Genetic Profiles of *Plasmodium falciparum* Isolates from a Therapeutic Efficacy Study on the Antimalarial Drug Dihydroartemisinin-Piperaquine in the West Sumba and Kupang Districts of East Nusa Tenggara Province, Indonesia.

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Abstract. Artemisinin resistance, first reported on the Thai-Cambodian border in 2009, has spread across Southeast Asia, characterized by delayed parasite clearance. Extended artemisinin-based combination therapy (ACT) regimens have shown efficacy where standard 3-day treatments failure. In Indonesia, dihydroartemisinin-piperaquine (DHA-PPQ) has been the first-line treatment for uncomplicated Plasmodium falciparum malaria since 2010. This study evaluates the efficacy, safety, and parasite clearance times of DHA-PPQ in West Sumba and Kupang Districts, East Nusa Tenggara, while assessing single-nucleotide polymorphisms (SNPs) in the Pfk13 and Pfcrt genes, which are linked to artemisinin and piperaquine resistance, respectively. Following WHO guidelines, 382 cases were screened, with 41 eligible for 42-day clinical and parasitological monitoring. Molecular analyses utilized PCR and real-time PCR. All cases demonstrated adequate

clinical and parasitological response (100% APCR). No mutations were detected in 21 Pfk13 SNPs associated with artemisinin resistance or in Pfcrt codons M343L, C350R, G353V, and I356L linked to piperaquine resistance. However, 10 of 41 samples (24.39%) exhibited Pfpm2 gene amplification, indicating early piperaquine selection pressure. DHA-PPQ remains effective, but Pfpm2 amplification underscores the need for continuous molecular surveillance and exploration of extended ACT regimens to sustain malaria control.

Keywords: *Plasmodium falcifarum*, DHA-PPQ, *Pfk13*, *Pfcrt*, *Pfpm2*

1 Introduction

Malaria is an infectious disease caused by the parasite *Plasmodium* spp., which is naturally transmitted through the bite of female *Anopheles* mosquitoes. Five species of *Plasmodium* have been identified as the causative agents of malaria in humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi*. This disease continues to pose a significant global health challenge. According to the World Health Organization (WHO), the global burden of malaria is substantial. In 2023, the WHO reported approximately 263 million cases in 83 malaria-endemic countries and regions, resulting in around 597,000 deaths [1]. Indonesia, a tropical country, faces ongoing challenges with malaria, particularly in its eastern provinces. Data from the Ministry of Health of the Republic of Indonesia indicates that throughout 2024, there have been 543,965 cases and 132 deaths from malaria. Most cases are reported in Papua Province, Central Papua, South Papua, and the Papua Mountains [2].

The malaria control program in Indonesia has significantly reduced malaria in 67% of 514 districts and cities. However, significant challenges remain, especially in endemic areas such as Papua and East Nusa Tenggara Provinces [3]. One aspect of the malaria control effort still relies on the use of antimalarial drugs, and the discovery of increasing treatment failures in uncomplicated falciparum malaria prompted a shift to artemisinin-based combination therapy (ACT). The ACTs, which combine two types of short-acting artemisinin drugs with a long-acting partner drug [4], have become the primary treatment option for malaria [1]. Since 2010, Indonesia has recommended Dihydroartemisinin-Piperaquine (DHA-PPQ), which demonstrates excellent efficacy and tolerability against multi-resistant *Plasmodium falciparum* [5, 6].

The first case of *Plasmodium falciparum* resistance to artemisinin was detected in 2009 at the Thailand-Cambodia border, and cases of resistance spread rapidly throughout mainland Southeast Asia [7]. The occurrence of resistance to *Plasmodium falciparum* poses a risk of reducing the malaria disease burden and threatens malaria control efforts [1, 4, 8, 9]. Using molecular markers to monitor the emergence and spread of parasite resistance to antimalarial drugs is an effective method for tracking resistance [8, 9]. Artemisinin resistance can be identified by observing the speed of parasite clearance and the presence of single-nucleotide polymorphisms (SNPs) in the *Plasmodium falciparum kelch 13 (Pfk13)* gene as resistance markers [4]. To date, 21 *Pfk13* SNPs have been validated both *in vitro* and *in vivo* [10].

