High prevalence of Pf dhfr–Pfdhps quadruple mutations associated with sulfadoxine–pyrimethamine resistance in Plasmodium falciparum isolates from Bioko Island, Equatorial Guinea

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Abstract

Background: Sulfadoxine–pyrimethamine (SP) is recommended for intermittent preventive treatment of malaria in Africa. However, increasing SP resistance (SPR) affects the therapeutic efficacy of the SP. As molecular markers, Pf dhfr (dihydrofolate reductase) and Pf dhps (dihydropteroate synthase) genes are widely used for SPR surveillance. This study aimed to assess the prevalence of Pf dhfr and Pf dhps genes mutations and haplotypes in Plasmodium falciparum isolates collected from Bioko Island, Equatorial Guinea (EG).

Methods: In total, 180 samples were collected in 2013–2014. The single nucleotide polymorphisms (SNPs) of the Pf dhfr and Pf dhps genes were identified with nested PCR and Sanger sequencing. The genotypes and linkage disequilibrium (LD) tests were also analysed.

Results: Sequences of Pf dhfr and Pf dhps genes were obtained from 92.78% (167/180) and 87.78% (158/180) of the samples, respectively. For Pf dhfr, 97.60% (163/167), 87.43% (146/167) and 97.01% (162/167) of the samples carried N51I, C59R and S108N mutant alleles, respectively. The prevalence of the Pf dhps S436A, A437G, K540E, A581G, and A613S mutations were observed in 20.25% (32/158), 90.51% (143/158), 5.06% (8/158), 0.63% (1/158), and 3.16% (5/158) of the samples, respectively. In total, 3 unique haplotypes at the Pf dhfr locus and 8 haplotypes at the Pf dhps locus were identified. A triple mutation (CIRNI) in Pf dhfr was the most prevalent haplotype (86.83%), and a single mutant haplotype (SGKAA; 62.66%) was predominant in Pf dhps. A total of 130 isolates with 12 unique haplotypes were found in the Pf dhfr and Pf dhps combined haplotypes, 65.38% (85/130) of them carried quadruple allele combinations (CIRNI-SGKAA), whereas only one isolate (0.77%, 1/130) was found to carry the wild-type (CNCSI-SAKAA). For LD analysis, the Pf dhfr N51I was significantly associated with the Pf dhps A437G (P < 0.05).

Conclusion: Bioko Island possesses a high prevalence of the Pf dhfr triple mutation (CIRNI) and Pf dhps single mutation (SGKAA), which will undermine the pharmaceutical effect of SP for malaria treatment strategies. To avoid an
Background
Malaria is a major global public health concern particularly in sub-Saharan Africa, with 219 million cases of malaria and approximately 435,000 deaths in 2017 [1]. Most of the severe clinical cases and deaths were caused by Plasmodium falciparum. Furthermore, pregnant women and children under 5 years old are the main victims of falciparum malaria. To alleviate the global malaria burden in a susceptible population, sulfadoxine–pyrimethamine (SP) is recommended by the World Health Organization (WHO) for use as intermittent preventive treatment in pregnant women (IPTp) and infants (IPTi) in malaria-endemic regions [2].

Equatorial Guinea (EG) is a hyperendemic area of year-round malaria transmission [3], and the population is more frequently exposed to episodes of malaria [4]. Recent studies demonstrated P. falciparum parasites are the predominant species in EG, leading to approximately 291,700 cases in 2016; 15% of the deaths from this species were in children under 5 years old [5]. The authorities have deployed a series of measures that include effective anti-malarial drugs, vector control and case management for malaria control [6]. In 2004, The Bioko Island Malaria Control Project (BIMCP) was initiated on Bioko Island [7]. That project succeeded in reducing the infection rate, anaemia and child mortality [6]. Subsequently, similar measures have been adopted and were applied on mainland EG by the Equatorial Guinea Malaria Control Initiative (EGMCI) in 2007 [8]. In EG, SP has been used as a second-line treatment in cases of uncomplicated falciparum malaria for several decades. Furthermore, it was administered as the partner drug with artesunate as a first-line drug because of chloroquine treatment failure and as a malaria prophylaxis since 2004 [9], which may have led to P. falciparum isolates undergoing sustainable selection pressure. Soon afterwards, SP was replaced by artemisinin-based combination therapy (ACT) in response to widespread drug resistance in 2009, but it still remains the only choice for IPTp [10]. Of even greater concern, SP resistance (SPR) had already evolved in most African countries before SP was implemented as the recommended treatment. To ensure the prophylactic efficacy of this approach and to support the national anti-malarial policy, large-scale screening and surveillance of SP drug resistance is highly recommended [11].

