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Trends in *Plasmodium falciparum* resistance markers to sulfadoxine-pyrimethamine and amodiaquine over ten years of seasonal malaria chemoprevention in Moissala Health District, Chad

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Abstract

Background

Seasonal Malaria Chemoprevention (SMC) has been implemented in Moissala Health District, southern Chad, since 2013 using the standard regimen of sulfadoxine-pyrimethamine (SP) and amodiaquine (AQ). Although not the sole determinant, SMC can play an important role in generating parasite drug resistance. Three studies spanning a ten-year period were conducted to monitor evolving trends of molecular markers of resistance to SP and AQ in implementation areas.

Methods

In 2014, 2021, and 2023, a total of 136, 256, and 219 blood samples, respectively, were collected from children with clinical malaria residing in eight health zones. Samples were analysed for known molecular mutations associated with emerging *Plasmodium falciparum* resistance to SP (*dhfr* N51I, C59R, and S108N; *dhps* A437G and K540E*)* and to AQ (*pfcrt* K76T and *pfmdr-1* N86Y).

Results

The proportion of triple *dhfr* mutants was very high in 2014 and 2021 (100% and 96.9%, respectively), but significantly lower in 2023 (83.9%, $p < 0.001$). The proportion of quadruple mutants (triple *dhfr* + *dhps* A437G) significantly increased from 28.0% in 2014 to 41.0% in 2021 and 47.9% in 2023 ($p < 0.001$). The proportion of quintuple mutants (triple *dhfr* + double *dhps*) was low and did not significantly increase over the years studied (7.6%, 2.8%, and 5.9% in 2014, 2021, and 2023, respectively). The proportion of samples with the *pfcrt* K76T mutation decreased from 44.6% in 2014 to approximately 11% in 2021 and 2023, while the proportion of samples with the *pfmdr-1* N86Y mutation remained consistently low across all three studies. One sample in 2014 exhibited all seven point-mutations investigated, while none were detected in samples in 2021 or 2023.

Conclusion

Surveillance of molecular markers of resistance conducted over a ten-year period in Moissala Health District indicates that SP and AQ remain effective despite prolonged use. However, the rise in quadruple mutants – linked to partial SP resistance – is concerning, and monitoring is needed to detect any increase in quintuple mutants, which confer stronger resistance. These findings underscore the importance of sustained molecular surveillance to guide policy decisions and enable timely adaptations of SMC strategies as resistance patterns evolve.

Keywords Malaria, Sulfadoxine-pyrimethamine, Amodiaquine, Molecular markers of resistance, *Plasmodium falciparum*, Seasonal malaria chemoprevention, Chad

Background

Seasonal malaria chemoprevention (SMC) has been an integral component of Chad's malaria control policy since 2013, shortly after the World Health Organization (WHO) endorsed SMC as a cost-effective, safe, and operationally feasible intervention for protecting children in areas with seasonal malaria transmission [1–3].

SMC consists of the intermittent administration of full treatment courses of antimalarial drugs during the malaria transmission season. The primary aim is to maintain therapeutic drug concentrations in the bloodstream, thereby clearing existing parasites and preventing new infections during the peak of malaria transmission [4]. The WHO currently recommends using a combination of sulfadoxine-pyrimethamine (SP) and amodiaquine (AQ) [2]. Initially, the target population consisted of children aged 3 months to 5 years living in areas with a transmission season of four months or less [2]. However, recent WHO guidance allows for a more tailored approach based on local malaria epidemiology, providing countries with greater flexibility to extend the age range to include older children and to implement SMC in areas with longer malaria transmission seasons [5].

In Chad, SMC has been implemented using SPAQ under the supervision of the National Malaria Control Programme (NMCP), in districts situated along the country's central latitude where peak malaria transmission lasts three to four months [6]. SP is also used in Chad as intermittent preventive treatment during pregnancy (IPTp). AQ is used in combination with artesunate as the first-line treatment for uncomplicated malaria in areas not covered by SMC, whereas artemether-lumefantrine is the first-line treatment in SMC-covered areas [7]. Although implementing SMC in southern Chad was not a priority in the national health policy in 2013, Médecins Sans Frontières (MSF) received authorisation from the Chadian health

authorities to initiate SMC in the southern health district of Moissala, located near the border with the Central African Republic (Fig. 1). First introduced in 2013 in eight health zones, SMC was progressively scaled up to encompass all remaining health zones, achieving full coverage of the Moissala district by 2015. Between 2013 and 2020, four monthly distribution rounds were conducted annually from July to October. Since malaria transmission in southern Chad lasts longer than in central regions of the country, and in alignment with the updated the WHO recommendations [5], a fifth round was added in November starting in 2021. In 2023, the estimated target population was 132,000 children aged 3 months to 5 years (84,600 and 47,400 residing on the west and east side of the Chari River, respectively) (Fig. 1). This figure likely underestimated the actual population, as an average 137,000 doses of SPAQ were administered per round.

