Genetic Epidemiology Use Cases for Malaria Control Programmes: A Methodology

V1.2; 18 January 2023

Olivo Miotto
Principal Investigator,
Genetic Reconnaissance in the Greater Mekong Subregion (GenRe-Mekong)

TABLE OF CONTENTS

Introduction

Executive Summary

Recent advancements in sequencing and genotyping technologies have made it possible to obtain comprehensive genomic data from large numbers of individuals, thus enabling the development of the field of **Genetic Epidemiology**: the study of genetic variation in populations. This has major impact in the control of infectious diseases, where evolutionary forces cause rapid changes in pathogen populations, which can now be monitored and studied through the use of high-throughput methods such as next-generation sequencing (NGS). In malaria, this field has advanced rapidly in the last decade, during which it has become a tool for understanding, monitoring and controlling the emergence in the parasite population of antimalarial drug resistance, and in particular artemisinin resistance, a global threat to malaria control and elimination efforts. Such advances have been particularly beneficial in the Greater Mekong Subregion (GMS) where markers of artemisinin and piperaquine resistance have been discovered, and the dynamics of evolution and spread have been revealed, chiefly through the use of high-throughput genetic epidemiology.

The decreasing costs of high-throughput technologies, their wider availability, and advances in the laboratory techniques, have supported a continuous increase in the volume of processed samples: the MalariaGEN P. falciparum Community Project published its first analysis of 212 parasite genomes in 2012;[1] ten years later, version 7 of this data resource contains over 20,000 samples.[2] Such scaling-up has encouraged denser and more frequent sampling of the parasite across endemic regions, leading to **Genetic Surveillance** projects able to estimate epidemiological parameters, such as the prevalence of drug resistance, with increasingly high spatial and temporal resolution. The construction of surveillance datasets makes it possible to monitor changes in prevalence, track gene flow and the spread of strains, and study epidemiological phenomena such as outbreaks.

These new information streams have great potential to provide strategic knowledge about the evolution of parasite populations, and inform interventions aimed at elimination. In particular, they derive their value from a study of parasite data, which is considerably easier and cheaper to collect than accurate clinical data from human patients (e.g. detecting a drug resistance mutation is much simpler that monitoring a patient for several days to investigate the efficacy of the drug). However, if this new information is to have a high impact, it must be used by decision-makers in the public health bodies that coordinate malaria control and elimination operations. These bodies are typically Health Ministry departments, which we refer to as **National Malaria Control Programmes (NMCPs)**, although department names may vary from country to country. NMCP staff are typically trained in public health, but are rarely experts in genetics, by the very nature of their job. It is therefore paramount that genetic data from surveillance and genetic epidemiology be translated to information that is relevant to their decision-making domain (e.g. estimating of the risk of an antimalarial drug failure is more informative to NMCPs than the presence of a mutation in a sample). In addition, NMCP officers will rarely be familiar with the sort of phenomena and patterns that genetic epidemiologists find informative, and findings must be interpreted if they are to be actionable. At the same time, most genetic epidemiologists have limited understanding of NMCP activities and requirements, making it difficult to make their messages impactful and usable.

The present document aims at helping to bridge these gaps, and encouraging the translation of genetic data into public health information, to support malaria elimination. We describe a framework in which public health activities and genetic epidemiology activities are separately identified, formally described, and connected to each other by the translational activities that must take place to add value to the genetic data. The framework is produced and maintained by the GenRe-Mekong project, and leverages on the project's experience working with several NMCPs in the GMS, providing genetic

data from large-scale genetic surveillance at public health facilities.[3] We built on an earlier catalogue of genetic epidemiology use cases relevant to malaria elimination,[4] which we have reorganized, re-elaborated and extended, providing implementation details describing the technologies, methods and techniques, as well as the sampling strategies and outputs that may be useful to the programmes. Crucially, we treated these **Genetic Epidemiology Use Cases** as only one half of the framework, since they do not by themselves address the knowledge gap between genetic epidemiology and public health decision making. To address this, we produced a catalogue of **Programme Use Cases**, which capture programme activities within which the outputs of Genetic Epidemiology Use Cases can provide value. Within the Programme Use Cases, we strive to identify what decisions are made; what information will influence the decisions; which Genetic Epidemiology Use Cases can produce such information; and what form the outputs produced must take, in order to be meaningful and actionable by the programme officers.

The conceptual methodology presented will inform researchers designing epidemiological studies and genetic surveillance; assessors of project proposals; and Control Programmes aiming to understand how technology contributes to their control and elimination activities. We hope that, by bringing together scientific analyses with public health activities, we will stimulate the growth of large-scale open repositories of cross-border epidemiological data, which will benefit future elimination efforts.

Acknowledgements

We thank all the participants of the 2018 GenRe-Mekong meeting on Genetic Surveillance Use Cases, who helped shape this document with their ideas and experiences. We are grateful to Kim Johnson, Varanya Wasakul and Tess Verschuuren for thoroughly reviewing V1.2 of this document, and helping with the editing. Also, many thanks to Vikki Simpson and Dominic Kwiatkowski for many useful discussions, and for their encouragement.

PART I - Use Case Methodology

Background

Overview

This report documents a methodology for the deployment of genetic epidemiology for the purpose of informing malaria control and elimination programmes in the GMS. This methodology comprises the components that shape the production and delivery of knowledge derived from field samples (parasitized blood samples from patients). In this methodology, we have identified core **Genetic Epidemiology Use Cases** which use Analysis Methods to process genotype data from samples collected in the field, as well as data aggregated from open access Data Repositories. The Sampling Strategies employed in obtaining these samples, and the Genotyping Technologies used to produce analyzable genotype data, determine which methods can be used and what data can be produced. The resulting knowledge is then translated to an appropriate form before being transferred to **Programme Use Cases**, which are activities leading to decision-making on malaria control and elimination interventions.

Framework Objectives

1. Identify, catalogue and detail the Genetic Epidemiology Use Cases, providing implementation details, reviewing technology options and listing analytical methods used.
2. Review genotyping technologies used, their limitations and applications, and how they are applied in different Genetic Epidemiology Use Cases.
3. Describe the different sample types and sampling strategies and how they affect the analysis methods used in different Epidemiology Use Cases.
4. Identify, catalogue and detail the Programme Use Cases, and describe how they relate to the Genetic Epidemiology Use Cases and how knowledge is exchanged.

