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Study of the Relationship between the Presence of the Pfcrt 76T Mutation and the Therapeutic Efficacy of the Artesunate-Amodiaquine Combination in the Treatment of Uncomplicated *Plasmodium falciparum* Malaria in Burkina Faso

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ABSTRACT

The emergence and development of resistance of Plasmodium falciparum to antimalariques commonly used (chloroquine, sulfadoxine-pyrimethamine) led to the adoption of combinations based on artemisinin including artesunate amodiaquine, which has shown a good therapeutic efficacy in areas where amodiaquine monotherapy remains effective. The genetic mutation Pfcrt 76T has been validated as a marker that confers resistance to chloroquine. So far, there is no known marker associated with resistance to artesunate. Because of the structural similarity of amodiaquine with chloroquine, it is interesting to investigate the existence of an association between the presence of point mutation Pfcrt 76T and effectiveness of the association for artesunate amodiaquine, better monitoring of the development of resistance to ACT (Artemisinin-based Combination Therapies). We conducted a randomized clinical trial opened in two rural sites in Burkina Faso (Dande and Gourcy) during which we assessed the therapeutic efficacy of artesunate-amodiaquine and artemether-lumefantrine in patients with uncomplicated malaria and monitored them for 28 days. The study of polymorphism parasitic PCR has helped distinguish new infections from recrudescences (Real failures). In total, 47 patients were treated with artesunate and amodiaquine and included in this study. The cumulative treatment failure (clinical and parasitological)

was 14.89% (7/47). The study of parasitic polymorphism showed 12.77% (6/47) cases of recrudescence (real failure). A total 48.94% of patients were carriers of the mutation Pfcrt 76T before treatment and 66.67% among recrudescences. In Burkina Faso, artesunate-amodiaquine is still effective; there was no association of the point mutation Pfcrt 76T with clinical outcomes of the patients treated with artesunate-amodiaquine. Other point mutations may be investigated to help with these drugs' failure prediction.

1. INTRODUCTION

Malaria is a parasitic disease caused by protozoa of the genus *Plasmodium*, transmitted to humans by the bite of infected female mosquitoes of the genus *Anopheles*. Despite major advances in prevention and treatment, malaria continues to be, alongside AIDS and tuberculosis, one of the deadliest infectious diseases worldwide. According to the World Health Organization [1], *Plasmodium falciparum* alone is responsible for an estimated 2.7 million deaths annually, of which 80% - 90% occur in sub-Saharan Africa. The most vulnerable populations are children under five years of age and pregnant women.

In Burkina Faso, malaria remains the leading cause of morbidity and mortality. In 2005, it accounted for 35.12% of medical consultations, 40.83% of hospitalisations, and 37.5% of deaths. The burden is especially heavy among children under five, who represented 44.86% of consultations, 54.94% of hospitalisations, and 57.29% of deaths related to malaria [2].

A major factor contributing to the persistence of high malaria mortality is the emergence and spread of drug resistance. Since the late 1950s, *P. falciparum* has developed resistance to several first-line antimalarial drugs, notably chloroquine and sulfadoxine-pyrimethamine [3-5]. Molecular studies have shown that mutations in specific parasite genes are associated with resistance. For example, mutations in the *Pfcrt* gene are linked to chloroquine resistance [6], while those in *Pfdhfr* confer resistance to sulfadoxine-pyrimethamine [7]. The Pfcrt-K76T and Pfmdr1-N86Y polymorphisms, in particular, are considered reliable markers of chloroquine resistance.

Amodiaquine, although chemically related to chloroquine, does not share the same resistance mechanisms. The presence of the Pfcrt-K76T and Pfmdr1-N86Y mutations is not an absolute requirement for the development of resistance to amodiaquine [8]. As a result, amodiaquine remains effective in many endemic regions and is widely used because of its affordability, good tolerability, and efficacy against some chloroquine-resistant strains [9-12]. For these reasons, it has been combined with artemisinin derivatives as part of Artemisinin-based Combination Therapies (ACTs).

Despite its widespread use, no validated molecular markers have yet been identified that predict *P. falciparum* resistance to the artesunate-amodiaquine combination. Understanding the genetic basis of therapeutic failures is therefore a critical priority for malaria control programs.

Problem Statement

Malaria continues to be a major public health challenge in Burkina Faso, particularly among children under five years of age. The widespread emergence of drug-resistant *P. falciparum* strains has significantly limited the effectiveness of many conventional antimalarial treatments. Although the artesunate–amodiaquine combination remains an important component of national treatment strategies, the lack of reliable molecular markers to predict treatment failure hampers efforts to monitor and control resistance. The *Pfcrt* 76*T* mutation has been strongly associated with chloroquine resistance, but its role in amodiaquine and artesunate-amodiaquine resistance remains unclear. Investigating the relationship between this genetic marker and in vivo therapeutic outcomes may provide valuable insights into the mechanisms of resistance and contribute to improved malaria management in Burkina Faso.