SMC implementation in Moissala has been accompanied by monitoring and evaluation activities, including surveillance for genetic mutations associated with parasite resistance to SP and AQ. Furthermore, following the rollout of SMC, the entire health district transitioned to artemether-lumefantrine as the first-line treatment for uncomplicated malaria, replacing artesunate-AQ in accordance with WHO guidelines [5].

Plasmodium falciparum resistance markers correlate well with *in vitro* measures of SP and AQ resistance [8,9] and with clinical treatment outcomes [10–13]. Nevertheless, studies have shown that SMC with SPAQ continues to provide significant protection against clinical malaria, even in areas with a relatively high prevalence of resistance markers [14,15]. This illustrates that although resistance remains an important consideration, its impact on SMC efficacy must be viewed alongside other factors such as drug pharmacokinetics, parasite load, and acquired immunity [14,16,17].

In this context, monitoring the evolution of molecular markers remains a useful tool for tracking the emergence and dissemination of potential resistance, and for guiding future policy for SMC [5].

Two recent studies in southern Chad reported a low prevalence of molecular markers associated with reduced susceptibility to lumefantrine and AQ [18], and no quintuple mutant parasites (see definitions below) were detected [19]. However, data on antimalarial drug resistance were unavailable in Moissala Health District, which is so far the only district in southern Chad where SMC has been implemented.

This paper summarizes the findings of three studies conducted between 2014 and 2023 in the health district of Moissala, which report trends of the dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genotypes (markers of *P. falciparum* resistance to SP), and the *P. falciparum* chloroquine resistance transporter (*pfcrt*) and *P. falciparum* multidrug resistance 1 (*pfmdr-1*) genotypes (markers of resistance to AQ) [2,10].

Methods

Study design and setting

Three cross-sectional surveys were conducted during the low malaria transmission season: from February to April 2014, in June 2021, and in May 2023. The study protocols were consistent across all surveys maintaining uniformity of design, study sites, participant recruitment, and laboratory analysis procedures.

The study sites comprised eight health centres serving as reference facilities of the health zones where MSF first introduced SMC in 2013. These zones include Bekourou, Gon, Gonhongon, and Koldaga on the west side of the Chari River, and Bekamba, Boukinaoua, Dembo, and Gabian on the east side (Fig. 1). *Plasmodium falciparum* malaria diagnosis was confirmed using a histidine-rich protein 2 (HRP2) rapid diagnostic test.

Sample size

Sample sizes were determined assuming an expected proportion of quintuple mutants (defined below) of 8% in 2014, and 15% in 2021 and 2023, with a ±5% margin of precision, and an allowance of 10% for non-analysable samples. Based on these parameters, the required sample sizes were 126 children in 2014, and 218 children in both 2021 and 2023.

Sample collection and analyses

Blood collected via finger-prick was spotted on Whatman™ 3MM chromatography paper from children under 15 years of age, residing in the specified health zones. Eligible participants were those presenting to one of the designated reference health centres with a diagnosis of clinical malaria, defined as fever or a history of fever in the preceding 72 hours, confirmed by rapid diagnostic test. Receipt of SMC was not an inclusion criterion, and therefore this information was not recorded.

Blood spot samples were air-dried, labelled, and stored in individual plastic envelopes at room temperature prior to shipment to the Malaria Research and Training Centre laboratory in Bamako, Mali where DNA analyses were performed.

