Artemisinin combination therapy fails even in the absence of *Plasmodium falciparum kelch13* gene polymorphism in Central India

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Artemisinin is the frontline fast-acting anti-malarial against *P. falciparum*. Emergence and spread of resistant parasite in eastern-India poses a threat to national malaria control programs. Therefore, the objective of our study is to evaluate the artesunate-sulfadoxine-pyrimethamine efficacy in Central India. 180 monoclonal *P. falciparum*-infected patients received standard ASSP therapy during August 2015–January 2017, soon after diagnosis and monitored over next 42-days. Artemisinin-resistance was assessed through in-vivo parasite clearance half-life (PC1/2), ex-vivo ring-stage survivability (RSA), and genome analysis of *kelch13* and other candidate gene (*pfcrt, pfmdr1, pfatpase 6, pfdhfr* and *pfdhps*). Of 180 *P. falciparum* positive patients, 9.5% showed increased PC1/2 (> 5.5 h), among them eleven isolates (6.1%) showed reduced sensitivity to RSA. In 4.4% of cases, parasites were not cleared by 72 h and showed prolonged PC1/2 (5.6 h) (P < 0.005) along with significantly higher RSA (2.2%) than cured patients (0.4%). None of day-3 positive isolates contained the *pfkelch13* mutation implicated in artemisinin resistance. Parasite recrudescence was observed in 5.6% patients, which was associated with triple *dhfr–dhps* (*A16I51R59N108I164–S436G437K540G581T613*) combination mutation. Emergence of reduced sensitivity to artesunate-sulfadoxine-pyrimethamine, in central India highlighted the risk toward spread of resistant parasite across different parts of India. Day-3 positive parasite, featuring the phenotype of artemisinin-resistance without *pfkelch13* mutation, suggested *kelch13*-independent artemisinin-resistance.

Drug resistant *P. falciparum* is one of the major factors for death in malaria. 445,000 deaths and an estimated 216 million confirmed malaria cases—including an increase of about 5 million cases over and above what was recorded in 2015-were reported in 2016⁶. Malaria transmission in India potentially occurs through either *P. falciparum* or *P. vivax* infections¹⁴. In India, 844,558 malaria cases were reported in 2017, of which 529,530 cases were *P. falciparum* positive⁴. North-eastern states and central Indian states contributed 80% of the total cases⁸. Chhattisgarh, one of the states in central India, contributed the second highest malaria incidence in India over the years⁸. National vector borne disease control program (NVBDCP) had launched artemisinin-based combination therapy (ACT) to wipe-out the burden of chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) resistant malaria in 2009⁷–⁹. Success of ACT depends on a combination of fast-acting short half-life artemisinin derivatives with late-acting longer half-life 4-aminoquinolines or antifolates¹⁰. Global mortality and morbidity associated with malaria were considerably reduced after the introduction of ACT, but emergence and subsequent spread of artemisinin-resistant parasites in the Greater Mekong sub-region had seriously threatened the global malaria control and elimination progress¹¹–¹⁴. Artemisinin resistance is characterized by drug failure reflected in slow parasite clearance as assessed by increased in vivo parasite clearance half-life (PC1/2) along with reduced sensitivity of ex-vivo ring-stage parasites to artemisinin¹⁵–¹⁷. Genome based transfection studies proved the
association of artemisinin resistance with kelch13 gene-polymorphism which was directly linked with increased ex-vivo ring-stage survivability and prolonged in-vivo PC1/2 (> 5.5 h)18–21. Previous genome-based reports suggested that polymorphism in pfdhfr, pfdhps, pfmdr1, pfcrt and P. falciparum ferredoxin (pfdf) genes also exhibited potential role on artemisinin resistance12–21. In addition to this, presence of day-3 parasite above 10% of day-0 parasitemia indicated a potential treatment failure. The level of PCR adjusted cure rates after 28 days treatment follow-up below 90% against the WHO recommended first line therapy for uncomplicated P. falciparum called for its reassessment22.

Artesunate-sulfadoxine-pyrimethamine (ASSP) is the drug of choice against P. falciparum in India excepting the north-eastern states8. Declining efficacy of ASSP was previously reported from these states and eastern India26–28. The emergence and spread of partial artemisinin resistant parasites were previously reported from West Bengal and eastern state of India27,28. Partial artemisinin resistance was associated with the failure of partner drugs in combination14,24. Prevalence of mutations in molecular markers (pfdhfr and pfdhps) associated with partner drug resistance (sulfadoxine–pyrimethamine) was previously reported from different parts of India including central India8,29–33. Therefore, the aim of this study was to critically examine the ASSP efficacy through in-vivo, ex-vivo and genome-wide variation studies in central India.

Results

Study population. A total of 1856 febrile patients were screened; of them, 199 patients (10.7%) were detected P. falciparum positive. Mean age of P. falciparum-infected persons was 30.7 years (range 8–69 years). 193 patients (193/199) were identified as monoclonal P. falciparum infection and received standard ASSP therapy. Patient characteristics on enrolment of the study were presented in Table 1. 180 patients (180/193) had successfully completed the 42 day’s follow-up. As we have only considered monoclonal P. falciparum infection, therefore patients with P. vivax infection (254/1856) and polyclonal falciparum (6/199) infections were excluded. A detailed information regarding patient selection (inclusion and exclusion criteria) were presented in Fig. 1.