Non-objectives and Limitations

- Non-objective: to be a complete and exhaustive catalogue of technologies and techniques.
- Limited to *Plasmodium falciparum* (may be extended to cover *P. vivax* at a later stage).
- Limited to DNA-based genetic technologies (not covering transcriptomics, serology, etc.)
- Limited to approaches that yield concrete results presently, or within the timeframe of the WHO GMS elimination strategy.[5]
- Limited to approaches that can produce information deemed to be useful to Control Programmes presently (i.e. not covering research that does not appear to have direct immediate application).
- Because of the focus on the GMS experience, some use cases may be more relevant to mid- to low-transmission intensity endemic areas.

Relationship to previous work

This methodology builds on a report[4] produced by the University of Washington's Global Health Strategic Analysis and Research Training Program (START), and commissioned by the Bill & Melinda Gates Foundation. The START report defined seven use cases for applying molecular epidemiology methods for use in malaria elimination settings. The Genetic Epidemiology Use Cases in that report were reviewed, described and extended, based on the GenRe-Mekong experience. In the present framework, we added to the use cases descriptions of the technologies, methods and techniques, sampling strategies, and outputs. We have also identified a set of Programme Use Cases that make use of the data produced by the Genetic Epidemiology Use Cases; the relationship between the two

sets of use cases is critical to maximizing information flow and the impact of scientific evidence on the public health decision-making process.

Part of the Genotyping Technologies section from a previous version of this document has been included in a publication.[6] It has since been updated and modified.

Methodology components

We describe a methodology for the deployment of genetic epidemiology for the purpose of informing malaria control and elimination programmes. Figure 1 shows the components of this methodology, and how they interact to form strategies for transforming field samples into knowledge to inform decision-making.

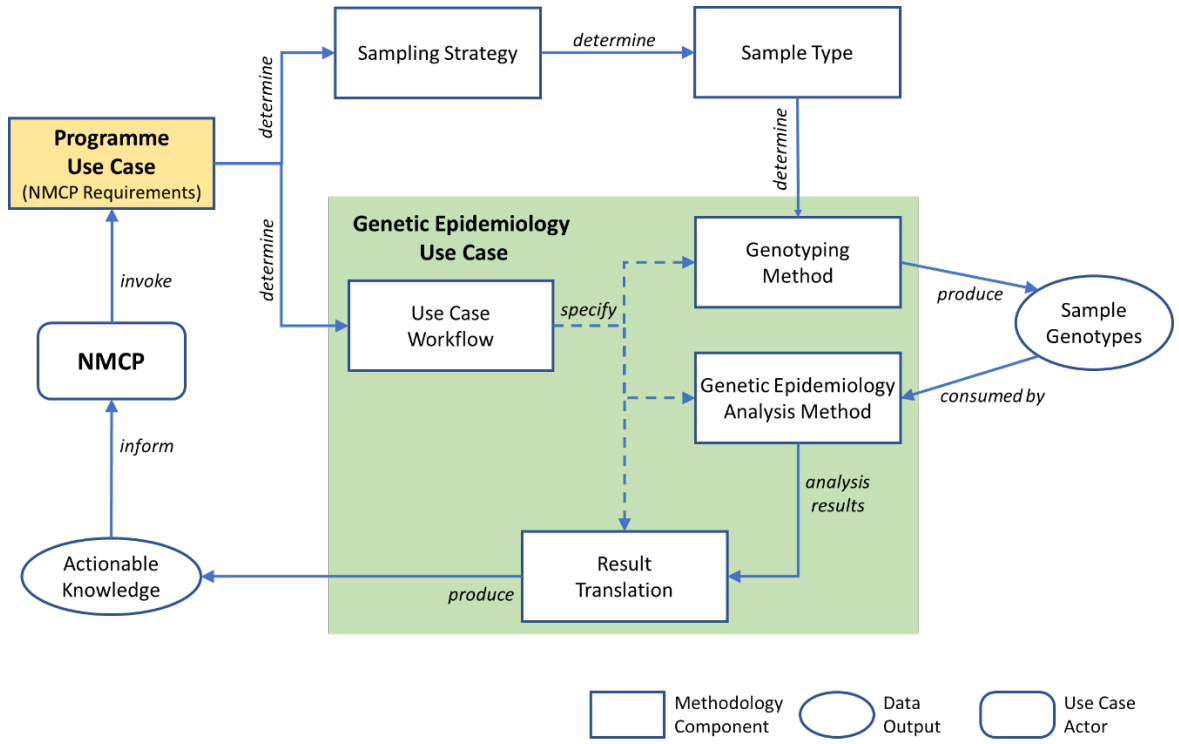

Figure 1 - Methodology components and their relationships. The two types of use cases (Programme Use Cases and Genetic Epidemiology Use Cases) are shaded in yellow and green respectively.

The methodology components are:

- **Programme Use Cases**: public health activities involving decision-making by programme officers, which are informed by Actionable Knowledge provided by Genetic Epidemiology Use Cases.
- **Sampling strategies** and **sample types**: parameters controlling the number, frequency, provenance and nature of the samples from which the analyzed genotypes are derived.
- **Genotyping Methods**: technologies that support genotype data production from field sample, and determine the resolution and coverage of the data to be analyzed.
- **Analysis Methods**: analytical and computational techniques employed by the Genetic Epidemiology Use Cases.
- **Result Translations**: activities aimed at converting results from analysis methods to outputs that can be used by NMCPs- may include reports, maps, visualizations, etc.

- **Genetic Epidemiology Use Cases**: activities that use genotypes from field samples to produce knowledge aimed to inform control and elimination interventions. They consist of a workflow that specified Genotyping Methods, Analysis Methods and Result Translations methods.

Use Cases

Concepts

The term "**use case**" originates from the field of software engineering, where it is used to specify systems with complex functionality. Ivar Jacobson and colleagues devised use cases as a way to partition this complexity into smaller standalone "stories" that could be implemented separately, but taken together they would form the whole functionality.[7] Use cases have been successfully used to specify and design software for over two decades. In the present framework the term "use case" is used as an analogy, to describe a usage scenario (in the activities of NMCPs or of genetic epidemiologists) that can be isolated and fully specified. Similarly, we use the word "system" as an analogy to mean a set of genetic epidemiology and surveillance capabilities provided by one or more operators, who apply analytical techniques to genetic data.

Programme Use Cases and Genetic Epidemiology Use Cases

In their original definition, use cases require an Actor, who initiates the use case, provides inputs and receives outputs. In other words, the Actor is a user without which the software system has no reason to exist. Analogously, we identify Programmes (a shorthand for NMCP officers) as the Actors of the use cases in this document: they ask questions, review evidence and take decisions as a result.

