MOLECULAR PROFILES OF MULTIPLE ANTIMALARIAL DRUG RESISTANCE MARKERS IN PLASMODIUM MALARIA AND PLASMODIUM VIVAX IN THE HYDERABAD REGION, INDIA.

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ABSTRACT

Emergence and spreading of antimalarial drug resistant malaria parasites are great hurdles to combating malaria. Although approaches to investigate antimalarial drug resistance status in Myanmar malaria parasites have been made, more expanded studies are necessary to understand the nationwide aspect of antimalarial drug resistance. In the present study, molecular epidemiological analysis for antimalarial drug resistance genes in Plasmodium falciparum and P. vivax from the Mandalay region of Myanmar was performed. Blood samples were collected from patients infected with P. falciparum and P. vivax in four townships around the Mandalay region, Myanmar in 2015. Partial regions flanking major mutations in 11 antimalarial drug resistance genes, including seven genes (pfdhfr, pfdhps, pfmdr-1, pfcrt, pfk13, pfubp-1, and pfcytb) of P. falciparum and four genes (pvdhfr, pvdhps, pvmdr-1, and pvk12) of P. vivax were amplified, sequenced, and overall mutation patterns in these genes were analyzed. Substantial levels of mutations conferring antimalarial drug resistance were detected in both P. falciparum and P. vivax isolated in Mandalay region of Myanmar. Mutations associated with sulfadoxine-pyrimethamine resistance were found in pfdhfr, pfdhps, pvdhfr, and pvdhps of Myanmar P. falciparum and P. vivax with very high frequencies up to 90%. High or moderate levels of mutations were detected in genes such as pfmdr-1, pfcrt, and pvmdr-1 associated with chloroquine resistance. Meanwhile, low frequency mutations or none were found in pfk13, pfubp-1, pfcytb, and pvk12 of the parasites. Overall molecular profiles for antimalarial drug resistance genes in malaria parasites in the secundarabad region suggest that parasite populations in the region have substantial levels of mutations conferring antimalarial drug resistance. Continuous monitoring of mutations linked with antimalarial drug resistance is necessary to provide useful information for policymakers to plan for proper antimalarial drug regimens to control and eliminate malaria in the country.

Keywords: Plasmodium falciparum; Plasmodium vivax; drug resistance genes; malaria; Anopheles

1. INTRODUCTION

Malaria is an acute febrile infectious disease caused by Plasmodium species parasites transmitted by female Anopheles mosquitoes. Despite the remarkable decline of global malaria incidences in recent years, approximately 241 million people still had malaria and more than 620,000 died in 2020 globally [1]. For effective control and elimination of malaria, accurate diagnosis followed by proper treatment with antimalarial drugs is important [2]. However, global efforts toward controlling malaria have been challenged by the emergence and widespread of antimalarial drug resistance. Antimalarial drug resistance of malaria parasites is acquired by mutations or duplications in target genes, which can confer reduced drug susceptibility. Up to now, multiple genes associated with antimalarial drug resistances have been identified and major mutations inducing the resistances have been characterized [3–5]. The Great Mekong Subregion (GMS) has been recognized as a breeding hub for antimalarial drug resistant malaria parasites. Emergence and spreading of parasites resistant to antimalarial drugs threaten recent outstanding achievements in malaria control and challenge the goal for malaria elimination in the GMS [6]. Moreover, there are concerns on the emergence of artemisinin resistant parasites in the GMS [7–11]. Myanmar is a country where both P. falciparum and P. vivax are prevalent. It has the largest malaria burden in the GMS [12]. Similar to other countries in the GMS, parasites resistant to multiple antimalarial drugs have been reported in the country [13–15]. Cur-rently, artemisinin-based combination therapy (ACT) and chloroquine (CQ) are applied as frontline treatment drugs for P. falciparum and P. vivax, respectively [12]. The unique geographical location of Myanmar, which connects GMS and South Asia, has also empha-sized its importance as a bridge to spread antimalarial drug resistant parasites from GMS to South Asia countries [16]. Molecular analysis of antimalarial drug resistance markers has been validated as one effective tool for surveillance of resistance [3–5]. These markers serve as valuable molecular blueprints for mapping drug resistance status and planning malaria control measures [17]. Currently, several molecular studies reporting drug resistant parasites have performed in border areas of Myanmar [18–28], but it is lacked in the central Myanmar. Therefore, in the present study we investigated on the prevalence of resistant parasites in the Central region of Myanmar. Using molecular profiling we report on the prevalence of resistant parasites in the central region, providing data that is important for the design of national malaria control strategy in Myanmar.

2. MATERIALS AND METHODS

2.1 Blood Samples- Blood samples were collected from patients who were infected with Plasmodium falciparum and P. vivax in four townships, Hyderabad, LB. Nagar, secundarabad, and Uppal, around Hyderabad region of India in 2022 [29] (Figure 1). The transmission intensity of malaria in the areas is low or hypo-endemic [1,20]. Plasmodium species were identified by microscopic examination of Giemsa stained thin and thick blood smears. Before antimalarial drug treatment, finger-prick blood samples were collected from the patients, spotted on Whatman 3 mm filter papers (GE Healthcare, Pittsburg, PA, USA), air-dried, and kept individually in sealed plastic bags at room temperature until further analysis. Informed consents were obtained from all patients before blood sampling. The study protocol was reviewed and approved by the Ethics Committee of the Ministry of Health, Myanmar (97/Ethics 2015) and the Biomedical Research Ethics Review Board of Inha University School of Medicine, Republic of Korea (INHA 15-013).

2.2 Amplification of Antimalarial Drug Resistance Genes- A total of 129 P. falciparum isolates (71 from Hydarabad, 6 from LB.Nagar, 20 from secundarabad, and 32 from Uppal) and 138 P. vivax isolates (112 from Hydarabad, 11 from secudarabad, and 15 from Uppal) were analyzed in this study. Parasite genomic DNA was extracted from the blood spots using the Blood DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Parasite species in the blood samples were further confirmed by species-specific nested polymerase chain reaction (PCR) for 18S ribosomal RNA (rRNA) gene [29,30]. The partial regions flanking major mutations associated with antimalarial drug resistance in P. falciparum and P. vivax marker genes were amplified by nested PCR, respectively. P. falciparum dihydrofolate reductase (pfdhfr), dihydropteroate synthase (pfdhps), multidrug resistance protein 1 (pfmdr-1), chloroquine resistance transporter (pfcrt), kelch propeller protein 13 (pfk13), cytochrome b (pfcytb), and ubiquitin carboxyl-terminal hydrolase 1 (pfubp-1) and P. vivax dihydrofolate reductase (pvdhfr), dihydropteroate synthase (pvdhps), multidrug resistance protein 1 (pvmdr-1), and kelch domain-containing protein (pvk12) were included. The primers used to amplify each drug resistance gene of P. falciparum and P. vivax were summarized in Table S1. Ex Taq DNA polymerase (Takara, Otsu, Japan) with proof-reading activity was used in all PCR steps to minimize the nucleotide misincorporation. The PCR products were analyzed on 1.5% agarose gel. Each PCR product was purified from the agarose gel and ligated into T&A cloning vector (Real Biotech Corporation, Banqiao City, Taiwan). Each ligation mixture was transformed into Escherichia coli DH5α competent cells and positive clones were selected by colony PCR using nested PCR primers for each gene. The plasmids were purified from the selected E. coli clones. The nucleotide sequences of the cloned gene fragments in the plasmids were analyzed by automatic Sanger sequencing method in both directions using M13 forward and M13 reverse primers. To verify the sequence accuracy, plasmids from at least three independent E. coli clones form each gene fragment in each isolate were sequenced. The nucleotide sequences of genes reported in this study have been deposited in the Genbank database following the accession numbers: pfcrt (OM981378–OM981466), pfcytb (OM981467–OM981568), pfdhfr (OM981569–OM981663), pfdhps (OM981664–OM981756), pfk13 (OM981757–OM981831), pfubp-1 (OM982024–OM982081), pfmdr-1 (OM981832–OM981927, OM981928–OM982023), pvmdr-1 (OM982314–OM982403), pvdhfr (OM982082–OM982174), pvdhps (OM982175–OM982252), and pvk12 (OM982253–OM982313).