Several studies suggest that mutations in the *Plasmodium falciparum chloroquine resistance transporter* (*Pfcrt*) gene are linked to chloroquine (CQ) resistance. The PfCRT protein is a transporter located in the membrane of the parasite's digestive vacuole, functioning as a

regulator of pH within the vacuole [11]. The mutation at position K76T is strongly associated with chloroquine resistance. Additionally, new mutations in *Pfcrt* linked to piperaquine (PPQ) resistance have been identified at positions 343, 350, 354, and 356 [4, 10, 12]. Another molecular marker is copy number variation (CNV) in the *PfPlasmepsin 2* (*Pfpm2*) gene, which encodes the Plasmepsin 2 enzyme involved in hemoglobin degradation, particularly in hemozoin formation. This gene is considered a major target of PPQ. Several studies have reported increased copy number of the *Pfpm2* gene in endemic areas [13, 14], including a previous report from Papua, Indonesia, which was identified in three out of four re-infection cases [5]. This suggests that the new infecting strain may exhibit potential resistance to piperaquine, potentially leading to late treatment failure. Given this increased risk of treatment failure with ACTs, regular and close molecular monitoring is necessary to assess the efficacy of DHA-PPQ in supporting malaria control strategies. This study aimed to evaluate parasite clearance days and assess the efficacy and safety of DHA-PPQ. Additionally, it investigated the presence of SNPs in the *Pfk13 gene* associated with artemisinin resistance, SNPs in the *Pfcrt gene,* and examined the copy number of the *Pfpm2* gene associated with piperaquine resistance in *P. falciparum* samples collected. The findings of this study provide critical insights into the molecular mechanisms of antimalarial drug resistance, paving the way for the development of rapid molecular diagnostic assays to detect resistance markers in real-time, and highlighting the need for continuous genomic surveillance to monitor the spread of resistant *Plasmodium falciparum* strains, thereby supporting the optimization of treatment policies and global malaria elimination efforts

2 Methods

2.1 Ethics document

This study was approved by the Health Research Ethics Committee of the Faculty of Medicine at Hasanuddin University in Makassar, Indonesia, No. 503/UN4.6.4.5.31/PP36/2022, and by the Health Research Committee of the National Research and Innovation Agency (BRIN) in Jakarta, Indonesia, No. 140/KE.03/SK/07/2024.

2.2 Research location and sampling

The DHA-PPQ antimalarial drug efficacy test study was conducted in malaria-endemic areas of West Sumba (coordinates: 119° 6' 43.61" E - 119° 32' 5.87" E and 9° 22' 24.27" S - 9° 47' 50.14" S) and Kupang (coordinates: 10°36'14" S - 10°39'58" S and 123°32'23" E - 123°37'01" E) districts, East Nusa Tenggara Province, in 2023 as described in Fig. 1.