Parasite clearance phenotype. Based on parasite clearance time (PCT), we classified four different parasite clearance phenotypes. We defined, PCT ≤ 36 h, as rapid-clearing parasite (RCP); PCT > 36– ≤ 48 h as parasite-clearance normal (PCN); PCT > 48– ≤ 72 h as delayed-clearing parasite (DCP); and PCT > 72 h as very slow-clearing parasite (VSCP) (Fig. 2A). We found low median PC1/2 in RCP (2.6 h; 95% CI 2.3–3 h) and PCN phenotype (2.8 h; 95% CI 2.4–3.2 h). We recorded very high median PC1/2 in VSCP phenotype (5.6 h; 95% CI 5.5–5.7 h) and higher PC1/2 in DCP phenotype (4.2 h; 95% CI 3.7–4.8 h) (Fig. 2B). 17 patients (9.5%) showed prolonged PC1/2 (> 5.5 h) (8/52, DCP; and 9/9 VSCP phenotype) (Table 2). We observed a significant statistical difference in PC1/2 among these four parasite phenotypes (Kruskal–Wallis Test, p = 0.0042).

Plasma availability of dihydroartemisinin. We measured artemisinin exposure in patients through detection of plasma DHA. Mean plasma DHA was recorded as 4052 nM, (95% CI 3925.8–4128.1) at 1.5 h and 2137.5 nM, (95% CI 2069.1–2194.8) at 3 h after ASSP exposure. Among 180 patients, 178 patients (98.8%) detected plasma DHA. Mean plasma DHA was recorded as 4052 nM, (95% CI 3925.8–4128.1) at 1.5 h and 2137.5 nM, (95% CI 2069.1–2194.8) at 3 h after ASSP exposure. Among 180 patients, 178 patients (98.8%) detected plasma DHA. Mean plasma DHA was recorded as 4052 nM, (95% CI 3925.8–4128.1) at 1.5 h and 2137.5 nM, (95% CI 2069.1–2194.8) at 3 h after ASSP exposure. Among 180 patients, 178 patients (98.8%) detected plasma DHA.

ACT efficacy. We observed persistence of parasite after 72 h of ASSP exposure in 9 patients (5%), with high median axillary temperature of 38 °C (95% CI 37.8–38.2 °C), corresponding patients also showed prolonged median PC1/2 of 5.6 h (95% CI 5.5–5.7 h). Among them, 8 patients represented adequate plasma DHA level. Of them, 7 patients showed reduced sensitivity to DHA (in vitro) and designated as early ACT failure cases.

We also observed reappearance of infections in 12 patients (6.7%) during 42-days follow-up. After PCR correction through analysing the msp1, msp2, and glurp genes, we further confirmed the existence of 10 (5.5%) true recrudescence cases among 12 parasite reappearance cases. Crude cure-rate (PCR uncorrected) after ASSP therapy was recorded 89.44% (Kaplan–Meier estimate; 95% CI 83.78–93.35) whereas PCR adjusted cure-rate after day 42 was recorded 90.56% (95% CI 85.07–94.23). We found artemether-lumefantrine (AMLF) rescue therapy was successful without any report of treatment failure (Tables 3, 6).

In vitro drug susceptibility. 156 (86.7%) clinical isolates were adapted for their in vitro susceptibility to DHA, sulfadoxine, and pyrimethamine (Table 4). We observed reduced sensitivity to DHA in 11 (7%) isolates. Of those, 7 (77.8%) belonged to VSCP phenotype (mean RSA9–3h = 2.8%; 95% CI 2.4–3.1) and 4 (7.7%) belonged

| Patient characteristics | Bhilai including durg |
|-------------------------|-----------------------|
| Age (year)              | 30.74 (95% CI 17–58)  |
| Sex ratio (women/men)   | 73/107                |
| Axillary temperature on day 0 (°C) | 39.22 °C (95% CI 38.16–40.19) |
| Parasite density (parasite/µL) | 44,152 (95% CI 9632–78,810) |
| Mean hemoglobin (g/dL)  | 12.3 (95% CI 10.2–14.8) |
| Hematocrit              | Male: 47.1% (95% CI 45.8–49.5) Female: 38.8% (95% CI 37.4–41.7) |

Table 1. Patient characteristics on enrolment of the study.
to DCP phenotype (mean RSA(0–3 h) = 1.9%; 95% CI 1.8–2) (Table 4). Lower mean RSA(0–3 h) was recorded in RCP (0.2%; 95% CI 0.1–0.3) and PCN phenotype (0.2%; 95% CI 0.2–0.3), and were highly sensitive to DHA (p = 0.092).

We observed prevalence of SP resistant parasite. 79 (50.6%) clinical isolates were identified as pyrimethamine resistance whereas 72 (46.1%) isolates were sulfadoxine resistant (Supplementary Fig. 1A, Supplementary Table 1). Only 22 (14.1%) isolates were sensitive to pyrimethamine and 36 (23.1%) were sensitive to sulfadoxine.

Two microscopists showed good conformity during assessment of slides (Pearson correlation r = 0.81, p < 0.005).