Figure 1. Blood samples collection site map. Blood samples of malaria patients were collected in the four townships, Hyderabad region, India, in 2022.

2.3 Sequence Polymorphism Analysis- The nucleotide and deduced amino acid sequences of each antimalarial drug resistance gene were annotated and analyzed with the Editseq, SeqMan, and Megalign programs in the DNASTAR package (DNASTAR, Madison, WI, USA). P. falciparum 3D7 pfdhfr (XM_001351443), HB3 pfdhps (PfHB3_080016200), 3D7 pfmdr-1 (XM_001351751), 3D7 pfcrt (XM_001348968), 3D7 pfk13 (PF3D7_1343700), 3D7 pfcytb (PF3D7_MIT02300), and 3D7 pfubp-1 (PF3D7_0104300) were used as wild type reference sequences. For P. vivax, Sal I pvdhfr (PVX_089950), Sal I pvdhps (XM_001617159), Sal I pvmdr-1 (AY618622), and Sal I pvk12 (PVX_083080) were used as reference sequences for P. vivax genes.

2.4 Statistical Analysis-The comparison of haplotype frequency in each gene population between townships was performed to analyze precision and the extent of difference in the proportions. Chi- square test with 95% confidence intervals (CI 95%) and p value were calculated by using Graphpad Prism ver. 8.0.2 (San Diego, CA, USA).

3. RESULTS

3.1 Molecular Profiles of Antimalarial Drug Resistance Genes in Myanmar P. falciparum

Molecular Profiles of pfdhfr and pfdhps- The pfdhfr and pfdhps were successfully amplified from 95 and 93 Myanmar P. falciparum isolates, respectively. Both genes in the parasites showed high frequencies of mutations related to sulfadoxine-pyrimethamine (SP) resistance (Figure 2). For pfdhfr, C59R and S108N showed the highest frequencies of 98.9%, respectively, and followed by I164L (71.6%) and N51I (69.5%) (Figure 2A). These mutations were concurrent in the isolates, making diverse haplotypes. The quadruple mutation, AIRNL, was the most prevalent haplotype (45/95, 47.4%) followed by triple mutations, ANRNL (23/95, 24.2%) and AIRNI (21/95, 22.1%), and double mutation ANRNI (5/95, 5.3%) (Figure 2B). These mutant haplotypes were not evenly distributed in four townships. The frequency of each haplotype also differed by township (Figure 2C). The AIRNL showed the highest prevalence in Hydarabad (23/47, 48.9%), LB.Nagar (5/6, 83.3%), and secundarabad (8/18, 44.4%). However, ANRNL was more prevalent in Tha Beik Kyin (13/24, 54.2%). The AIRNI was detected only in Hydarabad (15/47, 31.9%) and secundarabad (6/18, 33.3%). For pfdhps, validated mutations associated with SP resistance were also detected with high frequencies: S436A (57.0%), A437G (97.8%), K540E (90.3%), K540N (3.2%), and A581G (38.7%) (Figure 2A). These mutations were also concurrent in isolates. Meanwhile, A613S was not detect in Myanmar pfdhps. Triple mutations AGEAA and SGEGA were predominant ones in India pfdhps, accounting for 51.6% (48/93) and 31.2% (29/93), respectively (Figure 2B). Mutant haplotypes including quadruple mutation (AGEGA), triple mutations (SGNGA and AGNAA), and double mutations (SGEAA, SGKGA, and SAKAA) were also identified, but their frequencies were low ranged from 1.1 to 4.3%. These mutant haplotypes were unevenly distributed in the four townships and significant difference in frequencies of the haplotypes were observed ($\chi2 = 22.76$, $p = 0.0016$, CI 95%) (Table S2, Figure 2C). AGEAA and SGEGA showed high prevalence in all four townships, while AGEGA was found in only Naung Cho with a frequency of 6.5% (3/46). Diverse haplotypes were identified in Hydarabad and

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secundarabad, but not statistically different ($\chi 2$ = 34.55, p = 0.0754, CI 95%) (Table S2). However, LB.Nagar and Uppal showed simple haplotype compositions having only 2 or 3 distinct haplotypes. Synergistic effect of combined mutations in pfdhfr and pfdhps on SP resistance level has been classified previously: pfdhfr N51I/C59R/S108N + pfdhps A437G for partial resistance, pfdhfr N51I/C59R/S108N + pfdhps A437G/K540E for full resistance, and pfdhfr N51I/C59R/S108N + pfdhps A437G/K540E/A581G for super resistance [31]. Combined mutations in pfdhfr and pfdhps were identified in P. falciparum isolates only from Hydarabad and secundarabad, where more diverse haplotypes of mutations were detected. None of these combinations for partial resistance was found in India P. falciparum isolates. The combination considered as conferring full resistance was detected in three isolates (1 from Hydarabad and 2 from secundarabad). Super resistance was predicted for 8 isolates from hydarabad and 1 isolate from secundarabad (Table 1). Besides these validated mutations for SP resistance, diverse minor mutations were also observed in Myanmar pfdhps and pfdhfr (Table S3)

(A)