Objectives

The present study aims to evaluate the relationship between the Pfcrt 76T mutation and treatment

outcomes in patients receiving the artesunate-amodiaquine combination. Specifically, the objectives are to:

- Determine the prevalence of clinical and parasitological failures of *P. falciparum* to the artesunate-amodiaquine combination in the study population.
- Assess the prevalence of the Pfcrt 76T mutation in the same population.
- Establish the association between the Pfcrt76 T mutation and in vivo resistance of *P. falciparum* to the artesunate-amodiaquine combination.

2. MATERIAL AND METHODS

Study site and population

The study was conducted in Dandé and Gourcy, two rural areas located in the west and north of the country, respectively. Gourcy serves as a sentinel site for malaria surveillance, while Dandé is an agricultural area characterized by higher water availability. The study took place between October and December 2006, which corresponds to the peak malaria transmission season.

Study Design and Participants

We conducted a randomized clinical trial comparing Artesunate-Amodiaquine (AS-AQ) with Artemether-Lumefantrine (AL). During the study period, the research team was based in health centres located in the participating localities. All patients of both sexes who were residents of these areas met the inclusion criteria and provided informed consent, which made them eligible for enrollment.

Patients presenting with clinical features suggestive of uncomplicated malaria underwent a physical examination and parasitological confirmation using thick and thin blood smears for detection of asexual forms of *Plasmodium falciparum*. Individuals with positive test results were further assessed for eligibility based on the World Health Organization [13] criteria for uncomplicated *P. falciparum* malaria and subsequently enrolled into the study.

Inclusion criteria

Subjects with a confirmed parasitological diagnosis were then selected according to the following inclusion criteria:

- Age greater than or equal to 6 months;
- History of fever in the last 24 hours (or axillary temperature > 37.5˚C);
- Parasite density between 2000 and 200,000 parasites/μL;
- Mono-specific infection with Plasmodium falciparum;
- No known allergy to the study drug;
- No signs of danger and/or severe malaria;
- No other obvious causes of fever;
- Provision of informed consent from the child's parent or guardian;
- No antimalarial medication taken during the last two weeks.

Data Collection and Patient Follow-up

Study Procedures

Patients were followed at the clinic on days 1, 2, 3, 7, 14, 21, and 28, as well as on any additional day if they reported illness. At each visit, a complete clinical examination was performed to assess new symptoms or the progression of existing symptoms. Thick and thin blood smears were prepared to detect asexual forms of *Plasmodium falciparum* and gametocytes. For molecular analysis, two to three drops of blood were collected on Whatman filter paper at each visit, except on day 1. Haemoglobin concentration was measured at inclusion and at the final follow-up visit **Table 1**.

Criteria for assessing treatment efficacy

Treatment efficacy was assessed according to the criteria contained in the [1] protocol as follows:

Early Treatment Failure (ETF)

- Appearance of danger signs or severe malaria on days 1, 2, or 3 in the presence of parasitaemia;
- Parasitaemia on day 2 higher than on day 0, regardless of axillary temperature;
- Parasitaemia on day 3 and axillary temperature greater than or equal to 37.5˚C;
- Parasitaemia of at least 25% of that on day 0.

Table 1. **Patient follow-up and collection of biological samples.**

Day	J0	J1	J2	J3	J7	J14	J21	J28	Other days
Thick drops and thin smears	X		X	X	X	X	X	X	X
Confetti for PCR	X		X	X	X	X	X	X	X
Haemoglobin level	X							X	X
Clinical examination	X	X	X	X	X	X	X	X	X

PCR = Polymerase Chain Reaction; J0 = the first visit, before the first dose is given; J1 = first day; J2 = second day; J3= third day; J7 = seventh day; J14 = fourteenth day; J21 = twenty-first day; J28 = twenty-eight days.

Late Therapeutic Failure (LTF) is divided into two categories:

Late Clinical Failure (LCF):

- Appearance of danger signs or severe malaria after day 3 in the presence of parasitaemia and in the absence of any previous criteria for ETF.
- Axillary temperature above 37.5˚C; or history of fever in the previous 24 hours between days 4 and 28 in the presence of parasitaemia without meeting the criteria for early treatment failure.

Late Parasitological Failure (LPF)

Presence of parasitaemia on day 28 with an axillary temperature < 37.5˚C, in the absence of any criteria for LCF or LPF.

Adequate Clinical and Parasitological Response (ACPR)

Absence of parasitaemia on day 28 regardless of temperature without meeting the criteria for ETF, LCF or LPF.

