Int J Mol Epidemiol Genet 2024;15(3):22-30
www.ijmeg.org /ISSN:1948-1756/IJMEG0155182

Original Article

P. falciparum genetic markers associated with drug resistance from patients with treatment failure in the Southern part of Senegal in 2017

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Received December 29, 2023; Accepted June 11, 2024; Epub June 15, 2024; Published June 30, 2024

Abstract: Artemisinin Combination Therapies (ACT) stand as the most potent antimalarial treatments. In response to the emergence of ACT-resistant malaria parasites in Southeast Asia, the World Health Organization (WHO) has recommended continuous monitoring of the effectiveness of ACT and other antimalarials. To address this need, we collected dried blood spots from malaria patients during a 42-days drug efficacy trial evaluating the efficacy of Artesunate plus Amodiaquine (ASAQ), Artemether Plus Lumefantrine (AL) and Dihydroarthemisinine plus Piperaquine (DHAPQ) on simple *P. falciparum* malaria in 2017. Blood samples were collected on Day 0, prior to the patients' initial ACT dose, and on any days of recurrent parasitemia. Genetic markers such as *Merozoite Surface Protein* 1 *(MSP1)* and *Merozoite Surface Protein 2 (MSP2)* were genotyped to differentiate between recrudescence and re-infestation cases. Furthermore, PCR Single Specific Oligonucleotide Probes combined with-ELISA platform (PCR-SSOP-ELISA) and PCR-RFLP techniques were used to identify *Pfcrt* 72-76 mutant haplotype and *Pfmdr1*_86Y allele associated with chloroquine and amodiaquine resistance, respectively. Out of the 320 patients enrolled in the study, only 43 (13.43%) experienced relapses. Upon PCR correction, our analysis revealed that recrudescent infections affected 13 patients, with 8 in the ASAQ group, 5 in the AL group, and none in the DHAPQ group. Notably, no early treatment failures (within the first 3 days of treatment) were observed, and all recurrences occurred between Day 21 and Day 42. The prevalence of the *Pfcrt* wild-type haplotype CVMNK and *Pfmdr N86* allele was 67.03% and 97.70%, respectively. In contrast, the mutant types CVIET and 86Y were found at 32.97% and 2.3%, respectively. The high prevalence of the CVMNK wild haplotype suggests that the parasites remain sensitive to chloroquine, while the low prevalence of the 86Y mutants indicates continued effectiveness of amodiaquine. Furthermore, the low prevalence of strains exhibiting the combination of CVIET and 86Y suggests that the use of multiple antimalarials is valuable for resistance control. Notably, none of the relapse cases carried the 86Y mutation or the combination of 86Y and CVIET.

Keywords: Malaria, drug resistance marker, *Pfmdr*1_86, *Pfcrt* 72-76, RFLP, SSOP-ELISA

Introduction

Malaria remains a prominent global public health concern. In 2021, 247 million malaria cases were reported in 84 endemic countries [1]. In 2015 out of the 610,026 deaths attributed to parasitic diseases, malaria was responsible of 439,026 deaths [2], making malaria the deadliest among these diseases. Unfortunately, no efficient malaria vaccine alone exists at present, and the most effective treatment approach is Artemisinin Combination Therapy (ACT) [3]. The recent emergence of artemisinin-resistant (ART) parasites in Southeast Asia and the potential for this resistance to spread from western Cambodia to the Greater Mekong Sub-region [4] and Africa, as previously observed with chloroquine [5] and Sulphadoxine/pyrimethamine [6, 7] resistance, is a source of great concern. This surveillance of resistance is essential in the areas most affected by malaria such as sub-Saharan Africa, where almost all cases and deaths related to malaria are recorded (WHO, 2018). According to WHO in 2019, 92% of cases and 93% of deaths occur in sub-Saharan Africa and