If we define use cases as in terms of genetic epidemiology analyses, it is clear that they are not the right use cases for Programme actors, because the Programmes' activities and decisions are not formulated in genetic epidemiology terms. For example, a Programme would not ask: "What is the frequency of the *pfdhfr* 164 alleles in province A?" as a genetic epidemiologist would; rather, the Programme's question might be: "Will the frontline antimalarial be efficacious in province A next season?" The two questions are related, but:

a. the answer to the first question has to be translated into an answer to the second question;
b. the resulting knowledge has to be incorporated in a decision-making process related to the Programme's question, which is not part of the genetic epidemiology workflow; and
c. in most cases, Programmes cannot wait for fresh sample collections and genetic analyses to be conducted before their question is answered; therefore, the genetic epidemiology analyses need to be executed asynchronously from the Programme activities.

To model this dichotomy, we define two sets of Use Cases:

Programme Use Cases are initiated by Programmes (the actor), and capture public health activities leading to decision-making.
Decisions are informed by evidence provided by genetic epidemiology, which needs to be translated appropriately.

Genetic Epidemiology Use Cases are not directly initiated by a Programme actor, and are therefore *abstract* use cases. They perform genetic epidemiology analyses and provide evidence to be collected and stored in data repositories for access by Programme Use Cases.

Note that, since Genetic Epidemiology Use Cases cannot be invoked synchronously, they must execute independently; to use one more software analogy, they are similar to software services running continuously in the background. As a consequence, the methodology presented here clearly

works best when there is continual pre-planned generation of data, through organized execution of the Genetic Epidemiology Use Cases. This suggests that these use cases are best used as a tool for planning genetic surveillance as a set of analytical tasks.

Overview of Use Cases

Figure 2 shows an overview of the Use Cases identified, and their relationships. These use cases are detailed in later sections of this report.

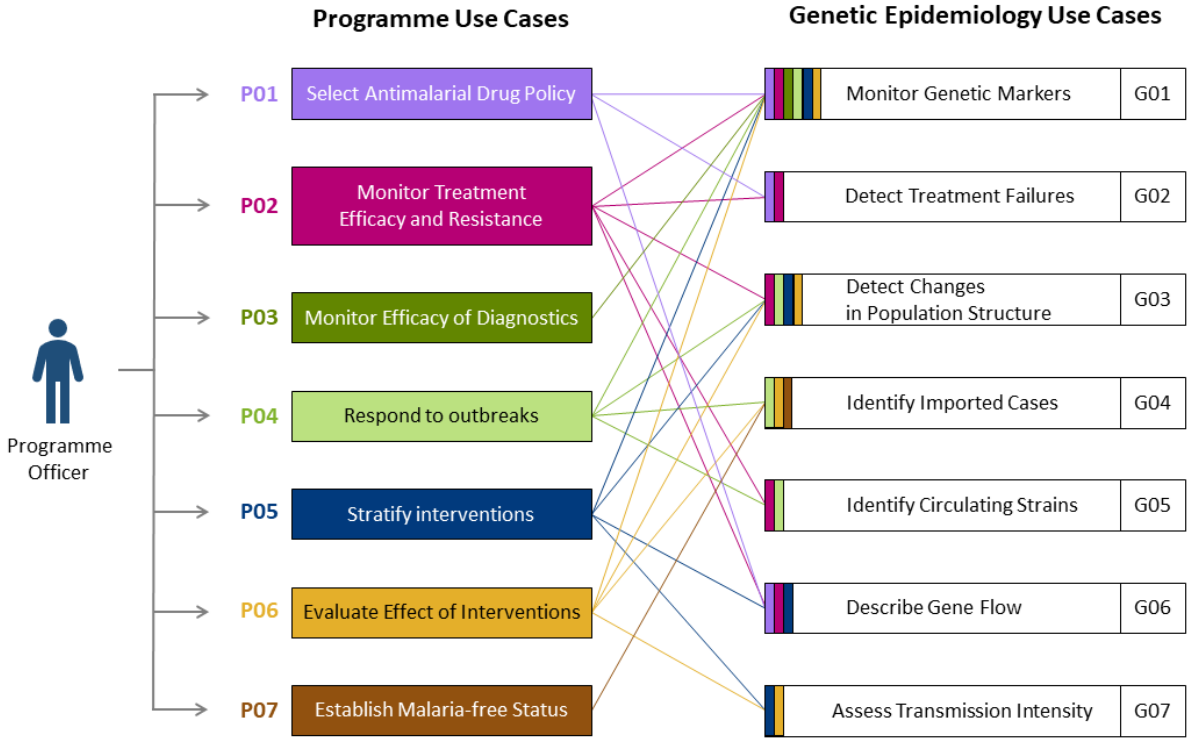

Figure 2 - Overview of the identified use cases and their relationships

Sample Types

This methodology focuses on *P. falciparum* parasites, and depends on the analysis of genotypes from parasite DNA. Therefore, the key source component is parasite DNA isolated from the blood of infected patients, since *Plasmodium* parasites are mostly found in red blood cells. Depending on the type of analysis, the DNA may need to have certain characteristics:

- Since all analyses consume a quantity of DNA, the sample must contain sufficiently abundant DNA to perform all the analyses. The minimum quantity depends on the type of analysis.
- Since the sample will be inevitably contaminated with the patient's (human) DNA, the level of contamination may need to be minimized for many applications.

Various techniques of parasite DNA amplification and purification are available that optimize the utility of available blood samples. The genotyping technologies mentioned in this section are described in detail in the section "Genotyping Technologies" later in this document.

Venous blood samples

The most versatile sample type for genetic analysis is blood from venous draws, which is typically collected in relatively large volumes (~200-5000 µL). Venous blood requires the intervention of

qualified medical staff, and refrigeration from the time of collection through to utilization. The typical quantities of parasite DNA extracted from this sample type can enable all genotyping techniques described in this document. Indeed, such high volumes of blood may allow the genotyping of very low parasitemias, when additional techniques are applied.

If venous blood samples are utilized for whole-genome sequencing (WGS), contamination of human DNA must be kept low, to maximize data output from parasite DNA. This can be done by filtering leukocytes by means of a mechanical filter, such as CF11 or Plasmodipur,[8] retaining only red blood cells. Unfortunately, this is a laborious task, and many laboratories are reluctant to undertake this type of processing.

A different, newer strategy is to amplify the parasite DNA using a *selective whole-genome amplification* (sWGA) technique, which leaves human DNA unaffected but amplifies the parasite DNA, enriching its proportion in the extracted DNA.[9] This technique does not require filtering at the time of collection, and can be applied as a standard step following DNA extraction.

Note that primer-based techniques, such as polymerase chain reaction (PCR) genotyping, quantitative polymerase chain reaction (qPCR), capillary sequencing and even amplicon sequencing do not require sWGA to be performed, as the PCR amplification use primers specific to *P. falciparum*, and will work in the presence of contaminating human DNA.

