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# Molecular Evidence of Drug Resistance in Asymptomatic Malaria Infections, Myanmar, 2015

## Technical Appendix

### Study Population

We conducted this study in Shwegyin Township, Bago Region (22°20′0″N, 95°56′0″E) in January 2015 (Figure 1). According to 2014 census data, 107,462 persons were living in an area of 2,440.1 $km^2$. Because of the nearby gold mine, many migrant workers were working in this area, and malaria was identified as one of the major diseases in Shwegyin (*1*). Since 2011, Shwegyin Township has been defined as a Tier I area of Myanmar artemisinin resistance containment because of the evidence of delayed parasite clearance in therapeutic efficacy studies conducted in Shwegyin during 2009–2010 (*2*,*3*) with molecular evidence of K13 mutations, an artemisinin-resistance marker (Nyunt et al., unpublished data). Shwegyin has been selected as one of the townships targeted for a malaria elimination program initiated in Myanmar. In 2001, 14.1% of outpatients and 20.1% of inpatients in Shwegyin Hospital were infected with malaria. The trend of prevalence has been decreasing, however, resulting in 11.3% of outpatients and 3.5% of inpatients infected with malaria in 2010 and 1.2% and 0.1%, respectively, infected with malaria in 2014.

### Study Procedures

We recruited the participants in this study by randomized cluster sampling in Shwegyin Township. Inclusion criteria included a minimum age of 6 years and being local residents in the study area for more than 3 years; both sexes were included. Because we aimed to know the status of asymptomatic infections in local residents, the migrant or mobile population was excluded. Persons who currently showed signs and symptoms of malaria were also not included in this study.

We obtained written informed consent from all the participants. All the patients with parasite infections detected by any method (rapid diagnostic test [RDT], microscopy, or molecular method) were treated according to the National Malaria Treatment Guideline. This study was approved by the Ethical Committee of the Department of Medical Research, Myanmar (approval no. 49/Ethics-2014). The study was also registered with ClinicalTrial.gov (identifier NCT02708199).

## Sample Size Determination

The prevalence of asymptomatic infection in the study population was unknown. Based on the previous studies (*4*,*5*) conducted in Southeast Asia, we assumed the maximum possibility of infection was 25% in the study site. A required sample size was calculated by anticipated population proportion of asymptomatic infection (25%) among the population of 107,462 (as of the 2014 census), with marginal error (2.5%), and 95% CI. The minimum sample size required was 1,141; in this study, 1,182 participants were involved.

## Sampling Procedure

Randomized cluster sampling method was used in this study. Shwegyin Township was selected according to the rationale described previously. Two of the 4 local health centers were randomly selected. All villages belonging to the local health centers were listed and 6 villages were randomly selected. Among these villages, a sampling interval was calculated to get the required blood samples in the villages, which we determined to be at least 191 per village. Household visits or meeting places were used to collect the samples, depending on the convenience of the participants.

## Laboratory Procedures

We collected 1 mL of venous blood from the participant's forearm under aseptic conditions, using a disposable syringe. The blood was used for detection of asymptomatic infections.

### Rapid Diagnostic Test

We detected malarial infection in all participants by *Pf*HRP2- and *Pv*-specific pLDH-based RDT (SDFK80; Standard Diagnostics, Gyeonggi-do, South Korea) and peripheral blood film examination to exclude malaria infection in the field. We used venous blood for malaria detection by RDT according to the manufacturer's instructions.