Parasite genetic architecture in relation to parasite clearance outcome. Only 9 isolates (5%) represented kelch13 mutation (Table 2). Substitution of alanine to valine at codon 675 was observed in 3 (1.7%) isolates (GenBank-N534312-N534314) (Supplementary Fig. 1B), corresponding isolates showed substantially higher RSA(0–3 h) (mean, 2.1%; 95% CI 1.9–2.3) (Table 2). Of those, one patient showed PC1/2 of 5.3 h while the remaining represented PC1/2 of 4.6 h and 4.9 h, respectively. Of whom, 1 was further developed recrudescence (Table 2). Isolates representing kelch13-A675V mutation strongly associated with reduced ex-vivo artemisinin-sensitivity but not showing any relationship in treatment outcome (P = 0.7, Kruskal–Wallis test) (Supplementary Fig. 1C). Another 6 (3.3%) patients contained N29L polymorphism, representing lesser PC1/2 (2.6 h, 95% CI 2.4–2.8 h) along with substantially lower RSA(0–3 h) (0.2%; 95% CI 0.1–0.2). Surprisingly, we observed the prevalence (16/17) of prolonged PC1/2 (> 5 h) related with wild pfkelch13 genotype (Table 2).

We found 11 patients (6.1%) with A623E mutation in pfatpase6 gene (Supplementary Fig. 1B). Of those, two isolates showed PC1/2 > 5.5 h. One of the corresponding isolates presented RSA(0–3 h) of 3.3% while others failed to

Figure 1. Schematic presentation of patient selection and entry criteria for randomization with ASSP. Monoclonal P. falciparum infections contained a single allelic form of infection i.e. either of msp1 or mspII or glurp. Polyclonal infections along with Pf, Pv mixed infections were excluded. Only P. falciparum monoclonal infections were selected for the study. Patients with additional P. vivax co-infection during the follow-up schedule were excluded. Patients, who had not completed the 42 days follow-up schedule, were eliminated.
On the day of parasite recurrence, all the LTFs received a standard dose of AMLF and all showed treatment success. Corresponding isolates showed prolonged PC1/2 (mean 5.6 h, 95% CI 5.5–5.7) which were significantly higher than the cured patients (mean-0.4%, 95% CI 0.4–0.5) (Table 2). Isolates containing polymorphism in pfkelch13 (N292L, K447E, and A675V) and pfatpase6 gene (E431K, and L452S) represented low to moderate PC1/2 (3.8–4.3 h) but not associated with early ASSP failure (ETF) (P = 0.74, Kruskal–Wallis test).

We observed prevalence of pfmdr1-N86Y (65%) and Y184F (50%) mutation (Supplementary Fig. 1D). A total of 48 patients represented N86Y mutation (Supplementary Table 1). Another 5 isolates (2.8%) contained pfatpase6-E431K polymorphism (L263K431A623A630S769), one of those isolates showed PC1/2 of 5.1 h (Table 2). Among 5 isolates, three were culture adapted and all were found sensitive to DHA (0.4%; 95% CI 0.4–0.5) (Table 2). Isolates containing polymorphism in pfkelch13 (N292L, K447E, and A675V) and pfatpase6 gene (E431K, and L452S) represented low to moderate PC1/2 (3.8–4.3 h) but not associated with early ASSP failure (ETF) (P = 0.74, Kruskal–Wallis test).

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**Molecular cause of day-3 positive parasite.** We identified 9 day-3 positive cases (Table 5). Of these, 8 represented adequate plasma DHA (mean 2037.2 nmol/L, 95% CI 1960–2180) after 3 h of ASSP exposure, corresponding isolates showed prolonged PC1/2 (mean 5.6 h, 95% CI 5.5–5.7) which were significantly higher as compared to PC1/2 (3.2 h; 95% CI 2.8–3.6) of cured patients (P < 0.005, Mann–Whitney U-test). Of whom 7 represented significantly higher RSA (mean-2.2%, 95% CI 1.7–2.8) than the cured patients (mean-0.4%; 95% CI 0.2–0.6). Perhaps these isolates were early ASSP failure cases. The mean axillary temperature [37.9 °C (95% CI 37.6–38.2 °C)] at day three was found very high in those 9 day-3 positive cases. Presence of parasites on day-3 represented significantly higher RSA (mean-2.2%, 95% CI; 1.7–2.8) than the cured patients (mean-0.4%; 95% CI 0.4–1.3) but were not associated with early ASSP failure (P = 0.68, Kruskal–Wallis test).

**Molecular characterization of parasite recrudescence.** After PCR corrections, we identified 10 cases of recrudescence. The mean parasite load on the day of recrudescence was found 8285/µl (95% CI 2726–13,843). The mean body temperature on the day of recrudescence was very high 38.6 °C (95% CI 38.2–39.0 °C). We observed 3 cases of parasite recrudescence within day-7 and another 7 cases between day-8 to day 42, corresponding isolates represented significantly higher IC50 for sulfadoxine and pyrimethamine. Parasite recrudescence was the true cause of late ASSP failure. We noticed combination of triple dhfr mutations (A16N51R59N108I164–A16N51R59N108I164–A675V, A16N51R59N108I164–A16N51R59N108I164–A436G437K540A581A613, and A675V-R59N108I164–A675V-R59N108I164–A16N51R59N108I164–A675V-R59N108I164) mutations associated with delayed-clearing parasite (PCT > 48–72 h) (P < 0.02), corresponding isolates showed moderate RSA (0.3–3.6) (0.78%, 95% CI 0.4–1.3) but were not associated with early ASSP failure (P = 0.68, Kruskal–Wallis test).