Targeting the P. falciparum enzymes dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), SP acts as a synergistic inhibitor of folate in the parasite [12, 13]. In vitro and in vivo studies have demonstrated that SPR is mainly conferred by amino acid point mutations at codons N51I, C59R, S108N, and I164L of PfDHFR and S436A, A437G, K540E, A581G, and A613S of PfDHPS [14]. These hotspot mutations are suggested to be gradually displayed with the increase of SPR [15]. Many clinical failures have been reported after SP treatment was found in Africa [16–18]. Thus, an urgent need exists to continue monitoring and assessing resistance in P. falciparum populations when determining whether to administer this drug for prevention.

On Bioko Island, IPTp was introduced in 2004 [7, 9], and the Ministry of Health has implemented the use of two doses of SP during pregnancy and antenatal care, starting from the second trimester and 1 month apart [19]. An assessment of the prevalence of mutations in P. falciparum genes related to SPR on Bioko Island is needed to provide complementary information for this preventive strategy. In the current study, an assessment of the prevalence of the PfDHFR and PfDHPS gene mutations and haplotypes was conducted on P. falciparum isolates collected from Bioko Island, EG.

Methods
Study area and samples collection
The study was performed in 2013–2014 on Bioko Island, the Insular Region of EG, where malaria is endemic and has continuous transmission throughout the year. Venous blood (3 ml) was collected from P. falciparum-infected patients and confirmed by thick and thin smears stained with diluted Giemsa. Additionally, positive blood spots were air dried, individually reserved in coded plastic bags with silica desiccant beads, and kept at room temperature for further molecular assessment.

Ethics statement
The Ethics Committees of Malabo Regional Hospital on Bioko Island gave scientific and ethical permission (EGCNGD-071). Consent was obtained from all persons or their legal guardians before sample collection.
DNA extraction and PCR
Genomic DNA was extracted from dried filtered bloodspots (DBS) by following the Chelex-100 extraction procedure described in the previous report [20]. The PfHdhfr and PfHdhps genes were amplified by nested PCR, and the conditions for amplification were as previously described [21]. The mutations of the PfHdhfr and PfHdhps genes in the amplified nested PCR products were purified and detected subsequently by Sanger sequencing (Genewiz, Soochow, China). All sequences were analysed using DNASTar (DNASTAR Inc., Madison, WI, USA).

Data analysis
All data were analysed with SPSS 18 (SPSS Inc., Chicago, IL, USA). The percentages of single nucleotide polymorphisms (SNPs) and haplotypes were calculated with a 95% CI as described previously [22]. Differences in allele prevalence were compared using Pearson Chi square test or Fisher’s exact test, when conditions were appropriate. To determine the association between the SNPs of the PfHdhfr and PfHdhps genes, linkage disequilibrium (LD) tests were performed for each possible pair-wise SNP implicated as a drug-resistant marker in the two genes by calculating the D’ and r2 values using Haploview 4.2 software [23]. P values, less than 0.05 indicated significance.

Results
General information
In total, 180 isolates were evaluated. Then, 167 and 158 samples were successfully amplified, sequenced and genotyped for the PfHdhfr and PfHdhps genes, respectively. Of these successfully sequenced isolates, 130 sequences without any mixed types in PfHdhfr and PfHdhps were analysed for combined genotypes.