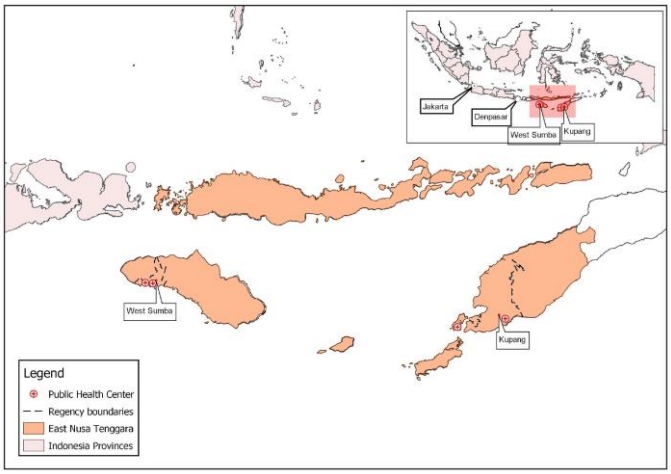

Fig. 1. Study sites

2.3 *Therapeutic Efficacy Study* (TES) Sample Set

The TES procedure follows the WHO criteria [15]. The criteria for patients included in this test were ages between 1 and 65 years, no symptoms or signs of complications, and confirmed malaria by microscopy with a parasitemia level greater than 500 parasites per microliter of blood for *Plasmodium falciparum*. Subjects meeting the inclusion criteria were treated with DHA–PPQ once daily for three days, administered orally. The following individuals were excluded from participation in the study: those who were pregnant, those with a history of allergy to the study drugs or their derivatives, those who had previously completed treatment with an anti-malarial drug within the preceding two weeks, and those with a medical history of untreated hypertension or chronic heart, kidney, or liver disease. Briefly, blood was collected from a finger prick and stored on Whatman 1 filter paper on the day of recruitment (Day 0), then on days 1, 2, 3, 7, 14, 21, 28, 35, and 42. The dried blood spot was then dried and placed in labeled plastic containers for storage at room temperature. Additionally, the participant's blood was dropped onto a glass microscope slide and examined under a microscope. A 3% Giemsa solution was made and used to stain blood smears for 60 minutes. Parasite density was calculated by counting parasites per 200 white blood cells on thick smears. A slide was deemed negative if, after examining 1000 white blood cells or 100 fields with at least 10 white blood cells each, no asexual parasites were observed. Two microscopists independently examined all slides and recorded parasite densities. A third microscopist reviewed any discrepancies between the initial readers regarding species identification and parasite density when the difference exceeded 50%. The final parasite density was obtained by averaging the two counts closest to each other.

2.4 DNA Extraction

Genomic DNA was extracted from dried blood spots (DBS) using the QIAamp DNA Mini Kit (Qiagen). The entire extraction process was conducted according to the protocol outlined

in the Qiagen manual [9]. After extraction, the DNA was stored at a minimum temperature of 20°C for later use in amplifying molecular markers.

2.5 Amplification of *Pfk13* and *Pfcrt* genes

Amplification of the *Pfk13* gene was performed using a *nested* PCR method with two pairs of primers in two separate PCR runs to evaluate mutations associated with artemisinin resistance. This approach was based on a species-specific amplification protocol for the propeller domain of the *Pfk13* gene [8], with minor modifications. The primers used in the nested PCR were K13 PCR F (5- GGGAATCTGGTGACAGC-3) and K13 PCR R (5-CGGAGTGACCAAATCTGGGA-3) for the first PCR, while K13 Nested F (5-GCCTTGTTGAAAGAAGCAGA-3) and K13 Nested R (5-GCCAAGCTGCCATTCATTTG-3) were used for the second. PCR was performed in a 25 µl reaction volume using My Taq HS Red Mix, which contained 12.5 µl of the mix, 0.25 µl of 40 pmol primers, and 2 µl of template DNA. The initial PCR conditions included 5 minutes of pre-denaturation at 95°C, followed by 30 cycles consisting of pre-denaturation at 95°C for 3 minutes, denaturation at 55°C for 1 minute, and extension at 72°C for 1 minute 30 seconds. A final extension step was performed for 10 minutes at 72°C. The PCR products were then analyzed using Sanger sequencing to identify mutations.

The *Pfcrt* gene was amplified from template DNA to evaluate PPQ-associated mutations using published protocols with minor modifications. The primer pairs used to detect the *Pfcrt* gene were Primer F (5'-CCATATAATTTTTCATTTTC-3') and Primer R (5'-GTTCTCTTACAACATCAC-3') [38]. Initial PCR conditions included a 5-minute pre-denaturation at 95°C, followed by 35 cycles consisting of pre-denaturation at 95°C for 3 minutes, denaturation at 60°C for 3 minutes, and extension at 72°C for 1 minute 30 seconds. The process concluded with a 10-minute post-extension step at 72°C. PCR products were then analyzed using Sanger sequencing to identify mutations.

2.6 *Real-time* PCR *for Pfpm2* gene *copy number* detection

Analysis of the *Pfpm2* gene was conducted using *real-time* PCR with specific primers and probes following protocols [8, 13] that have been previously published for measuring *copy number*. Amplification was carried out on MicroAmp 96-well plates (Applied Biosystems/IQ5 Bio-Rad). Reactions were performed in a 20 µl volume containing 10 µl Thunderbird Sybr Mix, 10 pmol of Forward and Reverse Primers (0.6 µl each), and 8.8 µl of template DNA. PCR conditions included an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 1 minute. A melt curve analysis was then performed: 95°C for 15 seconds, 68°C for 30 seconds, 86°C for 2 minutes, and a final step at 35°C for 2 minutes. Samples were run in duplicate with clone 3D7 as a positive control. Detecting *copy number* used the 2-ΔΔCT formula, comparing the threshold cycle (CT) values of the tested and reference samples. Results are considered invalid if the CT exceeds 33 or the *copy number* is less than 0.5 [8, 13].

