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# Surveillance of molecular markers for antimalarial resistance in Zambia: Polymorphism of Pfkelch 13, Pfmdr1 and Pfdhfr/Pfdhps genes

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ABSTRACT

Antimalarial resistance is an inevitable feature of control efforts and a key threat to achieving malaria elimination. Plasmodium falciparum, the deadliest of several species causing human malaria, has developed resistance to essentially all antimalarials. This study sought to investigate the prevalence of molecular markers associated with resistance to sulfadoxine-pyrimethamine (SP) and artemether-lumefantrine (AL) in Southern and Western provinces in Zambia. SP is used primarily for intermittent preventive treatment during pregnancy, while AL is the first-line antimalarial for uncomplicated malaria in Zambia. Blood samples were collected from household members of all ages in a cross-sectional survey conducted during peak malaria transmission, April to May of 2017, and amplified by polymerase chain reaction (PCR). Amplicons were then analysed by high-resolution melt following PCR to identify mutations associated with SP resistance in the P. falciparum dihydrofolate reductase (Pfdhfr) and P. falciparum dihydropteroate synthase (Pfdhps) genes and lumefantrine resistance in the P. falciparum multi-drug resistance 1 (Pfmdr1) gene. Finally, artemether resistance was assessed in the P. falciparum Kelch 13 (PfK13) gene using nested PCR followed by amplicon sequencing. The results showed a high frequency of genotypic-resistant Pfdhps A437G (93.2%) and Pfdhfr C59R (86.7%), N51I (80.9%), and S108N (80.8%) of which a high proportion (82.4%) were quadruple mutants (Pfdhfr N51I, C59R, S108N + Pfdhps A437G). Pfmrd1 N86Y, Y186F, and D1246Y - NFD mutant haplotypes were observed in 41.9% of isolates. The high prevalence of quadruple dhps/dhfr mutants indicates strong antifolate drug pressure from SP or other drugs (e.g., co-trimoxazole). Three samples contained PfK13 mutations, two synonymous (T478 and V666) and one non-synonymous (A578S), none of which have been associated with delayed clearance. This suggests that artemisinin remains efficacious in Zambia, however, the moderately high prevalence of approximately 40% Pfmdr1 NFD mutations calls for close monitoring of AL.

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**Fig. 1.** Map of Zambia showing the location of Southern and Western Provinces and the site where samples were collected.

## 1. Introduction

Antimalarial drug resistance is a key threat to eliminating malaria. Strategies to eliminate the disease include early case diagnosis and prompt treatment using an effective and efficacious antimalarial (MOH, 2017). For Zambia, mass drug administration (MDA) with an artemisinin-based combination therapy (i.e., dihydroartemisinin-piperaquine [DHAP]), given to populations of all ages with the exception of children under three months of age, has been added as an accelerator in Southern Province, an area selected for sub-national elimination (MOH, 2017). Intermittent preventive treatment during pregnancy (IPTp) with sulfadoxine-pyrimethamine (SP) is recommended by the World Health Organization in areas of Africa with moderate to high malaria transmission. While artemisinin-based therapies (ACTs) are used for treatment of malaria cases, namely artemether-lumefantrine (AL) for uncomplicated malaria with DHAP as an alternative, and injectable artesunate for severe malaria (MOH, 2014; WHO, 2015).

Resistance to SP has been reported in many countries, including Uganda (Mbonye et al., 2015), Tanzania (Baraka et al., 2015), Kenya (Hemming-Schroeder et al., 2018), Cameroon (Chauvin et al., 2015), and Zambia (Siame et al., 2015). Resistance to SP is mediated by mutations in two enzymes, namely Plasmodium falciparum dihydrofolate reductase (Pfdhfr), which confers resistance to pyrimethamine, and P. falciparum dihydropteroate synthetase (Pfdhps), which confers resistance to sulfadoxine (Cowman et al., 1988; Marks et al., 2005; Peterson et al., 1988). To reduce the likelihood of selection for resistance, it is recommended to use drug combinations (Majori, 2004) and regularly monitor drug efficacy.

Zambia was the first African country to use AL as a first-line treatment (Sipilanyambe et al., 2008). Between 1995 and 2005, chloroquine (CQ) and SP were the first- and second-line treatments, respectively, for uncomplicated malaria in Zambia (Bijl et al., 2000). In 2000, CQ resistance was wide spread, with some areas of the country reporting resistance in 58% of samples (Bijl et al., 2000).