https://doi.org/10.62347/RWLA6562

many of them were caused by *P. falciparum*. In response to this challenge, the World Health Organization (WHO) has launched the Global Plan for Artemisinin Resistance Containment, with the primary objective of preserving ACTs as effective treatments for *P. falciparum* malaria. The plan urges global and local partners to engage in containing and, if necessary, eliminating artemisinin resistance while preventing its dissemination to new regions. Losing the effectiveness of artemisinin to resistance, as noted with previous antimalarial, would represent a significant setback in the fight against malaria. The urgency is particularly acute because there are few alternatives with similar efficacy and tolerability to ACTs, and few alternatives exist for the immediate future [8]. In Senegal, malaria control primarily relies on chemotherapy and chemoprevention [9]. ACTs have been used for uncomplicated malaria since 2006, while chemoprevention commenced in 2004 with sulfadoxine-pyrimethamine for pregnant women. Due to its success, malaria chemoprevention has been extended to children aged 3 to 59 months in areas of high transmission, known as Seasonal Malaria Chemoprevention (SMC) [10]. This new strategy called Seasonal Malaria Chemoprevention (SMC) consists of multiple cycles (maximum 4 cycles) of Sulfadoxine-Pyrimethamine (SP) associated with amodiaquine (AQ) in children aged 3 to 59 months, at one month intervals, from the beginning of the transmission period [11]. In Senegal, due to the shift of the malaria transmission to children up to 5 years old, the strategy of SMC was extended to children under 10 years old. However, drug pressure has been shown to be one of the factors that would promote the resistance of *P. falciparum* to antimalarials used [8].

Thus, it appeared important to determine drug resistance markers associated with amodiaquine (AQ) and chloroquine (CQ) resistance of *P. falciparum*. In fact, AQ exerts enormous pressure on parasites since it has been used during SMC with SP and in combination with artesunate (AS) for malaria treatment [9]. Moreover, in order to reinforce the idea of the re-use of CQ issued by several authors [12, 13], it would be necessary to confirm a return of parasites susceptibility to chloroquine since it was abandoned in 2000. Evaluation of this resistance requires the use of innovative tools such as molecular biology to track Single nucleotide polymorphism (SNP) of *Plasmodium* genes associated with resistance to particular antimalarial. These SNPs are used as molecular markers to detect the emergence and spread of resistance in a given area, in order to determine the efficacy of parasite strains treatments and antimalarial drug resistance [8].

This study was conducted to assess the prevalence of amodiaquine and chloroquine resistance markers in *P. falciparum* among patients experiencing treatment failure during the monitoring of ACT effectiveness in the Mako and Tomboronkoto areas of Kedougou, southern Senegal in 2017. The specific objectives were to categorize clinical failure cases, confirm recrudescence or re-infestation using *MSP1* and *MSP2* PCR, determine the prevalence of wild-type CVMNT and mutant CVIET haplotypes of the *Pfcrt* 72-76 gene associated with *P. falciparum* susceptibility and resistance to chloroquine, and assess the prevalence of *Pfmdr1* SNPs (N86 and 86Y) linked to the sensitivity and resistance of P. falciparum to amodiaquine.

Material and methods

Study area

Our study was conducted in two distinct zones within the Kedougou region, separated by 8.9 kilometers: Mako (coordinates 12°52’0” N and 12°21’0” W) and Tomboronkoto (coordinates 12°48’0” N and 12°18’0” W). The Kedougou region covers an area of 9,954 square kilometers and is inhabited by a population of 89,481 [14]. Situated in the far southeastern part of Senegal, this region shares its borders with Mali to the east and Guinea to the south. It falls within the Sudanese and Guinean climatic zones, characterized by a wet savanna with an annual average rainfall ranging from 800 to 1,500 millimeters [9].

The predominant *Plasmodium* species in this region is *P. falciparum*, and the primary mosquito vectors responsible for malaria transmission include *An. gambiae*, *An. arabiensis, An. funestus*, and *An. nili* [9]. Malaria remains endemic year-round in the southern portion of the region, which exhibits the highest malaria prevalence and incidence rates in the country, with an incidence of 15%. The mortality rate for malaria is 6%, while infant mortality due to malaria stands at 4%. In response to this significant malaria burden, Seasonal Malaria Chemoprevention (SMC) has been implemented in this hyper-endemic area [10].

Samples collection and study design

Samples analysed in this study were collected from drug efficacy trial, evaluating the efficacy of ASAQ, AL, and DHAPQ, along with a molecular analysis of resistance markers integrated into the study design. In essence, individuals with uncomplicated falciparum malaria were enrolled and randomly assigned to receive AL, ASAQ, or DHAPQ. They were subsequently monitored over a 42-day period with scheduled assessments visits on days 3, 7, 14, 21, 28, 35, and 42. During each visit, blood samples were collected and thick and thin blood smears were prepared for microscopy identification and quantification. Additionally, finger prick blood samples were collected from each study participant and then preserved on Whatman filter paper 3MM, stored in ziplock bags with desiccant once they had dried. All Whatman filter papers collected on Day 0 (prior to the first ACT dose) and on any subsequent day showing recurrent parasitémie (Dx) (indicating either recrudescence or a new infection) were shipped to the central Parasitology Mycology laboratory in Dakar for genotyping of *MSP1*, *MSP2*, *Pfmdr1*, and *Pfcrt* genes.