**Discussion**

Emergence and spreading of partial artemisinin resistant parasites in eastern India along with late ACT failures in north-east India called for a systematic screening of ASSP in central India, as the second highest number of malarial infections was reported from the state of Chhattisgarh. We found the day-3 positive parasite with prolonged parasite clearance half-life (> 5.5 h), along with recrudescence cases. 9.4% of patients showed late ASSP failure.
A

Parasite density before drug administration (in %)

Parasite clearance time

- Rapid clearing parasite
- Parasite clearance normal
- Delayed clearing parasite
- Very slow clearing parasite

B

Different parasite group depending on parasite clearance time

Percentage (%) of different parasite phenotype

Parasite clearance half-life (in hour)

RCP, PCN, DCP, VSCP
Table 2. Distribution of different candidate genotype in relation to ASSP combination treatment. Underlined codons are mutant codons. ETF and LTF stand for early treatment failure and late treatment failure, respectively, whereas S, and RS stand for sensitive, and reduced sensitivity, respectively. Parasite clearance half-life was denoted as PC_{1/2}. *Patients did not present adequate plasma dihydroartemisinin concentration, therefore not considered as true day-3 positive case.

| No of isolates | kelch 13 | ATPase6 (260 + 431 + 623 + 630 + 769) | Pfmdr1 (86 + 184 + 1034 + 1042 + 1246) | PfLfsr (16 + 51 + 59 + 180 + 164) | Pfdhps (436 + 437 + 540 + 581 + 613) | Pfdhfr (72 + 76 + 326 + 356) | PC_{1/2} | Parasite clearance time | Recrudescence | Ex-vivo AS (RSA) sensitivity |
|----------------|----------|-------------------------------------|-----------------------------------|---------------------------------|---------------------------------|---------------------------------|----------|---------------------|---------------|-----------------------------|
| 21             | Wild     | LEAAS                               | NYSDND                            | ANC81                           | SAKAA                           | CVMNKNI                         | 21       | <5 h                | ETF (LTF)    | S                                           |
| 11             | Wild     | LEAAS                               | YYSND                            | ANC81                           | SAKAA                           | CVMNKNI                         | 11       | 7 h                 | S             | 18                                          |
| 6              | Wild     | LEAAS                               | NFSND                            | AICN81                          | SAKGA                           | SVMNTSI                         | 6        | 2 h                 | S             | 10                                          |
| 7              | Wild     | LEAAS                               | YYSND                            | ANC81                           | SAKAA                           | SVMNTSI                         | 7        | 4 h                 | S             | 4                                           |
| 24             | Wild     | LEAAS                               | YYSND                            | ANC81                           | SAKAA                           | SVMNTSI                         | 23       | 4 h                 | S             | 14                                          |
| 3              | Wild     | LEAAS                               | AICN81                           | SAKGA                           | SVMNTSI                         | 3                                | 1        | 2 h                 | S             | 3                                           |
| 17             | Wild     | LEAAS                               | YYSND                            | AICN81                          | SGKAA                           | SVMNTSI                         | 17       | 1 h                 | S             | 16                                          |
| 6              | N29L     | LEAAS                               | NFSND                            | AICN81                          | SGKAA                           | SVMNTSI                         | 6        | 1 h                 | S             | 6                                           |
| 10             | Wild     | LEAAS                               | YYSND                            | ANC81                           | SGKGT                           | SVMNTSI                         | 8        | 1 h                 | S             | 6                                           |
| 3              | Wild     | LEAAS                               | NFSND                            | AICN81                          | SGKAA                           | SVMNTSI                         | 3        | 1 h                 | S             | 6                                           |
| 5              | Wild     | LEAAS                               | NFSND                            | ANC81                           | SGKAA                           | SVMNTSI                         | 5        | 1 h                 | S             | 6                                           |
| 5              | Wild     | LEAAS                               | YYSND                            | ANC81                           | SGKAA                           | SVMNTSI                         | 4        | 1 h                 | S             | 6                                           |
| 21             | Wild     | LEAAS                               | YESND                            | AICN81                          | SGKGT                           | SVMNTSI                         | 15       | 1 h                 | S             | 15                                          |
| 8              | Wild     | LEAAS                               | YESND                            | AICN81                          | SGKGT                           | SVMNTSI                         | 6        | 1 h                 | S             | 6                                           |
| 3              | A675V    | LEAAS                               | NFSND                            | ANC81                           | SGKAA                           | SVMNTSI                         | 2        | 1 h                 | S             | 3                                           |
| 14             | Wild     | LEAAS                               | YESND                            | ANC81                           | SGKGT                           | SVMNTSI                         | 11       | 2 h                 | S             | 10                                          |
### Table 3. Summary of treatment After ASSP therapy. ACPR, ETF and LTF stand for adequate clinical parasitological response, early treatment failure and late treatment failure, respectively, whereas ASSP stand for artemisinin-sulfadoxine-pyrimethamine. *One patient did not attain the adequate plasma DHA concentration and in another patient, we failed to adapt the in vitro culture for RSA assay.* Initially reappearance of infection was observed in 12 patients (6.7%), but analyses of *msp1*, *msp2*, and *glurp* gene confirmed that among those 12 patients, 10 (5.5%) were true recrudescence (LTF) case.