Artemisinin resistance was first identified as a delayed clearance phenotype in South East Asia (Ashley et al., 2014; Noedl et al., 2008). The genetic basis for resistance was identified in the propeller domain of the P. falciparum Kelch13 (Pfk13) gene, which is located on chromosome 13, and encodes a 727 amino acid protein (Ariey et al., 2014). Currently, nine Pfk13 mutations have been validated as artemisinin-resistant markers, with an additional 11 associated with the resistant phenotype; 18 additional mutations have been isolated but not evaluated. The validated mutations include F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, and C580Y, while the 11 associated/candidates are P441L, G449A, C469F, A481V, P527H, N537I, G538V, V568G, P574L, F673I, and A675V (WHO, 2018). Some of these mutations have been identified in Africa countries, including, F446I, P574L, M476I, and A675V (Ocan et al., 2019). In African countries the overall prevalence of non-synonymous single nucleotide polymorphisms (SNPs) was 7.6%, as reported in a systematic review of studies published between 2014 and 2018 (Ocan et al., 2019). The most frequent allele observed in African countries is A578S, although it has not been associated with clinical or in vitro resistance to artemisinin or delayed clearance (Ménard et al., 2016; WHO, 2017).

A drug efficacy study conducted in Zambia in 2013 showed a few late parasitological failure (7%) and late clinical failure (3.5%), with no early clinical failures in younger patients treated with AL, suggesting that AL remains safe and efficacious for the treatment of uncomplicated P. falciparum malaria (Hamainza et al., 2014). The efficacy study suggested the need for further sentinel and periodic monitoring of AL efficacy in different areas of the country (Hamainza et al., 2014). Hence this study examines the mutations in PfK13 associated with artemisinin resistance.

P.falciparum multi drug resistance gene 1 (Pfmdr1) is one of the markers associated with decreased sensitivity to lumefantrine, particularly Pfmdr1 N86 (Venkatesan et al., 2014). Parasites carrying Pfmdr1 86 N gene are killed by treatment with AL but their increased tolerance of residual drug levels allows them to re-infect a person more rapidly after his treatment, compared to parasites carry 86Y form of the gene. (Hastings and Ward, 2005). This points to Pfmdr1 86 N as a

potential marker for lumefantrine resistance (Sisowath et al., 2005). The P-glycoprotein-1 homologue transporter encoded by Pfmdr1 affects the intra-parasitic concentration of some key antimalarials (Reed et al., 2000). With multiple genetic loci associated with differential drug resistance, this study sought to assess the presence of selected markers of resistance to two antimalarials that are currently widely used in Zambia, SP (markers Pfdhfr and Pfdhps) and AL (markers Pfk13 and Pfmdr1), using data from a cross-sectional survey conducted in Southern and Western provinces.

## 2. Materials and methods

### 2.1. Study area and participants

A cross-sectional, household survey to assess malaria elimination efforts was conducted in Zambia's Southern Province and Western Province during peak malaria transmission from April to May 2017 (Fig. 1). At the time of the survey, Southern Province had the lowest malaria prevalence in the country due to high coverage of various control measures including vector control, case management at facility and community levels, and targeted MDA with DHAP. In contrast, Western Province had a moderate to high malaria prevalence, with good vector control coverage and case management at the facility level (MOH, 2018).

### 2.2. Sampling

#### 2.2.1. Original survey sampling and sample collection

In Southern Province, a pre-existing sampling frame from another study was used (the 2017 Southern Province survey) (Eisele et al., 2015) whereby 52 households were randomly selected from each of the 60 health facility catchment areas. In Western Province, a de novo two-stage, cluster-randomized design was used, with the probability of selecting the primary sampling unity (standard enumeration area [SEA]) proportional to its relative size. The design was based on the 2010 census standard enumeration areas, with 25 households randomly selected from each of the 24 SEAs after listing all households in each SEA (Ministry of Health, 2015).

During the survey, three dried blood spots on filter paper were obtained via finger prick from each participant, labelled with a participant identification code, air dried, individually placed in tight closing medicine pack with silica gel, and transported to the molecular lab where they were stored at −20 °C. Malaria infection was diagnosed using rapid diagnostic tests (SD Bioline Malaria Ag Pf, Standard Diagnostics, Inc, Suwon city South Korea) on individuals of all ages in the sampled households, yielding 6977 samples across the two provinces.

#### 2.2.2. Study sampling

After excluding clusters with zero prevalence (by rapid diagnostic test), 13 clusters were randomly selected from both provinces, yielding 1567 samples to be screened for Plasmodium species via photo-induced electron transfer-polymerase chain reaction (PET-PCR). A subset of the positives was later assayed for genetic markers of drug resistance using high-resolution melt (HRM)-PCR. Only samples with P. falciparum infections from the PET-PCR analysis were included.