Molecular Studies

The molecular study consisted of two major analyses:

1) Genetic polymorphism of *msp*1 and *msp*2 genes in *Plasmodium falciparum*

2) Genotyping of the Pfcrt K76T allele

Both were performed using **Polymerase Chain Reaction (PCR)**, which involves four steps:

- DNA extraction
- Amplification
- Digestion
- Detection of products

Extraction of Parasitic DNA

(Method adapted from Plowe *et al.*, 1995, using Chelex-100)

Procedure:

- Cut confetti (filter paper with sample) and place in a microtube.
- Add 1 ml PBS (1X) + 50 µL of 10% saponin, soak overnight at 4˚C.
- Centrifuge briefly (8000 rpm, 5 sec). Discard supernatant.
- Wash with 1 ml PBS (1X), soak 30 min at 4˚C.
- Centrifuge, discard supernatant.
- Add 50 µL 20% Chelex-100 + 100 µL sterile double-distilled water.
- Heat at 95˚C for 10 min, vortex every 2 min.
- Place on ice, crush confetti with sterile tips.
- Centrifuge at 10,000 g for 5 min.
- Transfer supernatant, centrifuge again.
- Collect supernatant (DNA extract) and store at –20˚C.

DNA Amplification (Nested PCR)

Nested PCR Principle:

- Two successive PCR rounds.
- First PCR → amplifies target region.
- Second PCR (nested) → uses new primers within the first amplicon, increasing sensitivity and specificity.

PCR Components

- **DNA Template & Primers**
  - DNA is double-stranded, containing target fragment.
  - Primers: Hybridisation temperature (Th) **40˚C - 70˚C**.
    - <40˚C non-specific products.
    - 70˚C no amplification.
- **Taq Polymerase**
  - Thermostable enzyme (optimum 72˚C - 80˚C).
  - Synthesises complementary DNA strands.
  - Too little → weak amplification.
  - Too much → non-specific amplification.
- **dNTPs (dATP, dCTP, dGTP, dTTP)**
  - Supply nucleotides for DNA synthesis.
  - Equal concentrations required.
  - Lower concentrations → higher specificity.
  - Higher concentrations → risk of non-specific products.
- **PCR Buffer**
  - Ensures primer hybridisation and enzyme function.
  - Contains salts (e.g., KCl for hybridisation, gelatin for Taq stability).
- **$MgCl_2$**
  - Essential cofactor for Taq polymerase (Table 2).
  - Affects primer binding, specificity, and fidelity.
  - Stabilises dNTP substrates.

Table 2. Reaction mixture for the first and second series of PCRs of Pfcrt 76T.

Reagents	Concentration	Volume to be withdrawn (µl)
H20	-	15.3
Primer sens	10 µM	0.5
Primer antisens	10 µM	0.5
Buffer	10X	2.5
dNTP	2 mM	2.5
$MgCl_2$	25 mM	1.5
Tag polymerase	5 U/µl	0.2
DNA solution	Unknown	2
Final volume	-	25

The reaction mixture was incubated in a thermocycler according to the program shown in Table 3. For the second PCR series, the mixture was prepared with the CQR-A and CQR-B primers, to which 2 µl of the product from the first amplification was added. This mixture was then incubated in a thermocycler under the program specified in Table 3 below.

Table 3. Amplification programmes and primer sequences for Pfcrt-K76T analysis.

Gene and PCR stage	Primes (5' → 3')	Amplification programme
*Pfcrt T*76	CRT-1: (21bp)	94˚C × 3min
PCR1	GACG AGCG TTAT AGAGAATTA	(94˚C × 305 47˚C × 1 min 72˚C × 1.5) × 40 cycles
	CRT-2: (20bp)	72˚C × 3 min
	C CAGTAGTTCTTGTAAGACC	Concertation at 4˚C
*Pfcrt T*76	CQR-A: (21bp)	94˚ C × 5 min
PCR2	TGTGC TCATGTG TTTAAACTT	(94˚ C × 30 s 52˚C × 4 min 72˚C × 1 min) × 30 cycls
	CQR-B: (23bp)	72˚ × 3 m
	CAAAACTATAGTTACCAATTTTG	Concertation at 4˚C

Pfcrt = *Plasmodium falciparum* chloroquine resistance transporter.

Digestion of DNA by the restriction enzyme ApoI (NEB)

Digestion is carried out at a temperature of 50˚C for 2 hours in a thermocycler or incubator. The Apo1 enzyme (NEB) will only cut the wild-type strain (HB3), leaving the mutant strain (Dde2) intact.