Parasite genomic DNA from all dried blood spot samples was extracted using a QIAamp DNA blood kit (Qiagen, Valencia, CA), following the kit's dried blood spot protocol. Polymorphisms in the *P. falciparum dhfr*, *dhps, Pfcrt,* and *Pfmdr-1* genes associated with resistance to SP and AQ, more specifically polymorphisms *Pfdhfr* N51I, *Pfdhfr* C59R, *Pfdhfr* S108N, *Pfdhps* A437G, *Pfdhps* K540E, *Pfcrt* K76T, and *Pfmdr-1* N86Y, were detected using nested mutation-specific PCR, as described in previous studies [20–25]. Briefly, in primary PCR, 20.0 µl reaction mixture consisting of 12.125 µl nuclease free water, 5 µl of buffer, 2.5 µl of dNTPs, 0.152 µl of each forward and reverse primers, 0.125 µl Taq polymerase, and 5.0 µl of DNA template was used. PCR was conducted in a thermocycler (BioRad PT-100). Primer names, sequences, and conditions of amplification are described in Additional file 1.

Definitions of combined mutations

Combined mutations were defined as follows. Triple *dhfr* mutant: mutations in three codons of the *dhfr* marker (N51I, C59R, and S108N); double *dhps* mutant: mutations in two codons of the *dhps* marker (A437G and K540E); quadruple mutant: combined triple *dhfr* mutation and *dhps* A437G mutation; quintuple mutant: combined triple *dhfr* mutation and double *dhps* mutation.

Statistical analysis

The analysis aimed to estimate the proportion of each molecular mutation. In addition to single mutations, the proportion of combined mutations (as per definitions here above) was also estimated. Samples yielding mixed results, indicating the presence of both wild-type and mutant alleles, were classified as mutant. Samples without a conclusive result were excluded from the denominator prior to calculating estimates.

All analyses were conducted using R Statistical Software (v4.4.1; R Core Team 2021). Confidence intervals were set at 95% and calculated using the Clopper-Pearson method. Trends in the prevalence of resistance-associated mutations over time were assessed using the Cochran-Armitage trend test, implemented in the *DescTools* package, which is appropriate for evaluating changes in proportions across ordered groups [26]. Unless otherwise specified, p-values were derived using this method. Estimates were considered significantly different when their p-value was less than 0.05.

Results

A total of 136 samples were collected in 2014 (between 24 February and 4 April), 256 samples in 2021 (between 21 and 25 June), and 219 samples in 2023 (between 22 and 31 May). Table 1 details the number of samples collected per health centre.

Single and multiple mutations of *Plasmodium falciparum* resistance markers to SP

For SP resistance markers, the proportion of single mutations in the three *dhfr* codons ranged from 96.9% to 100% across all three years, except for a notable decrease in 2023 for *dhfr* S108N (87.4%). Consequently, the proportion of triple *dhfr* mutants was high in 2014 and 2021 (100% and 96.9%, respectively) but declined significantly to 83.9% in 2023 ($p < 0.001$) (Fig. 2).

The proportion of mutants in the two codons of the *dhps* marker was highly variable. The proportion of samples with the *dhps* A437G mutation increased from 27.9% in 2014 to 40.2% in 2021 and 59.3% in 2023. In contrast, the proportion of *dhps* K540E mutants was low in

2014 (11.8%) and remained low in 2021 and 2023 (8.9% and 8.3%, respectively). The proportion of double *dhps* mutants was also low (7.4%, 3.7%, and 6.1% in 2014, 2021, and 2023, respectively) with no significant trend ($p = 0.411$) (Table 2).

When combining the *dhfr* and *dhps* markers, the proportion of quadruple mutants (triple *dhfr* + *dhps* A437G) increased significantly from 28.0% in 2014 to 41.0% and 47.9% in 2021 and 2023, respectively ($p < 0.001$). Conversely, due to the persistently low prevalence of the *dhps* K540E mutation, the proportion of quintuple mutants remained low and showed no significant change over time (7.6%, 2.8%, and 5.9% in 2014, 2021, and 2023, respectively, $p = 0.278$) (Fig. 2).

Single and multiple mutations of *Plasmodium falciparum* resistance markers to AQ

For AQ resistance markers, the proportion of mutations in the *pfcrt* K76T decreased significantly from 44.6% in 2014 to 11.3% and 11.4% in 2021 and 2023, respectively ($p < 0.001$). A smaller but significant decline was observed for the *pfmdr-1* N86Y mutation, which decreased from 11.7% in 2014 to 3.8% and 5.6% in 2021 and 2023, respectively ($p = 0.025$) (Table 2). As a result of the low proportion of the *pfmdr-1* mutation, and the decreasing proportion of the *pfrct* mutation, the proportion of samples harbouring both *pfrct* and *pfmdr-1* mutations was low in 2014 (6.7%), decreased further in 2021, and was not detected in 2023 ($p < 0.001$) (Fig. 3).

