Genetic surveillance in the Greater Mekong Subregion to support malaria control and elimination

Mapping genetic markers to resistance status classification

V1.0, May 2020

Change History

Version	Date	Notes
1.0	18 May 2020	Released with Jacob CG *et al.* (2020)

Introduction

In the accompanying data release, we have classified all samples into different types of drug resistance based on published genetic markers including SNPs and copy number variations (CNVs). The methods of classification are heuristic and represent a best attempt based on the available data. Our aim is to improve the accuracy of classification in future data releases, as new sources of evidence become available on the relationship between genotype and drug resistance phenotype.

Each type of resistance was considered to be either *present*, *absent* or *unknown* for a given sample. We have not attempted to make a quantitative assessment of the level of drug resistance, which may depend on complex genetic interactions that remain poorly understood.

This is more problematic for some types of resistance than others. For example, the *crt* 76T allele is a very reliable marker of chloroquine resistance, whereas there are various degrees of resistance to sulfadoxine-pyrimethamine (SP) that are determined by complex interactions between multiple mutations in the *dhfr* and *dhps* genes. Where appropriate, we used the simplest approach, e.g. the *dhfr* 108N allele appears sufficient for clinically significant treatment failure, and so parasites were classified as pyrimethamine resistant if this mutation was present irrespective of other *dhfr* and *dhps* alleles.

This document describes the heuristic utilized for each of the following drugs or combination of drugs:

- Artemisinin
- Piperaquine
- Dihydroartemisinin-piperaquine
- Chloroquine
- Pyrimethamine
- Sulfadoxine
- Sulfadoxine-Pyrimethamine (SP) when used for the treatment of uncomplicated cases
- SP when used for Intermittent Preventive Treatment in Pregnancy (IPTp)

For each drug, the rules have priorities and are applied sequentially according to the "Step" column with the first successful criterion determining the classification and stopping the procedure. For example, a sample is only considered to be "Undetermined" for artemisinin resistance if it has no mutation from the lists of mutations given, or else if it has no mutation but at least one missing genotype call between amino acids 349 and 726. If there is both a non-synonymous mutation associated with resistance and any missingness, the sample will be considered resistant because the rule concerning the non-synonymous mutation is triggered before the rule concerning missingness.

The inferred resistance status classifications for all samples can be found on the resource page at https://www.malariagen.net/resource/29.

Artemisinin

Info on the drug: https://en.wikipedia.org/wiki/Artemisinin

Locus utilized: PF3D7_1343700 (*kelch13*)

Codons: 349-726

Workflow:

Step	Genetic change	Interpretation	Classification
1	441L, 446I, 449A, 452E, 458Y, 469Y, 469F, 476I, 479I, 481V, 493H, 515K, 522C, 527L, 537I, 537D, 538V, 539T, 543T, 553L, 561H, 568G, 574L, 575K, 579I, 580Y, 584V, 667T, 673I, 675V or 719N as homozygous call	Mutant – associated with delayed clearance	Resistant
2	441L, 446I, 449A, 458Y, 469Y, 469F, 476I, 479I, 481V, 493H, 515K, 522C, 527L, 537I, 537D, 538V, 539T, 543T, 553L, 561H, 568G, 574L, 575K, 579I, 580Y, 584V, 667T, 673I, 675V or 719N as heterozygous call	Mutant - heterozygous	Undetermined
3	252Q, 578S as homozygous or heterozygous with wild type call	Mutant - not associated	Sensitive
4	Any missing call in amino acids 349-726	Missing	Undetermined
5	No non-reference calls in amino acids 349-726	Wild-type	Sensitive
6	otherwise	Mutant - not in WHO list	Undetermined

References:

- World Health Organization. Artemisinin and artemisinin-based combination therapy resistance: status report. (2018) https://apps.who.int/iris/handle/10665/274362.

Piperaquine

Info on the drug: https://en.wikipedia.org/wiki/Piperaquine

Loci utilized: PF3D7_1408000 (*plasmepsin 2*) and PF3D7_1408100 (*plasmepsin 3*)

Codons: Amplification status of the genes

Workflow:

Step	Genetic change	Interpretation	Classification
1	Missing	Missing	Undetermined
2	Heterozygous duplication	Heterozygous mutant	Undetermined
3	Single copy	Wild type	Sensitive
4	Multiple copies	Mutant	Resistant

References:

- Amato, R. *et al.* Genetic markers associated with dihydroartemisinin–piperaquine failure in Plasmodium falciparum malaria in Cambodia: a genotype–phenotype association study. *Lancet Infect. Dis.* **17**, 164–173 (2017)

Dihydroartemisinin-piperaquine

Info on the drugs combination: https://en.wikipedia.org/wiki/Piperaquine/dihydroartemisinin

Loci utilized: PF3D7_1343700 (*kelch13*), PF3D7_1408000 (*plasmepsin 2*) and PF3D7_1408100 (*plasmepsin 3*)

Codons: *kelch13*, codons 349-726; and amplification of PF3D7_1408000 (*plasmepsin 2*) and PF3D7_1408100 (*plasmepsin 3*)

Workflow:

Step	Genetic change	Interpretation	Classification
1	Lack of WHO K13 mutant or single copy of *pm2/3*	Wild type for at least one component	Sensitive
2	WHO K13 mutant and multiple copies of *pm2/3*	Mutant	Resistant
3	otherwise	Unknown	Undetermined