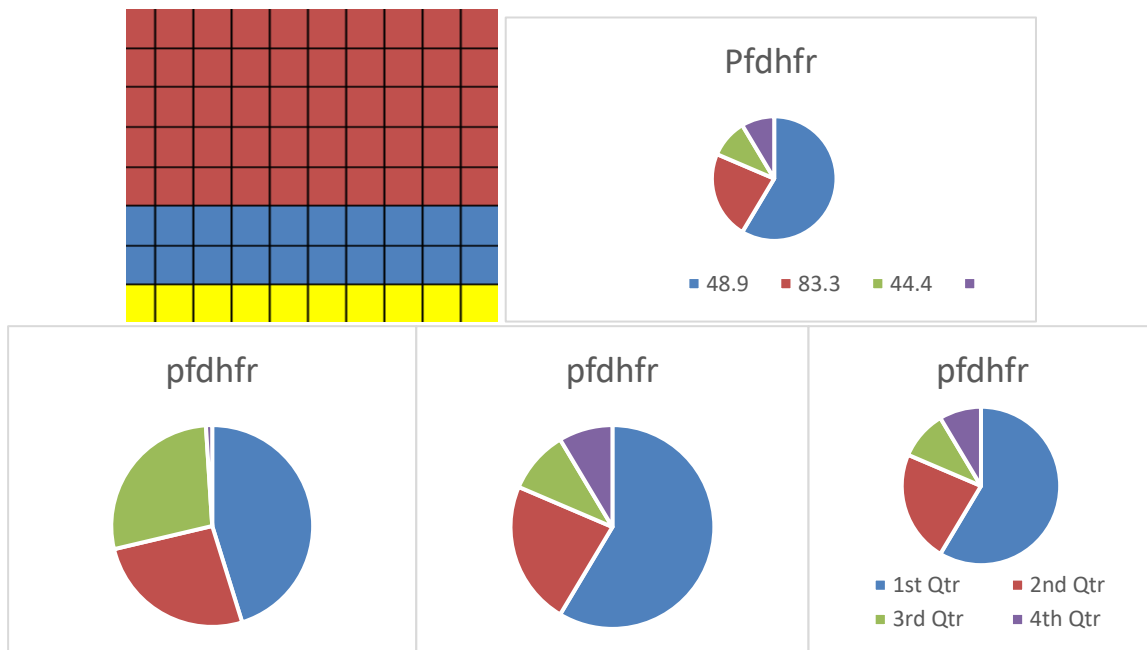| Gene	Mutation	Frequency(%)
Pfdhfr (N=95)	A16V	0.0
	N51I	69.5
	C59R	98.9
	S108N	98.9
	S108T	0.0
	I164L	71.6

(B)

Figure 2. Frequencies and distributions of dihydrofolate reductase (pfdhfr) and dihydropteroate synthase (pfdhps) in India P. falciparum isolates. (**A**) Overall prevalence of mutations identified in pfdhfr and pfdhps. (**B**) Overall frequency of haplotypes of pfdhfr and pfdhps. The amino acid codons in haplotypes corresponded to the amino acids specified in (**A**). (**C**) Proportion of haplotypes of pfdhfr and pfdhps in each township.

Table 1. Combination of mutations between genes related with multidrug resistance and resistance level of India P. falciparum.

Gene	Mutation	Hydarabad	secundarabad	LB.Nagar	Uppal
Combination		(n = 38)	(n = 17)	(n = 6)	(n = 19)
	N51I/C59R/S108N + A437G	0	0	0	0
pfdhfr + pfdhps	N51I/C59R/S108N + A437G/K540E	1 (2.6%)	2 (11.8%)	0	0

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	N51I/C59R/S108N + A437G/K540E/A581G	8 (21.1%)	1 (5.9%)	0	0

Gene combination	Mutations	Hydarabad	secundarabad	LB.Nagar	Uppal
		(*n* = 16)	(*n* = 11)	(*n* = 0)	(*n* =6)
pfmdr-1 + pfcrt	N86Y + K76T	1 (6.3%)	0	0	0

n: number of samples amplified pfdhfr + pfdhps or pfmdr-1 + pfcrt.

3.2 Molecular Profiles of pfmdr-1 and pfcrt

High frequency (56.2%) of mutations associated with CQ resistance was identified in 96 India pfmdr-1. Notable mutations found in India pfmdr-1 were Y184F (28.1%) and F1226Y (24.0%) (Figure 3A). Although N86Y, E130K, and S1034I mutations were also observed, they showed lower frequencies ranging from 1.0% to 4.2%. NEFSNFD and NEYSNYD haplotypes were identified with the same frequency of 24.0% (Figure 3B). Frequencies of NEFINFD, YEYSNFD, and NKYSNFD were 4.2%, 1.0%, and 3.1%, respectively. Different pfmdr-1 haplotypes were identified in P. falciparum from Hydarabad, secundarabad, and Uppal, but no mutant haplotype was identified in P. falciparum from LB.Nagar (χ^2 = 20.86, p = 0.1414, CI 95%) (Figure 3C, Table S2). In the case of pfcrt, very high levels of mutations linked to CQ resistance were found. All India pfcrt had major mutations of M74I/T, N75E, and K76T known to closely associated with CQ resistance. Frequencies of M74I, M74T, N75E, and K76T were 98.9%, 1.1%, 100%, and 100%, respec- tively (Figure 3A). These mutations resulted in two quadruple mutation haplotypes of CIET and CTET, with CIET being highly prevalent (88/89, 98.9%) (Figure 3B). CIET was found in all India P. falciparum isolates except for one isolate from Uppal, which had the CTET haplotype (Figure 3C). Combination of mutations in the two genes, pfmdr-1 N86Y + pfcrt K76T suspected to be associated with amodiaquine (AQ) and CQ resistance [32], was identified in one isolate from Hydarabad (Table 1). Besides these validated or associated mutations, a number of minor mutations were also found in pfmdr-1 and pfcrt of India P. falciparum (Table S3).

3.3 Molecular Profiles of pfk13, pfubp-1, and pfcytb

A total of 75 sequences of pfk13 were obtained from Myanmar P. falciparum isolates. Although wild type pfk13 was prevalent (54/75, 72.0%), four mutations (F446I, N458Y, R561H, and P574L) associated with artemisinin resistance were identified. F446I was the most common mutation accounting for 17.7%, followed by P574L with a frequency of 6.7% (Figure 4A). INYRIRPC was the most prevalent mutant haplotype with a frequency of 18.7%, followed by FNYRIRLC (6.7%) (Figure 4B). Two mutant haplotypes, FYYRIRPC and FNYRIHPC, accounted for 1.3%, respectively. These mutant haplotypes were detected in the isolates from Naung Cho, Mandalay, and Tha Beik Kyin, but not in isolates from Pyin Oo Lwin (χ^2 = 16.86, p = 0.1549, CI 95%) (Table S2). Haplotype diversity was greater in Hydarabad and India (Figure 4C). Sequence analyses of 102 pfcytb and 58 pfubp-1 sequences revealed no mutation associated with drug resistance (V739F, V770F, or E1528D in pfubp-1 or Y268N/S/C in pfcytb) in Myanmar isolates. Beyond these validated mutations, minor mutations were identified in pfk13, pfubp-1, and pfcytb of India P. falciparum (Table S3).

3.4 Summary of Mutations in Multiple Antimalarial Drug Resistance Genes

Although not all antimalarial drug resistance genes were successfully amplified in all Myanmar P. falciparum isolates, comparative analysis of molecular profiles of the five genes except pfubp-1 and pfcytb in each isolate suggested a high prevalence of multiple drug resistance of the parasite, implying resistance against at least two different antimalar- ial drugs (Figure 5). Among the 129 P. falciparum isolates analyzed, 9 shared validated mutations in pfdhfr, pfdhps, pfmdr-1, pfcrt, and pfk13, suggesting their potent resistance against multiple antimalarial drugs including SP, CQ, and artemisinin. Concurrent muta- tions in genes associated with SP and CQ resistance were also identified 85 in India