### 2.3. Laboratory methods

Molecular analysis was conducted at the National Malaria Elimination Centre Reference Laboratory in Lusaka, Zambia. Parasite genomic DNA was extracted using a QIAamp DNA minikit (Qiagen, Hilden, Germany) according to manufacturer instructions, with modifications. A 6 mm (approximately 13.8 µl of whole blood) punch was extracted, either individually if the rapid diagnostic test result was positive or in a pool of 10 if the rapid diagnostic test was negative. Extracted DNA was eluted in 100 µl of elution buffer before being tested by PET-PCR as previously described (Lucchi et al., 2013). Positive pools were de-convoluted as required. Samples were stored long-term at −20 °C.

Extracted DNA was pre-amplified using diluted (0.2x) pooled forward and reverse primers for Pfdhfr, Pfdhps, and Pfmdr1 using TaqMan PreAmp Master Mix (Life Technologies, Foster City, CA, USA) as previously described by (Daniels et al., 2012). The total reaction volume depended on the quantity of DNA. A 10 µl reaction volume was used for samples with a PET-PCR cycle threshold (Ct) value > 35, whereas a 20 µl reaction volume was used for samples with a Ct < 35. The pre-amplified DNA then was cleaned using a genomic DNA clean and concentrator-10 kit (Zymo Research, Tustin, CA, USA), as per the manufacturer's instructions.

The pre-amplified and cleaned samples were then amplified in a 5:1 asymmetric PCR, and HRM analysis performed on a LightScanner 380 system (Roche, Basel Switzerland) with 3′ mismatched probes specific to mutations in Pfdhfr (N51I, C59R, I164L, and S108N), Pfdhps (S436F, A437G, K540E/N, and A581G), and Pfmdr1 (N86Y, Y184F, and D1246Y). 3D7, Dd2, HB3, and Tm90C6B were used as positive controls for mutant and wild-type alleles; however, Pfdhps K540E/N only had a control for the sensitive allele (K540). All PCR amplifications were performed using 2.0 µL of LightScanner Master Mix (BioFireDefense LLC, Salt Lake City, UT, USA), 2.5 µL of the pre-amplified template (1:20 dilution), and 0.5 µL of primers and probes. The final primer concentrations for a 5.0 µL total reaction volume were 0.5 µM of excess primer, 0.1 µM of limiting primer, and 0.4 µM of the 3′ mismatched probe. The following amplification conditions were used: 95 °C denaturation for 2 min, 45 cycles of 94 °C for 5 s and 66 °C (or 63 °C for Pfdhfr S108N and Pfmdr1 D1246Y) for 30 s, and a pre-melt cycle of 5 s each at 95 °C and 37 °C. Probe binding was measured as the change in fluorescence against the sample temperature from 40 °C to 90 °C (Daniels et al., 2012; Obaldia et al., 2015).

The PfK13 gene was amplified via nested PCR using the primary PCR primers PfK13_PCR_F 5′-CGGAGTGACCAAATCTGGGA-3′ and PfK13_PCR_R 5′-GGGAATCTGGTGGTAACAGC-3′ and nested PCR primers PfK13_N1_F 5′-GCCAAGCTGCCATTCATTTG-3′and PfK13_N1_R 5′-GCCTTGTTGAAAGAAGCAGA-3′. For both primary and nested reactions, a 25 µL and 50 µL reaction volume was used, respectively, containing a 1x final concentration buffer of 2.5 mM of $MgCl_2$, 20 nM of deoxyribonucleotide triphosphates (dNTPs), 1.25 of UTaq® polymerase (Solis BioDyne, Tartu, Estonia), and 250 nM of each primer, with 5 µL of template (primary) and 1 µL of template (nested). Reactions were undertaken on a MyCycler™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) as follows: 15 min at 95 °C, 30 cycles of 30 s at 95 °C, 2 min at 58 °C, 2 min at 72 °C, and a final extension of 10 min at 72 °C, for the primary reaction. For the nested reaction, the conditions were 15 min at 95 °C, then 40 cycles of 30 s at 95 °C, 1 min at 60 °C, 1 min at 72 °C, and a final extension of 10 min at 72 °C.

Inqaba Biotechnology Industries in South Africa cleaned up the amplicons using the ZR-96 DNA Sequence Clean-up Kit Catalogue No. D4053 (Zymo Research, Tustin, CA, USA) before injecting them onto an ABI 3500XL Genetic Analyser (Applied Biosystems, Foster City, USA) for sequencing. Due to the cost of sequencing, only 83 samples were sent.