Dried blood spots (DBS)

Small blood samples can be collected in the form of dried blood spots (DBS) on filter paper. These consist of a small volume of peripheral blood (typically 20-60 µL) usually drawn from a small finger prick; the draw can safely be conducted by non-medical staff, such as village malaria workers. The blood is deposited on a piece of filter paper, to form a number of spots (typically 2-3); the filter paper is subsequently air-dried and can be stored in a dry container at room temperature. The transport and logistics of DBS samples are straightforward, not requiring cold chains.

The relatively small quantities of parasite DNA extracted from such a sample means that only relatively simple PCR-based tests have a high probability of success. However, the application of sWGA yields sufficient parasite DNA to enable most DBS samples to be used in all genotyping methods, including WGS.

Spent Rapid Diagnostic Tests (RDT)

In cases where malaria is confirmed by means of a Rapid Diagnostic Test (RDT), the patient's finger is pricked to collect a small quantity of blood which is then processed by the RDT device. Removing the test strip from the spent RDT device in order to extract parasite DNA is an attractive idea, since it would simplify the sample collection Standard Operating Procedures (SOPs), and spare the patient from a second finger prick to collect a DBS sample. This extraction method has been successfully attempted.[10] Unfortunately, it presents a number of difficulties: the strip is contaminated with diagnostic reagents; the quantity of blood is very small; the blood is distributed across the strip, rather than concentrated in a spot; the RDT devices are not designed for easy removal of the strip. Even with sWGA, RDT strips are less reliable parasite DNA sources than DBSs.

Further research work is needed. Redesigns of RDT devices, to enable separation and collection of red blood cells, and easy processing of the content of the RDT device, may help leverage on these ubiquitous items for genetic epidemiology in the future.

Sampling Strategies

Sampling Sites

A key decision to be made is selecting the sites where sampling will take place. In a framework where samples are collected in collaboration with Control Programmes, Programme officers will most likely drive the selection of sites. While researchers may be keen on selecting sites where certain epidemiological phenomena are most likely to be observed, in practice the choice of sites will usually be determined by more pragmatic parameters. A site should be sufficiently equipped to collect the types of samples needed. As a rule of thumb, DBS can be collected at most public health sites, such as district health centres, as long as the personnel has been adequately trained. Venous blood samples, on the other hand, are best collected in hospitals where refrigeration, centrifuges and medical staff are available. In addition, a site should ideally be adequately connected by road, to facilitate the supply chain of collection material and the collection and routing of samples.

Clearly, dense geographical sampling may produce the best maps of malaria epidemiology, but each additional site adds to the complexity and cost of collection. It is important that site selection is reviewed realistically against the epidemiological questions that are to be answered.

Sampling Activities

Ideally, one would like to sample all clinically relevant cases, but this has to be balanced against cost and available funding. In mid- or low-transmission countries, relatively low prevalence of infections may allow such broad collections. For example, sample collections at public health facilities in the GMS routinely yield a few thousand/hundreds samples a year in each country, which creates a useful dataset and can realistically be managed with the funding available to GenRe-Mekong, given the relatively low cost of collecting DBSs sample (as opposed to venous blood samples).

In high-transmission settings, however, high numbers of cases may demand a more sophisticated sampling strategy, and require some limits to be imposed on sample numbers. Generally sample sizes will have to be determined according to the use that is made of the samples, using typical statistical calculations used by clinical trials. For example, to estimate allele frequencies of genetic markers, a surveillance project would need to determine an acceptable confidence interval, and calculate the number of samples required to achieve such confidence.

Sampling Metadata

Most genetic epidemiology use cases described herein require minimal accompanying metadata; usually, only the date and geographical location of collection are required (the geographical location may need to be structured, e.g. province, district, etc., to allow meaningful sample aggregation). In general, clinical data are not required, except for specific use cases:

- Use Case G02 “Detect Treatment Failure” requires multiple samples from the same patient to be linked, so that recurrences can be analyzed to estimate treatment failure rates.

Sampling Frequency

The sampling period and frequencies must be determined according to the questions being investigated. For example, prevalence changes can only be detected if multiple consecutive collections are performed, and seasonal patterns can only be observed if sampling takes place all year round. Continual, routine collections are the most informative, but clearly this must be weighed against budgeting constraints.

Genotyping Technologies

Many phenotypic traits (such as resistance to an antimalarial drug) are controlled by biological mechanisms involving proteins, which are produced by translating gene sequences. Genetic variants introduce changes in these gene sequences, which produce changes in the proteins and consequently changes in phenotype. Therefore, we are often interested in monitoring the presence of mutations that we know to be associated to particular phenotypes. In addition, we may be interested in creating a parasite's "genetic profile" by detecting the presence or absence of multiple mutations, so that parasites can be compared to each other.

Genotyping is performed to determine the state (or *allele*) of a genetic variant- a feature of the genome that varies between individuals. Most often, one is interested in variants that affect the structure or the abundance of a protein. Variants are typically investigated by comparing the tested DNA sequence to that of a reference strain, which may be considered a *wild-type* strain; alleles diverging from the reference are considered *mutations*. In malaria parasites, we are generally interested in three main types of variants: *single nucleotide polymorphisms* (SNP), which are changes of a single base in the DNA sequence; *short indels*, where short DNA sequences are added to or removed from a gene; and *copy number variations* (CNV), where additional copies of one or more genes are found in an individual, causing higher expression of a protein.

Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) is one of the simplest methods to detect SNP alleles. It involves fragmenting a sample of DNA by restriction enzymes, which recognize and cut DNA at specific short sequences (digestion). By choosing restriction enzymes that match the sequence containing a SNP, fragments of identifiably different lengths are produced by each of the two alleles (one allele matches the enzyme allowing the cut, the other one does not). The alleles are detected by running the fragments through an electrophoresis gel, which separated fragments by mass.

Characteristics	
Sample Type	Venous blood, DBS
Processing Requirements	Basic molecular biology laboratory
Analytical Requirements	Published protocols for interpreting results, not automated

Suitable	Not suitable
• **best suited for monitoring a single mutation (or very few) and a relatively small number of samples**	• unsuitable for large panels of drug resistance loci

Advantages	Disadvantages
• relatively cheap • widely available: can be done in endemic countries • relatively simple to design and execute	• one SNP at a time • laborious, not suitable for high-throughput • relatively slow compared to other methods, including PCR • "analog" rather than "digital" output: electrophoresis gels often difficult to interpret • spurious banding can give bogus results

PCR SNP Genotyping

PCR is a widely-used technique used to amplify segments of DNA through several repeated cycles, generating thousands of copies of the DNA sequence. A common way to detect a SNP alleles is to perform nested PCR as follows. At a given locus (e.g. a gene where the SNP to be genotyped is

located), DNA primers are used to cut the region to be amplified. A second set of primers covers the SNP position, and binds only if a specific allele is present. The PCR products are then detected by running them through an electrophoresis gel, where the presence or absence of bands reveals the allele carried by the sample.