Prevalence of individual point mutations in PfHdhfr and PfHdhps
A high prevalence of PfHdhfr mutant alleles was detected in the analysed samples. The two major mutations, N51I (97.60%; 163/167) and S108N (97.01%; 162/167), showed similar prevalence, followed by C59R (87.43%; 146/167). The C59R mutant allele showed lower prevalence compared to N51I and S108N (χ2 = 6.141, P = 0.013; χ2 = 6.082, P = 0.014). No mutation was identified at positions 50 and 164. The key mutation of PfHdhps linked to sulfadoxine resistance at codon A437G was predominant at 90.51% (143/158), while the prevalence of the S436A mutation was found to be 20.25% (32/158), and the K540E, A581G and A613S mutations were less frequent, occurring at rates of 5.06% (8/158), 0.63% (1/158) and 3.16% (5/158), respectively. The A437G mutation occurred at a significantly different rate compared with S436A (χ2 = 153.837, P < 0.001), K540E (χ2 = 234.34, P < 0.001), A581G (χ2 = 259.538, P < 0.001), and A613S (χ2 = 249.161, P < 0.001). Similar to A437G, the S436A occurred at a significantly different rate compared with K540E (χ2 = 17.929, P < 0.001), A581G (χ2 = 34.142, P < 0.001) and A613S (χ2 = 24.726, P < 0.001) (Table 1).

Prevalence of PfHdhfr and PfHdhps haplotypes
In the reconstitution of the haplotypes, 3 and 8 distinct genotypes were observed in PfHdhfr and PfHdhps, respectively, and mixed genotypes were also found in both genes. For PfHdhfr, only 1.2% (2/167) of the isolates were wild type CNCSI whereas 86.83% (145/167) carried the triple mutation CIRNI. The double mutant CICNI occurred with low prevalence at 5.99% (10/167). The overall prevalence of the mixed haplotypes was 5.99% (10/167). The overall prevalence of the mixed haplotypes was 5.99% (10/167) as follows: 0.6% (1/167) CNC/RSI, 4.19% (7/167) CIC/RSI, 0.6% (1/167) CJRS/NI, and 0.6% (1/167) CN/IC/RS/NI. For PfHdhps, the single mutated haplotype SGKAA was present in 62.66% (99/158) of the samples, followed by the double mutant haplotypes AGKAA in

Table 1 Prevalence of PfHdhfr and PfHdhps SNPs in Plasmodium falciparum isolates from Bioko Island, Equatorial Guinea

| Gene     | SNP | Wild type n (% 95% CI) | Mutation n (% 95% CI) | Mixed type n (% 95% CI) |
|----------|-----|------------------------|-----------------------|------------------------|
| PfHdhfr  | 51  | 3 (1.80, 0.22 to 3.82) | 163 (97.60, 95.28 to 99.92) | 1 (0.60, 0.57 to 1.77) |
|          | 59  | 12 (7.19, 3.27 to 11.11)| 146 (87.43, 82.4 to 92.46) | 9 (5.39, 1.97 to 8.81) |
|          | 108 | 3 (1.80, 0.22 to 3.82) | 162 (97.01, 94.43 to 99.59) | 2 (1.20, 0.45 to 2.85) |
| PfHdhps  | 436 | 115 (72.78, 65.84 to 79.72)| 32 (20.25, 13.98 to 26.52) | 11 (6.96, 2.99 to 10.93)  |
|          | 437 | 12 (7.59, 3.46 to 11.72) | 143 (90.51, 85.94 to 95.08) | 3 (1.90, 0.23 to 4.03) |
|          | 540 | 146 (92.41, 88.28 to 96.54) | 8 (5.06, 1.64 to 8.48) | 4 (2.53, 0.08 to 4.98) |
|          | 581 | 152 (96.20, 93.22 to 99.18) | 1 (0.63, 0.6 to 1.86) | 5 (3.16, 0.43 to 5.89) |
|          | 613 | 153 (96.84, 94.11 to 99.57) | 5 (3.16, 0.43 to 5.89) | 0 (0.00) |

SNPs single nucleotide polymorphisms, n number, CI confidence interval
10.76% (17/158), whereas only one isolate exhibited the triple mutated haplotype SGEAA. Of the remaining samples, 5.7% (9/158) harboured AAKAA, 4.43% (7/158) SGEAA, 0.63% (1/158) SGKAS, and 2.53% (4/158) AGKAS. The overall prevalence of mixed haplotypes was 11.39% (18/158) as follows: 0.63% (1/158) S/A AAKAA, 3.8% (6/158) S/AGKAA, 1.9% (3/158) SGK/EAA, 0.63% (1/158) SGK/GA, 1.27% (2/158) AGK/GA, 0.63% (1/158) S/AGK/GA, 1.9% (3/158) S/AAGK/GA, and 0.63% (1/158) SGK/EAGA (Table 2).