2.7 Analysis

Data analysis was performed using BioEdit software (Abbott, CA, USA) to examine and correct the DNA sequences of the *Pfk13* and *Pfcrt* genes to ensure their accuracy and identify polymorphism points. Alignment visualization was performed with Unipro UGENE ver 49.1 (Unipro, Novosibirsk, Russia). Analysis was also conducted with Microsoft Office Excel to

detect copy number variation and to evaluate the intraspecific nucleotide base variation between samples.

Results

In the West Sumba district, out of 7807 people screened through passive and active case detection, 323 tested positive for malaria. A total of 36 *Plasmodium falciparum* cases met the inclusion criteria. In the Kupang district, out of 3578 people screened, 59 tested positive for malaria. A total of 6 *Plasmodium falciparum* cases met the inclusion criteria.

The baseline characteristics of TES participants in West Sumba and Kupang districts are presented in Table 1.

Table 1. Baseline characteristics of study participants for *Plasmodium falciparum* cases

Variable	Primary Health Centers		
	West Sumba		Kupang
	Kabukarudi	Gaura	
Number of registered patients (n)	21	15	6
Age (Years)			
-Mean (SD)	16.6 (7.9)	20.3 (17.4)	37.7 (11.6)
-Reach	4 - 36	4 - 66	21 - 52
Hb (g/dL) [mean (SD)]	11.6 (0.8)	12.3 (1.5)	13.4 (0.7)
-Reach	10 - 13.8	10.3 - 15.1	12.7 - 14
Gender			
-Male [n (%)]	16 (76.2%)	13 (86.7%)	2 (33.3%)
-Female [n (%)]	5 (23.8 %)	2 (13.3%)	4 (66.7%)
Body Temperature [$^{(o)}C$, mean (SD)]	38.6 (0.4)	37.8 (1.1)	37.8 (0.8)
-Reach	38 - 39	36.3 - 39.9	37 - 39.2
Parasite Density	5281	10861	5719
-Reach	480 - 154950	780 - 106320	1980 - 22740

A total of 41 DBS samples were collected from uncomplicated falciparum malaria patients in East Nusa Tenggara in 2023 to analyze genotyping profiles of molecular markers associated with DHA-PPQ treatment resistance. The samples were assessed for clinical and parasitology effectiveness of DHA-PPQ during a 42-day follow-up period. Kaplan-Meier analysis of microscopic readings showed no cases with treatment failure classified as *Adequate Clinical and Parasitological Response* (ACPR) or 100% cure in samples from both districts, as shown in Table 2.

Table 2. Falciparum phenotyping results from Sumba and Kupang districts during 42 days of follow-up

Study Location	Total Subjects	Results (Number of patients)				
		ETF	LCF	LPF	ACPR	LTU

Sumba	36	0	0	0	35	1
Kupang	6	0	0	0	6	0

Early Treatment Failure (ETF); Late Clinical Failure (LCF); Late Parasitological Failure (LPF); Adequate clinical parasitological response (ACPR); and Lost to follow-up (LFU)

2.8 *Single-nucleotide polymorphisms* (SNPs) of the *Pfk13* gene

Based on the results of *nested* PCR performed on 36 West Sumba samples and 6 Kupang samples, followed by Sanger sequencing, all amplicons were successfully generated. The analysis and alignment of 21 SNP resistance markers, which are reported to be associated with resistance to artemisinin, did not reveal any mutations in the samples examined (Figure 2).

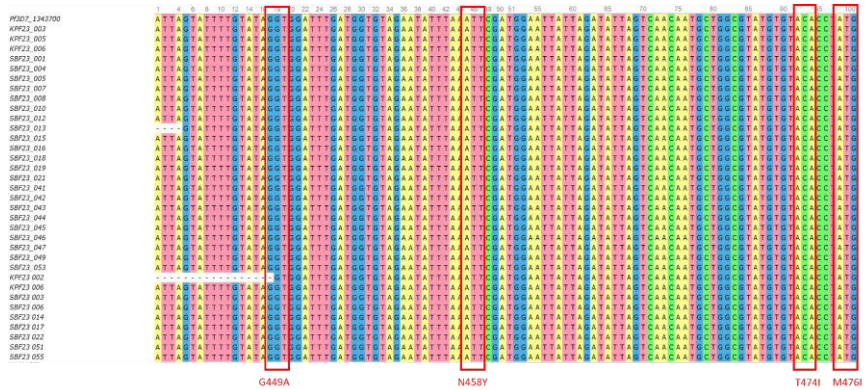

Figure 2A. Alignment results of SNPs already observed. G449A, N458Y, T474I, M476I