In 2014, one sample exhibited all seven point-mutations analysed, whereas no such cases were observed in samples from 2021 or 2023.

Geographical variation in quadruple mutants

Given the significant increase in quadruple mutants over time, an additional analysis explored differences between the west and east sides of the Chari River (Fig. 4). No statistically significant differences were observed in 2014 and 2021. However, in 2023, the prevalence of quadruple mutants was significantly higher on the west side (56.3%) compared to the east side (40.8%, Fisher's exact $p = 0.041$).

Discussion

The three studies undertaken over 10 years have documented clear trends in the prevalence of the molecular markers of resistance for SP and AQ in Moissala Health District, where SMC has been in place since 2013. Given that this evaluation focused on the eight health zones with the longest history of SMC – and thus likely under the highest SPAQ drug selection pressure – the findings presented here may represent the most pronounced resistance profiles currently observable in Moissala Health District.

The findings present a mixed picture: while they provide some reassurance regarding the current use of SP and AQ for SMC in Moissala, they also highlight emerging signs that could cast doubt on the long-term viability of these treatments.

The most notable and concerning trend is the progressive increase in *dhps* A437G mutants and, consequently, in quadruple mutants, which rose from approximately one third of samples in 2014 to nearly half by 2023. Although this increase is significant, it is not unprecedented in other contexts where SMC was deployed. Whereas no increase in the A437G mutation was observed in Ouelessebougou (Mali) after 3 years of SMC implementation (74.5% at baseline and 64.5% three years later) [27], a trend toward mutant selection was observed after only

three rounds of SMC in a clinical trial in Burkina Faso (56.8% at baseline and 62.5% post-SMC) [28], and similarly after three years of large-scale SMC deployment in Senegal (71.4% before and 82.4% after) [29]. In comparison, the increase observed in Moissala over the span of ten years was more gradual. Nonetheless, a continued and steady rise in the *dhps* A437G mutation is likely in the coming years, as long as SMC with SPAQ remains in use.

The higher proportion of quadruple mutants on the west side of the Chari River warrants attention. Although the underlying cause remains unclear, the greater number of SMC treatment doses administered on the west side of the river over the years—compared to the east side—may have increased the potential for selection of resistant parasite strains [30]. Conversely, the consistently low prevalence of the *dhps* K540E mutation over time is reassuring. Similar patterns have been reported in Burkina Faso and Senegal [28,29], as well as in other West African countries [31], suggesting a geographical east–west gradient. Indeed, the situation contrasts sharply with East Africa, where the *dhps* K540E mutants have become predominant [32], prompting avoidance of SP [33]. Importantly, the proportions observed in Chad remain well below the WHO threshold of 50% prevalence for the K540E mutation, which is considered acceptable for continued use of SP as a malaria preventive treatment. Recent modelling work suggests that SMC with SPAQ leads to the relatively slow spread of quintuple mutants and remains effective at preventing clinical malaria despite this spread [34]. However, the steady increase in the proportion of the quadruple mutant, which confers partial resistance to SP, underscores the need for close monitoring to detect any early surge in *dhps* K540E prevalence, as this would indicate a critical increase in quintuple mutants and a much stronger resistance profile [10,32].

Although AQ lacks specific molecular resistance markers, *pfcrt* K76T and *pfmdr-1* N86Y mutations have been associated with *in vivo* resistance to this drug when used for treatment [12,35]. Their low prevalence in the 2021 and 2023 studies provides reassurance for the continued use of AQ in Moissala.

The decline in the prevalence of *pfcrt* K76T from nearly half of the samples in 2014 to about 10% in recent years is noteworthy, but consistent with documented reductions following chloroquine withdrawal in multiple countries [36,37]. The *pfmdr-1* marker was low in 2014 and reduced even further in 2021 and 2023, suggesting that AQ is not losing its efficacy. The availability of baseline data collected prior to SMC implementation, as well as the inclusion of additional mutations such as *dhfr* I164L and *dhps* A581G [38,39], would have enabled a more comprehensive assessment of the evolution of resistance; the inclusion of additional mutations will be incorporated in future studies in Moissala.