P. falciparum DNA extraction

DNA was extracted from filter paper by Chelex-100 method described [15]. Briefly, 1X PBS with 0.5% saponin was added to small pieces of blood-impregnated filter paper, shake for 10 min (150 rpm) and incubated at room temperature overnight. The resulting supernatant was removed, and the filter paper was washed twice with PBS buffer. Subsequently, a mixture of 150 μL of milli-Q H_2O and 75 μL of a 20% Chelex solution (prepared by dissolving 5 g of Chelex in 25 ml of milli-Q H_2O) was added to the wells of a 96-well deep plate, and the plate was securely sealed. The deep plate was then subjected to two rounds of boiling for 8 minutes (2 cycles of 4 minutes each) and allowed to cool for 10 minutes at room temperature. After 5 minutes centrifugation, 50 μL of solution containing DNA of the resulting supernatant was carefully transferred to new 96-well PCR plates, with care taken to leave the Chelex in the original deep well plate. The new 96-well PCR plate with DNA was then frozen at -20°C.

Genotyping of MSP1 and MSP2 genes

A nested PCR approach was employed to analyze polymorphism in two variable loci, specifically merozoite surface proteins *MSP1* and *MSP2*, in order to distinguish recrudescence between new infections, following a previously described method [16]. In essence, DNA fragments generated from the amplification of the baseline sample (D0) and from the day of recurrent parasitemia (Dx) were compared based on the size and number of bands observed, taking into consideration *MSP1* and *MSP2 allelic families*. Cases were classified as recrudescence if they exhibited at least one matching band between the baseline sample (D0) and the sample from the day when the parasites reappeared (Dx), for either of the two markers. Conversely, patients were categorized as new infections when no common bands were found between the samples D0 and Dx. It is important to note that cases with recurrent parasitemia classified as new infections, rather than recrudescent infections, were not considered as clinical failures.

Pfcrt and Pfmdr1 genotyping

Pfcrt 72-76 amplification and analysis: A nested PCR described by Djimde and others [17] was used to amplify fragments of the *Pfcrt* gene. The only modification was that primers TCRD2 in the *Pfcrt* nested PCRs were biotinylated at the 5-end by the supplier (www.mwg-biotech.com). The 20-mL *Pfcrt* outer PCR mixture consisted of the primers P1/P2 (1 mM/primer), 10 TEMPase Hot StartMaster Mix (3.0 mM $MgCl_2$, 0.4 mM deoxynucleoside 5-triphosphate [dNTP], and 0.2 units/mL TEMPase Hot Start DNA Polymerase, Ampliqon III; VWR-Bie, Berntsen, Denmark), and 1 mL extracted DNA. The reaction mixture of the nested *Pfcrt* PCR was identical to the mixture of the outer PCR, and the primer set TCRD1/TCRD2-biotin was used. Genomic DNA preparation of laboratory isolates 3D7, Fcr3, K1 and 7G8 were included as references for wildtype CVMNK and mutant types CVIET haplotypes, respectively. Amplifications were performed in 96-well

PCR microplates. The nested PCR products were confirmed by running the controls by electrophoresis on a 1.5% agarose gel.

The SSOP-ELISA assay was performed for *Pfcrt* 72-76 haplotypes analysis. This method has been described by Alifrangis and others [18]. Briefly, biotin-conjugated nested PCR products were fixed on streptavidin-coated ELISA plates and incubated overnight at 4°C. After washing three times in washing buffer (1 + phosphate-buffered saline [PBS] with 0.05% Tween 20), digoxigenin-labeled oligonucleotide probes with specificity for the haplotypes of interest (CVMNK, CVIET, or SVMNT) were added to each plate and incubated for 1 hour at 53°C. The mixture was washed with high stringency at 60°C two times for 10 minutes before they were incubated for 1 hour with peroxidase-conjugated antidigoxigenin antibodies (Roche Diagnostics, Mannheim, Germany) and visualized by o-phenylene-diamine (OPD; Dako, Glostrup, Denmark). The SSOP is able to detect both single and mixed haplotypes with high specificity. For each analysis, parasite samples were categorized into single or mixed infections. Infections were considered to be single haplotype when only one was present at optical density (OD) values above the threshold of positivity. Conversely, samples were considered as mixed if OD values for both haplotypes were above the threshold of positivity. For statistical analysis purposes and adherence to Q-PCR data, parasites carrying the CVMNK haplotype only were classified as wild-type parasite infections, whereas parasite harbouring both CVMNK and CVIET haplotypes were considered as resistant parasite infections.

