Compendium of molecular markers for antimalarial drug resistance

Methods and thresholds (version 1.0, 8 December 2025)

Introduction

The *Compendium of molecular markers for antimalarial drug resistance* was developed to address the need for an evidence-based, up-to-date resource presenting genetic alterations associated with reduced susceptibility to antimalarial drugs.

The compendium summarizes and classifies molecular markers using evidence from three domains: **laboratory, clinical and genetic epidemiology**. These domains represent distinct but complementary lines of evidence that support the association between specific genetic alterations and drug resistance.

The approach builds on earlier classification efforts, including those applied to *PfKelch13* mutations and is designed to evolve as new evidence emerges. The compendium will be updated annually following a review of new evidence and, where applicable, revisions to molecular markers and methodologies.

Development process

The compendium was developed through a multi-step process:

1. **Development of predefined criteria and thresholds for evidence evaluation**. Thresholds were established to assess the quality and relevance of evidence within each domain. These were developed in consultation with independent experts convened by WHO. Heterogeneity in measurement and reporting across the published literature presented a significant challenge to standardization. The criteria were designed to balance scientific rigor with practical applicability across different sources of evidence and study designs of varying quality.
2. **Literature review**. Published laboratory, clinical, and genetic epidemiology data were reviewed and evaluated against these thresholds by a group of primary reviewers.
3. **Expert consultations**. Two rounds of expert review were conducted to refine and validate marker classifications, assess study quality, and consider contextual factors affecting interpretation. The experts also served as secondary reviewers for the initial assignment of markers.

Methods

To support a transparent and consistent evaluation, all genetic markers were classified according to predefined thresholds established for each of the three evidence domains: laboratory, clinical, and genetic epidemiology. Within each domain, different types of evidence were considered to assess causal relationships between genetic alterations and antimalarial drug resistance, as described in the section on Thresholds.

Markers that met the domain-specific thresholds were considered for inclusion in the compendium. Based on the combined assessment of evidence across domains, each genetic alteration was classified as a potential, candidate, or validated marker of antimalarial drug resistance (Figure 1 and Table 1).