### 2.4. Data analysis

Allele prevalence was analysed using Stata version 13 (College Station, TX, USA). Any sample that contained a mixed result (i.e. presence of both wild-type and mutant alleles) was scored as a mutant. Chi square tests were used to compare differences between the two provinces. The confidence interval was set at 95%, and a P-value of $< 0.05$ was considered statistically significant.

Multiple nucleotide sequence alignments were analysed by MacVector (Cambridge, UK) using the 3D7 PfK13 sequence (GenBank

accession no. XM001350122) as a reference to detect point mutations in the gene.

### 2.5. Ethical clearance

Ethical clearance was obtained from the regional committee for medical and health research ethics Western Norway (Ref no. 2016/1393/REK Vest), the University of Zambia biomedical research ethics committee (Ref no. 010–05–16), as well as from the Tulane university institutional review board (Ref no. 17–1,013,453). As this analysis was part of a larger study, ethical clearance for the larger study also was obtained from University of Zambia biomedical research ethics committee (Ref no. 010–12–17). Permission to use data was obtained from the national health research authority and from the Ministry of Health. All data were anonymized.

## 3. Results

### 3.1. Sample and isolate characteristics

A total 266 of 1567 isolates (17%) were positive for P. falciparum. Of these, 181 (68%) were tested and >70% successfully characterised for Pfdhfr, Pfdhps, and Pfmdr1 resistance markers (Table 1). For Kelch 13, 83 of the 266 samples (31%) were sent for analysis of which 70 returned high quality sequence data.

Of the 181 samples, 42 were from Southern Province and 139 were from Western Province. Due to the small sample size for Southern Province and the small non-significant difference in allele frequencies between the two provinces (S1 table), data were analysed collectively.

### 3.2. Pfdhfr and Pfdhps mutations

Mutant alleles (Note-mixed samples were scored mutant) were observed in PfdhpsA437G (93.2%) and Pfdhfr C59R (86.7%) N51I (80.9%) and S108N (80.8%). Other mutations were rare (e.g., PfdhpsK540E/N [4.7%] and A581G [2.6%]) or not observed (e.g., Pfdhfrcodon164 [0%]) (Table 2). The mixed samples were observed in the HRM PCR, where one sample had two different peaks indicating different melting points.

Looking at Pfdhfr and Pfdhps separately, 11 haplotypes were observed for each gene (Note-mixed sample were scored as mutant), from wild-type to double (Pfdhps) or triple (Pfdhfr) mutants. For Pfdhfr, the triple mutant N51I + C59R + S108N haplotype was the most prevalent (75.2%), and the double haplotype C59R + S108N was least prevalent (0.8%). For Pfdhps, the single haplotype A437G, also denoted as SGKA was most prevalent (86.5%) (Table 2).

Amongst 87 isolates with complete results for Pfdhfr and Pfdhps alleles tested, eight haplotypes of differing combinations of Pfdhfr and Pfdhps mutant alleles were identified (triple, quadruple, and quintuple), as shown in Table 3. The quadruple mutation N51I + C59R + S108N + A437G (Pfdhfr + Pfdhps) was the most prevalent (82.4%). From the haplotypes formed, no wild-type, single-mutant and double-mutant haplotypes were observed, nor were the known high-grade resistant septuple haplotypes.

**Table 1**
Overall frequency of individual Pfdhfr, Pfdhps, and Pfmdr1 mutations in isolates.

| Gene | Loci | Successfully assayed (N) | Mutant n (%) | 95% CI |
|---|---|---|---|---|
| Pfdhfr | N51I | 136 | 110 (80.9) | 73.3 – 87.1 |
| | C59R | 128 | 111 (86.7) | 79.6 – 92.0 |
| | S108N | 172 | 139 (80.8) | 74.1 – 86.4 |
| | I164L | 129 | 0 (0) | 0- 2.8 |
| Pfdhps | S436F | 146 | 2 (1.4) | 0.17 −4.9 |
| | A437G | 146 | 136 (93.2) | 87.8 – 96.7 |
| | K540E/N | 171 | 8 (4.7) | 2.0 −9.0 |
| | A581G | 154 | 4 (2.6) | 0.7 – 6.5 |
| Pfmdr1 | N86Y | 140 | 0 (0) | 0–2.6 |
| | Y184F | 132 | 57 (43.2) | 34.6–52.1 |
| | D1246Y | 150 | 3 (2.0) | 0.4–5.7 |