Importantly, SMC is not the sole driver of SP resistance. Other factors such as the administration of SP for IPTp [40], along with other drugs from the same class—such as trimethoprim-sulfamethoxazole used for both treatment and prevention—may have played a role in, and will likely continue to influence, the development of parasite resistance. Finally, molecular markers do not fully capture the chemopreventive effectiveness of SPAQ as multiple determinants influence its performance. Still, the trends presented here are helpful to inform country health authorities about the future scope of SP and AQ for SMC in Moissala Health District, and guide future studies.

Conclusion

The low proportion of quintuple mutants, the decrease of the *pfcrt* marker, and the consistently low proportion of the *pfmdr-1* marker across a decade-long series of three studies in Moissala, southern Chad, suggest that SP and AQ remain viable drugs for SMC, despite their prolonged use in the area. However, the steady increase of quadruple mutants, which confers partial resistance to SP, is a point of concern, and close monitoring is required in the coming years for the early detection of any critical increase of quintuple mutants which confers much stronger resistance to SP. Future studies in Moissala may benefit not only from continued *in vitro* monitoring of molecular markers of resistance, but also from the addition of an *in vivo* evaluation of chemoprevention efficacy. This combined approach would provide a more comprehensive and reliable understanding of local parasite resistance to the chemopreventive effects of SPAQ used in SMC.

Abbreviations

AQ	Amodiaquine
Dhfr	Dihydrofolate reductase
Dhps	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
MSF	Médecins Sans Frontières
NMCP	National Malaria Control Programme
PCR	Polymerase Chain Reaction
Pfcrt	*Plasmodium falciparum* chloroquine resistance transporter
Pfmdr-1	*Plasmodium falciparum* multidrug resistance 1
SMC	Seasonal malaria chemoprevention
SP	Sulfadoxine-pyrimethamine
WHO	World Health Organization

Declarations

Ethics approval and consent to participate

The three studies independently received ethical approval from the Chad National Bioethics Committee (No: 0415/PR/PM/MSP/SE/SG/DGAS/DSPELM/DMTNT/PNLP/14, No: 006/CMT/PCMT/PMT/MESRI/SE/DGM/CNBT/SG/21 and No: 034/PT/PM/MERSI/SE/SG/CNBT/SG/2023).

The study in 2014 was also approved by the Comité de Protection des Personnes Ile de France XI (Ref: CPP 14027). The studies in 2021 and 2023 were also approved by the MSF Ethics Review Board (Ref: 2120 and 2329).

Prior to study participation, written consent was obtained from the child's parent/guardian. In addition, verbal assent was obtained from children 8 to 14 years old.

Consent for publication

Not applicable.

Availability of data and materials

Data from the three studies were transmitted to the Worldwide Antimalarial Resistance Network collaborative platform (https://www.wwarn.org) and are available in accordance with the platform policy.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

FG was the principal investigator and coordinated the implementation of the three studies. MSID, FK, JS, PO, and SS helped edit the protocols and facilitated the implementation of the studies. SD and AAD contributed to the study design and oversaw the molecular analyses of the samples and interpretation of the data. The manuscript was drafted by FG and all authors contributed to the revision and approval of the final version.

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Tables and Figures

Figure 1: Location of Moissala Health District in Chad (*top left*).

Boundaries of Moissala Health District (green) and of the health zones participating in the molecular studies of *P. falciparum* resistance (darker green) (*right*)