Table 2 Prevalence of Pfdfhr and Pfdhps haplotypes in Plasmodium falciparum isolates from Bioko Island, Equatorial Guinea

| Gene | Category | Haplotype | n (%, 95% CI) |
|------|----------|-----------|---------------|
| Pfdfhr (n = 167) | Wild type | CNCSI | 2 (1.20, −0.45 to 2.85) |
| | Double mutant | C/CNI | 10 (5.99, 2.39 to 9.59) |
| | Triple mutant | CIRNI | 145 (86.83, 81.7 to 91.96) |
| | Mixed type | CN/CE/RS/NI | 1 (0.60, −0.57 to 1.77) |
| Pfdhps (n = 158) | Wild type | SAKAA | 2 (1.27, −0.48 to 3.02) |
| | Single mutant | S/AAGKAA | 9 (5.70, 2.08 to 9.32) |
| | Double mutant | AGKAA | 99 (62.66, 55.12 to 70.2) |
| | Triple mutant | AGKAS | 1 (0.63, −0.6 to 1.86) |
| | Mixed type | S/AAGKAA | 1 (0.63, −0.6 to 1.86) |

Pfdfhr and Pfdhps allele combinations

When the Pfdfhr and Pfdhps haplotypes were combined, 12 genotypes were verified and are shown in Table 3. Quadruple mutant haplotypes with a triple Pfdfhr and a single Pfdhps mutation (CIRNI-SGKAA) was the most common at 65.38% (85/130). One sample at the Pfdfhr and Pfdhps loci was fully a wild type. The second prevalent haplotype was CIRNI-AGKAA with a frequency of 12.31% (16/130). The quintuple mutation (CIRNI-SGEAA) and sextuple mutation (CIRNI-SGEGA) were found in 4.62% (6/130) and 0.77% (1/130) of the isolates, respectively. The occurrence of other combined haplotypes was generally low: 0.77% (1/130) CNCSI-SGKAA, 4.62% (6/130) CICNI-SGKAA, 0.77% (1/130) CIRNI-SAKAA, 0.77% (1/130) CICNI-SGKAA, 5.38% (7/130) CIRNI-AAGKAA, 0.77% (1/130) CIRNI-SGKAA, and 3.08% (4/130) CIRNI-AGKAA.

Linkage disequilibrium (LD) test for Pfdfhr and Pfdhps haplotypes

The LD pattern for each SNP in the Pfdfhr and Pfdhps genes was assessed (Fig. 1). For the Pfdfhr gene, base substitution mutations of T152A, T175C and G323A were related to the single amino acid mutations of N51I, C59R, S108N, respectively. Similarly, the T1482G, C1486G, A1794G, and G2013T in the Pfdhps gene category.
in the other SNPs of either the Pfdhps or Pfdhfr and the value is 0.68. No such association was detected in N51I was significantly associated with the C1486G, Pfdhps found among the SNPs located in both the Pfdhfr and A613S, respectively. The gene indicated mutations of S436A, A437G, K540E, and A613S, respectively.

Several statistically significant associations were found among the SNPs located in both the Pfdhfr and Pfdhps genes (Fig. 1). For the Pfdhfr gene, base substitution mutations of T152A, T175C, and G323A are related to single amino acid mutations of N51I, C59R, and S108N, respectively. Similarly, the T1482G, C1486G, A1794G, and G2013T in the Pfdhps gene are related to mutations of S436A, A437G, K540E, and A613S, respectively. According to the four-gamete test, these SNPs are divided into two blocks (black frame). The number in the square indicates a D’ value. The square with dark red and light red indicates a linkage that was statistically significant (P < 0.05). The square with Cambridge blue indicates a linkage is present but is not statistically significant (P > 0.05). The square with white indicates no linkage is present.