Characteristics	
Sample Type	Venous blood, DBS, RDT
Processing Requirements	Laboratory equipped with PCR
Analytical Requirements	Published protocols for interpreting results, not automated

Suitable	Not suitable
• best suited for monitoring a single mutation (or very few)	• unsuitable for large panels of drug resistance loci

Advantages	Disadvantages
• relatively cheap • widely available: can be done at all labs with PCR equipment, including many in endemic countries • can give a result rapidly on a small number of samples and of tests, and individual cases	• one SNP at a time • laborious, not suitable for high-throughput • "analog" rather than "digital" output: electrophoresis gels often difficult to interpret • spurious banding can give bogus results • costs rise rapidly with number of samples and tests

Microsatellite genotyping

Microsatellite genotyping is a commonly used method for characterizing genomic sites that are known to contain DNA repeat regions (i.e. stretches of DNA where the same sequence is repeated a variable number of times). At each locus (gene), PCR is used with primers to amplify the region containing the repeat sequence. The resulting product has a length (and therefore a mass) which is dependent on the number of repeats in this particular parasite. The PCR products are separated according to their mass by running them through an electrophoresis gel; the number of repeats is estimated by comparison to a reference sample with a known number of repeats.

Characteristics	
Sample Type	Venous blood, DBS, RDT
Processing Requirements	Laboratory equipped with PCR
Analytical Requirements	Published protocols for interpreting results, not automated

Suitable	Not suitable
• best suited for monitoring a small number of sites (e.g. for comparing two samples for identity)	• unsuitable for large panels of drug resistance loci

Advantages	Disadvantages
• relatively cheap • widely available: can be done at all labs with PCR equipment, including many in endemic countries • can give a result rapidly on a small number of samples and of tests, and individual cases	• laborious, not suitable for high-throughput • "analog" rather than "digital" output: electrophoresis gels often difficult to interpret • spurious banding can give bogus results • costs rise rapidly with number of samples and tests • difficult to classify samples with complex infections

Quantitative PCR (qPCR)

qPCR is a variant of PCR used to simultaneously detect a specific DNA sequence and determine the relative abundance of this sequence relative to a standard. Therefore, it is commonly used to estimate the number of copies of a gene. The quantitative estimation is carried out by measuring the fluorescence from dyes in the PCR equipment itself, so no electrophoresis is required. Copy number is measured by comparing the abundance of DNA in the tested gene against that in the reference gene.

Characteristics	
Sample Type	Venous blood, DBS, RDT
Processing Requirements	Laboratory equipped with qPCR equipment (less common than PCR)
Analytical Requirements	Published protocols for interpreting results, not automated

Suitable	Not suitable
• best suited for testing copy numbers of specific genes	• unsuitable for high-throughput
Advantages	**Disadvantages**
• suitable for copy-number genotyping • can give a result rapidly on a small number of samples and of tests, and individual cases	• equipment not as widely available as PCR • more costly than standard PCR • costs rise rapidly with number of samples and tests • difficult to classify samples with complex infections

Capillary Sequencing

This established method produces DNA sequences for a genome segment that has previously been amplified (e.g. by nested PCR). Thus, primers can be standardized for a locus of interest, and the output is suitable to detect any mutations in the amplified region (typically a few hundred bases long). This makes the method particularly suitable for detecting heteroallelic mutations, such as those responsible for artemisinin resistance in *pfkelch13*, and for verifying new detected mutations. Although sequencers are needed, there are several companies to which this type of sequencing can be outsourced at a moderate price. However, it is costly to apply to many regions of a single sample, and requires relatively large amounts of DNA.

Characteristics	
Sample Type	Venous blood, DBS
Processing Requirements	Laboratory equipped with PCR equipment; sequencing can be outsourced
Analytical Requirements	Requires skills in reading sequencing traces

Suitable	Not suitable
• heteroallelic genes	• unsuitable for high-throughput
Advantages	Disadvantages
• can easily be outsourced to external companies • relatively inexpensive for single locus • can give a result rapidly on a small number of samples and of tests, and individual cases	• expensive when applied to several loci • equipment not as widely available as PCR • no cheaper to do than genotyping single SNPs

High-throughput Technologies

Several technologies are able to produce genotypes at a multitude of loci simultaneously, and are therefore well suited to monitoring broad panels of variations of interest, such as drug resistance mutations, or barcoding SNPs. These technologies vary substantially in cost per sample, equipment cost and availability, and flexibility; in general, equipment tends to be costly and mostly available in larger specialized labs. Also, they produce a higher volume of results which, in practice, often cannot be processed manually, but require substantial processing. Generally, these technologies tend to be most cost-effective for large projects and large sample sets. Currently available high-throughput technologies are rather diverse, but they might be grouped into broad classes:

- High-throughput Genotyping technologies, which are able to call alleles at a large number of variants in a sample, but do not produce sequences
- Short-read Sequencing technologies, which are able to produce sequences from large numbers of small genomic DNA fragments
- Long-read Sequencing technologies, which produce sequences from larger DNA fragments

High-throughput Genotyping

High-throughput Genotyping technologies can genotype large number of variants in a sample. They generally require that primers are designed for each variant, and do not provide sequences for other regions of the genome. Although these technologies can provide very useful datasets, they generally require significant upfront investments; as a result, the falling price of whole-genome sequencing technologies has made them less popular. Several high-throughput genotyping technologies are available, the following being two notable examples:

- **SNP microarrays** use microarray chips that contain primers that bind to specific DNA sequences (the loci to be genotyped). Scans of the chip to detect dye fluorescence gives a readout of the allele at each of the loci. Microarrays can vary in number of genotyped loci, up to several thousand, increasing in cost as the number of loci grows. However, they require substantial design effort, they require commitment to use a given number of chips, and they are inflexible once the design is finalized. In addition, they require expensive equipment for processing, although manufacturers may provide processing.
- **Agena (Sequenom)** uses primers to isolate short DNA sequences that contain the SNP position to be genotyped. The amplified products are then separated by mass using very sensitive mass spectrometers able to discriminate by the difference in mass between nucleotides. This method is suitable for relatively small panels of SNPs (of the order of 40-200 SNPs), and running costs are low because of the high degree of multiplexing supported. Once the primers are designed, SNPs can still be added and/or removed, but this requires multiplex design skills. In addition, Agena hardware is expensive, generally only available in specialized centres, and specialized expertise is relatively hard to come by.