### Malaria Microscopy

We followed the World Health Organization standardized protocol (*6*) for malaria microscopy. Briefly, 10% Giemsa was used to stain thick and thin blood films in the field for initial screening. In the main laboratory, another set of thick and thin blood films was stained with 3% Giemsa stain for confirmation and validation of the result. We prepared a fresh Giemsa stain dilution at least once a day and possibly more often, depending on the number of slides processed. We examined the Giemsa-stained thick and thin blood films at a magnification of 1,000× to identify the parasite species and to determine the parasite density. We calculated the parasite density, expressed as the number of asexual parasites per microliter of blood, by dividing the number of asexual parasites by the number of leukocytes counted and then multiplying by an assumed leukocyte density (6,000 leukocytes/μL). All slides were stained and checked by World Health organization–certified microscopists and validated by an expert microscopist. A blood slide was considered negative when examination of 1,000 leukocytes revealed no asexual parasites. Two qualified microscopists read all the slides independently, and parasite densities was calculated by averaging the 2 counts. Blood smears with discordant results (differences between the 2 microscopists' results in species diagnosis, in parasite density of >50%, or in the presence of parasites) were reexamined by a third, independent microscopist, and parasite density was calculated by averaging the 2 counts closest to each other.

### Molecular Detection

We adapted the pooling strategy described previously (*7*). In brief, 20 μL from each of 10 samples was combined to make 1 pool of 200 μL whole blood. These pooled blood samples were designated for DNA extraction with QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) and eluted into a final volume of 100 μL. Ten microliters of the DNA eluent was used for *Plasmodium* genus-specific amplification of the 18S rRNA gene, as described elsewhere (*8*). Only *Plasmodium*-positive pools were included to do individual DNA extraction again; 1 μL of

the DNA eluent was used for individual genus and species identification for asymptomatic malaria infection by using genus and specific primer pairs (*8*).

### Artemisinin Resistance Molecular Markers for *P. falciparum* Infection

We analyzed all the samples that tested positive for *P. falciparum* malaria infection for artemisinin-resistance molecular markers (*9,10*) such as K13 kelch genes (PF3D7_1343700), *pfarps10* (PF3D7_1460900.1), *pffd* (PF3D7_1318100), and *pfmdr2* (PF3D7_1447900) by using the pairs of primers (Table 1. The details for amplification of these targets are available at our institutional web page (http://kmrl.kangwon.ac.kr/).

We performed amplification with an Accupower Premix (Bioneer, Daejon, South Korea) in a final volume of 20 μL. The final volume included 250 nmol/L of each primer, 0.25 mmol/L of each dNTP, 10 mmol/L Tris-HCl (pH 9.0), 30 mmol/L $MgCl_2$, 1.0 units of Taq polymerase, and 2 μL of genomic DNA template.

For nested-1 PCR amplification of the K13 kelch propeller gene, initial denaturation at 95°C for 5 min was followed by 35 cycles at 95°C for 30 s, 58°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 10 min. Using 1 μL of the nested-1 PCR product as a template, we applied the same conditions for the nested-2 PCR except for an annealing temperature of 60°C for 1 min and 72°C for 1 min with 30 cycles.

For nested-1 PCR amplification of the *pfarps10* gene, initial denaturation at 95°C for 5 min was followed by 35 cycles at 95°C for 30 s, 58°C for 1 min, 72°C for 1.5 min, and a final extension of 72°C for 10 min. Using 1 μL of the nested-1 PCR product as a template, we applied the same conditions for the nested-2 PCR except for an annealing temperature of 62°C for 1 min and 72°C for 1 min with 30 cycles.

For nested-1 PCR amplification of the *pffd* gene, initial denaturation at 95°C for 5 min was followed by 35 cycles at 95°C for 30 s, 62°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. Using 1 μL of the nested-1 PCR product as a template, we applied the same conditions for the nested-2 PCR except for an annealing temperature of 60°C for 1 min and 72°C for 1 min with 30 cycles.

For nested-1 PCR amplification of the *pfmdr2* gene amplification, initial denaturation at 95°C for 5 min was followed by 35 cycles at 95°C for 30 s, 58°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 10 min. Using 1 μL of the nested-1 PCR product as a template, we applied the same conditions for the nested-2 PCR except for an annealing temperature of 60°C for 1 min and 72°C for 1 min with 35 cycles.