In order to digest the nested PCR product, 10 µL of a reaction solution with APO1 (NEB) was prepared Table 4. To this reaction solution, we added 10 µL of the nested PCR amplification product. The entire mixture (20 µL) was placed in an Eppendorf microtube and incubated at 50˚C in a water bath. APO1 has optimal activity at 50˚C.

Table 4. Composition of the reaction medium for digestion with APO1.

	Concentration	Volume to be withdrawn
H20	-	6.8
Neb3	10 X	2.0
BSA	100 X	0.2
APOI (NEB)	10 U/µl	1.0
Nested PCR product	Unknown	10
Final volume		20.0

Amplification product disclosure

Electrophoresis:

Migration is performed on 2.5% agarose gel:

- Dissolve 2 g of agarose in 100 ml of 1X TBE;
- Boil this mixture for 3 minutes in a microwave oven;
- Add 7.5 µl of ethidium bromide to the melted agarose;
- Pour the gel into the mould and wait for it to solidify;
- Remove the combs and place the gel with the mould in the migration tank containing 0.5X TBE; the gel must be submerged;
- Cut the parafilm paper;
- Place 3 µl of dye on the parafilm for each sample to be migrated;
- Place 5 µl of the molecular weight marker in the first well;
- Mix between 15 and 18 µl of digestion product with the dye and place in the well;
- Migrate at between 80 and 90 volts for 1.5 to 2 hours;

- Remove the gel from the mould and place it under the UV device for photography;
- Mark the corresponding sample number in front of each band.

Interpretation of the photograph:

A good reaction is indicated by the presence of bands consistent with those expected. The size of the expected product and that of the positive controls must be checked for consistency. The Apo I enzyme (NEB) only cuts wild-type strains. Bands of the same size as the wild-type control (100 bp) correspond to strains sensitive to chloroquine.

On the other hand, those of the same size as the resistant control (134 bp) correspond to strains resistant to chloroquine. Bands that appear at both controls correspond to mixed strains.

The expected size for the amplification products is 537 bp for the first amplification and 134 bp for the second.

Determination of genetic polymorphism of msp1 and msp2

Reaction mixture and amplification programme for msp1 and msp2

For msp1 and msp2, the constituents (volume and concentration of reagents) are identical except for the sequences of the primer pairs.

The components of the reaction mixtures for the first and second PCRs of msp1 and msp2 are shown in Table 5. The product of the first amplification serves as the DNA source for the second amplification.

Table 5. Composition of reaction mixtures for the first and second PCRs.

Reactive	PCR 1		PCR 2	
	Concentration	Volume (µl) to be collected	Concentration	Volume (µl) to be collected
H_2O ultra pure		19,525		20,025
Primer sens	50 µM	0.05	50 µM	0.05
Primer antisens	50 µM	0.05	50 µM	0.05
Buffer	10X	2.5	10X	2.5
dNTPs	20 mM	0.25	20 mM	0.25
$MgCl_2$	25 mM	1.5	25 mM	1.5
Taq polymerase	5 U/µl	0.125	5 U/µl	0.125
DNA extract	unknown	1	unknown	0.5
		Final volume = 25 µl		

dNTPs = Deoxyribonucleoside triphosphates.

During the first PCR, the primer pairs used were: 01/02 for msp1 and S3/S2 for msp2; during the second PCR, primers N1/N2 and S1/S4 were used for msp1 and msp2, respectively. The 25 µl, contained in a sterile microtube, are incubated in a thermocycler (Master cycler gradient) under a program specific to each gene Table 6.

Interpretation of results

1) Experimental Setup Recap

- **Nested PCR products**: Amplified DNA fragments are loaded onto a **2.5% agarose gel** (good for re-solving relatively small DNA fragments).
- **Ethidium Bromide (EtBr)**: Intercalates between DNA bases, fluoresces under **UV light at 622 nm emission** → allows visualization of DNA bands.
- **Loading dye**: Provides density (so the sample sinks in the well) and tracking dyes to monitor migration.
- **Electrophoresis conditions**: 80 V, 350 mA, 3 hours in 1X TBE buffer → separates DNA fragments based on size.