P. falciparum isolates.

3.5 Molecular Profiles of Antimalarial Drug Resistance Genes in India P. vivax

Molecular Profiles of pvdhfr and pvdhps

The pvdhfr was successfully amplified from 93 India P. vivax isolates. The majority of these isolates carried mutations related to SP resistance. F57L/I, S58R, T61M, and S117N/T were found in 60.2%, 74.2%, 61.3%, and 92.5% of samples, respectively (Figure 6A). A total of eight distinct haplotypes of pvdhfr having quadruple, double, or single mutation were observed in India pvdhfr, with IRMT being the most prevalent (31/93, 33.3%), followed by LRMT (25/93, 26.9%) (Figure 6B). Frequencies of FSTN, FRTN, and wild type FSTS

were 12.9%, 11.8%, and 9.7%, respectively. Meanwhile, frequencies of FRTT, and FSTT, and FSMN were low, ranging from 1.1% to 2.2%. Distributions of these mutant haplotypes were differed by township, but not significantly different ($\chi^2 = 22.62$, p = 0.0068, CI 95%) (Table S2). The IRMT was prevalent in Hydarabad and secundarabad, whereas LRMT was predominant in Uppal (Figure 6C). Greater haplotype diversity was found in P. vivax isolates from Hydarabad and Uppal than those from Secundarabad. For pvdhps, S382A, A383G, K512E, and A553G were detected in 78 India P. vivax isolates

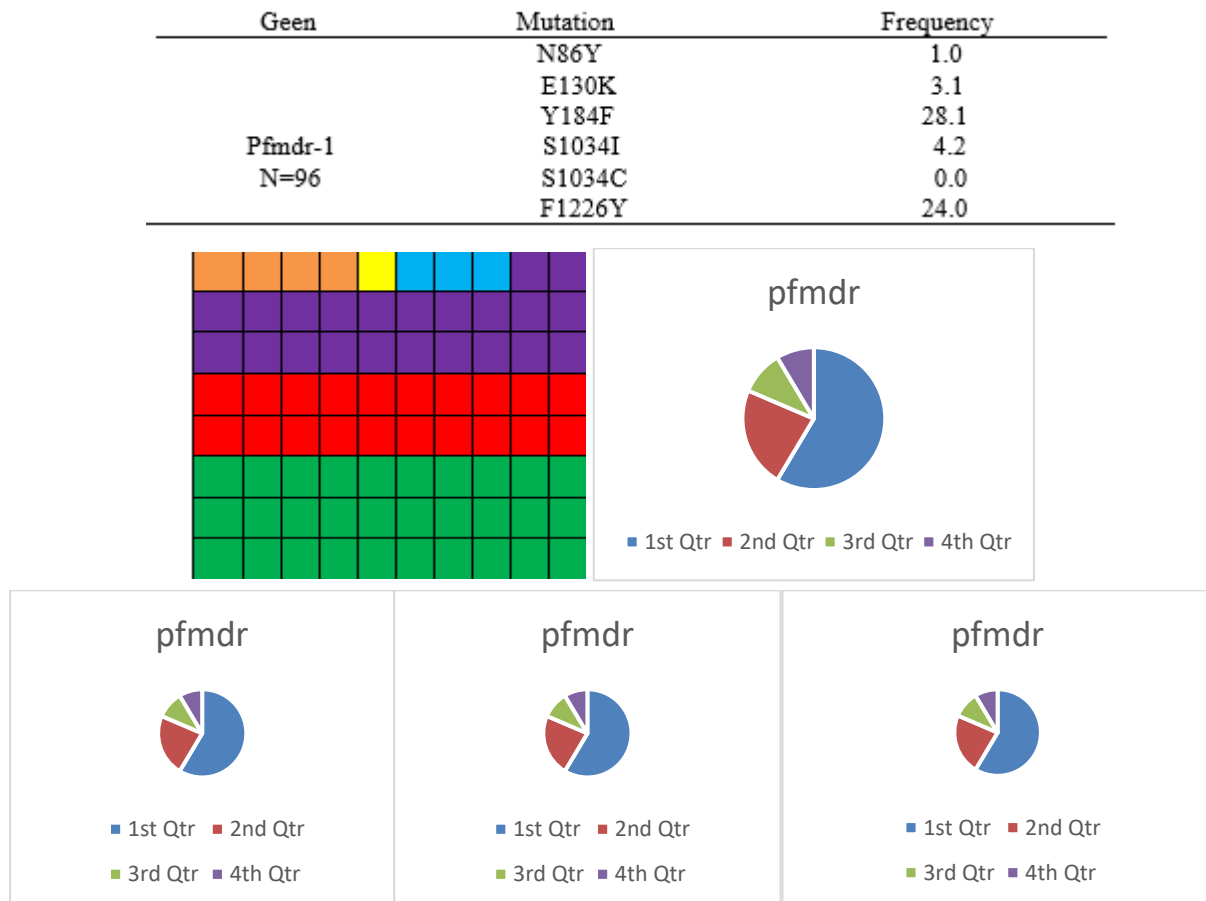| Geen	Mutation	Frequency
Pfmdr-1 N=96	N86Y	1.0
	E130K	3.1
	Y184F	28.1
	S1034I	4.2
	S1034C	0.0
	F1226Y	24.0

Figure 3. Frequencies and distributions of multidrug resistance 1 (pfmdr-1) and chloroquine resistance transporter (pfcrt) in India P. falciparum isolates. (**A**) Overall prevalence of mutations identified in pfmdr-1 and pfcrt. (**B**) Overall frequency of haplotypes of pfmdr-1 and pfcrt. The amino acid codons in haplotypes corresponded to the amino acids specified in (**A**). (**C**) Proportion of haplotypes of pfmdr-1 and pfcrt in each township.

with frequencies of 9.0%, 89.7%, 3.8%, and 73.1%, respectively (Figure 6A). There was no V585R detected in Myanmar pvdhps. These mutations generated five different haplotypes of India pvdhps harboring triple mutations (AGKGV), double mutations (SGKGV and AGKAV), and single mutation (SGKAV) (Figure 6B). The SGKGV was the most prevalent haplotype, having a frequency of 69.2%. Frequencies of SGKAV, AGKAV, and AGKGV were 11.5%, 5.1%, and 3.9 %, respectively. Meanwhile, the frequency of SAKAV (wild type) was 10.3%. The SGKGV was a prevalent haplotype in all three townships (Figure 6C). Similar to pvdhfr, greater haplotype diversity of pvdhps was detected in P. vivax isolates from Hydarabad and Uppal than from Secundarabad ($\chi^2 = 26.41$, p = 0.0009, CI 95%) (Table S2). Allele combination between pvdhfr and pvdhps harboring multiple mutations in these two genes is known to contribute to synergistic SP resistance [33]. Sixty-three India P. vivax isolates carried concurrent mutations in both pvdhfr and pvdhps (Table 2). Besides these major mutations, diverse minor mutations were also found in the genes (Table S4).