Figure 1. Marker classification framework

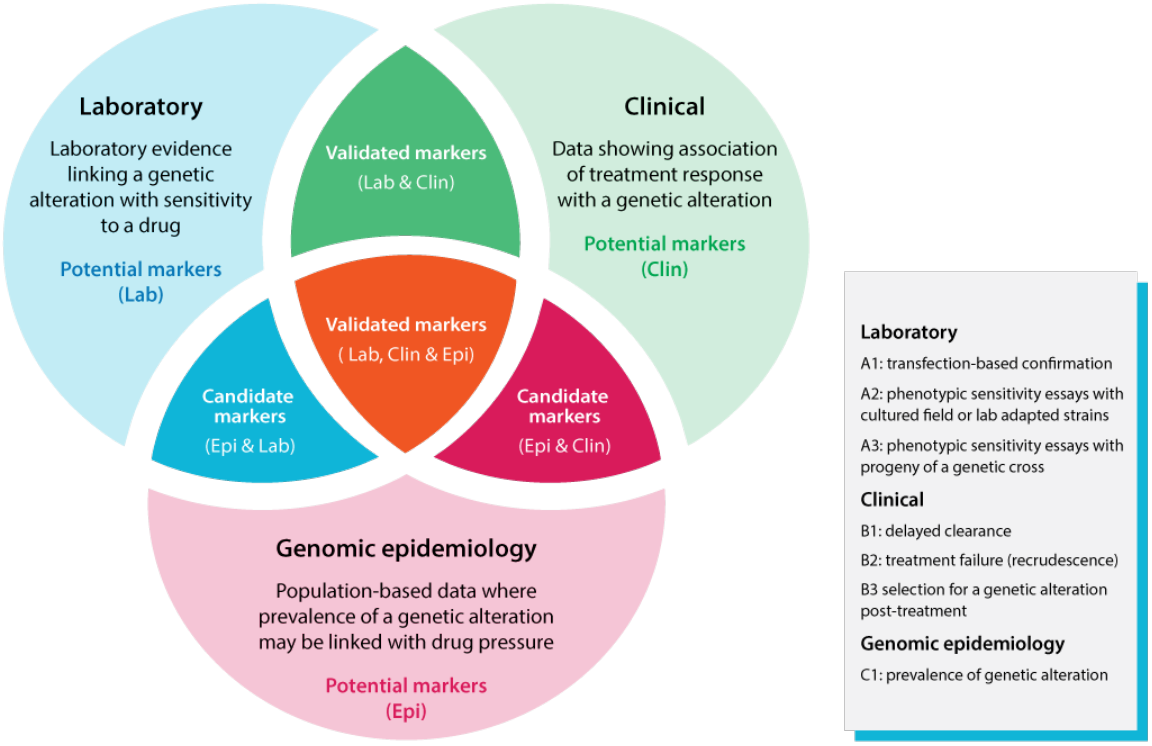

Table 1. Molecular marker classification levels based on combination of supporting evidence domains

Classification	Evidence domain(s) supporting association with drug resistance
Potential marker	Evidence from one domain only (Laboratory or Clinical or Genetic epidemiology)
Candidate marker	Evidence from two specific domains (Laboratory **and** Genetic epidemiology or Clinical **and** Genetic epidemiology)
Validated marker	Evidence from either two specific domains (Laboratory and Clinical) or from all three domains (Laboratory, Clinical, and Genetic epidemiology)

Thresholds

A. Laboratory evidence

Three types of evidence are considered in the assessment of laboratory data. These include studies demonstrating that a genetic alteration reduces parasite susceptibility to a drug *in vitro*, using transfected strains, field isolates or culture-adapted laboratory strains, or progeny derived from genetic crosses. Among these, data from transfected strains are regarded as stronger evidence, as they provide direct evidence of causality. Consequently, priority is given to studies using transfection and gene-editing techniques. When thresholds are met using these methods, additional *in vitro* data are not reviewed. In-vitro drug-pressure selection studies, where parasites acquire mutations following prolonged exposure to antimalarials, can provide supportive evidence of adaptive potential. While not proving direct causality, such findings may inform prioritisation of studies on candidate genes for further validation.

A1. Transfection-based confirmation

Threshold

- When comparing a culture-adapted, recombinant isogenic parasite line incorporating the genetic alteration – produced through transfection and gene-editing techniques – with a control isogenic line of the same strain, the threshold is met under the following conditions:
- *For markers associated with artemisinin partial resistance:* a significant difference ($p < 0.05$) in the Ring-Stage Assay ($RSA_{0–3h}$),[1] with a minimum survival of >1% in the gene-edited line compared to the control isogenic line (same strain).
- *For markers associated with piperaquine (PPQ) resistance:* a significant difference ($p < 0.05$) in the Piperaquine Survival Assay (PSA),[2] with ≥10% survival at 200 nM PPQ in the gene-edited line compared to the wild-type isogenic line.
- *For markers associated with resistance to other drugs:*
    - *For single point mutations, or alleles where multiple mutations are introduced*: a statistically significant ($p<0.05$) increase in half-maximal inhibitory concentration (IC_{50}) or 90% inhibitory concentration (IC_{90})[3] in the gene-edited line compared with the wild-type isogenic line.
    - *For copy number variations where transfection is used to overexpress the gene:* a statistically significant ($p<0.05$) increase in IC_{50} or IC_{90} in the gene-edited line compared with the wild-type isogenic line.
- For *P. vivax* (and rarely for *P. falciparum*) where transfection and expression of wild-type and mutant alleles are carried out in other Plasmodium species or in bacterial/yeast strains: a statistically significant ($p<0.05$) increase in IC_{50}, IC_{90}, effective dose for 50% inhibition (ED_{50}), effective dose for 90% inhibition (ED_{90}), or inhibition constant (K_i) in the mutant allele compared with the wild type.[4]

Requirements and reasons to disregard evidence or study

Not meeting evidence requirements:

- Not statistically significant ($p>0.05$) increase in phenotypic measurement (IC_{50}, IC_{90}, RSA or PSA).

Additional reasons to disregard the study:

- Lack of parental or isogenic controls.

[1] For details see Witkowski et al. Reduced artemisinin susceptibility of Plasmodium falciparum ring stages in western Cambodia. Antimicrob Agents Chemother. 2013 Feb;57(2):914-23. doi: 10.1128/AAC.01868-12.

[2] For details see Duru et al. Plasmodium falciparum dihydroartemisinin-piperaquine failures in Cambodia are associated with mutant K13 parasites presenting high survival rates in novel piperaquine in vitro assays: retrospective and prospective investigations. BMC Med. 2015 Dec 22;13:305. doi: 10.1186/s12916-015-0539-5.