**Table 2**
Pfdhfr and Pfdhps haplotypes in Southern Province and Western Province, Zambia.

| | Mutations | Single/ Multiple mutations | n (%) | 95% CI |
|---|---|---|---|---|
| Pfdhfr $n$ = 124 | | Wild type | 3 (2.4) | 0.5–6.9 |
| | C59R | Single | 2 (1.6) | 0.2–5.7 |
| | S108N | Single | 13 (10.5) | 5.7–17.3 |
| | C59R + S108N | Double | 1 (0.8) | 0.02–4.4 |
| | N51I + C59R | Double | 11 (8.9) | 4.5–15.3 |
| | N51I + C59R + S108N | Triple | 94 (75.2) | 67.3–83.0 |
| Pfdhps $n$ = 133 | | Wild type | 6 (4.5) | 1.7–9.5 |
| | A437G | Single | 115 (86.5) | 79.5–91.8 |
| | S436F + A437G | Double | 2 (1.5) | 0.2–5.3 |
| | A437G + A581G | Double | 3 (2.3) | 0.5–6.5 |
| | A437G + K540E/N | Double | 7 (5.3) | 2.1- 10.5 |

### 3.3. Pfk13 mutations

Three isolates from Western Province had mutations in the Pfk13 gene, of which two were synonymous and one was non-synonymous (Table 4).

### 3.4. PfMDR1 mutations

Of the approximately 150 samples that were typed at the Pfmdr1 locus, there was no evidence of the Pfmdr1 N86Y mutation, and Pfmdr1 D1246Y was observed in only three samples. In contrast, Pfmdr1 Y184F was identified in 43.2% of isolates (Table1). In terms of haplotypes, 97% of all samples were either wild-type NYD (55.3%) or single mutant NFD (42.0%) (Fig. 2).

## 4. Discussion

Drug-resistant malaria parasites pose a serious public health challenge for control and elimination of malaria. This study reports the prevalence of Pfdhfr, Pfdhps, Pfmdr1, and PfK13 gene mutations associated with drug resistance from field isolates collected in Western Province and Southern Province in Zambia in 2017 from cross-sectional household surveys. Surveillance of drug resistance markers allows for early detection of drug resistance and is vital for providing evidence for policy change.

Consistent with previous findings from Zambia (Siame et al., 2015) and the Democratic Republic of the Congo (Nkoli Mandoko et al., 2018; Ruh et al., 2018), in this study, Pfdhfr mutations had a high prevalence (~80%), whereas, except for A437G, Pfdhps mutants were relatively rare (<5%). The prevalence of the three Pfdhfr mutations (N51I, C59R, and S108N) increased from under 20% to approximately 80% between 1988 and 2005, after which prevalence remained high but did not reach 100% (Fig. 2). With the exception of a single survey in 2006, Pfdhps A437G prevalence also increased dramatically. These trends correlate well with a strong selection pressure until 2003 when SP was replaced by AL.

Mutations associated with SP resistance are evolutionarily acquired in a stepwise fashion. For Pfdhfr, selection occurs first at codon 108, then codon 51 or 59, and then others; while for Pfdhps, mutations occur first at codon 437 (Sirawaraporn et al., 1997). Looking at both genes, the quadruple mutant haplotype [Pfdhfr + Pfdhps (N51I + C59R + S108N + A437G)] dominated the isolates, with few quintuple mutants and no sextuple or septuple mutants. This quintuple haplotype has

**Table 3**
Pfdhfr and Pfdhps genotypes from 87 isolates with complete genotypes from Southern Province and Western Province, Zambia.

| | Pfdhfr mutations | | | | | Pfdhps mutations | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | 51 | 59 | 108 | 164 | 436 | 437 | 540 | 581 | | % (95% CI) |
| Triple | 1 | – | – | + | – | + | + | – | - | S108N + S436F + A437G | 1.2 (0.03 – 6.4) |
| | 1 | – | + | + | – | – | + | – | – | C59R + S108N + A437G | 1.2 (0.03 – 6.4) |
| | 1 | + | – | – | – | – | – | + | + | N51I + K540E/*N* + A581G | 1.2 (0.03 – 6.4) |
| | 1 | – | + | – | – | – | + | + | – | C59R + A437G + K540E/N | 1.2 (0.03 – 6.4) |
| | 2 | – | – | + | – | – | + | + | – | S108N + A437G + K540E/N | 2.3 (0.3 - 0.8.2) |
| | 6 | + | + | – | – | – | + | – | – | N51I + C59R + A437G | 7.1 (2.6 −14.7) |
| Quadruple | 70 | + | + | + | – | – | + | – | – | N51I + C59R + S108N + A437G | 82.4 (76 - 91) |
| Quintuple | 1 | + | + | + | – | + | + | – | – | N51I + C59R + S108N + S436F + A437G | 1.2 (0.03 – 6.4) |
| | 1 | + | + | + | – | – | + | – | + | N51I + C59R + S108N + A437G + A581G | 1.2 (0.03 – 6.4) |
| | 3 | + | + | + | – | – | + | + | – | N51I + C59R + S108N + A437G + K540E/N | 3.5 (0.7 – 10.0) |

been strongly linked to SP treatment failure (Wang et al., 1997). There was no evidence for Pfdhfr I164L, a rare mutation in Africa, that is also highly correlated with phenotypic resistance (Hyde, 1990).