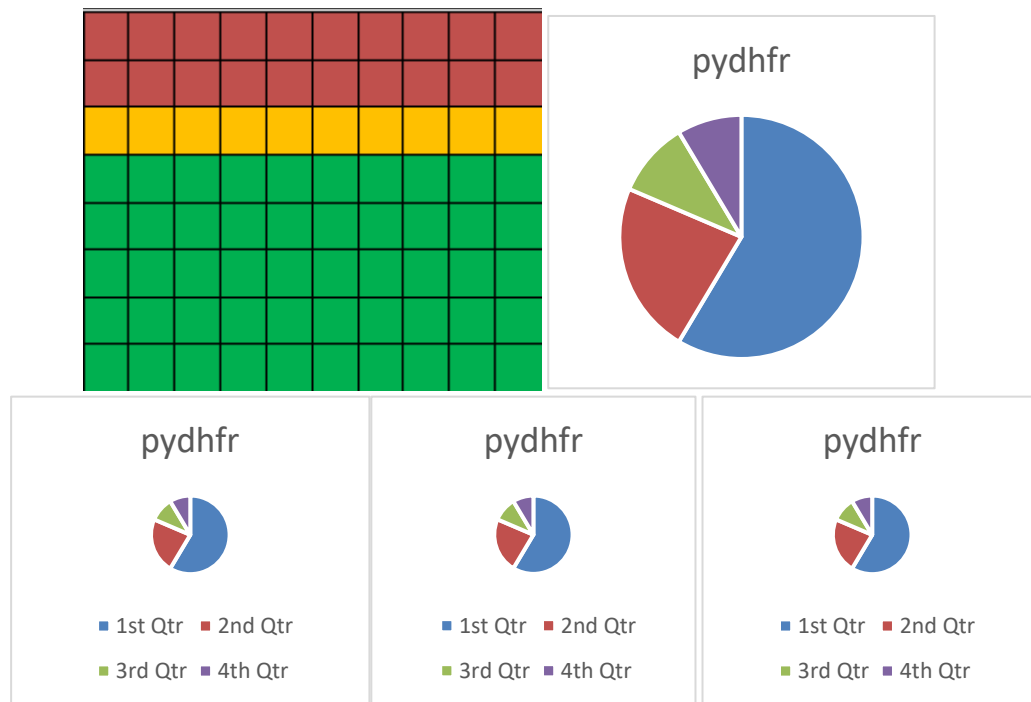| Gene	Mutation	Frequency (%)
Pfk13	F446I	17.7
	N458Y	1.3
	Y493H	0.0
	R539T	0.0
	R561H	1.3

Figure 4. Frequencies and distributions of Kelch 13 (pfk13), ubiquitin specific protease 1 (pfubp-1) and cytochrome b (pfcytb) in India P. falciparum isolates. (**A**) Overall prevalence of mutations identified in pfk13, pfubp-1, and pfcytb. (**B**) Overall frequency of haplotypes of pfk13, pfubp-1, and pfcytb. The amino acid codons in haplotypes corresponded to the amino acids specified in (**A**). (**C**) Proportion of haplotypes of pfk13, pfubp-1, and pfcytb in each township.
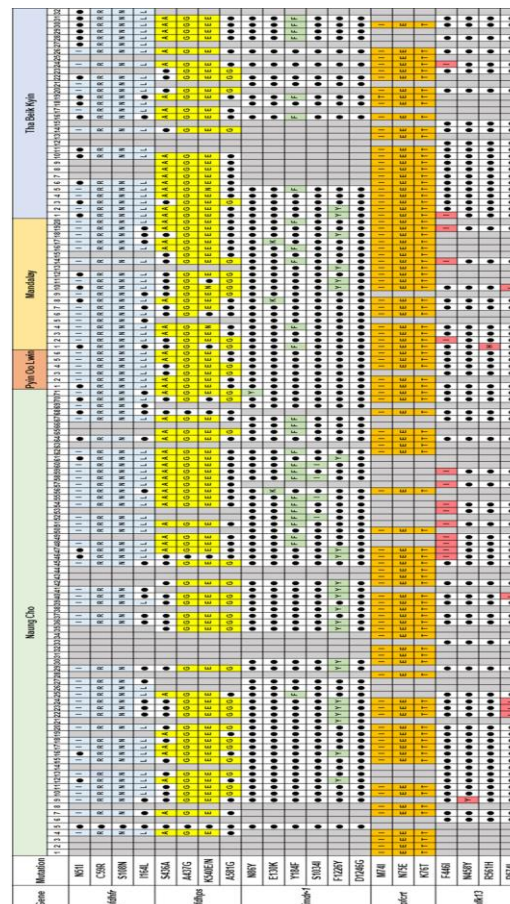

Figure 5. Combinational analysis of the mutations in five genes associated with antimalarial drug resistance in P. falciparum. Mutations in each gene highlighted with different colors and wild type residues shown as closed circles. Grey boxes represented samples failed to amplify.

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Table 2. Combination of mutations between genes related with multidrug resistance and resistant level of India P. vivax.

Gene Combination	Mutations	Hydarabad (n = 43)	secundrabad (n = 9)	Uppal (n = 12)
pvdhfr + pvdhp	F57I/S58R/T61M/S117T + S382A/A383G/A553G	1 (2.3%)	0	0
	F57I/S58R/T61M/S117T + A383G/A553G	19 (41.1%)	5 (55.6%)	1 (8.3%)
	F57L/S58R/T61M/S117T + A383G/A553G	7 (16.3%)	2 (22.2%)	5 (41.7%)
	F57I/S58R/T61M/S117T + S382A/A383G	0	0	0
	F57L/S58R/T61M/S117T + S382A/A383G	0	1 (11.1%)	0
	F57L/S58R/T61M/S117T + A383G	2 (4.7%)	0	1 (8.3%)
	F57I/S58R/T61M/S117T + A383G	1 (2.3%)	0	0
	S58R/S117N + A383G/A553G	6 (13.9%)	0	1 (8.3%)
	S58R/S117T + S382A/A383G/A553G	0	0	1 (8.3%)
	S58R/S117N + S382A/A553G	1 (2.3%)	0	0
	T61M/S117N + A383G/A553G	1 (2.3%)	0	0
	S58R/S117T + A383G/A553G	0	1 (11.1%)	0
	S117N + A383G/A553G	3 (7.0%)	0	0
	S58R/S117N + A553G	0	0	2 (16.7%)

n: number of samples amplified pvdhfr + pvdhps.

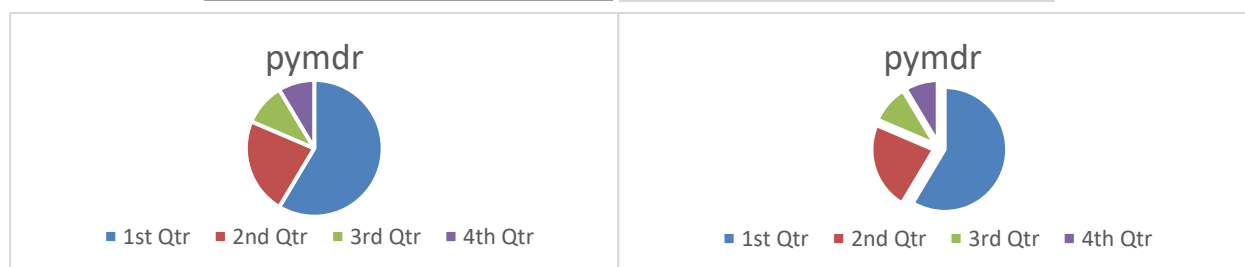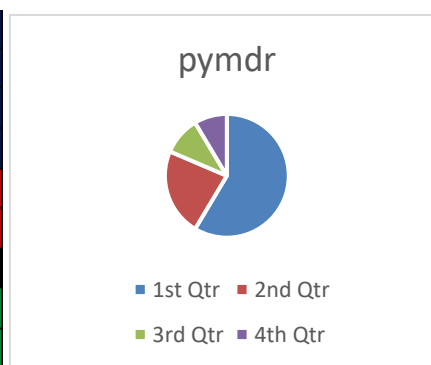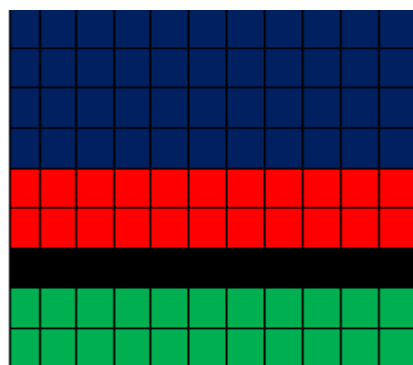| Gene	Mutation	Frequency (%)
Pvdhfr	F57L	25.8
	S58R	74.2
	T61M	**61.3**
	S117N	**25.8**
	S117T	**66.7**