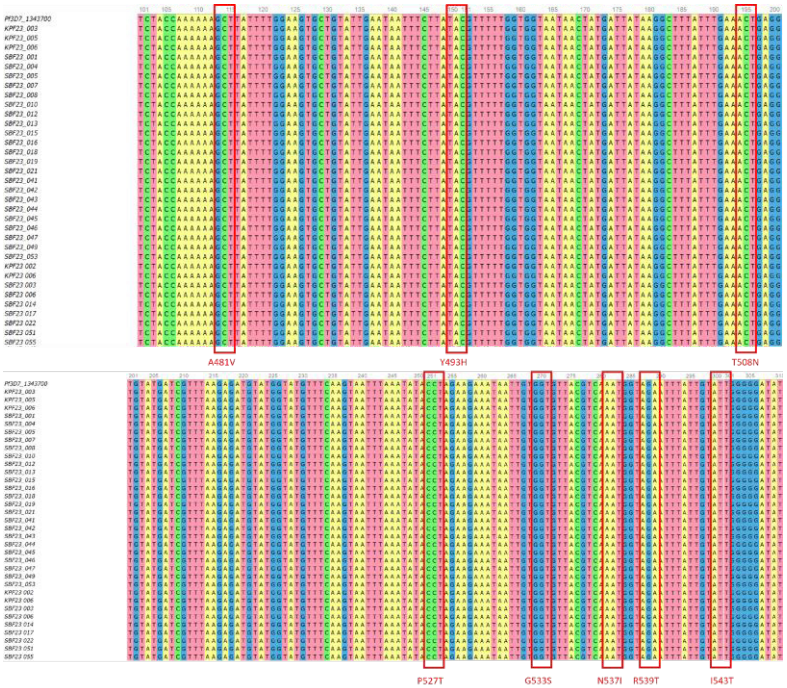

Figure 2B. Alignment results of SNPs already observed. A481V, Y493H, T508N, P527T, G533S, N537I, R539T, I543T

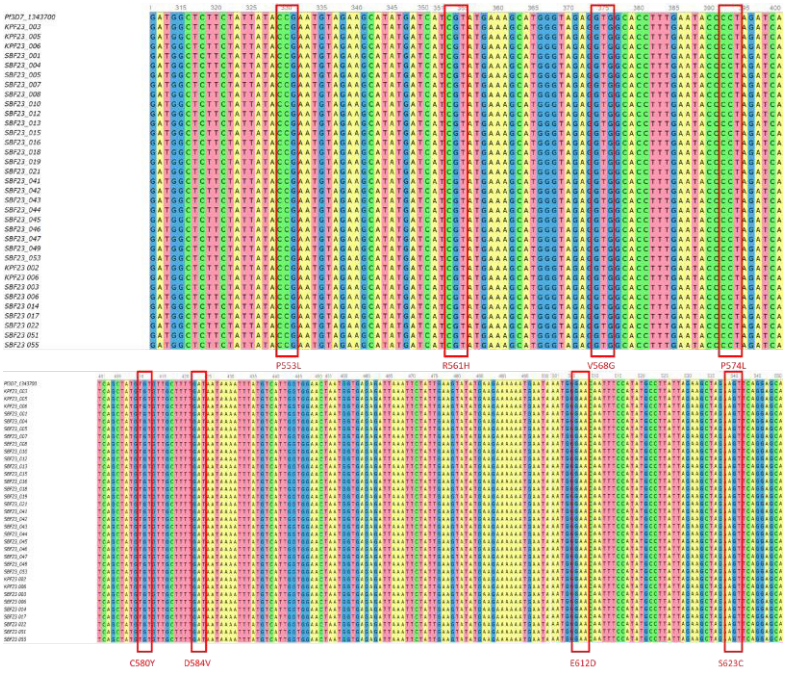

Figure 2C. Alignment results of SNPs already observed. P553L, R561H, V568G, P574L, C580Y, D584V, E612D, S623C.

