treatment, which may also impact, in addition to precipitation of resistance, on the outcome of the treatment under investigation.

**Conclusion**

The intense parasite transmission and the widespread presence of sub-curative drug levels in blood most likely constitute a predisposing environment for the selection and spread of resistant Pf strains. As resistance gradually expands and intensifies, it is also likely that the drug is taken more frequently and at higher doses. As a consequence, more intense drug pressure will then select for more resistant parasites. The chances of drug resistant parasites to be selected depends on several factors, and is higher for patients with lower immunity (e.g., young children), drugs with long residence times and resistance being conferred through single point mutations, and for infections with a large parasite biomass. In this scenario, the efficiency of treatment outcome also cannot be evaluated effectively as the clearance of parasites may be due to an additive effect of the current intake of treatment drug and the residual drug taken during previous episodes of malaria infection. To minimize the burden of drug resistance in the country, it essentially requires awareness in the community as well as amongst the physicians and paramedical staff on national drug policy for treatment of malaria.

The findings of this study are revealing and must be confirmed in other settings as they have potential implications for both clinical research, surveillance (treatment efficacy and safety outcome) and malaria control policy.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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