Figure 6. Frequencies and distributions of dihydrofolate reductase (pvdhfr) and dihydropteroate synthase (pvdhps) in Myanmar P. vivax isolates. (**A**) Overall prevalence of mutations identified in pvdhfr and pvdhps. (**B**) Overall frequency of haplotypes of pvdhfr and pvdhps. The amino acid codons in haplotypes corresponded to the amino acids specified in (**A**). (**C**) Proportion of haplotypes of pvdhfr and pvdhps in each township.

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3.6 Molecular Profiles of pvmdr-1 and pvk12

Moderate levels of mutations associated with CQ resistance were detected in pvmdr-1 of Myanmar P. vivax isolates. Two mutations, Y976F and F1076L, were identified in Myan- mar pvmdr-1 with frequencies of 10.0% and 33.3%, respectively (Figure 7A). Frequencies of FL haplotype and YL haplotype were 10.0% and 33.3%, respectively (Figure 7B). FL wasdetected only in Hydarabad isolates, while YL was commonly identified in all isolates from Hydarabad, secundarabad, and LB.Nagar (Figure 7C). Meanwhile, V552I, which was suspected to be associated with artemisinin resistance, was not detected in India pvk12. Diverse minor mutations were also observed in India, pvmdr-1 and pvk12 (Table S4).

3.7 Summary of Mutations in Multiple Antimalarial Drug Resistance Genes

Consistent with P. falciparum, not all antimalarial drug resistance genes were success- fully amplified in all Myanmar P. vivax isolates analyzed. However, comparative analysis of molecular profiles of the four genes in each P. vivax isolate implied substantial levels of multiple drug resistances in India isolates (Figure 8). Among the 138 P. vivax isolates analyzed, 21 had concurrent mutations in pvdhfr, pvdhps, and pvmdr-1, suggesting their potent roles in SP and CQ resistance.
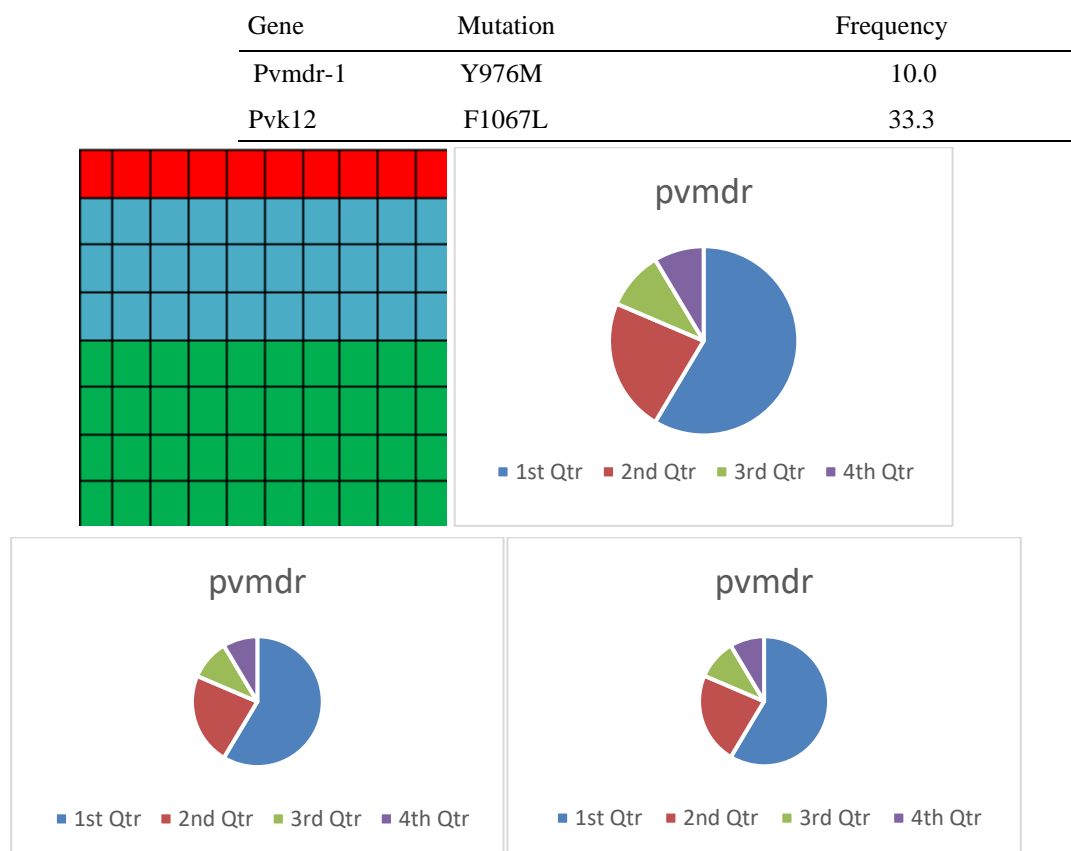
Gene	Mutation	Frequency
Pvmdr-1	Y976M	10.0
Pvk12	F1067L	33.3

Figure 7. Frequencies and distributions of multidrug resistance 1 (pvmdr-1) and kelch 12 (pvk12) in Myanmar P. vivax isolates. (**A**) Overall prevalence of mutations identified in pvmdr-1 and pvk12.(**B**) Overall frequency of haplotypes of pvmdr-1 and pvk12. The amino acid codons in haplotypes corresponded to the amino acids specified in (**A**). (**C**) Proportion of haplotypes of pvmdr-1 and pvk12in each township

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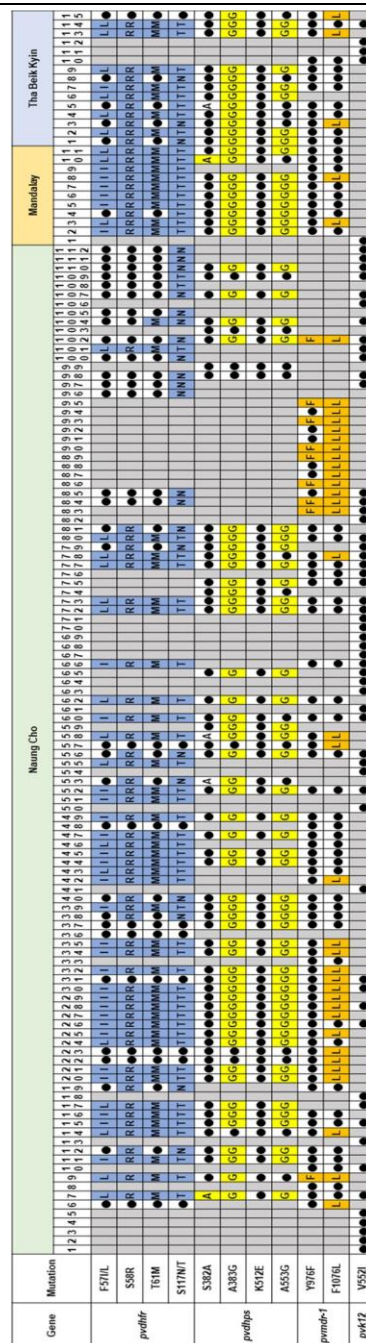
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Figure 8. Combinational analysis of the mutations in four genes associated with antimalarial drug resistance in P. vivax. Mutations in each gene highlighted with different colors and wild type residues shown as closed circles. Grey boxes represented samples failed to amplify.