| Case  | parasite load on day-0 (µL) | parasite load on day-3 (µL) | Fever on day 0 (°C) | Fever on day 3 (°C) | RSA (0–3 h) (mean) | IC₅₀ DHA (mean) | IC₅₀ pyrimethamine (mean) | IC₅₀ sulfadoxine (mean) |
|-------|---------------------------|-----------------------------|---------------------|-------------------|-------------------|-----------------|-----------------|-----------------|
| 1     | 4023                      | 2361                        | 19,368              | 59.4              | 0.17% (95% CI 0.14–0.25) | 45/45          | 51.5 (95% CI 43.7–59.4) | 206.1 (95% CI 185–229) |
| 2     | 4196                      | 2147                        | 8432                | 38.5              | 0.23% (95% CI 0.15–0.32) | 62/62          | 78.4 (95% CI 71.6–85.3) | 189.4 (95% CI 172–207) |
| 3     | 3833                      | 2051                        | 21,641              | 38.7              | 1.23% (95% CI 1.13–2.08) | 37/41          | 48.7 (95% CI 43.9–53.5) | 261.5 (95% CI 239–284) |
| 4     | 4077                      | 1952                        | 70,846              | 38.4              | 1.92% (95% CI 1.83–2.02) | 8/62           | 49.2 (95% CI 43.6–54.8) | 240.5 (95% CI 218–263) |
| 5     | 4086                      | 2052                        | 56,855              | 38.7              | 2.76% (95% CI 2.43–3.09) | 7/8            | 80.4 (95% CI 75.8–85.1) | 408.1 (95% CI 372–446) |
| 6     | 3981                      | 2114                        | 11,790              | 38.3              | 7.27% (95% CI 6.23–8.31) | 4/3            | 1887.45 (95% CI 1812.1–1963) | 28/62 (95% CI 25–31) |
| 7     | 3591                      | 1805                        | 44,181              | 38.9              | 5.5% (95% CI 4.6–6.4) | 16/17          | 608.17 (95% CI 570–646) | 364.0 (95% CI 330–398) |
| 8     | 955                       | 548                         | 9716                | 38.4              | 10% (95% CI 8.8–11.2) | 23/24          | 1887.45 (95% CI 1812.1–1963) | 28/62 (95% CI 25–31) |
| 9     | 4258                      | 2104                        | 36,847              | 39.5              | 68.42 ± 2.1          | 43/44          | 1920.5 (95% CI 1882.7–1958) | 12/45 (95% CI 11–13) |

### Table 4. In vitro drug susceptibility in different parasite phenotype. Individual ex-vivo ring stage survivability of parasite isolates was represented as RSA. Here S, IR, R and RS stand for Sensitive, Intermittent resistant, Resistant, and Reduced susceptibility, respectively. Parasite clearance time was denoted as PCT and Parasite clearance half-life was denoted as PC₁/₂.

| Different parasite phenotype | PC₁/₂ > 3 h | Culture adaptation | RSA (0–3 h) (mean) | IC₅₀ DHA (mean) | IC₅₀ pyrimethamine (mean) | IC₅₀ sulfadoxine (mean) |
|-----------------------------|-------------|--------------------|--------------------|-----------------|--------------------------|------------------------|
| Rapid clearing parasite (PCT ≤ 36 h) | 0/47 | 45/47 | 0.19% (95% CI 0.14–0.25) | 45/45 | 72.43 (95% CI 55.8–88.4) | 12/45 |
| Parasite clearance normal (PCT > 36 h ≤ 48 h) | 0/72 | 62/72 | 0.23% (95% CI 0.15–0.32) | 62/62 | 77.21 (95% CI 64.5–89.4) | 8/62 |
| Delayed clearing parasite (PCT > 48 ≤ 72 h) | 8/52 | 41/52 | 1.92% (95% CI 1.83–2.02) | 37/41 | 88.9 (95% CI 88–90) | 4/21 |
| Very slow clearing parasite PCT > 72 h | 9/9 | 8/9 | 2.76% (95% CI 2.43–3.09) | 7/8 | 1887.45 | 27/41 |

### Table 5. Distribution of parasite phenotype and genotypes in day-3 positive parasites. PCT represented Parasite clearance time (in h) and parasite clearance half-life was denoted as PC₁/₂ (in h). We have presented parasite density as PD. Individual ex-vivo ring stage survivability of parasite isolates was represented as RSA (0–3 h). Underline codons were mutant codon. In case 8, RSA (0–3 h) was recorded 0.94% with PC₁/₂ of 5.7 h while case 7 was failed to culture adaptation. The day-3 parasite load was ranging from 1 to 1.4% from that of day-0 parasitaemia.
Table 6. Phenotypic and genotypic characteristics of late treatment failure and evaluation of AMLF rescue therapy. Underlined codons are mutant codons. ACPR, ETF and LTF respectively stand for adequate clinical parasitological response, early treatment failure and late treatment failure, whereas ASSP stand for artesunate-sulfadoxine-pyrimethamine and AMLF stands for artemether-lumefantrine. Parasite load was expressed as number of parasite/ micro liter of blood. *After PCR correction, case 9 and case 11 were identified as the case of parasite re-infection.