*Pfmdr1_86 amplification and analysis: Pfmdr*1_86 was amplified by a nested PCR. For the first amplification the 19-µL PCR mixtures consisted of the primers mdr1 New rev1 4 µl of the primers FN1F1/FR1R1 (0.2 µM/primer), 10 TEMPase Hot StartMaster Mix (3.0 mM $MgCl_2$, 0.4 mM deoxynucleoside 5-triphosphate [dNTP], and 0.2 units/mL TEMPase Hot Start DNA Polymerase, Ampliqon III; VWR-Bie, Berntsen, Denmark), and 1 µl extracted DNA. The reaction mixture of the nested *Pfmdr*1 PCR was identical to the mixture of the first PCR, and the primer set FN2R2/FR2R2 was used. 3D7 (N86), Fcr3 (86Y) and 7G8 (N86-184F-1246Y) were used as positive controls.

Restriction enzyme (RFLP) was used for SNPs determination. Endonuclease *Afl*III had been obtained from New England BioLabs™, Roche Molecular Biochemicals™ and Stratagene™ respectively. Incubations of *P. falciparum* DNA samples with restriction enzymes were setup following the manufacturer's instructions. Following amplification of the fragments concerned, *Afl*III enzyme was used for *Pfmdr*1_N86Y SNPs determination. *Pfmdr*1_86 DNA was incubated with the *Afl*III enzyme overnight at 53°C. The mixture products was visualised on 2% agarose gel with ethidium bromide and visualised under UV (ultraviolet) light. Samples are classified as mutant type (86Y) when DNA fragment length was found at 346 bp and 175 bp while mutant type (N86) was found at 521 bp. Samples were classified as mixt if fragment length for wild and mutant types were found.

Statistical analysis

Clinical Data were double entered in Microsoft Excel database by two independent data entry clerks. Molecular data were entered independently from treatment outcomes in an Excel database. Statistical analysis was performed using R software, version R version 3.4.4 (R Core Team, 2018). OD values obtained from the ELISA reader were entered in a Microsoft Excel sheet, and the haplotype of *Pfcrt* 72-76 of each positive sample was determined. *Pfcrt* and *Pfmdr*1 genotype profile was determined by the presence or absence of wild/mutant alleles. Samples carrying both wild and mutant *Pfcrt* or *Pfmdr*1 alleles and for which related frequencies could not be determined were excluded from the analysis. Differences between groups were assessed using the Chi-square test or Fisher exact test for proportions and a *P*-value of less than 0.05 was considered as statistically significant.

Results

Population characteristics

A total of 320 individuals confirmed *P. falciparum* positive were enrolled in our drug efficacy trial. Among them 44 individuals were included in our molecular study. For this study, samples were collected from individual at D0 (before receiving any antimalarial treatment regiment) and Dx representing any

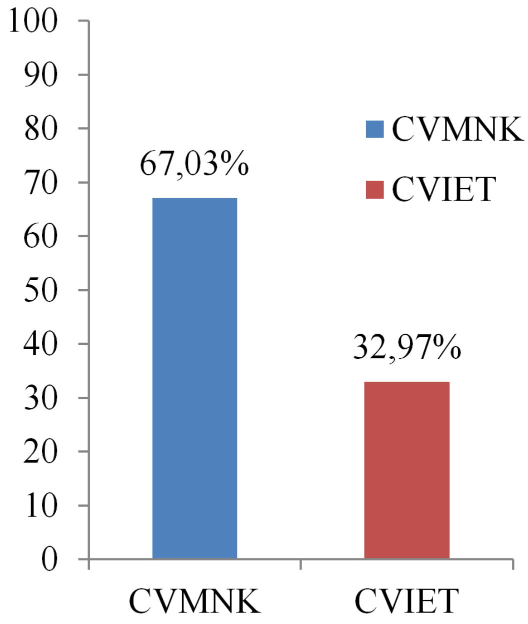

Figure 1. Prevalence of *Pfcrt* alleles.

day of recurrent parasitemia confirmed by microscopy examination. At inclusion, the mean parasitemia was 25,734.70 parasites/µl (± 22,368.20), with a minimum of 2,222 parasites/µl and a maximum of 88,313 parasites/µl. At the day of treatment failure, the mean parasitemia was 19,885.08 parasites/µl (± 26,014.88), with a minimum of 1,110 parasites/µl and a maximum of 91,554 parasites/µl.