We visualized the PCR products by 1% agarose gel electrophoresis stained with 0.05% Redsafe dye (iNtRON Biotechnology, Daejeon, South Korea). We purified the PCR products by using a MEGA quick-spin Total Fragment DNA Purification Kit (iNtRON Biotechnology) and sequenced them with primers from a commercial sequencing company (Genotech, Daejeon, South Korea). We compared all the nucleotide and amino acid sequences with the reference sequence of 3D7 version 3 (www.plasmodb.org) and aligned them by using software in the Lasergene Genomic Suite (MegAlign, version 7.1; DNAStar, Madison, WI, USA).

### Drug Resistance Molecular Marker Analysis for *P. vivax* Isolates

We further analyzed all the *P. vivax* isolates for *pvcrt-o* (*P. vivax* chloroquine resistance transporter gene, PVX_087980), *pvmdr1* (*P. vivax* multidrug resistance protein 1, PVX_080100), *pvdhps* (*P. vivax* hydroxymethyl pterinpyrophosphokinase dihydropteroate synthetase, PVX_123230), and *pvdhfr* (*P. vivax* dihydrofolate reductase thymidylate synthase, PVX_089950) by using the modified procedures described previously (*11*). We performed amplification by using an Accupower premix (Bioneer, Daejon, South Korea) in a final volume of 20 μL, which included 250 nmol/L of each primers, 0.25 mmol/L of each dNTP, 10 mmol/L Tris-HCl (pH 9.0), 30 mmol/L MgCl2, 1.0 units of Taq polymerase, and 2 μL of genomic DNA template and pairs of primers (Table 1).

For the *pvcrt-o* PCR amplification, initial denaturation at 94°C for 10 min was followed by 35 cycles at 94°C for 30 s, 60°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min for nested-1 PCR. Using 1 μL of the nested-1 PCR product as a template, we applied the same conditions for the nested-2 PCR except for an annealing temperature of 60°C for 45 s with 30 cycles of amplification.

For the *pvmdr1* PCR amplification, initial denaturation at 94°C for 10 min was followed by 35 cycles at 94°C for 30 s, 58°C for 45 s, 72°C for 2 min, and a final extension at 72°C for 10 min for nested-1 PCR. Using 1 μL of the nested-1 PCR product as a template, we applied the

same conditions for the nested-2 PCR except for an annealing temperature of 62°C for 45 s and extension at 72°C for 45 s with 33 cycles of amplification.

For the *pvdhps* PCR amplification, initial denaturation at 94°C for 10 min was followed by 40 cycles at 94°C for 30 s, 58°C for 45 s, 72°C for 1.5 min, and a final extension at 72°C for 10 min for nested-1 PCR. Using 1 μL of the nested-1 PCR product as a template, the same conditions were applied for the nested-2 PCR except for 35 cycles of amplification.

For the *pvdhfr* PCR amplification, initial denaturation at 94°C for 10 min was followed by 35 cycles at 94°C for 30 s, 58°C for 45 s, 72°C for 1 min, and a final extension at 72°C for 10 min for nested-1 PCR. Using 1 μL of the nested-1 PCR product as a template, we applied the same conditions for the nested-2 PCR except for an annealing temperature of 62°C for 30 s and extension at 72°C for 1 min with 30 cycles of amplification.

We visualized the PCR products by 1% agarose gel electrophoresis and staining with 0.05% Redsafe dye (iNtRON Biotechnology). We purified the PCR products by using a MEGA quick-spin Total Fragment DNA Purification Kit (iNtRON Biotechnology) and sequenced them with primers from a commercial sequencing company (Genotech).

## Sequence Analysis and Statistics

We compared all the nucleotide and amino acid sequences with the reference sequence of K13 kelch protein (gene ID: PF3D7_1343700), *pfarps10* (PF3D7_1460900.1), *pffd* (PF3D7_1318100), *pfmdr2* (PF3D7_1447900), *pvcrt* (PVX_087980), *pvdhps* (PVX_123230), *pvdhfr* (PVX_089950), and *pvmdr1* (PVX_080100) from www.plasmodb.org and aligned them by using software in the Lasergene Genomic Suite (MegAlign, version 7.1). We used SPSS software (version 22.0, IBM SPSS Statistics, Armonk, NY, USA) for all statistical analysis. We compared the frequency of mutations and haplotypes of the target genes among the groups by using $\chi^2$ and Fisher exact tests with a two-sided confidence interval at the 95% confidence level. The sequences were deposited at GenBank (accession nos. KX000945–KX000959 and KX384672–KX384687).