| Case | Drug | Day of recurrence | Parasite load on recurrence | Fever (°C) | PCR correction | pfk13 allele | pfldhfr allele | pfldhps allele | Rescue therapy with AMLF |
|------|------|-------------------|----------------------------|------------|----------------|--------------|---------------|----------------|--------------------------|
|      |      |                   |                            |            |                |              |               |                | ACPR | ETF | LTF |
| Case 1 | ASSP | 5                 | 3642/µL                    | 38.6       | Recrudescence  | Wild         | AICNI         | AGKAA          |                  |
| Case 2 | ASSP | 7                 | 5082/µL                    | 38         | Recrudescence  | Wild         | ANRNI         | AGKAA          |                  |
| Case 3 | ASSP | 21                | 27,419/µL                  | 39.1       | Recrudescence  | N29L         | AICNI         | AGKAA          |                  |
| Case 4 | ASSP | 11                | 11,026/µL                  | 39.4       | Recrudescence  | Wild         | AICNI         | AGKAA          |                  |
| Case 5 | ASSP | 7                 | 4957/µL                    | 38.4       | Recrudescence  | A675V        | ANRNI         | AGKAA          |                  |
| Case 6 | ASSP | 28                | 570/µL                     | 37.9       | Recrudescence  | Wild         | ANRNI         | SGKGT          |                  |
| Case 7 | ASSP | 35                | 7350/µL                    | 38.1       | Recrudescence  | Wild         | ANRNI         | SGKGT          |                  |
| Case 8 | ASSP | 32                | 512/µL                     | 38.3       | Recrudescence  | Wild         | ANRNI         | SGKGT          |                  |
| Case 9 | ASSP | 28                | 51,154/µL                  | 40.1       | Re-infection   | Wild         | AIRNI         | SGKGT          |                  |
| Case 10 | ASSP | 21                | 16,822/µL                  | 39.3       | Recrudescence  | Wild         | ANRNI         | AGKAA          |                  |
| Case 11 | ASSP | 35                | 3092/µL                    | 38         | Re-infection   | Wild         | AIRNI         | SGKGT          |                  |
| Case 12 | ASSP | 14                | 5168/µL                    | 38.8       | Recrudescence  | Wild         | AIRNI         | SGKGT          |                  |

Figure 3. In vitro IC$_{50}$ of pyrimethamine and sulfadoxine in relation to individual dhfr-dhps genotype: Here PYR and SDX respectively stand for “pyrimethamine, and sulfadoxine.” The blue line (corresponding to 2000 nM of PYR) represented the in vitro PYR resistance, while the red line (corresponding to 3000 nM of SDX) represented the in vitro SDX resistance. We observed prevalence of SP resistant parasites. The isolates presenting triple dhfr and dhps mutation (AIRNI-SGKGT; ANRNI-SGKGT) and double dhfr and dhps combination mutation (AICNI-AGKAA) represented very high IC$_{50}$ for pyrimethamine and sulfadoxine and proved to be highly resistant to PYR and SDX (P < 0.01). Isolates contained ANRNI-AGKAA, AICNI-SGKAA, and ANRNI-AAKAA mutations exhibited moderate to high IC$_{50}$ for pyrimethamine and sulfadoxine but never connected with recrudescence (P = 0.73). Pyrimethamine sensitive and sulfadoxine resistant 3D7 strain was used as a control strain.
prolonged PC_{1/2} (> 5.5 h) which was an alarming sign. Prolonged parasite clearance was an indicator of decreased efficacy of fast-acting artemisinin within ACT which perhaps led to ACT failure.\textsuperscript{11,16,19} We observed the median PC_{1/2} of very slow-clearing parasite (VSCP) (5.6 h) and delayed-clearing parasite (4.2 h) phenotype were significantly higher from the PC_{1/2} of Thailand–Myanmar border (3.7 h) and lower from Western Cambodia (5.9 h).\textsuperscript{13,14} Identification of these VSCP parasite phenotypes, confirmed the emergence of parasites that became less sensitive to artemisinin in vivo.\textsuperscript{12} In spite of this, we also observed isolates with reduced sensitivity to DHA in vitro. The corresponding isolates of VSCP phenotype (5%) represented higher RSA_{0–3 h} (2.2%) than the cured patients (0.46%), which proved those VSCP phenotypes were less sensitive to artemisinin in vitro. Previous reports from Thailand suggested that increased viability of ring-stage parasites (RSA_{0–3 h} > 1%) was strongly associated with elevated PC_{1/2}.\textsuperscript{13} Recent reports suggested that apart from reduced efficacy of fast-acting artemisinin drugs, failure in late-acting combinations also contributes towards the increment of PC_{1/2}.\textsuperscript{14} Thus, the reduced artemisinin sensitivity along with elevated PC_{1/2} of the ring stage parasite resulted in reduced sensitivity to ASSP combination therapy. However, most studies in India reported no evidence of less susceptibility to artemisinin in vivo or in vitro.\textsuperscript{14–16} Some recent studies reported the spreading of parasites with reduced sensitivity to artemisinin in vivo in eastern India.\textsuperscript{27,28} We have identified 9, day-3 positive cases; among them, 7 (3.9%) patients were confirmed as ETF whereas 10 (5.6%) patients were identified as true recrudescence (LTF) cases (Table 2). Given the results of our study, although the numbers of treatment failure cases were not very high, we confirmed the emergence of reduced susceptibility to ASSP combination in this part of India. Nevertheless, findings of reduced efficacy of ASSP in Central India suggested the possibility of emergence of resistant parasites in the near future.