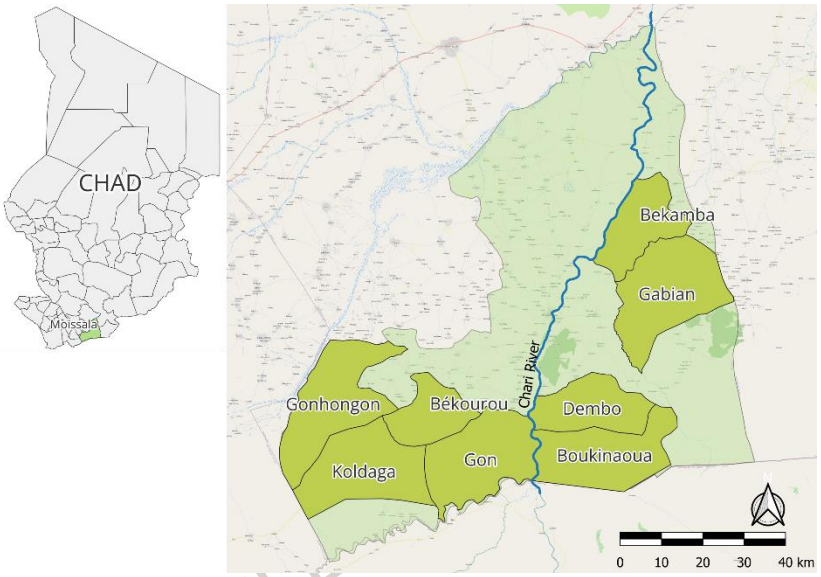

Figure 1 note: The map shows the boundaries of Moissala Health District in 2014. While the district was later divided into three health districts (Moissala, Dembo, and Bekourou), the boundaries of the health zones remained unchanged.

Table 1: Number of samples collected per health centre and year

	2014	**2021**	**2023**	**All years**
Bekamba	20	28	37	85
Bekourou	17	32	35	84

Boukinaoua	26	35	24	85
Dembo	37	53	30	120
Gabian[1]	0	39	22	61
Gon	16	35	30	81
Gonhongon	9	20	17	46
Koldaga	11	14	24	49
All health centres	136	256	219	611

[1] No samples were collected from the Gabian health centre in 2014 due to security concerns

Table 2. Trends in proportions of single and combined *P. falciparum* molecular marker mutations

	2014		**2021**		**2023**		***p-value***
	n/N	***%***	**n/N**	***%***	**n/N**	***%***	
***Dhfr* N51I**	135/135	*100.0*	214/214	*100.0*	208/214	*97.2*	*0.041*
***Dhfr* C59R**	133/133	*100.0*	249/249	*100.0*	204/209	*97.6*	*0.060*
***Dhfr* S108N**	136/136	*100.0*	216/223	*96.9*	180/206	*87.4*	*<0.001*
***Dhps* A437G**	38/136	*27.9*	100/249	*40.2*	128/216	*59.3*	*<0.001*
***Dhps* K540E**	16/136	*11.8*	22/247	*8.9*	18/217	*8.3*	*0.259*

***Pfcrt* K76T**	54/121	*44.6*	26/230	*11.3*	24/211	*11.4*	*<0.001*
***Pfmdr-1* N86Y**	13/121	*10.7*	9/240	*3.8*	11/197	*5.6*	*0.025*
Triple dhfr	132/132	*100.0*	186/192	*96.6*	162/193	*83.9*	*<0.001*
Double dhps	10/136	*7.4*	9/241	*3.7*	13/214	*6.1*	*0.411*
Quadruple	37/132	*28.0*	77/188	*41.0*	91/190	*47.9*	*<0.001*
Quintuple	10/132	*7.6*	5/181	*2.8*	11/188	*5.9*	*0.278*

Note: Samples with no results were excluded from the denominator

p-values were calculated using the Cochran-Armitage trend test

Figure 2: Proportion of samples with combined mutations in the *dhfr* and *dhps* markers

Legend: The small square shows the central estimate; the bars show the 95% confidence interval calculated using the Clopper-Pearson method.