Table 6. **Summary table of amplification programs for each gene.**

*MSP*1	1st amplification.	O1: 5'CACATGAAAGTTATCAAGAACTTGTC-3' O2: 5'-GTACGTCTAATTCATTTGCACG-3'	94˚C × 3 min; (94˚C × 25 s; 50˚C × 35 s; 68˚C × 2 min 30 s) × 30; 72˚C × 3 min
	2nd amplification.	N1: 5'-GCAGTATTGACAGGTTATGG-3' N2: 5'-GATTGAAAGGTATTTGAC-3'	94˚C × 3 min; (94˚C × 25 s; 50˚C × 35 s; 68˚C × 2 min 30 s) × 30; 72˚C × 3 min
*MSP*2	1st amplification.	S3: 5'-GAAGGTAATTAAAACATTGTC-3' S2: 5'-GAGGGATGTTGCTGCTCCACAG-3'	94˚C × 3 min; (94˚C × 25 s; 42˚C × 1 min; 65˚C × 2 min) × 30; 72˚C × 3 min
	2nd amplification.	S1: 5'-GAGTATAAGGAGAAGTATG-3' S4: 5'-CTAGAACCATGCATATGTCC-3'	94˚C × 3 min; (94˚C × 25 s; 50˚C × 1 min; 70˚C × 2 min) × 30; 72˚C × 3 min

MSP1, 2 = Merozoite Surface Protein 1, 2.

2) Gel Visualization

- After electrophoresis, **DNA bands** appear as fluorescent signals.
- Each **band position** corresponds to the **size (in bp)** of the fragment.
- By comparing **D0 (day of inclusion)** vs **D-failure (day of treatment failure)** samples, one can assess whether the parasite DNA profile is the same or different.

3) Interpretation of Banding Patterns

This method is commonly used in **malaria genotyping** (e.g., to distinguish recrudescence from reinfection).

- **Recurrence (Recrudescence):** The banding pattern (number and size of DNA fragments) at D-failure is **identical or highly similar** to D0 → indicates that the **same strain persisted** despite treatment.
- **New Infection (Reinfection)**: The banding pattern at D-failure shows **different bands** compared to D0 → indicates infection by a **different strain** acquired after treatment.
- **Indeterminate**: If the banding patterns are **partially overlapping** (some bands in common, some different) or unclear due to poor resolution → interpretation cannot be made with certainty.

Non-exhaustive example of gel interpretation

Status	Features
New infection	The number and size of bands on Day 0 and Failure Day are different.
Resurgence	The sizes of the bands on Day 0 and Failure Day are identical.
Undetermined	Negative PCR for D0 or Day of failure

For a given patient, the status is determined based on the polymorphism of msp1 and msp2.

Data analysis

We used EXCEL 2003 software to enter the data. Statistical analysis was performed using Epi Info 6.04 and SPSS 11.0 software.

The chi-square test and Fisher's exact test were used to compare proportions and measure the association between certain variables. A p-value of <0.05 was considered statistically significant.

Ethical considerations

Prior to the start of the study, this protocol was submitted to and approved by the institutional ethics committee of the MURAZ/IRSS center.

Patients were informed that their participation in the study was entirely voluntary and that the information collected by the team was strictly confidential. They were also allowed to withdraw from the study at any time if they wished.

3. RESULTS

Characteristics of the Study Population

Between October and December 2006, a total of 497 patients were examined at two sites. Of these, 286 tested positive for thick blood smears under microscopic examination, corresponding to a plasmodial index of 57.54%. A total of 167 patients were enrolled in the study and treated either with Artesunate-Amodiaquine (AQ/AS, n = 67) or artemether-lumefantrine (AL, n = 100). During follow-up, 60 patients were excluded for various reasons (including repeated vomiting, withdrawal of consent, and loss to follow-up): 20 from the AQ/AS group and 40 from the AL group Figure 1.