These findings suggest past or current pressure to select for SP-resistant mutants, consistent with results from previous studies (Depoortere et al., 2004; Mkulama et al., 2008; Siame et al., 2015) (Figs. 2 and 3). As SP is available without a prescription and is widely used to self-medicate, despite its official exclusive use in IPTp (Alifrangis et al., 2014), while antifolate drugs like co-trimoxazole are commonly used to manage bacterial infections and prevent opportunistic infections amongst HIV patients in Zambia (Khalil et al., 2003), the opportunity for selection pressure exists. However, selection is not absolute, as not all mutations are fixed in the population and higher-order mutants appear to be absent. Taken together, the results suggest that SP could still provide a benefit (e.g., in IPTp (Mace et al., 2015; Siame et al., 2015; Tan et al., 2014). It is likely still efficacious (Tan et al., 2014) and any additional mutations would threaten its utility. In addition, the major predictors for IPTp failure (Pfdhps E540 and A581 or the quintuple haplotype) were rare (< 4%). WHO recommends stopping IPTp with SP if Pfdhps K540E is over 95% and Pfdhps A581G is over 10% (WHO, 2013) and introduction of IPTi-SP in areas where mutations at codon 540 in the Pfdhps gene are 50% or less (WHO, 2011).

Three mutations not linked to late parasite clearance were detected in PfK13; non-synonymous A578S and synonymous T478 and V666. A578S has been observed at low frequencies in Africa (WHO, 2018), for instance in Gabon (Voumbo-Matoumona et al., 2018), Kenya (Muwanguzi et al., 2016), Mali (Ouattara et al., 2015), and Uganda (Conrad et al., 2014) and was also previously identified in Zambia in 2016 (Hawela et al., unpublished data). A578 has not been associated with clinical or in vitro resistance to artemisinin (Ménard et al., 2016). The synonymous mutation V666 has been identified in Nigeria (Oboh et al., 2018) and Mozambique (Gupta et al., 2018), and T478 has been reported in Kenya and Cote d'Ivoire (Kamau et al., 2015). Critically, none of these mutations alter protein function, suggesting that artemisinin remains highly unlikely. In addition, from a genetic perspective, the observed low frequency of the mutations could be attributed to negative selection and since P. falciparum is not a single population, there is evidence of local population expansions could lead to genes that show different mutations at low frequencies (Pacheco et al., 2019).

Pfmdr1 haplotype NFD associated with reduced susceptibility to

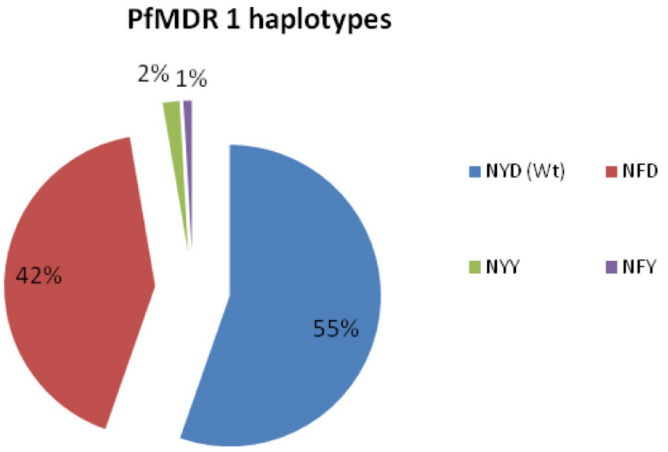


**Fig. 2.** P. falciparumPfmdr1 haplotypes in Southern and Western Provinces of Zambia.

Lumefantrine was found as 42.0%, consistent with finding from Ghana (Osarfo et al., 2018) and Mainland Tanzania (Ishengoma et al., 2019). This result is also consistent with reports from Ghana (Osarfo et al., 2018) and Rwanda (Zeile et al., 2012). While, to the best of our knowledge, AL remains efficacious in Zambia (Banda et al., 2019), the presence of these mutations associated with lumefantrine resistance calls for close monitoring of AL.