Among the study population, males constituted the majority at 55.81% (24 out of 43), while females represented 44.19% (19 out of 43). The sex ratio was 1.26. The mean age of the study participants was 9.58 years (± 6.22), with ages ranging from 1 to 29 years. When categorizing patients into two age groups, less than 10 years (0-10 years) was predominant 58.13% (25 out of 43). This age group represents the target age group for Seasonal Malaria Chemoprevention (SMC) in Senegal. Meanwhile, 41.87% (18 out of 43) were older than 10 years.

Additionally, distribution of geographical therapeutic failure cases showed that 51.16% (22 out of 43) were found in Tomboronkoto, while 48.84% (21 out of 43) were from Mako with no statistical significant difference (P=0.88). Most of treatment failure cases (53%; 23/43) had been treated with ASAQ while 46.51% had received AL with no statistical significant difference (P=0.65).

PCR efficacy

All samples were amplified successfully for *MSP1*, *MSP2* and *Pfmdr*1_86 genes. However, for *Pfcrt* gene, 77% (67/87) of samples were successfully amplified; among them 33 samples were collected at D0 and 34 samples at day of relapse (Dx).

Clinical and parasitological responses

Out of 320 patients included in drug efficacy trial, 111 were randomly allocated to receive ASAQ, 113 to receive AL and 96 to receive. The parasitological failure rate, without PCR correction, was 13.43% (43/320).

After a 42-day follow-up, genotyping of *MSP1* and *MSP2 genes* showed that 13 patients were classified as recrudescent infections, among them 8 cases were noted in ASAQ group, 5 cases in AL group, and none in DHAPQ group (P=0.41). In contrast, 30 patients were classified as new *P. falciparum* infections; with 10 cases in AL group and 56 cases in ASAQ group. Results show that no early treatment failure (within the first 3 days of treatment) was observed, all treatment failures were noted between Day 21 and Day 42.

Prevalence of Pfcrt 72-76 haplotypes

Our findings revealed that 67.03% of P. falciparum strains carried out the CVMNK wild-type haplotype, while 32.97% of the strains the CVIET mutant haplotype (**Figure 1**). By comparing prevalence of mutant haplotype associated with chloroquine resistance at D0 and Dx (post-treatment), a slightly higher prevalence at Dx (38.46%) than at D0 (27.91%) was noted with no statistically significant difference (P=0.20) (**Figure 2**).

Prevalence of Pfmdr1_86Y allele

Overall only 2.3% of the parasites in our study carried the *Pfmdr1*_86Y mutant allele associated with Amodiaquine resistance, while 97.7% of parasites carried out the *Pfmdr1*_86N wild-type allele (**Figure 3**). Interestingly, our results further revealed that the *Pfmdr1*_86Y mutation was exclusively detected in patients who had received treatment with the ASAQ combination

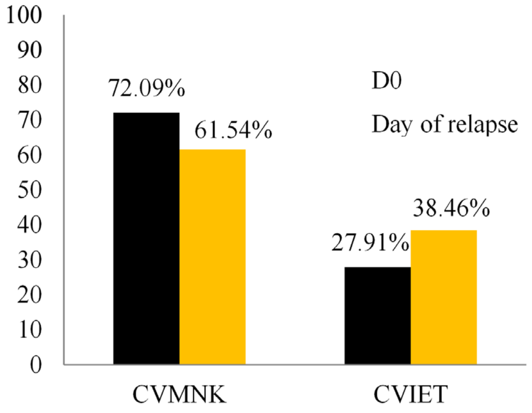

Figure 2. Prevalence of *Pfcrt* alleles at D0 and Day of relapse.