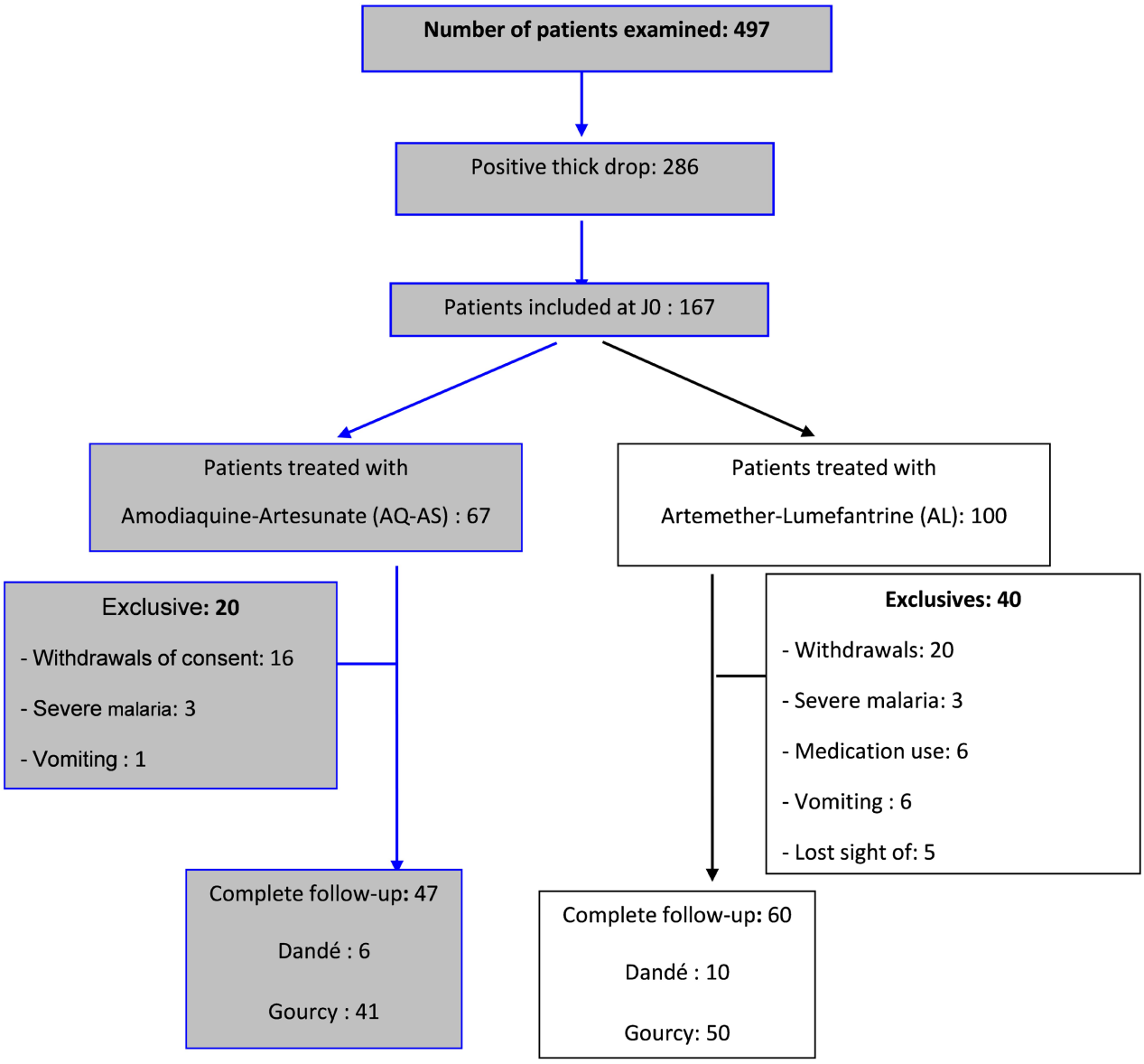

Figure 1. Study profile.

During the follow-up, 60 patients were excluded for various reasons (repeated vomiting, withdrawal of consent, lost to follow-up…) including 20 for AQ/AS and 40 for AL (Figure 1). In the Amodiaquine-Artesunate (AQ-AS) arm, 20 students were excluded for various reasons:

- 16 patients had left the study due to withdrawal of consent, being lost to follow-up or other reasons;
- 3 cases of severe malaria;
- 1 case of vomiting;

In the Artemether-Lumefantrine (AL) arm, 40 students were excluded for various reasons:

- For withdrawals of consent: 20 patients;
- For severe malaria: 3 patients;
- Due to medication intake: 6 patients;
- Due to vomiting: 6 patients;
- Lost to follow-up: 5 patients.

Characteristics of the study population

Among the 47 patients included, the majority (78.72%, 37/47) were aged between 6 and 59 months. The overall age range was 6 months to 30 years, with a mean age of 3.85 ± 5.21 years.

Female patients represented 55.32% (26/47) of the study population.

Temperature

At inclusion, patient temperatures ranged from **36.1˚C to 40.2˚C**, with an overall mean of **38.2˚C ± 1.03˚C**. Among children under 5 years of age, the mean temperature was **38.4˚C**, compared to **37.7˚C** in those over 5 years. The average temperature showed minimal variation between genders.

Parasite Density

The geometric mean parasite density at inclusion was **12,751.12 trophozoites/µL**, ranging from **2000 to 110,068 trophozoites/µL**. The mean parasite density was **12,617.3 trophozoites/µL** in children over 5 years of age, compared with **12,086.8 trophozoites/µL** in children under 5 years of age.

Hemoglobin level

The mean hemoglobin level was **9.10 ± 2.27 g/dL**, ranging from **5** to **14.8 g/dL**. In patients older than 5 years, the mean value was **11.38 g/dL**, whereas in those younger than 5 years, it was **8.10 g/dL**. Overall, **55.3% (26/47)** of patients were anemic at inclusion.