[3] For details see page 19 of Report on antimalarial drug efficacy, resistance and response: 10 years of surveillance (2010-2019). Geneva: World Health Organization; 2020 (https://apps.who.int/iris/handle/10665/336692).

[4] See for instance: Cortese JF, Plowe CV. Antifolate resistance due to new and known Plasmodium falciparum dihydrofolate reductase mutations expressed in yeast. Mol Biochem Parasitol. 1998 Aug 1;94(2):205-14. doi: 10.1016/s0166-6851(98)00075-9.

A2. Phenotypic sensitivity assays with field isolates or culture-adapted laboratory strains (if there is no evidence from transfection studies)

Threshold

For in-vitro activity studies of field isolates or culture-adapted laboratory strains, the threshold is met under the following conditions:

- *For markers associated with artemisinin partial resistance:* >1% survival using the $RSA_{0–3h}$ (or >2 standard deviations above the mean value for wild-type parasites from the same area) in at least five individual isolates or strains with a given genetic alteration.
- *For markers associated with piperaquine resistance:* in the PSA, ≥10% survival at 200 nM PPQ (or >2 standard deviations above the mean value for wild-type parasites from the same area) in at least five individual isolates or strains with a given genetic alteration.
- *For markers associated with resistance to other drugs:*
    - a statistically significant increase in IC_{50} or IC_{90} in at least five individual isolates or strains with a given genetic alteration compared to the mean value for wild-type parasites from the same area.

A3. Phenotypic sensitivity assays with progeny of a genetic cross (if there is no evidence from transfection studies)

For in vitro activity studies of the progeny of a genetic cross, the threshold is met under the following condition:

- A statistically significant increase in the RSA or PSA, or IC_{50} or IC_{90} values (see above), in at least five recombinant progeny parasite clones expressing the genetic alteration of interest, compared with recombinant progeny expressing the wild-type allele or copy number variant. This applies to genetic loci that, through genetic mapping (such as quantitative-trait-loci analysis), have been associated with a phenotypic shift in susceptibility.

Requirements and reasons to disregard evidence or study

Not meeting the evidence requirements:

- Not a statistically significant ($p>0.05$) increase in phenotypic measurement (IC_{50}, IC_{90}, RSA or PSA).
- Fewer than five recombinant progeny parasite clones tested for antimalarial drug in vitro activity.

Additional reasons to disregard the study:

- No statistically significant difference in susceptibility between the parental lines used in the genetic cross.

B. Clinical data

Three types of evidence are considered in the evaluation of clinical data: delayed parasite clearance in patients, treatment failure (recrudescence), and selection of genetic variants post-treatment. Interpreting treatment response in relation to artemisinin-based combination therapy (ACTs) is particularly complex in this context, as it requires understanding the distinct roles, pharmacokinetics, and pharmacodynamics of each component of the ACTs.

When treating with ACTs, reduced sensitivity to artemisinin typically manifests as delayed parasite clearance, whereas resistance to the partner drug is often associated with treatment failure.

Establishing a causal link between a genetic alteration and treatment response is challenging, especially in studies using pooled data, where differences in parasite genetic backgrounds and other confounding factors can obscure the contribution of specific mutations.

B1. Delayed clearance

Delayed clearance is a measure of artemisinin partial resistance and is only considered for artemisinin-based antimalarial medicines.

Threshold

A statistically significant association ($p < 0.05$) between the presence of a mutation and delayed parasite clearance, defined as a parasite clearance slope half-life of $\geqslant$5 hours or the presence of parasitaemia at 72 ($\pm$ 2) hours in a minimum of 20 clinical cases.[5]

Requirements and reasons to disregard evidence or study

Not meeting the evidence requirements:

- No statistically significant association ($p>0.05$) between the presence of a mutation and delayed parasite clearance.

Additional reasons to disregard the study:

- Not all treatment doses were given under direct observation.

B2. Treatment failure (recrudescence)

Treatment failures are considered for all antimalarial medicines, including artemisinin-based combination therapies.

Threshold

Treatment failures[6] statistically associated with the presence of a genetic alteration at the start of treatment. PCR correction to distinguish recrudescence from reinfection in *P. falciparum* should be conducted according to WHO guidance[7] or using other advanced genotyping methods. For *P. vivax* and *P. ovale*, any parasite recurrence within 28 days post-treatment is considered a treatment failure, due to difficulties in distinguishing recrudescences from new infections and relapses.