Single-nucleotide polymorphisms **(SNPs) of the** ***Pfcrt*** **gene**

From 41 *Plasmodium falciparum* samples, *the PcrtT* gene was successfully amplified, followed by Sanger sequencing and alignment. No mutations associated with resistance to the *Pfcrt* gene were found in all regions linked to resistance to PPQ (M343L, C350R, G353V, and I356L/T), as shown in Figure 3.

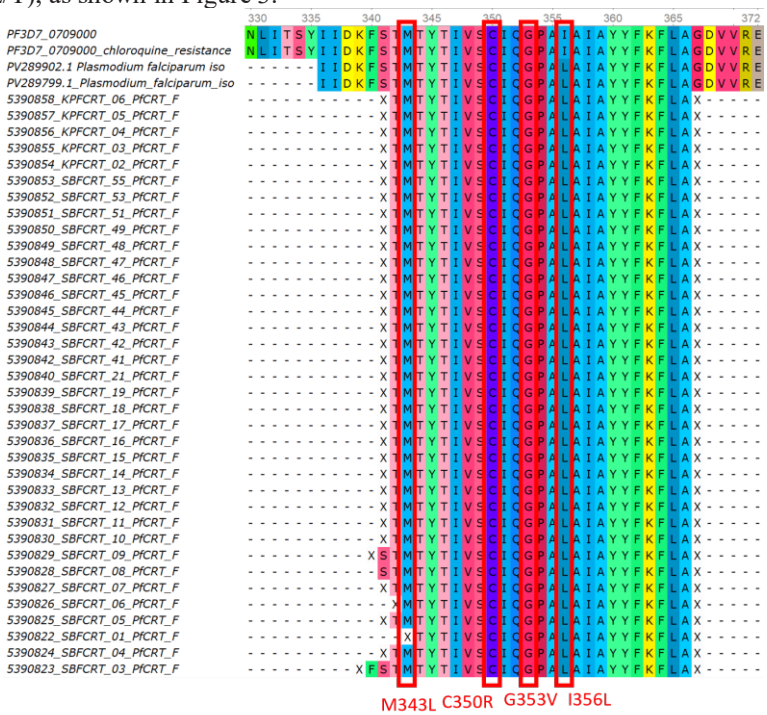

Figure 3. Alignment of the *Pfcrt* gene at positions M343L, C350R, G353V, and I356L are associated with PPQ resistance.

2.9 *Copy number variation* (CNV) of the *Pfpm2* gene

Copy numbe*r* analysis of the *Pfpm2* gene was performed on 41 DNA samples that were successfully amplified using *real-time* PCR. Ten out of 41 samples (24.39%) showed multiple copy numbers, while 31 samples (75.61%) exhibited a single copy number. This detection was based on the threshold value (CT) *of* copy number >1.5, which suggests a possible increased tolerance or resistance of the parasite to PPQ, as shown in Table 3.

Table 3. Copy number variation of *Pfpm2* gene analysis results

Sample code	*Plasmepsin 2* (*PfPM2*) *copy number variation*	
	Copy number	*Classification*
SBF23001	1.67	Multiple
SBF23002	1.16	Single
SBF23003	0.89	Single
SBF23004	0.32	Single
SBF23005	1.19	Single
SBF23006	0.55	Single

SBF23007	1.22	Single
SBF23008	1.15	Single
SBF23009	1.55	Multiple
SBF23010	1.20	Single
SBF23011	0.73	Single
SBF23012	0.45	Single
SBF23013	1.58	Multiple
SBF23014	1.16	Single
SBF23015	1.43	Single
SBF23016	1.81	Multiple
SBF23017	1.89	Multiple
SBF23018	1.10	Single
SBF23019	1.31	Single
SBF23020	0.96	Single
SBF23021	1.81	Multiple
SBF23041	1.07	Single
SBF23042	0.66	Single
SBF23043	1.05	Single
SBF23044	1.13	Single
SBF23045	1.30	Single
SBF23046	1.25	Single
SBF23047	1.04	Single
SBF23048	1.76	Multiple
SBF23049	1.65	Multiple
SBF23051	2.10	Multiple
SBF23052	1.58	Multiple
SBF23053	1.23	Single
SBF23054	0.90	Single
SBF23055	1.70	Multiple