Gametocyte density

Among the 47 patients who completed follow-up, only one presented with gametocytes on the day of inclusion, corresponding to a prevalence of **2.13% (1/47).**

Clinical and Parasitological Results

Table 7 below presents the prevalence rates obtained after treatment with AQ/AS. Across all study sites, the average prevalence of treatment failure was **14.89% (95% CI: 6.2 - 28.3)**. No significant variation in treatment failure prevalence was observed between sites (**p = 0.45**).

Table 7. **Analysis of treatment efficacy by site.**

Location	Gourcy (n = 41)	Dande (n = 6)	Total (N = 47)
ETF	0	0	0
LCF	0	0	0
LPF	6 (14.63%)	1 (16.67%)	7 (14.89%)
GTF	6 (14.63%)	1 (16.67%)	7 (14.89%)
ACPR	35 (85.37%)	5 (83.33%)	40 (85.11%)

ETF = Early Treatment Failure; LCF = Late Clinical Failure; LPF = Late Parasitological Failure; GTF = General Therapeutic Failure; ACPR = Adequate Clinical and Parasitological Response.

No cases of early treatment failure were observed. Regardless of the study site, all recorded failures were

classified as late parasitological failures. The overall therapeutic efficacy was 85.11%, with site-specific rates of 85.37% in Gourcy and 83.33% in Dandé.

Given the late onset of these failures, an analysis of parasite polymorphism will be necessary to differentiate between recrudescences and new infections. This will provide a more accurate estimate of the true treatment failure rate.

Clinical and parasitological results by site after PCR adjustment

The prevalence of treatment failure due to relapse was **12.8% (95% CI: 8.1 - 17.5)** in the study population. Moreover, no significant influence of the study site on the prevalence of treatment failure was observed Table 8.

Table 8. **Analysis of treatment efficacy after adjustment for PCR.**

Location (N = 47)	Gourcy (n = 41)	Dande (n = 6)	Total
ETF	0	0	0
LCF	0	0	0
Resurgence	0	0	0
New infections	0	0	0
LPF	6 (14.6%)	1 (16.7%)	7 (14.9%)
Resurgence	5 (12.2%)	1 (16.7%)	6 (12.8%)
New infections	1 (2.4%)	0	1 (2.1%)
GTF	6 (14.6%)	1 (16.7%)	7 (14.9%)
ACPR	35 (85.4%)	5 (83.33%)	40 (85.1%)

The polymorphism analysis of *msp*-1 and *msp*-2 revealed that six (6) of the seven (7) PCR-positive clinical cases were due to recrudescent parasites, while only one (1) case, detected at the Gourcy site, represented a new infection. Thus, among all treatment failures, 14.3% were reinfections and 85.7% were recurrences, a statistically significant difference (p = 0.03).

When stratified by age, four (4) of the six (6) recurrences occurred in children under 5 years of age, compared to two (2) cases in children aged 5 years and above. This difference was not statistically significant (P = 0.56).

Prevalence of the Pfcrt-K76T allele

The prevalence of the *Pfcrt* 76T mutation prior to AS/AQ therapy was 48.9% (95% CI: 34.6 - 63.2) in the study population. By site, prevalence was 48.8% in Gourcy and 50% in Dandé, with no significant difference between the two locations (P = 0.70). Similarly, prevalence did not vary significantly according to age or sex (P = 0.94) (Table 9).

Table 9. **Pfcrt association and clinical response to treatment.**

	Mutations at Pfcrt 76T		Total
	Present	Absent	
GTF***	4 (66.7%)	2 (33.3%)	6
RCPA	19 (46.3%)	22 (53.7%)	41
Total	23 (48.9%)	24 (51.1%)	47

***Due to resurgence.

Prevalence of mutations in subjects who failed treatment

To assess whether the presence of the *Pfcrt* 76T mutation prior to treatment was associated with failure of the AS/AQ combination, we compared the prevalence of the mutant allele between cases of treatment failure and RCPA cases. Among the six recrudescent isolates, four (66.7%) carried the *Pfcrt* 76T mutation, whereas 19 of 41 (46.3%) RCPA isolates also harbored this mutation. Statistical analysis showed no significant association between the presence of the *Pfcrt* 76T allele and treatment failure due to parasite recrudescence (P = 0.94).

4. DISCUSSION

The therapeutic efficacy of the combination of artesunate and amodiaquine

Analysis of the prevalence of failure rates for the Artesunate-Amodiaquine combination indicates that this treatment maintains a relatively high efficacy. However, our results demonstrate higher failure rates compared to those reported by other authors in the region. For instance, [10] reported a 100% efficacy rate for this combination in Bobo-Dioulasso.