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**Technical Appendix Table 1.** Pairs of primers used for amplification of the drug resistance markers for asymptomatic malaria, Myanmar, 2015

| Target gene | PCR | Primer | Primer sequences, 5′→3′ |
|---|---|---|---|
| K13 (PF3D7_1343700) | Nested 1 | K13_1R | CGG AGT GAC CAA ATC TGG GA |
| | | K13_3NF | AGC GGA AGT AGT AGC GAG AA |
| | Nested 2 | K13_2F | GCC AAG CTG CCA TTC ATT TG |
| | | K13_3R | GCC TTG TTG AAA GAA GCA GA |
| *pfarps10* (PF3D7_1460900.1) | Nested 1 | PfARPS_F3 | TGC GAC TTT TAG GGT GTG GA |
| | | PfARPS_N2R | CAT GGT ACC ACT TTT TCT TTT CCA |
| | Nested 2 | PfARPS_F1 | TTG TAG CAG GCC CAA TTC CC |
| | | PfARPS_N2R2 | TCT GGG TAA TTT GAC ATT CAT |
| *pffd* (PF3D7_1318100) | Nested 1 | PfFD_F1 | AGT TGT TCT ACA TGC GCA GC |
| | | PfFD_R1 | AAT GTG CGC TTG TAG TGC AT |
| | Nested 2 | PfFD_F2 | TGC GCA GCA AAA TTA GTC GAA |
| | | PfFD_R2 | CAT TCC CCA TTT CAA TCA TAT CCA |
| *pfmdr2* (PF3D7_1447900) | Nested 1 | Pfmdr2_F1 | TTT GTG GCC AAG CAA AAG GA |
| | | Pfmdr2_R1 | TCT TTG TCG TTC TCC TCC TGA |
| | Nested 2 | Pfmdr2_F8 | AGA GGT ACC GAG AGT GCT AA |
| | | Pfmdr2_R8 | AGA GCA CAT GTT GTA CCT GGT T |
| *pvcrt-o* (PVX_087980) | Nested 1 | Pvcrt_N2F | ACG GAA TCA ACC CGA ATC CA |
| | | Pvcg10_R | AGT TTC CCT CTA CAC CCG |
| | Nested 2 | Pvcrto_F | TCC TTG CCG CTG ATT CTA CG |
| | | Pvcrto_R | GGT AAC GTT CAT CGG GGG TT |
| *pvmdr1* (PVX_080100) | Nested 1 | Pvmdr1_F3 | GGA TAG TCA TGC CCC AGG ATT G |
| | | Pvmdr1_R1 | CTT ATA TAC GCC GTC CTG CAC |
| | Nested 2 | Pvmdr1_F3 | GGA TAG TCA TGC CCC AGG ATT G |
| | | Pvmdr1_R3 | CAT CAA CTT CCC GGC GTA GC |
| *pvdhps* (PVX_123230) | Nested 1 | Pvdhps_F1 | AGG AAG CCA TTC GCT CAA C |
| | | Pvdhps_B | GAG ATT ACC CTA AGG TTG ATG TAT C |
| | Nested 2 | Pvdhps_D | GGT TTA TTT GTC GAT CCT GTG |
| | | Pvdhps_B | GAG ATT ACC CTA AGG TTG ATG TAT C |
| *pvdhfr* (PVX_089950) | Nested 1 | Pvdhfr_F1 | ATG GAG GAC CTT TCA GAT GTA TT |
| | | Pvdhfr_N1R | CGG GTT TTT CTC CCC CAC TT |
| | Nested 2 | Pvdhfr_F1 | ATG GAG GAC CTT TCA GAT GTA TT |
| | | Pvdhfr_R1 | CCA CCT TGC TGT AAA CCA AAA AGT CCA GAG |