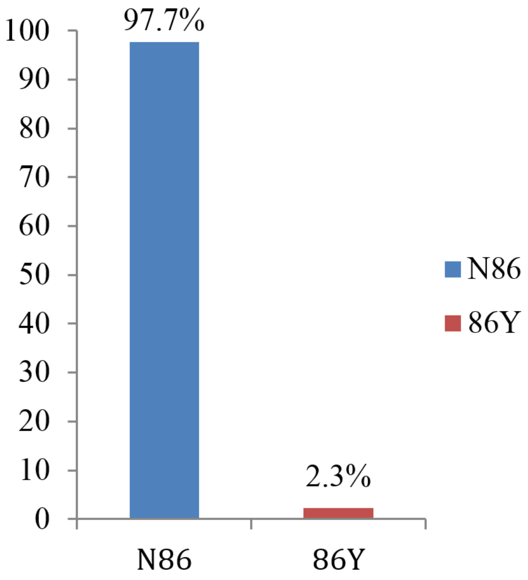

Figure 3. Prevalence of *Pfmdr*1_86 alleles.

(**Figure 4**). According to age and treatment regiment, no *Pfmdr*1_86Y was found in treatment failure cases. Indeed, *Pfmdr*1_86Y was only found in under 10 years old re-infected patients.

Prevalence of combination CVIET and Pfmdr1_86Y

Parasites harbouring both CVIET haplotype and 86Y mutation were detected at a prevalence of 2.30%. These patients were treated with ASAQ combination and were all under 10 years old. Notably, in the cases of treatment failures, all parasites harbouring CVIET haplotype in combination with 86Y mutation were found in re-infestation cases (**Figure 5**).

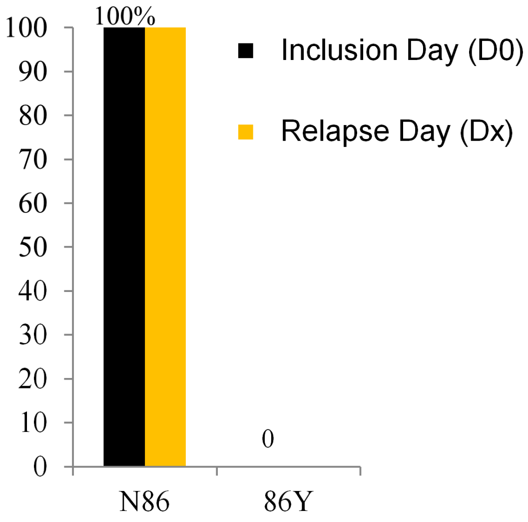

Figure 4. Prevalence of *Pfmdr*1_86 alleles at D0 and Dx.

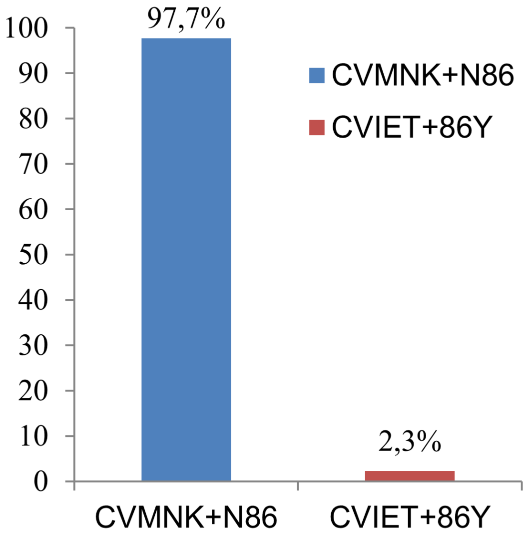

Figure 5. Prevalence of the combinations CVMNK+N86 and CVIET+86Y.

Discussion

Malaria persists as the most fatal parasitic disease globally. Without an effective antimalarial vaccine, the primary approach to managing uncomplicated malaria cases relies on Artemisinin Combination Therapeutics (ACTs), proven to be highly efficacious. However, the emergence and spread of artemisinin-resistant parasites in Southeast Asia and the potential spread to Africa raise concerns about the sustained efficacy of these drugs. Consequently, the World Health Organization (WHO) recommends regular monitoring of antimalarial efficacy, encompassing ACTs, SP, and AQ, and tracking resistance in endemic regions. In this context, an assessment of the prevalence of mutations in the *Pfcrt* and *Pfmdr1* genes associated with *P. falciparum* resistance to chloroquine (CQ) and amodiaquine (AQ) was conducted among patients experiencing treatment failures after ACT treatment.