Notes:

- The classification here is determined by the classification from the Artemisinin and Piperaquine sections above
- Only one “Sensitive” classification from Artemisinin or Piperaquine classifications is needed to determine sample is “Sensitive”, even if other classification is “Resistant” or “Undetermined”

Chloroquine

Info on the drug: https://en.wikipedia.org/wiki/Chloroquine

Locus utilized: PF3D7_0709000 (*crt*)

Codon: 76

Workflow:

Step	Genetic change	Interpretation	Classification
1	76 K/T heterozygote	Heterozygous mutant	Undetermined
2	76 missing	Missing	Undetermined
3	K76	Wild type	Sensitive
4	76T	Mutant	Resistant
5	otherwise	Unknown mutant	Undetermined

References:

- Djimdé, A., Doumbo, O. K., Steketee, R. W. & Plowe, C. V. Application of a molecular marker for surveillance of chloroquine-resistant falciparum malaria. *Lancet* 358, 890–891 (2001).

Notes:

- There is evidence linking SNPs in the gene *mdr1* with some level of chloroquine resistance. However, their impact on clinical phenotypes and the interactions with *crt* mutations is not fully understood hence the decision to not use that in the classification at this stage.

Pyrimethamine

Info on the drug: https://en.wikipedia.org/wiki/Pyrimethamine

Locus utilized: PF3D7_0417200 (*dhfr*)

Codon: 108

Workflow:

Step	Genetic change	Interpretation	Classification
1	108 S/N heterozygote	Heterozygous mutant	Undetermined
2	108 missing	Missing	Undetermined
3	S108	Wild type	Sensitive
4	108N	Mutant	Resistant
5	otherwise	Unknown mutant	Undetermined

References:

- Cowman, A. F., Morry, M. J., Biggs, B. A., Cross, G. A. & Foote, S. J. Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of Plasmodium falciparum. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 9109–13 (1988)

Notes:

- The allele 108N appears to be necessary and sufficient for clinical resistance to pyrimethamine. However different set of mutations in the gene have been associated with various levels of resistance. To date the link between those mutations and the clinical outcome is not completely understood hence the decision to not consider those mutations in the classification.

Sulfadoxine

Info on the drug: https://en.wikipedia.org/wiki/Sulfadoxine

Locus utilized: PF3D7_0810800 (*dhps*)

Codon: 437

Workflow:

Step	Genetic change	Interpretation	Classification
1	437 A/G heterozygote	Heterozygous mutant	Undetermined
2	437 missing	Missing	Undetermined
3	A437	Wild type	Sensitive
4	437G	Mutant	Resistant
5	otherwise	Unknown mutant	Undetermined

References:

- Triglia, T., Wang, P., Sims, P. F. G., Hyde, J. E. & Cowman, A. F. Allelic exchange at the endogenous genomic locus in Plasmodium falciparum proves the role of dihydropteroate synthase in sulfadoxine-resistant malaria. *The EMBO Journal* **17**, (1998)

Notes:

- Although the 3D7 sequence translates as a G at 437, this is thought to be the mutant allele, i.e. 3D7 does not have the wild type allele.

Sulfadoxine-Pyrimethamine (uncomplicated malaria treatment)

Info on the drugs combination: https://en.wikipedia.org/wiki/Sulfadoxine/pyrimethamine

Locus utilized: PF3D7_0417200 (*dhfr*)

Codons: 51, 59 and 108

Workflow:

Step	Genetic change	Interpretation	Classification
1	N51 or C59 or S108	Not triple mutant (at least one allele is WT)	Sensitive
2	51I and 59R and 108N, all homozygous	Mutant	Resistant
3	otherwise	Missing or unknown combination	Undetermined

References:

- Nzila, A. M. *et al.* Towards an Understanding of the Mechanism of Pyrimethamine-Sulfadoxine Resistance in Plasmodium falciparum: Genotyping of Dihydrofolate Reductase and Dihydropteroate Synthase of Kenyan Parasites. **44**, (2000)

Notes:

- Also known as the “triple mutant” and associated with “partial resistance”
- Only need one wild-type allele to determine sample is “Sensitive”, even if other alleles are mutant or missing

Sulfadoxine-Pyrimethamine (IPTp)

Info on the drugs combination: https://en.wikipedia.org/wiki/Sulfadoxine/pyrimethamine

Locus utilized: PF3D7_0417200 (*dhfr*) and PF3D7_0810800 (*dhps*),

Codons: *dhfr*, codons 51, 59, 108 and 164 and *dhps*, codons 437, 540, 581 and 613

Workflow:

Step	Genetic change	Interpretation	Classification
1	*dhfr*: N51 or C59 or S108 or *dhps*: A437 or K540 or all of *dhfr*:I164 and *dhps*:A581 and *dhps*:A613	Not sextuple mutant (at least one allele is WT)	Sensitive
2	*dhfr*: 51I and 59R and 108N and *dhps*: 437G and 540E and one of *dhfr*:164L, *dhps*:581G, *dhps*:613S or *dhps*:613T with all mutants homozygous	Sextuple mutant	Resistant
3	otherwise	Missing or unknown combination	Undetermined

References:

- Naidoo, I. & Roper, C. Mapping 'partially resistant', 'fully resistant', and 'super resistant' malaria. *Trends Parasitol.* **29**, 505–515 (2013)

Notes:

- Also known as the "sextuple mutant" and associated with "super resistance"