**Technical Appendix Table 2.** Characteristics of 28 patients with asymptomatic malaria infection, Myanmar, 2015

| Patient no. | ID | Village | Age, y | Sex | Occupation | PCR result | Microscopy result, parasites/µL |
|---|---|---|---|---|---|---|---|
| 1 | A954 | Let Pa Dan | 59 | F | Dependent | *P. vivax* | NS* |
| 2 | A878 | Let Pa Dan | 24 | F | Farmer | *P. vivax* | NS |
| 3 | A887 | Let Pa Dan | 23 | F | Farmer | *P. vivax* | NS |
| 4 | A894 | Let Pa Dan | 52 | M | Manual worker | *P. vivax* | NS |
| 5 | A903 | Let Pa Dan | 31 | F | Farmer | *P. vivax* | NS |
| 6 | A905 | Let Pa Dan | 19 | M | Student | *P. vivax* | NS |
| 7 | A910 | Let Pa Dan | 17 | M | Manual worker | *P. vivax* | NS |
| 8 | A808 | Maung Yone | 43 | M | Farmer | *P. falciparum* | NS |
| 9 | A799 | Maung Yone | 32 | M | Manual worker | *P. malariae* | NS |
| 10 | A850 | Maung Yone | 56 | M | Dependent | *P. malariae* | NS |
| 11 | A762 | Maung Yone | 28 | F | Farmer | *P. vivax* | NS |
| 12 | A857 | Maung Yone | 40 | M | Dependent | *P. vivax* | NS |
| 13 | A356 | Sate Ka Lay | 52 | M | Manual worker | *P. falciparum* | NS |
| 14 | A349 | Sate Ka Lay | 56 | F | Dependent | *P. vivax* | NS |
| 15 | A383 | Sate Ka Lay | 21 | M | Student | *P. vivax* | NS |
| 16 | A353 | Sate Ka Lay | 25 | M | Manual worker | *P. vivax* | 580 |
| 17 | A377 | Sate Ka Lay | 41 | M | Manual worker | *P. vivax* | NS |
| 18 | A409 | Tha-Yet-Chaung | 35 | M | Company staff | *P. vivax* | NS |
| 19 | A541 | Tha-Yet-Chaung | 26 | F | Farmer | *P. falciparum* | NS |
| 20 | A548 | Tha-Yet-Chaung | 29 | F | Farmer | *P. vivax* | NS |
| 21 | A624 | Tha-Yet-Chaung | 25 | M | Farmer | *P. vivax* | 1,200 |
| 22 | A661 | Tha-Yet-Chaung | 29 | M | Manual worker | *P. vivax* | NS |
| 23 | B1095 | Wae Gyi | 17 | M | Taxi cycle driver | *P. falciparum* | NS |
| 24 | A968 | Wae Gyi | 18 | M | Manual worker | *P. vivax* | NS |
| 25 | A031 | Win Ka Nane | 26 | F | Farmer | *P. vivax* | NS |
| 26 | A035 | Win Ka Nane | 9 | M | Student | *P. vivax* | NS |
| 27 | A107 | Win Ka Nane | 45 | M | Manual worker | *P. vivax* | NS |
| 28 | A032 | Win Ka Nane | 30 | M | Manual worker | *P. vivax* | NS |

*NS, not seen by microscopy.

**Technical Appendix Table 3.** Mutations in drug resistance molecular markers for 4 patients with asymptomatic *P. falciparum* malaria, Myanmar, 2015

| Patient no. | ID | K13 (kelch gene)* | *pfarps10* | *pffd* | *pfmdr2* |
|---|---|---|---|---|---|
| 1 | A356 | P574**L** | V127**M** | D193**Y** | T484**I** |
| 2 | A541 | C580**Y** | V127**M** | D193**Y** | T484**I** |
| 3 | A808 | Wild | Wild | D193**Y** | T484**I** |
| 4 | B1095 | Wild | Wild | Wild | T484**I** |

*Mutant amino acids are shown in bold. All of the sequences are aligned with 3D7 sequences retrieved from plasmodb.org.