Analysis of the *MSP1* and *MSP2* gene polymorphism revealed that among the 43 cases of therapeutic failures confirmed by microscopy, 30.23% (13/43) were categorized as recrudescence cases, while 69.77% (30/43) were re-infected cases (new infection). These results align with previous findings in Senegal and the prevalence of recrudescence cases noted during our study was higher than results obtained in Central and Western Senegal [19, 20]. This difference could be due to the difference of malaria transmission between study areas.

The absence of early therapeutic failure (within the 3 days of ACT treatment), suggests that parasites are sensitive to artemisinin derivatives [21]. However, the occurrence of recrudescence between Day 21 and Day 42 suggests potential resistance development to partner drugs [22].

Globally, prevalence of the chloroquine resistant haplotype CVIET and sensitive haplotype CVMNK on the *Pfcrt* 72-76 gene were 32.97% and 67.03% respectively, in our study area. Our results were comparable to previous studies conducted in southern Senegal where authors noted prevalence of 65.40% and 34.60% for wild-type and mutant haplotypes respectively [23]. These results were consistent with previous studies in southern Senegal, indicating a re-emergence of sensitivity of *P. falciparum* strains to chloroquine several years after withdrawal of chloroquine for the management of uncomplicated malaria cases in Senegal. Variations from findings in Benin where authors noted that 93.90% of analysed samples carried out the 76T mutation after several years after chloroquine withdrawal may be attributed to self-medication practices despite chloroquine withdrawal [24]. In addition, our results showed that the prevalence of the CVIET haplotype was higher after recrudescence (Day x) than at inclusion (Day 0). This increase noted between the inclusion and the day of failure could probably be due to the drug pressure of treatment.

For the polymorphism of the *Pfmdr*1_86 gene, our results showed a high prevalence of the wild-type N86 allele (97.70%) and a low prevalence of the 86Y mutant allele (2.30%). An earlier study conducted in the Thies region showed the same trend with a high prevalence (98%) of the wild-type N86 allele [25]. Similarly, a study conducted in Burkina Faso also showed a high prevalence (91.70%) of the wild-type N86 allele [26]. This high prevalence of the wild-type N86 allele found in Senegal and in the sub-region was noted after the withdrawal of CQ in the management of malaria and the adoption of ACTs (ASAQ, AL and DHAPQ) for the treatment of uncomplicated malaria. Moreover, the high prevalence of N86 found in our study area could be explained by the use of AL in this zone because it has been shown that the AL combination leads to a selection of the N86 allele [27].

Moreover, the *Pfmdr*1_86Y allele was only found in re-infestation cases after treatment with the ASAQ combination, specifically in individuals under 10 years old. This occurrence may be due to the selective pressure exerted by amodiaquine, used in combination with SP for malaria chemoprevention in this age group in southern Senegal. This is confirmed by previous studies indicating that the *Pfmdr*1_86Y mutation was predominant in the seasonal malaria chemoprevention (smc) group compared to the control group [28].

When examining the prevalence of parasites carrying both the *Pfcrt* mutant haplotype (CVIET) and the *Pfmdr1_86Y* mutation, a low prevalence of this combination (2.30%) was observed. This may be attributed to the multiple ACT combinaison use for malaria management in Senegal, as these combinations favour different *Pfcrt 72-76* and *Pfmdr1 86* alleles [8].

Conclusion

Artemisinin Combination Therapeutics has significantly reduced malaria morbidity and mortality. Nonetheless, the emergence of artemisinin-resistant parasites and the potential for their spread pose a threat to malaria control efforts. WHO's recommendation for resistance surveillance in malaria-endemic regions is crucial. Results indicate an increased prevalence of the CVMNK haplotype associated with *P. falciparum* susceptibility to chloroquine. Additionally, a low prevalence of the 86Y mutant allele associated with *P. falciparum* amodiaquine resistance was observed, suggesting that amodiaquine remains effective against *P. falciparum* strains. However, the low prevalence of *Pfmdr1_86Y* and the rise in chloroquine-sensitive strains may be attributed to the alternating use of various therapeutic combinations in malaria management.

Acknowledgements

We would like to express our gratitude to all the study participants, particularly the study population and administrative authorities, the entire staff of the Parasitology and Mycology Department in Senegal at the University Cheikh Anta Diop, Dakar.

Disclosure of conflict of interest

None.

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