ETF generally occurs due to reduced efficacy of fast-acting artemisinin in combination therapy.\textsuperscript{18} The mechanism behind the reduced susceptibility to artemisinin is not fully clear, but genome wide analyses, \textit{ex-vivo} RSA, and transfection studies suggested that artemisinin resistance is predominantly related to \textit{pfkelch13} gene polymorphism.\textsuperscript{13,14,21} Interestingly, predominance of wild \textit{pfkelch13} allele was observed in this parasite population of Central India. Despite the direct correlation with \textit{pfkelch13} polymorphism, we had identified 7 (3.9%) ETF cases with prolonged PC_{1/2} as well as increased ex-vivo RSA_{0–3 h} survivability, without \textit{pfkelch13} polymorphism. However, the findings of our study were quite uncommon. Our observations from this study suggest several novel aspects of artemisinin-resistance. Firstly, the \textit{kelch13}-independent artemisinin-resistance which was previous reported only in Thailand.\textsuperscript{24,37,38} Although, we identified 3 isolates with \textit{pfkelch13}-A675V polymorphism which showed positive association with increased ex-vivo RSA_{0–3 h} but not with treatment outcome like previous reports from in north-east India and southern Rwanda.\textsuperscript{39,40} The results do not represent outliers, as we studied a statistically valid number of patients. Secondly, the absence of \textit{pfkelch13} polymorphism suggests definitive roles for other genetic factors in reduced artemisinin-sensitivity and emergence of artemisinin-resistance. Of note, polymorphism in \textit{Ca}-ATPase6 gene (\textit{pfatpase6}) had some role in reducing susceptibility to artemisinin.\textsuperscript{42} However, the likelihood of this mechanism has been debated.\textsuperscript{43} Like the observations from Thai-Cambodian border,\textsuperscript{24} we have not so far found any definitive correlation between \textit{pfatpase6} mutation and ASSP efficacy in India. Thirdly, \textit{pfcrt}, \textit{pfmdr}, and \textit{pfld} polymorphisms plausibly represent the genetic background required for the onset of \textit{pfkelch13} polymorphism, as mutations in those genes showed strong association with the beginning of \textit{pfkelch13} mutation during selection of artemisinin-resistance.\textsuperscript{18,22,23,27,28} Indeed, in our study, day-3 positive cases demonstrated acquired mutations in \textit{pfcrt} (K76T, I356T) and \textit{pfmdr1} (N86Y, Y184F) genes similar to the findings reported in eastern and north-India.\textsuperscript{27,37} A recent report showed \textit{P. falciparum} strains in South-East Asia having some genetic attenuation to develop novel mutations that caused artemisinin-resistance.\textsuperscript{42} Fourthly, the precise genetic architectures in relation to reduced artemisinin-sensitivity in the East, North-East, South-West, and Central India are plausibly different owing to extensive variations in socio-demographic, environmental, seasonal and parasite-vecor relationships.\textsuperscript{36,27,39}

We detected 10 recrudescence cases in our study. The clinical manifestations of recrudescence were related with less susceptibility of longer acting partner drug (SP), as evidence, parasite isolates showed acquired combination mutation in \textit{pfdhfr} – \textit{pfdhps} gene.\textsuperscript{27,38} Isolates representing sextuple or quintuple \textit{dhfr} – \textit{dhps} combination mutations (\textit{A}_{108}N, \textit{R}_{530}S, \textit{N}_{106}S, \textit{G}_{146}S, \textit{S}_{436}G, \textit{G}_{540}S, \textit{T}_{613}), \textit{A}_{108}N, \textit{R}_{530}S, \textit{N}_{106}S, \textit{G}_{146}S, \textit{S}_{436}G, \textit{G}_{540}S, \textit{A}_{581}A, \textit{T}_{613} and \textit{A}_{108}N, \textit{R}_{530}S, \textit{N}_{106}S, \textit{G}_{146}S, \textit{S}_{436}G, \textit{G}_{540}S, \textit{A}_{581}A, \textit{A}_{613}) exhibited very high IC_{50}s for pyrimethamine and sulfadoxine, proving true resistance towards SP. Quadruple \textit{dhfr} – \textit{dhps} combination mutations with reduced SP sensitivity was previously reported from Chhattisgarh.\textsuperscript{44} Despite the high prevalence of molecular markers associated with SP resistance, treatment failure rate especially LTFs were much less in number. The sensitivity of artemisinine over this parasite population was still very high. Artemisinin derivatives within the ASSP were therefore able to kill most of the parasite and reduce the burden of partner drugs. That is how all treatment failure cases recovered after AMLF therapy suggesting that a standard six-dose-AMLF could be a potential second-line treatment against three-dose ASSP failure.

In conclusion, emergence of reduced sensitivity to artemisinin and predominance of SP resistant parasites suggested us for the evaluation of national drug policy as artemether–lumefantrine could be successor of ASSP. Finally, a National Malaria Eradication Program requires urgently for centralized and synchronized implementation of new drug-combinations and tracking of genetic mutations that might lead to higher level of resistance to artemisinin and its partner drug.

**Methods**

**Study population.** We conducted the study at Bhilai (21.21° N, 81.38° E) and Durg (21.19° N, 81.28° E), districts of Chhattisgarh, India during August 2015–January 2017. Chhattisgarh had contributed the second highest malaria incidence in 2014.\textsuperscript{9} The inclusion criteria were age ≥ 3 years, axillary temperature ≥ 37.5 °C, shivering, headache during the past two days or more, and no recent history of anti-malarial medication. All participants were screened for \textit{P. falciparum} infection by microscopic examination of Giemsa-stained thick and
thin blood smears. Quantification of parasitaemia was performed by counting the number of parasites per 8000 WBC. We usually count at least 200 fields using 100× oil immersion objectives. Every field generally comprises 4–5 WBCs. Parasites/µl blood was determined as (parasites/WBCs) × 8000 (WBC count/µl of blood). The minimum detectable parasitaemia was 40 parasites/µl of blood. *pfmspI* (MAD20 and K1) and *pfmspII* (3D7 and FC27) alleles were screened to detect the clonality of infection. Patients having signs and symptoms of severe malaria, pregnant women, infants and poly-clonal *P. falciparum* infections were excluded. The experimental design and protocols were duly approved by Vidyasagar University, Human ethical committee (VU/HEC 15-0051). We strictly followed the WHO and WWARN (World Wide Anti-malarial Resistance Network) guideline along with the Helsinki protocol. We obtained duly signed informed consents from each patient and patient’s guardian for minor (child patient) (Fig. 1 for patient selection details).