### *4.1. Study limitations*

All samples were PCR pre-amplified, which could have introduced an amplification bias for certain alleles. Pfdhfr 540 only had a single control (wild type) and with results showing additional peaks, it was difficult to discriminate between K540E and K540N, hence the assignment of K50E/N. The study did not assess piperaquine resistance despite the use of DHAP for the MDA study, however at the time of field work for the surveys, it had only been used for three years and only in a few areas in Southern Province.

## 5. Conclusions

A high proportion of samples in our study contained multiple antifolate-resistant markers, although higher-order combinations most

**Table 4**
Proportion of polymorphisms observed in the kelch 13 gene in isolates from Southern Province and Western Province, Zambia.

| Target gene Pfk13 | Pfk13 Codon | Locus Nucleotide | n (%) | Reference sequence | Mutant sequence | Reference amino acid | Mutant amino acid |
|---|---|---|---|---|---|---|---|
| | 478 | 1434 | 1 (1.4) | ACC | ACG | Threonine | Threonine |
| | 578 | 1732 | 1 (1.4) | GCT | TCT | Alanine | Serine |
| | 666 | 1998 | 1 (1.4) | GTA | GTC | Valine | Valine |

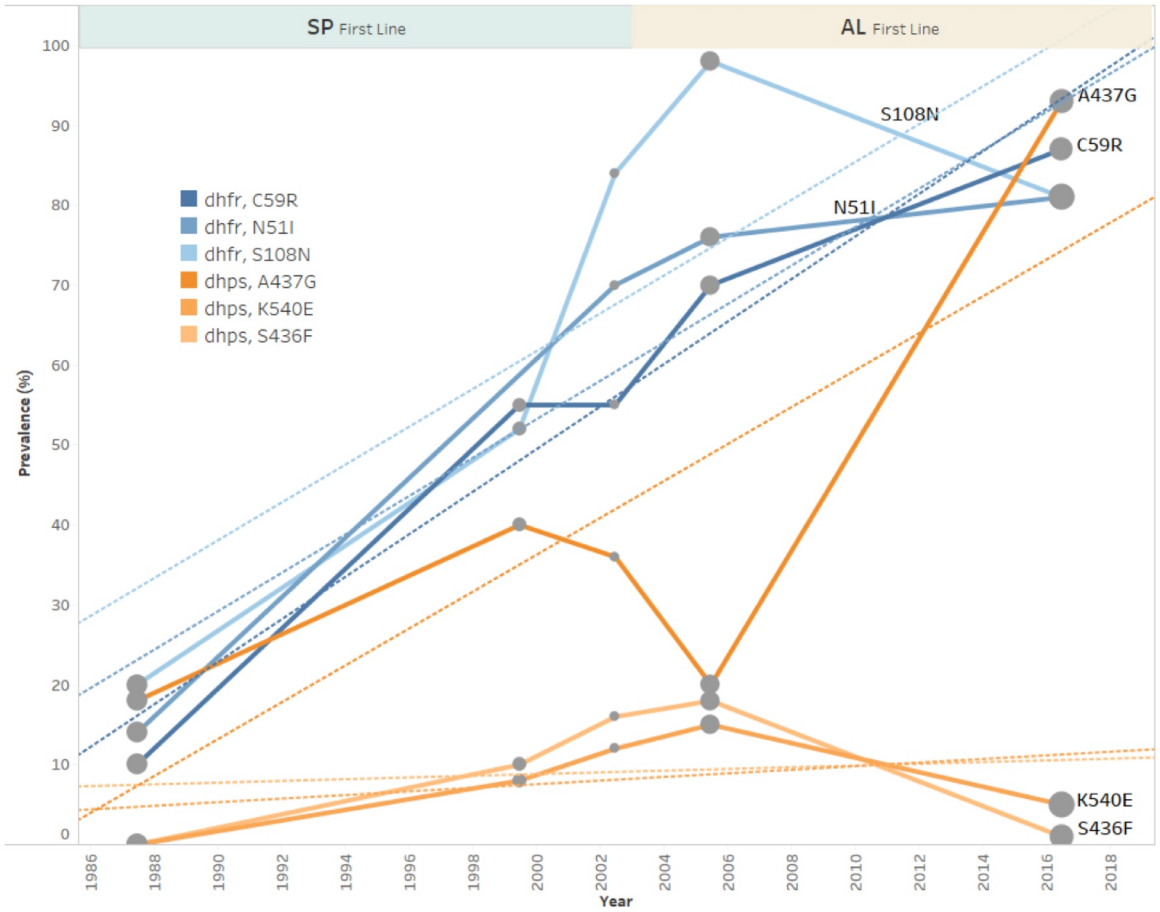


**Fig. 3.** Prevalence (%) of mutations in Pfdhfr (blue) and Pfdhps (orange) in Southern and Western Province of Zambia between 1988 and 2017. The size of the points is relative to the number of samples tested (min = 25, max = 172). The first line treatment is indicated in the bar at the top of the page.