Characteristics	
Sample Type	Venous blood, DBS with sWGA
Processing Requirements	Specialized laboratory setup, e.g. with Agena, microarray equipment, Illumina MiSeq
Analytical Requirements	Requires specialized pipeline for calling genotypes

Advantages	Disadvantages
• automated, high-throughput • can be used on large sample sets • "digital" rather than "analog" output: clear and unambiguous allele readouts • cost is lower than that of producing the same results from non-high-throughput technologies	• need high-throughput setup/equipment • less easy to run ad-hoc on small sets • cost may be perceived as high, depending on application (especially setup costs,)

Whole Genome Sequencing Technologies

WGS technologies allow the whole of the pathogen genome (or selected amplified segments) to be sequenced with high-throughput techniques that allow the multiplexing of several samples in a single experiment. WGS generally requires DNA to be broken into segments (whose optimum length depends on the technology), and produces sequences of nucleotide calls (reads) which are then assembled by means of specialized software pipelines, often using a reference genome as a scaffold. Detected differences are used to discover SNPs *de novo*, and variants are genotyped by counting the number of assembled reads that carry a given allele. If sufficient reads are obtained to cover the whole genome multiple times, sequencing errors can be corrected and within-sample variability can be accounted for. The high density of genotype variations, the variety of obtainable measures, and the ability to reconstruct haplotypes make WGS is a complete platform for genetic surveillance of malaria. At present, there are concerns that WGS is still rather expensive for large-scale surveillance, but the cost of sequencing a sample is constantly decreasing, while the ability to multiplex samples in a single experiment is increasing, making WGS an increasingly attractive proposition.

One important aspect by which WGS technologies differ is the length of the reads that can be generated. Short-read sequencing has been extremely popular in the last 10-15 years and has dramatically driven down the cost of sequencing. Long-read sequencing presents many advantages, but has historically been less popular due to cost. Lately, however, the availability of low-cost long-read platforms has widened the choice for malaria epidemiology.

Short Read Sequencing

Next-generation short-read sequencing (NGS) such as Illumina, produce WGS data with very high throughput at an ever-falling cost per sample. In Illumina sequencing, genomic DNA is randomly broken into fragments of a few hundred bases, and sequences of about 100-150 bases (*reads*) can be read at each end of the fragment. The pairing of Illumina reads allows more accurate mapping when assembling these reads against a reference genome. Illumina reads have low error rates, making it possible to call genotypes even when the coverage across the genome is relatively low (e.g. 10x coverage).

In the context of malaria, the *P. falciparum* genome has extreme AT-richness, and high numbers of low complexity regions such as repeats, whose length may be comparable to that of a read. As a result, many areas of the genome are not amenable to reliable assembly with Illumina data. Even so, Illumina sequencing of *P. falciparum* can produce high-quality genotypes for hundreds of thousand SNPs, providing high-resolution panels for comparison, and excellent coverage of drug-resistance loci. In addition, the random nature of read coverage makes it possible to estimate gene copy numbers, as well as detect deletions and insertions.

Although Illumina sequencers are now affordable by many labs, even in malaria endemic countries, this technology requires extensive computing infrastructure, and specialized software pipelines for assembling genomes, calling genotypes and analyzing the results. In endemic countries, this can be problematic both in terms of the computing equipment and of the specialized manpower required. For these reasons, the technology platform must be accompanied by a suitable accessible analytical informatics pipeline, capable of producing standardized results with limited computing resources. If running such pipelines on low-end computers is not a viable proposition, an alternative approach is to make them available through a shared *cloud computing* platform, which require less investment in large computing infrastructure and in-depth technical know-how.

Characteristics	
Sample Type	Venous blood, DBS with sWGA
Processing Requirements	Labs with an Illumina sequencer, such as MiSeq, or better
Analytical Requirements	Requires specialized pipeline for genome assembly, discovering SNPs and calling genotypes

Suitable	Not suitable
• High-throughput • For high-resolution analyses	• ad-hoc runs on small sets

Advantages	Disadvantages
• automated, high-throughput • "digital" rather than "analog" output: clear and unambiguous allele readouts • massive amounts of data can help compensate for genotype missingness, complex infections etc. • high degree of multiplexing reduces cost • sequence data can be used for many applications	• requires lab equipped with sequencer • requires substantial data processing capabilities, infrastructure and know-how

Long Read Sequencing

Long-read sequencing (NGS) such as that offered by Oxford Nanopore Technologies (ONT) or Pacific Biosciences Technologies (PacBio), WGS data with very high throughput. In this section, we will focus on ONT, which has the most interesting characteristics for genetic surveillance of malaria in endemic settings.

Differently from Illumina, ONT does not read both ends of a short fragment; rather, it takes a fragment of any length, and reads it one nucleotide at a time by detecting electrical current as the DNA molecule passes through a small pore. Therefore, ONT reads can be considerably longer than those from Illumina, making their assembly potentially more straightforward even in problematic regions of the genome (e.g. intergenic regions). In addition, long reads can reveal structural variants such as amplifications, large deletions or rearrangements (which could be particularly useful for the AT-rich genome of *P. falciparum*). One downside of ONT sequencing are its error rates, which are considerably higher than those produced by Illumina, such that higher coverage of the genome is required to minimize genotyping errors.

A major advantage of ONT is the small size of the hardware, and its ease of deployment when compared to the complex and expensive process of setting up an Illumina sequencer. This reduction in setup costs and procurement may make ONT solutions very attractive in endemic countries, although the increased coverage requirements partly offset these cost advantages.

Characteristics	
Sample Type	Venous blood, DBS with sWGA
Processing Requirements	Labs with an Oxford Nanopore sequencer, such as a MinION or better; this can potentially be powered by a laptop computer. For genetic surveillance project, a larger setup may be required, such as a GridION with a dedicated PC
Analytical Requirements	Requires specialized pipeline for genome assembly, discovering SNPs and calling genotypes

Suitable	Not suitable
• High-throughput • For high-resolution analyses • For detecting structural variants	• Running very large surveillance operations (longer processing times)
Advantages	**Disadvantages**
• "digital" rather than "analog" output: clear and unambiguous allele readouts • massive amounts of data can help compensate for genotype missingness, complex infections etc. • sequence data can be used for many applications • long reads allow analysis of copy number variations, structural variants, intergenic regions • small size of hardware	• requires lab equipped with sequencer (but less so than Illumina) • requires substantial data processing capabilities, infrastructure and know-how • higher error rates may require higher coverage of samples

Genotyping Strategies for Surveillance

To summarize the gamut of technologies available, simpler technologies that cover individual genotypes can address specific use cases, while high-throughput technologies can simultaneously address different questions by genotyping whole genomes, at a higher cost and complexity of setup and informatics. When setting up a general-purpose genetic surveillance platform, geared up to answer a variety of Programme Use Cases, WGS technologies are arguably the most versatile. However, performing WGS on each individual sample may be unaffordable, highly resource-intensive and overkill for many use cases. For this reason, some surveillance projects (including GenRe-Mekong) have adopted genotyping strategies that use high-throughput sequencing in a relatively low-cost manner, producing data that can approximate WGS data, albeit at a lower resolution. We present two such approaches here: Amplicon Sequencing and Genetic Barcoding.