A study conducted in the central region revealed a failure rate of 7.94% [14]. This difference from our results could be explained by the phenomenon of fluctuation over time. Given that chemoresistance is a dynamic phenomenon that evolves over time and space, it is understandable that this failure rate increased between these two periods. In fact, our results are comparable to those published in other regions; for example, in 2004 [12], Grandesso *et al.* reported a failure rate of 13.49%. Results reported from Central and East Africa also indicate similar rates [15]. However, other authors have reported lower rates. Between 2000 and 2005, average cure rates of 97% [16] were observed in southern Senegal. [17] showed that ACT (AL and alternatives) remained highly effective in Mali. Similarly, [18] in Côte d'Ivoire also reported that AS-AQ and AL demonstrated clinically adequate cure rates (~95%). [19] reported that dihydroartemisinin-piperaquine (DHAP) demonstrated PCR-corrected cure rates exceeding 90% and was generally well tolerated in the Ghanaian sites where it was studied. This difference from our results could be explained by the heterogeneity of the geographical distribution of P. falciparum strains.

We also found a higher failure rate in the 6- to 59-month age group compared to the 5-year-old and older age group, but this difference could be explained by the difference in the degree of immunity that changes with age [20-22]. However, this observation was not verified in the present study, as there was no statistical association between age and therapeutic failure with AS/AQ. This association could probably be established if immunogenetic studies were conducted.

Prevalence of Pfcrt 76T mutations in the study population

The 76T mutation of the *Pfcrt* gene, which encodes a transporter protein located in the digestive vacuole of *Plasmodium falciparum* [6], was detected in 48.9% (23/47) of samples collected prior to treatment, with a 95% CI of [34.6 - 63.2]. Although the proportion of mutant alleles varied across study sites, these differences were not statistically significant (P > 0.05).

A study conducted by [23] in Bobo-Dioulasso reported higher proportions than ours (61.4%). In the Anjouan Islands, where transmission appears to be significant, 88% of isolates carried the mutant profile 76T [24]. These differences could be explained by variations in circulating strains and/or temporal fluctuations of the phenomenon.

Nevertheless, our findings are consistent with those of [25] in the Niger Valley, where a prevalence of 50% was reported.

Since amodiaquine was used less frequently than chloroquine, these mutations were most likely selected by chloroquine pressure. Moreover, because chloroquine and amodiaquine share the same mode of action, some chloroquine-resistant mutant strains may also exhibit resistance to amodiaquine.

The relationship between the presence of Pfcrt 76T mutations and prediction of clinical outcomes

"In our study, the prevalence of the mutation was high among both patients who experienced parasite recrudescence and those who achieved therapeutic success. Therefore, no significant association was found between the presence of the mutation and clinical outcome (P > 0.05)."

Several studies have investigated the prevalence of the Pfcrt-K76T mutation as a molecular marker for assessing the effectiveness of artemisinin-based combination therapies (ACTs) in Burkina Faso in particular, and in Africa more broadly [26, 27]. In addition, the Kelch13-propeller (K13) marker, which has been associated both *in vivo* and *in vitro* with resistance to artemisinin and its derivatives, requires regular monitoring [28]. This molecular marker of artemisinin resistance was first reported in 2014 and subsequently adopted by the global scientific community [29].

As part of efforts to characterize K13 gene mutations across Africa, 3,257 *Plasmodium falciparum* isolates were collected between 2011 and 2019 from eleven malaria-endemic countries: Gambia, Sierra Leone, and Burkina Faso in West Africa; Chad, the Central African Republic, the Republic of Congo, and Equatorial Guinea in Central Africa; and Burundi, Tanzania, Rwanda, and Somalia in East Africa. Of these, 98% (3179/3257) carried the wild-type K13 allele, sometimes with synonymous (non-coding) substitutions. In total, 35 unique non-synonymous K13 mutations were identified, though only a small subset has been validated as mediators of artemisinin resistance [30].

It is well established that the reduced sensitivity of *P. falciparum* to ACTs could compromise progress made in malaria control. Beyond K13, other genetic determinants are also implicated in resistance. Point mutations in the Pfmdr-1 gene on chromosome 5, which encodes a multidrug resistance protein, and in the Pfcg2 gene on chromosome 7, have been associated with decreased drug susceptibility [31].

5. CONCLUSIONS

Amodiaquine-artesunate is an artemisinin-based combination therapy that has proven effective in Burkina Faso, which supports its selection by the National Malaria Control Program for the treatment of uncomplicated malaria.

Moreover, the high prevalence of the **Pfcrt 76T** mutation (48.9%) and the persistently high efficacy of the free combination (treatment failure rate = 12.8%) indicate that this mutation is not associated with clinical outcomes in treated patients.

Future efforts to identify predictive markers of resistance to this combination should therefore focus on novel genetic markers or on combinations of several known markers.

LIMITATIONS OF THE STUDY

Although this topic remains relevant today, this study was conducted more than twenty years ago.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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