clearly associated with treatment failure were missing. This suggests that while SP is likely still efficacious, any additional mutations would threaten its utility due to increased parasite resistance. While no markers for artemisinin resistance were identified, a significant proportion of the study population contained a mutation associated with reduced sensitivity to lumefantrine, which calls for close monitoring of AL.

Monitoring genotypic markers of resistance during the routine therapeutic efficacy studies recommended by WHO will be critical in ensuring malaria infections continue to be treated with an efficacious drug.

## 6. Author contributions

Lungowe Sitali: conceptualization, data curation, formal analysis, investigation, methodology, project administration, visualisation, writing-original draft, writing-review and editing. Mulenga C. Mwenda: formal analysis, investigation, methodology, project administration, validation, visualisation, writing-original draft writing-review and editing. John M. Miller: data curation, formal analysis, fund acquisition, investigation, methodology, project administration, resources, writing-original draft -writing-review and editing. Daniel J. Bridges: data curation, investigation, methodology, project administration, software, writing-original draft, writing -review and editing. Moonga B. Hawela: formal analysis, investigation, methodology, writing-original draft, writing,-review and editing. Busiku Hamainza: formal analysis, investigation, methodology, writing-original draft, writing -review and editing. Mutinta Mudenda-Chilufya: formal analysis, investigation, methodology, project administration, resources, writing-original draft, writing -review and editing. Elizabeth Chizema-Kawesha: formal analysis, investigation, methodology, project administration, resources, writing-original draft, writing-review editing. Rachel F. Daniels: investigation, methodology, software, project administration, visualisation, writing-original draft, writing,-review and editing. Thomas P. Eisele: data curation, investigation, methodology, resources, writing-original draft, writing-review and editing. Audun H. Nerland: investigation, methodology, project administration, software, visualisation, writing-original draft, writing-review and editing. James Chipeta: conceptualization, formal analysis, investigation, methodology, project administration, visualisation, supervision, writing-original draft, writing-review and editing. Bernt Lindtjorn: conceptualization, data curation, fund acquisition, investigation, methodology, project administration, visualisation, supervision, writing - original draft, writing-review and editing.

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## 8. Author statements

With reference to the above caption, we wish to submit our manuscript entitled “Surveillance of antimalarial drugs in Zambia: Surveillance of Molecular Markers for Antimalarial Resistance in Zambia: Polymorphism of Pfkech 13, Pfmdr1 and Pfdhfr/dhps genes ” for your consideration in Acta Tropical Journal after attending to all the comments.

The findings in this manuscript add to the body of knowledge in the sense that they give an update on the resistance markers in Zambia. The findings show that there moderate Sulfadoxine-Pyrimethamine resistance and absence of Artemisinin resistance markers. This information is likely to be of great interest to the health systems in Zambia and to the international scientific community.

The authors have read the manuscript and approved the final version.

## CRediT authorship contribution statement

**Lungowe Sitali:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing - original draft, Writing - review & editing. **Mulenga C. Mwenda:** Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing - original draft, Writing - review & editing. **John M. Miller:** Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing - original draft, Writing - review & editing. **Daniel J. Bridges:** Data curation, Investigation, Methodology, Project administration, Software, Writing - original draft, Writing - review & editing. **Moonga B. Hawela:** Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Busiku Hamainza:** Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Mutinta Mudenda-Chilufya:** Formal analysis, Investigation, Methodology, Project administration, Resources, Writing - original draft, Writing - review & editing. **Elizabeth Chizema-Kawesha:** Formal analysis, Investigation, Methodology, Project administration, Resources, Writing - original draft, Writing - review & editing. **Rachel F. Daniels:** Investigation, Methodology, Software, Project administration, Visualization, Writing - original draft, Writing - review & editing. **Thomas P. Eisele:** Data curation, Investigation, Methodology, Resources, Writing - original draft, Writing - review & editing. **Audun H. Nerland:** Investigation, Methodology, Project administration, Software, Visualization, Writing - original draft, Writing - review & editing. **James Chipeta:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Visualization, Supervision, Writing - original draft, Writing - review & editing. **Bernt Lindtjorn:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Visualization, Supervision, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2020.105704.

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