Amplicon Sequencing

This is a specific application of high-throughput next-generation sequencing, to produce data outputs similar to those from high-throughput genotyping platforms. DNA segments at multiple loci are selected by a set of primers, prior to sequencing, so that only a fraction of the whole genome is sequenced. The resulting reads are assembled on the reference genomes only at the selected loci, where the SNP genotypes can be called. Since only a fraction of the genome is sequenced, a large number of samples can be multiplexed in a single experiment, thus lowering dramatically the cost of individual genotypes. In other words, amplicon sequencing leverages on the high-throughput qualities of next-generation sequencing but avoids the increased cost and computational requirements of WGS by focussing on a fraction of the genome.

This strategy is largely technology-independent (e.g. they can run on Illumina, ONT and other platforms), and can be implemented on the smaller sequencers that are rapidly becoming available in endemic countries, at an affordable cost. In addition, this method allows the set of tested loci to be extended relatively simply, by the addition of new primers, without the complex design steps that are necessary for Agena or microarrays. However, it still requires specialized informatics pipelines and must therefore rely either on local know-how, or on standardized amplicon sequencing platforms that provide their own pipelines.

In summary, amplicon sequencing provides a high-throughput genotyping platform with the advantages of WGS technology at low cost for laboratories that possess an entry-level sequencer.

Characteristics	
Sample Type	Venous blood, DBS
Processing Requirements	Labs with a sequencer, such as MiSeq or minION, or better
Analytical Requirements	Requires specialized pipeline for genome assembly, and calling genotypes

Suitable	Not suitable
• high-throughput	• for high-resolution genetic data

Advantages	Disadvantages
• "digital" rather than "analog" output: clear and unambiguous allele readouts • high coverage is possible at a reasonable cost, making it easier to correct sequencing errors • flexible and extensible • low incremental per-sample cost	• requires lab equipped with sequencer • requires substantial data processing capabilities, infrastructure and know-how

Genetic Barcoding

Many methods for genetic epidemiology of malaria require genomes to be compared, to establish the degree of similarity between individual parasites. This is used, for example, to estimate genetic distance, identify clusters of similar parasites, detect expanding strains, reconstruct ancestry, etc. Genome comparisons are typically conducted by comparing alleles at multiple loci across the genome. When using WGS data, these comparisons can be performed on tens or hundreds of thousand variants ("high resolution"). When using a platform that can only generate a smaller number of genotypes, one may instead select a panel of genotyping targets, so as to assay a limited number of loci that are informative about variations, and therefore provide a "surrogate" of the whole genome for the purposes of selected analysis task. The higher the resolution of these panels (known as "genetic barcodes"), the more accurate the estimates produced by comparative analyses, which means that not genetic barcodes may only be of use for a given analysis methods if they have suitable resolution. Therefore, it is useful to classify barcode panels by their genome resolution.

- **Low Resolution barcodes** typically consist of SNP panels that are simultaneously genotyped by a high-throughput genotyping technology, or by amplicon sequencing. The term "barcode" comes from the practice of stringing together the genotyped alleles to form a "barcode" string. Examples of genetic barcodes are those used by Broad Institute,[11,12] by GenRe-Mekong[3,13] and in *P. vivax* surveillance.[14] Because a low number of SNPs is used, these barcodes have relatively low discriminatory power, and may only be used in selected methods. Typically, the panel SNPs are chosen from amongst the most variable in the genome, to maximize the amount of information produced by comparisons, but other criteria may be applied (e.g. geographic distribution).
- **Microhaplotype panels** also use genotypes from a relatively small number of loci, but these loci are selected for the presence of multiple closely clustered SNPs (microhaplotypes). This means that each locus may produce a greater variety of alleles than single SNPs, increasing the barcode resolution. The higher number of alleles can be used to test for identity with higher confidence, and may provide evidence of identity by descent and recombination patterns.
- **High Resolution SNP panels** are produced by WGS (or by SNP microarrays), and extend the concept of "barcode" to very large sets of genotypes, typically including several thousand SNP locations. Comparisons using these large SNP panels typically produce considerably more accurate estimates of genetic distance than are possible with low-resolution barcodes, and can reveal subtle features of population genetics.

PART II - Programme Use Cases

In this section, we described in detail the following Programme Use Cases:

- **P01 - Select antimalarial drug policy**
- **P02 - Monitor treatment efficacy and resistance**
- **P03 - Monitor efficacy of diagnostics**
- **P04 - Respond to outbreaks**
- **P05 - Stratify interventions**
- **P06 - Evaluate effects of interventions**
- **P07 - Establish Malaria-free Status**

P01 - Select antimalarial drug policy

Purpose

This use case reviews and revises the current choice of first-line and alternative antimalarials. The Programme evaluates evidence on the efficacy of currently deployed and alternative therapies, and decides which drug(s) should be used in the next deployment period. These decisions may be taken nationally, or at regional or even local level if the policy needs to be heterogeneous. Although it is related to P02 “Monitor treatment efficacy and resistance”, the present use case is focussed on assessing the options available for treatment for the near future.

Rationale

The use of antimalarials is generally regulated by a country’s Ministry of Health (MoH), who decide on a list of therapies to be deployed in the country. This may include frontline and backup therapies, therapies for infections from different parasite species, and therapies for uncomplicated and severe malaria. In addition, policy may differ between different areas of the country, e.g. because of different levels of efficacy of the same drug. The decision of which drug(s) to use has significant implications in terms of large-scale procurement, old stock disposal, warehousing, distribution, organizational and training needs, among others. It is desirable that policy decisions remain unchanged for several seasons, and therefore it is crucial that choices are evidence-based, using information on drug efficacy, and on the risk of emerging resistance to the selected antimalarials.

Programmatic Questions

- Are the currently used therapies efficacious in all regions where they are deployed?
- Which alternative therapies can be deployed with high efficacy?
- Is there a high risk that the current or alternative therapies may fail in the short term?
- Are there regions where differentiated therapeutic regimen should be recommended?