3 Discussion

The rising failure rate of single-antimalarial drug treatments prompted the WHO to adopt *Artemisinin-based Combination Therapy* (ACT). ACT is known for its ability to kill parasites quickly and is effective against both sexual and asexual stages [4, 5]. Since 2004, Indonesia has used ACT to treat malaria, and in 2010, DHA-PPQ was established as the first-line treatment in the country [5, 6]. DHA-PPQ shows good tolerance; artemisinin derivatives are believed to rapidly clear parasites, while PPQ can eradicate residual parasites [6, 8]. The first report of artemisinin treatment failure appeared on the Thai-Cambodian border in 2009 [4] and soon spread across Southeast Asia.

This study aimed to evaluate the clinical and parasitological effectiveness, as well as the molecular profile of resistance markers, in patients with falciparum malaria. The Therapeutic Efficacy Study (TES) conducted in East Nusa Tenggara province, Indonesia, in 2023, showed

that DHA-PPQ remains effective because all patients who completed the observation period until day 42 achieved *Adequate Clinical and Parasitological Response* (ACPR 100%).

Molecular analysis of the *Pfk13* gene among the samples revealed *no mutations* in *21* SNPs associated with resistance, which is the main marker for artemisinin resistance [6, 10]. This supports the evidence that there has been no sign of artemisinin resistance emerging in East Nusa Tenggara province, particularly in West Sumba and Kupang districts, so far.

Both CQ and PPQ are antimalarial drugs that accumulate in the digestive vacuole. Their mechanism of action likely involves disrupting heme detoxification. Mutations in PfCRT, such as I356L/T, can alter the transporter's structure and function, potentially affecting its ability to move CQ and PPQ out of the vacuole. Reduced transport may lead to less drug buildup inside the vacuole, decreasing their effectiveness. Although I356L/T mutations have been linked to CQ resistance, their impact on PPQ resistance is more complex. It is hypothesized that these mutations might contribute to PPQ resistance, but the level of resistance can vary depending on the specific mutation and the parasite's genetic background. Other mutations can also influence how I356L/T mutations affect PfCRT and related genes involved in drug resistance, such as pfmdr1. The emergence and spread of PfCRT mutations, including I356L/T, highlight the need for ongoing molecular monitoring of *P. falciparum* to track resistance development and guide treatment decisions.

Analysis of PPQ is associated with the presence of SNPs at codons M343L, C350R, G353V, and R371I in the *Pfcrt* gene, as demonstrated in previous studies [5]. A mutation was identified at position 356L of the Pfcrt gene, associated with chloroquine resistance, in the analyzed samples. However, no 356T mutation, associated with piperaquine resistance, was detected. However, this study also did not find mutations at these positions. The absence of mutations suggests that resistance to PPQ from DHA-PPQ therapy has not significantly developed in the West Sumba and Kupang districts. In contrast, in the *Pfpm2* gene, which is also associated with PPQ resistance, *the* copy number variation (CNV) results from 10 out of 41 samples (24.39%) in Table 3 indicate the presence of multiple copies that could potentially affect PPQ sensitivity or resistance in the parasite population studied [14]. Although no treatment failures were observed, the presence of multiple copies in the *Pfpm2* gene remains a concern, as it has been linked to PPQ resistance in several Southeast Asian countries. Multiple copies of *Pfpm2* gene suggest possible selective drug pressure in the field, given that DHA-PPQ has been used in Indonesia as the first-line treatment for over a decade. The study's limitation is its small sample size, especially in the Kupang district. Therefore, further research with a broader range of areas and samples is necessary to better monitor resistance in a more representative manner.

4 Conclusion

This study demonstrates that DHA-PPQ treatment remains highly effective against uncomplicated *Plasmodium falciparum* malaria in West Sumba and Kupang districts, with a 100% cure rate and no cases of treatment failure. Molecular analysis revealed no mutations in the *Pfk13* or *Pfcrt* genes associated with artemisinin and piperaquine resistance. However, about 24.39% of the samples exhibited multiple copy numbers in the *Pfpm2* gene, which could indicate early selection pressure against PPQ and should be monitored regularly. This study highlights the importance of ongoing molecular surveillance and the exploration of extended ACT regimens to prevent resistance, providing critical insights for malaria control strategies.

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The authors declare that they have no conflicts of interest.

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