Discussion

The rapid and widespread development of anti-malarial drug resistance is directly influencing and hindering the process of malaria control, prevention and elimination [24]. Surveillance with molecular markers has allowed the early detection of drug resistance susceptibility and may provide fundamental information for drug policy [25]. The current study displays the mutations and haplotypes of the Pfdhfr and Pfdhps genes from isolates collected from the general population on Bioko Island, thus allowing the degree of SPR in this malaria hotspot to be inferred.

The results demonstrate that Pfdhfr polymorphism associated with SPR persists at high frequency. A high prevalence of the Pfdhfr N51I mutation in 97.60% and the S108N mutation in 97.01% of the samples was found among the P. falciparum population on Bioko Island (Table 1), and these mutations also had been found at a very high level (97.9 and 99.1%, respectively) in the Democratic Republic of Congo (DRC) in 2008 [26]. For C59R, the level was significantly lower than for N51I and S108N, similar to observations in the mainland of EG [4]. Like neighbouring countries, Pfdhfr I164L, which is related to high-grade SPR, has been reported at low proportions (1.4%) in rural areas of the EG mainland [4, 27]. Fortunately, this mutation was not found in any isolates within the study. Although the mutations of Pfdhfr C50R and I164L are not found in the present data, the high prevalence of three well-characterized mutations in Pfdhfr (N51I, C59R, S108N) indicate the P. falciparum isolates from Bioko island display high pyrimethamine resistance that needs to be addressed by the EGMCI. For the Pfdhps haplotypes, 86.83% of the isolates carried the Pfdhfr triple mutation (CIRNI) (Table 2) and was reported in 80% of P. falciparum infections in 2005 from the mainland of EG, 100% in 2005 in Cameroon [28], and 72.4% in Gabon [29]. This triple mutation is an important SPR indicator, but its detrimental effects may be largely compromised by an absence of the Pfdhfr I164L mutation [30, 31]. The frequency of the Pfdhfr double mutant CICNI was 5.99% (Table 2), and this genotype has a lesser degree of resistance compared with the triple mutation CIRNI [29]. For the dominant mutant haplotype CIRNI (86.83%) and the double mutant haplotype CICNI (5.99%), the results are consistent with previous studies in EG and Central Africa [4, 10, 32, 33]. If the CIRNI haplotype is found concurrently with the Pfdhps mutations, it is associated with a high level of resistance [34]. The reported prevalence of the Pfdhfr triple mutation was also lower than those previously reported at the site where the proportion of the Pfdhfr triple mutation reached a frequency of 97%. Only 1.2% of the isolates...
(2/167) were a pure Pf dhfr wild type (CNCSI) (Table 2). The results indicate that almost all samples collected harbour pyrimethamine resistance.

Compared with the mutations of the Pf dhfr gene, the mutations of the Pf dhps gene exhibit a relatively low prevalence, except for the A437G mutation (90.51%, 143/158) (Table 1), which is also common in other EG regions and several African countries [27, 31, 35]. This mutation has been reported to occupy the key position of the initial mutation of sulfadoxine resistance, and its resistance increases along with the augmentation of other mutations in Pf dhps [36]. Although the prevalence of S436A is significantly lower than that of A437G, it is higher than for other mutations, including K540E, A581G and A613S. In Central Africa, the Pf dhps K540E mutation was less prevalent, which was also confirmed in this study (5.06%, 8/158) (Table 1). This mutation is more common in East Africa, particularly in Tanzania [37] and Uganda [17]. The WHO has recommended that IPT with SP should be abandoned in areas where the K540E mutation has been detected at >95% and Pf dhps the A581G mutations are detected at >10% because it could be ineffective [11]. Fortunately, only 5.06% (5/158) of the isolates showed the Pf dhps K540E mutation, and 0.63% (1/158) of the isolates harboured the A581G mutation in current survey (Table 1). The relatively low prevalence of these mutations suggests that IPT-SP can possibly be efficacious on Bioko Island, EG. The A613S mutations were detected in 3.16% (5/158) of the isolates, which is consistent with reports in Central African countries, including the DRC [27] and Cameroon [29]. For the Pf dhps haplotype, the single-mutant SGKAA haplotype predominates in our results (62.66%) (Table 2), similar to observations made in Gabon [38] and the DRC [39]. AGKAA is present in 10.76% of the isolates (Table 2), and an increased trend was detected in Gabon between 2013 and 2014 [38]. Parasites with double- and triple-mutant Pf dhps haplotypes were observed at a low frequency (Table 2), suggesting a low tendency in the emergence and development of the sulfadoxine resistance alleles.