4. DISCUSSION

Antifolate drugs represented by SP have been extensively deployed in India in the past decades. However, they have been withdrawn due to widespread use of resistant Plasmodium species against these drugs. As expected, extremely high levels of mutations strongly associated with SP resistance were detected in both P. falciparum and P. vivax analyzed in this study. P. falciparum isolates from secundarabad region had major mutations that were strongly associated with SP resistance in pfdhfr and pfdhps with high frequencies. In the case of pfdhfr, these mutations were commonly identified as concurrent mutations rather single mutations in the gene. Frequencies of quadruple mutation (AIRNL), triple mutation (AIRNI or ANRNL), and double mutation (ANRNI) were comparable to or slightly different from those of P. falciparum collected from northern (Telangana State) and western (Telangana State) India [25,34]. Concurrent mutations were also identified in pfdhps. Frequencies of the mutations were similar to or slightly higher than those of P. falciparum collected from northern (Telangana state State) and western (Telangana stateState) India [25,34]. However, more diverse haplotypes of pfdhps were identified in P. falciparum isolates collected from secundarabad region. Combined mutations in pfdhfr and pfdhps have been recognized as reliable

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predictors for SP treatment failure [31,35]. These mutations were also detected in India isolates analyzed in this study. Similar proportions of these combinations have been reported in northern India, Thailand and Cambodia [25,36]. High rates of pvdhps and pvdhfr mutations in India P. vivax have been reported previously [17,19,25]. High levels of mutations associated with SP resistance were also observed in pvdhps and pvdhfr in P. vivax isolates from secundarbad region, which were comparable to parasites from southern (Kayah, Mon, and Kayin), northern (Laiza, Kachin State), and western (Sagaing and Buthidaung) Myanmar [19,25]. These molecular profiles for SP-resistant Plasmodium population in Myanmar should be underscored in that mutations in dhfr and dhps conferring SP resistance to both P. falciparum and P. vivax were still highly preserved in the parasite population of Myanmar, albeit antifolate drugs were not used for malaria treatment in recent few decades. The reason for why selective pressure still acting to maintain dhfr and dhps mutations for SP resistance in Myanmar Plasmodium population is currently unclear. Sulfa based drugs such as cotrimoxazole, sulfamethoxazole and trimethoprim used to treat bacterial infections can be one cause. Further comprehensive studies are needed to elucidate it. CQ had been largely applied in Myanmar to treat both falciparum malaria and vivax malaria in the past. However, CQ resistant P. falciparum began to be reported in Myanmar in 1970s and has rapidly spread throughout the country [37]. Mutations in pfcrt are primary indicators for CQ resistance, especially K76T mutation [38,39]. They can also influence susceptibility to quinine (QN), mefloquine (MQ), halofantrine (HF), and amodiaquine (AQ) [40]. High levels of mutations including M74I, N75E, and K76T were detected in Myanmar pfcrt analyzed in this study. Moreover, all P. falciparum isolates harbored the triple mutation, CIET or CTET. These values were greater than those of P. falciparum isolates collected from northern (Laiza and Banmauk; 76.5%) and western (Paletwa; 95.8%) Myanmar [25]. Meanwhile, frequencies of two validated major mutations linked to CQ resistance in pfmdr-1, Y184F and F1226Y, were relatively low in samples analyzed in this study. Concurrent mutations in pfcrt (K67T) and pfmdr-1 (N86Y, Y184F, S1034I, N1042D, and D1246Y) are known to increase CQ resistance [41]. Combination of pfcrt K76T and pfmdr-1 N86Y was identified in only one isolate from Naung Cho, while combined mutations of pfcrt K76T and pfmdr-1 Y184F were detected with a frequency of 22.5%, which was lower than that in P. falciparum isolates collected from northern Myanmar (Laiza and Banmauk: 76.4%), but higher than that in P. falciparum isolates collected from western Myanmar (Paletwa: 12.5%) [25]. The N86Y/Y184F mutation in pfmdr-1 also has proposed to decrease parasite susceptibility to lumefantrine and MQ by enhancing digestive vacuole transport efficacy [42]. This double mutation was not identified in pfmdr-1 in the parasites analyzed in this study, suggesting lumefantrine and MQ may still applicable as partner drugs for ACT in Mandalay region. These results collectively indicate that substantial levels of CQ-resistant P. falciparum are prevalent in Myanmar, although the drug was withdrawn from the country in the 1970's for radical cure of falciparum malaria. Nonetheless, CQ is still continuously used as a frontline treatment drug for vivax malaria in Myanmar. This might have contributed to a stable maintenance of pfcrt mutations in Myanmar P. falciparum population. Although CQ is still the drug of choice for vivax malaria in Myanmar, CQ-resistant P. vivax was first detected in Myanmar in the 1990s [37]. Declined therapeutic responses of P. vivax to CQ have been recently reported in the China-Myanmar border and southern Myanmar [21,43–46]. Although controversial as molecular markers for CQ resistance in P. vivax still remains, pvmdr-1 and pvcrt-o have been queried since these two genes are orthologues of pfmdr-1 and pfcrt, which are validated molecular markers for CQ resistance in P. falciparum [3,47]. The Y967F in pvmdr-1 is a major mutation that can decrease in vitro sensitivity of CQ in P. vivax. Double mutation of Y976F/F1076L in pvmdr-1 can significantly increase CQ resistance [48,49]. The frequency of FL double mutation in this study was 11.8%. Different levels of these mutations were reported in P. vivax isolates from other regions of Myanmar, including Y976F in southern (Kawthaung and Shwegyin, 20.9%), northern (Laiza, 3.8%), and western (Buthidaung, 1.7%) and F1076L in western (Buthidaung; 63.3%), southern (Kawthaung and Shwegyin; 45.9%), and northern (Laiza, 78.8%) [20,50]. These findings imply that Myanmar P. vivax population also has molecular profiles for potent CQ resistance, albeit frequencies of these mutations differed by region. Myanmar occupies an important geographical location in containment of artemisinin-resistant parasites, as the country has the largest malaria burden in GMS and bridges GMS and South Asia. Furthermore, artemisinin resistance risk areas are challenging for malaria control due to high rates of migration at border areas, remote forested and mountainous areas, and reliance on private health care providers [51]. ACT regimen has changed several times given the selection of resistance markers to the partner drugs. ACT (artemether–lumefantrine [AL], dihydroartemisinin–piperaquine [DHA-PPQ] or artesunate-mefloquine [AS-MQ]) has been presently adopted as the frontline treatment for uncomplicated falci-parum malaria [1,52]. In Mandalay region, the first line ACT for uncomplicated P. falci-parum is AL and the alternative ACT is AS-MQ [53]. Delayed parasite clearance against artemisinin has been recognized to be associated with mutations in pfk13. Nine muta-tions (F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, and C580Y) in pfk13 are validated or associated with in vivo and in vitro ACT resistance in P. falciparum [6]. Of