Requirements and reasons to disregard evidence or study

Not meeting evidence requirements:

- No statistically significant association ($p>0.05$) between the presence of a mutation and treatment failures

Additional reasons to disregard the study:

- Major deviation from WHO guidance in PCR correction to distinguish recrudescence from reinfection
- Major methodological deviations from WHO TES protocol

[5] Threshold for artemisinin resistance is currently under review. If changed, any impact will be addressed in the annual review of the compendium.

[6] For the definition of side 7 see Methods for surveillance of antimalarial drug efficacy. Geneva: World Health Organization; 2009 (https://apps.who.int/iris/handle/10665/44048).

[7] For details see Informal consultation on methodology to distinguish reinfection from recrudescence in high malaria transmission areas: report of a virtual meeting, 17–18 May 2021. Geneva: World Health Organization; 2021 (https://iris.who.int/handle/10665/348385).

- Not all treatment doses given under direct observation

B3. Evidence of selection for genetic alterations acquired post-treatment

Selection of genetic alteration acquired post-treatment is considered for all antimalarial medicines.

Threshold

A statistically significant ($p < 0.05$) increase in the proportion of the genetic alteration detected in parasites post-treatment compared with baseline (pre-treatment) proportion in at least two separate cohorts of patients.

Requirements and reasons to disregard evidence or study

Not meeting the evidence requirements:

- No statistically significant association ($p > 0.05$) between increase in genetic alterations in parasites post-treatment compared with baseline (pre-treatment), or findings limited to a single patient cohort.

Additional reasons to disregard the study :

- None

C. Genetic epidemiology

A genetic alteration that confers reduced susceptibility to a drug may gain a selective advantage under drug pressure, leading to its increased prevalence in the population. However, assigning causality to a specific genetic alteration that is spreading can be complex. The likelihood that a genetic change will be selected for is influenced by several factors, including the fitness cost of the alteration (which can vary depending on the parasite's genetic background), the mixture of antimalarial drugs used in a region, and the intensity of malaria transmission in the area. **This measure is only relevant for antimalarial medicines that are widely used in the population, across both public and private sectors.**

In some cases, a genetic alteration may spread not due to its own selective advantage, but because it is linked to another selected mutation. The extent to which these changes translate into reduced drug efficacy varies by population and region, with stronger correlations typically observed in non-immune populations. Nevertheless, once a mutation reaches a prevalence of ≥5%, it is considered to have the potential to spread further and may warrant closer monitoring. Only prevalence of genetic alterations is considered in the evaluation of genetic epidemiological data.

C1. Prevalence of genetic alteration

Selection of genetic alterations at population level is considered for antimalarial medicines that are or have been widely used.

Threshold

- **For markers already supported by clinical and/or laboratory evidence:** the genetic alteration has a prevalence $\geqslant$ 5% at a study site, based on a single study or survey. The study's sample size and the prevalence must be sufficiently large to minimize the likelihood of spurious or chance findings.
- **For other markers not supported by clinical and/or laboratory data:** the genetic alteration must have a prevalence $\geqslant$ 5% at a study site, based on a single study or survey, and must be associated with a specific drug pressure. This association must be demonstrated in a study showing either:
    - A statistically significant ($p < 0.05$) increase in mutation prevalence over time following the introduction of the drug; or

- A statistically significant ($p < 0.05$) higher mutation prevalence in geographical areas where the specific drug is in use compared to other areas where the drug is not in use.

Requirements and reasons to disregard evidence or study

Not meeting the evidence requirements:

- Studies or surveys that do not include enough samples to detect meaningful signals. In studies or surveys with ≥70 samples, a prevalence of at least 5 % serves as a practical benchmark for reliable detection.

Additional reasons to disregard study or evidence:

- Prevalence studies on markers not supported by clinical and/or laboratory data that do not include appropriate historical or geographical comparison groups without drug exposure, or that fail to account for major confounding factors influencing allele frequency.

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Compendium of molecular markers for antimalarial drug resistance

The Compendium of molecular markers for antimalarial drug resistance provides a consolidated and up-to-date source of knowledge on genetic alterations associated with antimalarial drug resistance.

For more information on the compendium, please visit: https://www.who.int/tools/compendium-of-molecular-markers-for-antimalarial-drug-resistance