The combination of the Pf dhfr and Pf dhps mutant alleles generated 12 different haplotypes in the present survey (Table 3). Only one wild-type haplotype (CNCSI-SAKAA) was found in this study (Table 3). The quadruple mutant (CIRNI-SGKAA) was predominant, with a prevalence of 65.38% (Table 3), which is higher than reports from mainland EG (54%) [4]. The saturation of the Pf dhfr triple mutants could further induce the Pf dhps mutants, and thus, the presence of quadruple mutants (CIRNI-SGKAA) was common [40]. Although quintuple mutant genotypes (CIRNI-SGEGA) are highly linked to SP failure [34], this mutant was detected at a rate of 4.62% (Table 3). WHO recommends surveillance for this genotype and inhibition of IPTp-SP when the prevalence of this quintuple mutant exceeds 50% [31]. To date, this quintuple mutant is less than 10% in other areas of EG [4, 10]. Previous in vitro studies demonstrated that the quadruple mutant (CIRNI-SGKAA) has a less deleterious effect on SP-IPT than the quintuple mutant genotypes (CIRNI-SGEGA) [41]. Notably, the ‘super resistant’ alleles (CIRNI-SGEGA) may render SP ineffective [42], but these were detected in only one isolate. Although this occurrence is low, sustainable monitoring for SPR and avoiding the growth of super resistance alleles are still critical.

Although the LD analysis of the SNPs between the Pf dhfr and Pf dhps genes showed a strong linkage between N51I and A437G, those main SNPs of the Pf dhfr and Pf dhps genes form two independent LD blocks, respectively. These results indicate that the mutations located in the Pf dhfr and Pf dhps genes have relative independence. However, combined chemotherapy will likely lead to the occurrence and progress of resistance gene mutations even though the Pf dhfr and Pf dhps genes are located on different chromosomes [40]. For the Pf dhfr gene, T152A, T175C, and G323A develop as a block. When distributed in the Pf dhfr gene, these SNPs exhibit strong linkage, particularly of N51I and S108N (D’: 0.71–1, P < 0.05). For the Pf dhps gene, the T1482G, C1486G, A1794G, and G2013T were found in an LD block. Although the SNPs in Pf dhps gene show weak linkage and no significant differences (P > 0.05), strong linkages were also commonly detected from S436A and other mutations, including A437G, K540E and A613S. Notably, the study had weaknesses, including the small sample size and the lack of full-length DNA sequences for the Pf dhfr and Pf dhps genes showed a strong linkage between these genes [43]. Although the LD analysis of the SNPs between the Pf dhfr and Pf dhps genes have relative independence. These results indicate that the mutations located in the Pf dhfr and Pf dhps genes form two independent LD blocks, respectively. These results indicate that the mutations located in the Pf dhfr and Pf dhps genes have relative independence. However, combined chemotherapy will likely lead to the occurrence and progress of resistance gene mutations even though the Pf dhfr and Pf dhps genes are located on different chromosomes [40].

Conclusions

The results of this study indicate that this area had a high prevalence of the Pf dhfr triple mutation (CIRNI) and the Pf dhps single mutation (SGKAA), which could undermine the efficacy of SP for chemoprevention strategy. To
avoid increases in SPR, continuous molecular monitoring and additional control efforts are urgently needed.

Authors’ contributions

JL conceived and designed the experiments. ML and JTC coordinated the field collections of patient isolates. JTC, JUME, RAM and MMOO carried out microscopic examination. YY and TTJ performed the experiments. JL, WM, XKD, and HBT analysed the data. JL and TTJ wrote the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets analysed in this study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Current study was approved by the ethics committee of Malabo Regional Hospital in Bioko Island. The informed consent was obtained from all participants.

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