**ASSP efficacy.** Those patients who fulfilled the inclusion criteria received the quality assured standard ASSP dose (supplied by Ministry of Health and Family-Welfare) of 4 mg/kg body weight artesunate once daily for 3 days and a single dose of 25 mg/kg body weight sulfadoxine along with 1.25 mg/kg bodyweight pyrimethamine on first day, under the supervision of medical officer. Trained microscopists checked the thin blood smears at an interval of 6 h, until the patients became parasite free. We estimated the PC_{50} by parasite clearance estimator. Patients who developed renal failure and those who vomited the drug were withdrawn from the study and sent for further care. We performed the follow-up evaluations on day 7, 14, 21, 28, 35, and 42 days after initial therapy, and responses were classified accordingly. We performed unscheduled follow-up when symptoms of malaria reappear. Patients, not responding to ASSP, received artemether-lumefantrine rescue therapy (6 tablets, each containing 40 mg AM and 240 mg LF) and were further followed-up for next 42 days.

**Plasma artemisinin testing.** We collected 500 µl of intra-venous blood just before, at 1.5 h and 3 h (± 30 min) after initiation of ASSP therapy. We obtained plasma samples immediately and stored at –20 °C. We evaluated the plasma dihydro-artemisinin (DHA) to validate the sporadic drug exposure by liquid chromatography as stated previously.

**In vitro drug sensitivity testing.** We adapted *P. falciparum* clinical isolates in vitro as described previously. After culture adaptation parasites were allowed to proliferate for 72 h before doing the antimalarial drug (sulfadoxine, pyrimethamine, and chloroquine) exposure. We performed sensitivity testing of anti-malarial by hypoxanthine incorporation assay in triplicate, according to our standard laboratory protocol. As following the standard guideline, we defined criteria for sulfadoxine, IC_{50}< 640 nM, susceptible; IC_{50}> 640– ≤ 3000 nM, intermediate; and IC_{50}> 3000 nM, resistant. For pyrimethamine, we defined IC_{50}< 100 nM, susceptible;  IC_{50}> 100– ≤ 2000 nM, intermediate; and  IC_{50}> 2000 nM, resistant. For chloroquine, we defined IC_{50}< 100 nM, susceptible; and IC_{50}> 100 nM, resistant. We used pyrimethamine sensitive and sulfadoxine resistant 3D7 strain as quality-control strain.

**Ring-stage survival testing.** We performed ring-stage survival (RSA) assay in triplicate after culture adaption of clinical isolates as described earlier. We treated 0–3 h post-invasive, highly synchronized early ring-stage parasites with 700 nMoles of dihydro-artemisinin for 6 h, followed by washing with RPMI-1640 for three times and further cultivated for another 6 h. We measured parasite survival rates by microscopic examination of 10,000 RBCs per treatment replicate in Giemsa-stained thin blood smears.

**DNA extraction and sequencing.** We extracted parasite DNA from frozen blood aliquots (200 µl) using Mini blood-kit (Qiagen). We performed nested PCR to amplify pfkelch13 gene using Kelch13-F and Kelch13-R primers according to our standard laboratory protocol. We also performed nested PCRs to amplify pfATPase6, pfdhfr, pfhdps, pfmdr1, and pfcr gene as described previously. For pfATPase6 gene amplifications, we used primer-pairs (5’TGGTTAATAAACTCCCG3’ and 5’TATCTCCTTAG-CACCACCC3; for 250–500 codon; 5’AAGAAGATATAATCCAGA2’ and 5’AAATAACGTATA-CCAGCC3’; for 520–800 codon). We sequenced the amplicons directly using the BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems), and were run on 3730xl genetic analyzer. We used online translation tool (http://www.expasy.org) to translate the sequences. We queried single nucleotide polymorphism of individual sequences by using a nucleotide database with BLAST.

**Statistical analysis.** We expressed our data as a univariate median; mean ± SEM. Fisher’s exact test along with regression analyses were performed to correlate the treatment efficacies with molecular genotyping. We used the Clopper-Pearson method to calculate the 95% confidence intervals. Data were compared between two groups by Mann–Whitney U-test while Kruskal–Wallis-test was used to compare among more than two groups. We considered p < 0.05 statistically significant. All statistical analyses were performed through Graph Pad in-Stat 3.0 and Origin 6.1.

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Author contributions
S.S.D., B.S. and A.K.H. contributed to the designing and conceptualization of the manuscript. A.K.H. and S.D. supervised in vivo ASSP therapy. In vivo data analysis and writing of the article were done by S.D., S.M. (Sayan-tani Mandal) and A.K.H. A.K., S.M. (Subhankar Manna) and S.R.M. (Samaresh Mandal) analysed the in vitro data and wrote the article. S.S.D. and A.K. contributed to genome analysis and interpretation of the data. S.S.D., A.K.H. and B.S. drafted the manuscript. All authors have approved the final version of the manuscript.

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