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these 9 mutations, 4 mutations (F446I, N458Y, R561H, and P574L) were found in pfk13 of P. falciparum isolates analyzed in this study, with F446I showing the highest prevalence of 17.7%. This value was lower than those of previously reported P. falciparum isolates from Pakistan (41.9%) and China-India border (55.9%), but higher than those from pakistan (15.5%) and southern India including India-Nepal border (less than 10%) [15,22,25,54]. The P574L (6.3%) was also detected with comparable or slightly lower

frequency compared to a previous report on isolates collected from the India-China border [55]. Interestingly, C580Y, the key mutation of artemisinin resistance, was not iden- tified in P. falciparum isolates analyzed in this study. This mutation has been reported in limited areas of India, southern (India-Nepal border) and northern (Telangana State) with a frequency of 11.4% linked to other two pfk13 mutations, R561H and F446I [20,56]. Several therapeutic efficacy studies on artemisinin-based combinations such as AL, AS-MQ, and DHA-PPQ for the treatment of uncomplicated P. falciparum in Myanmar have been performed [34,54–56]. Efficacy of these antimalarial drugs is likely to remain high in Myanmar albeit pfk13 mutations were reported in some regions [55,56], suggesting ACT is still effective in the country. In addition, pyronaridine-artesunate also displayed high efficacy for both uncomplicated P. falciparum and P. vivax malaria, implying it can be included in the national malaria treatment protocols of Myanmar [56]. Molecular mechanism for artemisinin resistance of P. vivax has not been clearly elucidated yet. However, V552I mutation in pvk12, an orthologous of pfk13, was supposed to be associated with artemisinin resistance of the parasite [57]. In areas where P. falciparum and P. vivax are co-endemic, these two species can share the same mosquito vectors and human hosts and they are often subject to similar forces of natural selection [2,58]. Therefore, wide employment of ACT to cure P. falciparum infections might exert collateral selective pressure to P. vivax populations. Although P. vivax might have been exposed to higher selective pressure by artemisinin for the last few decades after ACT was introduced, V552I wasnot detected in pvk12 of any Myanmar P. vivax isolates analyzed, albeit 48 other minor mutations were discovered. V552I was not reported in Cambodia or Myanmar P. vivax population either [26,57,59–61]. Considering limited knowledge on pvk12 and mutations associated with artemisinin resistance in the gene, further study is needed to determine the role of these mutations with artemisinin resistance.

Mutations in pfubp-1 is also known to contribute to artemisinin resistance of P. falci- parum [62]. Especially, mutations at D1525E and E1528D in pfubp-1 are likely to be closely associated with delayed parasite clearance [63]. These mutations were not observed in pfubp-1 of P. falciparum analyzed in this study. Although a few non-synonymous muta- tions including H1459R, W1470C, D1522G, N1548D, and H1550R/L were detected with low frequencies, it is currently unclear whether these mutations could confer artemisinin resistance in P. falciparum. This needs to be investigated further. Mutations in pfcytb of P. falciparum can induce treatment failures of atovaquone against P. falciparum by inhibiting parasite mitochondria electron transport mechanism [64]. A previous study has suggested that Y268N/S/C in pfcytb is related to resistance of atovaquone-proguanil (Malarone) in P. falciparum [65]. This mutation was not detected in P. falciparum isolates analyzed in this study, coinciding with a previous study on Myanmar P. falciparum isolates [66]. How- ever, continuous monitoring this mutation in Myanmar P. falciparum should be necessary as Malarone is a drug that has widely used for chemoprophylactic purposes, especially for travelers.

Overall molecular profiles of multiple antimalarial drug resistance genes in Myanmar P. falciparum and P. vivax populations suggest mild geographical heterogeneity in Myanmar. However, overall rates of mutations validated or associated with SP and CQ resistances are likely to be maintained at high or substantial levels. Meanwhile, mutations conferring ACT- resistance remained at relatively low levels. Non-neglected levels of parasite population carrying multiple drug resistance characters were also found. Increasing genetic diversity of P. falciparum and P. vivax, albeit marked reduction of recent malaria incidences, raises a concern for dynamic changes of genetic structure and expansion of genetic heterogeneity of Plasmodium population in Myanmar [67,68]. Increasing movement of human populations may facilitate changes of parasite transmission patterns and spreading of drug-resistant Plasmodium populations in the country. Asymptomatic cases are also a great concern since asymptomatic patients serve as silent reservoirs to continue transmission of malaria and antimalarial drug resistant parasites [23,26,29]. Similar to other countries in the GMS,

P. vivax is becoming a predominant species in Myanmar with recent decrease of falciparum malaria cases. Mixed infections are also found frequently [29,69]. Continuous molecular surveillance for antimalarial drug resistance in Plasmodium parasites nationwide would be necessary to update and reset guidance for the use of antimalarial drugs in Myanmar.

5. CONCLUSIONS

High or substantial levels of mutations in antimalarial drug resistance genes were detected in both P. falciparum

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and P. vivax isolates collected from the secundarabad region, central India. Non-neglectable proportions of combined mutations in SP- and CQ- resistance genes in parasite populations suggest that multiple drug resistance parasites are prevalent in secundarabad region. However, mutations in pfk13, pfubp-1, and pvk12 were identified at low frequency or were absent, suggesting that artemisinin resistance might not be a great concern, at least in the secundarabad region of India in 2015. But further assessment of artemisinin resistance in larger numbers of samples would be necessary to trace the current change in the frequencies of the mutations in the region. No mutation in pfcytb also suggests that atovaquone may be effective against Myanmar P. falciparum so far. Frequency of mutations in the genes analyzed in this study differed slightly by township. It

can be caused by the different size of samples in each township. Considering that these two parasites, P. falciparum and P. vivax, share the same vectors and human hosts and that they are affected by collateral selective pressure of antimalarial drugs, continuous monitoring of antimalarial drug resistances in both parasites is necessary. The limitations of this study are the small sample size per region and time of sampling, which limits the applicability and generalizability of the study findings to contemporary surveillance programs at current. However, similar antimalarial drug resistance profiles in the central region of India with border areas of the country emphasize the necessity of continuous monitoring in the larger areas to provide useful information for policy makers to design proper antimalarial drug strategy for effective control and elimination of malaria in India.

Supplementary Materials: The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/microorganisms10102021/s1, Table S1: PCR primers to amplify

P. falciparum and P. vivax drug resistance genes; Table S2: The distribution of mutant haplotypes of antimalarial drug resistance between each township in Myanmar P. falciparum and P. vivax isolates; Table S3: Minor mutations identified in drug resistance genes of Myanmar P. falciparum; Table S4: Minor mutations identified in drug resistance genes of Myanmar P. vivax [70–75].

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