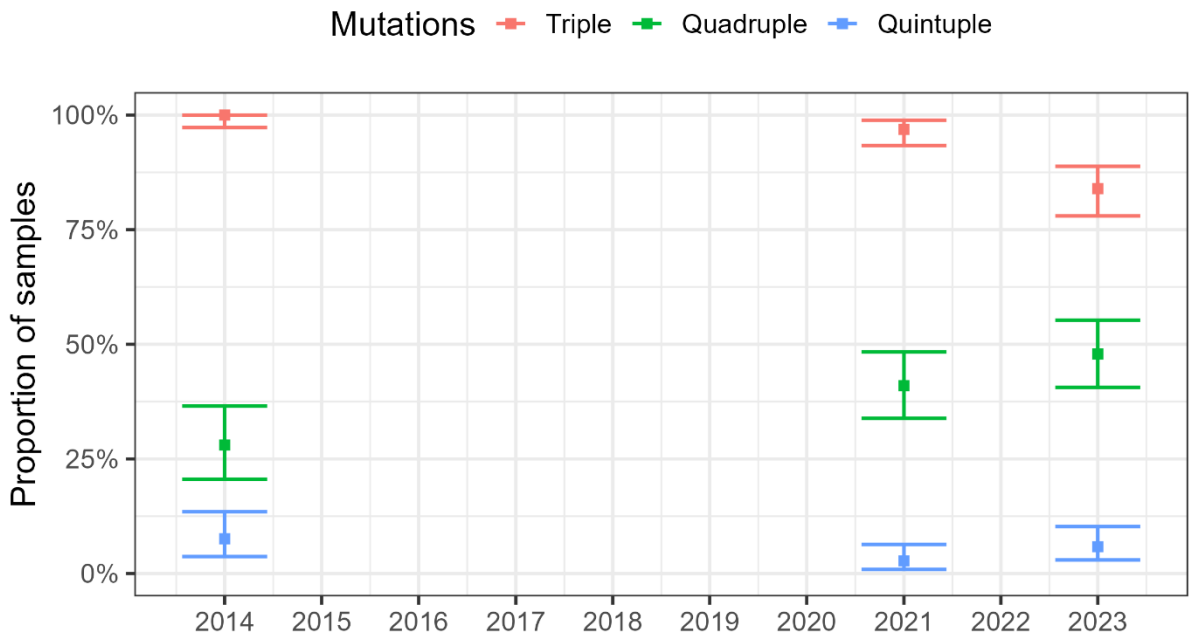

Figure 3: Proportion of samples with combined mutations in the *pfcrt* and *pfmdr-1* markers

Legend: the small square shows the central estimate; the bars show 95% confidence interval calculated using the Clopper-Pearson method.

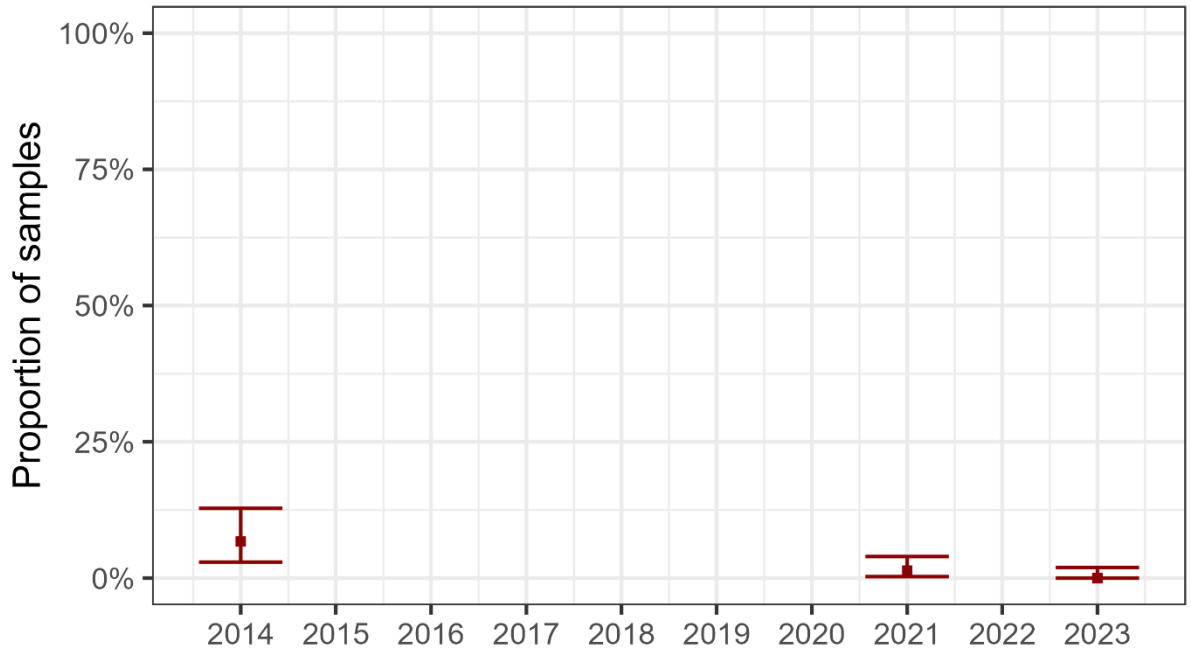

Figure 4: Proportion of samples with quadruple mutants by side of the Chari River

Legend: The small square shows the central estimate; the bars show the 95% confidence interval calculated using the Clopper-Pearson method.