Flow of Events

1. Use case is triggered either periodically at set review times, or when the Programme gathers evidence of failures of currently used therapies (e.g. from use case P02).
2. The Programme and other agencies (government departments, research organizations, NGOs, WHO) convene a review of available evidence.
3. After all available evidence is reviewed, either no change is actioned, or the policy is revised to use the selected therapies and moved to the implementation stage.
4. The use case ends.

Current Approaches

Currently, evidence gathered is sparse and incomplete:

- An important input often comes from Therapeutic Efficacy Studies (TES), conducted in collaboration with the WHO on an *ad hoc* basis, which test clinical efficacy at selected sites in the country. While these are very useful high-quality surveys- indeed, often the best evidence available from a clinical perspective- they have a number of limitations:
    - high costs
    - limited territorial coverage
    - irregular frequency
    - needs long planning lead times
    - relatively small sample sizes
    - narrow range of drugs tested
    - providing limited information towards alternative treatments

- Results of collaborative local/national research studies, or by international groups (e.g. TRAC) may provide additional evidence, which also tends to be focussed on few sites and a specific set of therapies.
- Reports of treatment failures, either anecdotal or systematically collected by health facilities, will also be analyzed. These are limited to observations about the frontline therapy. Depending on circumstances, it may not be possible to routinely verify treatment failure by PCR, or track patients for recurrences. In addition, in resource-poor endemic areas it may be problematic to link infections if the patient presents at different facilities.
- In some cases, surveys of drug resistance mutations are conducted. This has been more common in regions affected by artemisinin resistance and as such, often limited to *kelch13* typing.

Integration with Genetic Epidemiology Use Cases

The following Genetic Epidemiology Use Cases may inform the present use case:

- G01 “Monitor Genetic Markers” provides maps and site statistics of resistant allele frequency; it may cover a broad range of drugs besides the first-line therapy. Therefore, these outputs support evaluation of alternative therapies, in addition to monitoring the predicted efficacy of the current frontline. Changes in allele frequency over time may also be used to assess whether drug resistance is spreading in a given geography, and therefore the use of the antimalarial should be avoided.

- G02 “Detect Treatment Failure” detects failures of the current first-line treatment at health centre level, with verification by genotyping. When applied as a systematic surveillance activity, it can provide:
    - Maps of failure rate statistics to indicate regions where emergence of drug resistance is probable, and frontline therapies may be failing.
    - Supporting evidence for individual case investigations.

- G06 “Describe Gene Flow” provides reports on the spread of drug resistant strains, and is informative of the risk of future increases in failure rates. This use case provides maps predicting the routes of gene flow, and therefore informs the Control Programme about areas that are at risk even though they are currently resistance-free (because they are on a putative route where drug resistance has started advancing).

Current Gaps and Research Needs

- The detection and monitoring of treatment failures requires genetic surveillance to be able to link multiple samples originating from the same patient, i.e. D0 and recurrence samples. In general, this requires a rigorously enforced patient ID systems, such as a national health card number. In addition, it is common for patients to use a different health facility when experiencing a recurrence. In these cases, cases can only be connected if there is a networked computerized medical records system in addition to the IDs. In many endemic regions this is not available.
- It is also desirable for treatment failure data to be uploaded into the data management systems used by programmes, so that they can be analyzed and visualized on dashboards, alongside other epidemiology data. Dashboard implementations may differ in different countries, so a platform-neutral solution is desirable.

P02 - Monitor treatment efficacy and resistance

Purpose

This use case captures the activities performed by Programmes in the monitoring of the efficacy of their frontline and alternative treatments, and of the emergence and prevalence of resistance. Although it is related to P01 "Select Antimalarial Drug Policy", the present use case is focussed on gathering evidence that the drug policy may need revising. The use case is conducted in all endemic regions of the country.

Rationale

Changing drug policy is difficult and costly, requiring procurement, warehousing, distribution, training, etc. Therefore, drug policy reviews are unlikely to occur unless there is evidence that the current therapies are failing, or that there is a high risk that they will fail in the near future. This requires Programmes to continually monitor the efficacy of first-line treatments in endemic areas, and respond to the emergence of resistance. Such monitoring demands considerable effort; therefore, programmes must balance thoroughness with availability of resources.

All forms of monitoring have important limitations, and no one strategy can give the complete answer in all cases. Wherever possible, one should use multiple approaches and consider all evidence jointly. In this section we consider the contribution of genetic epidemiology data alongside that of clinical studies, *in vitro* studies and health facilities reporting. Ideally, Control Programmes should be able to combine all these.

Programmatic Questions

- Are there significant levels of treatment failures when using the current first-line therapy?
- Is there an emergence of resistant strains that may soon cause failures?
- Is there developing resistance to one of the components of a combination therapy, such that there are no failures, but the risk is increasing?
- Is there evidence of epidemiological changes (e.g. outbreak of a single strain) that suggests resistance to drugs?

Flow of Events

1. Use case takes place routinely by carrying out surveillance activities, efficacy studies and research studies.
2. Periodically, NMCP officials review the evidence from all these sources. If the evidence reveals a drop in efficacy, or a rise in drug resistance (exceeding some previously defined threshold), the officials will evaluate the risks and, if deemed necessary, trigger Use Case P01 "Select antimalarial drug policy".
3. The use case ends.

Current Approaches

Currently, frequently used methods are based on monitoring clinical failures; such monitoring may be systematic across the public health system or, more frequently, sporadic in the form of efficacy studies. Anecdotal evidence (e.g. of unconfirmed failures) is often an important addition to more formal study results.

- **Therapeutic Efficacy Studies (TESs)** are periodically conducted in collaboration with the WHO, to test efficacy to frontline therapy at selected sites in the country. Clinical failures are confirmed by PCR testing. Because of their high cost and resource requirements, TESs have a number of limitations: limited territorial coverage, small sample sizes, narrow range of drugs tested. Not all TESs include the detection of genetic variants associated with failure.
- **Reporting systems from health centres (HCs)** may be in place in certain countries, giving access to a larger number of cases. In several settings, however, malaria patients are not followed up

after treatment, and therefore data on failures may be incomplete and unreliable (e.g. no system for connecting a recurrence to the original case; patients returning to a different HC; inconsistent patient identification; etc.). Furthermore, failures may not be confirmed by lab testing, and the HC staff may not be able to distinguish a recrudescence from a reinfection.

- Local, national or international **clinical research studies** may focus on careful and formal testing of specific drug response phenotypes, which may be determined *in vivo* (e.g. measuring parasite clearance rates in patients under artemisinin treatment[15]) or *in vitro* (e.g. measuring IC50 to a specific drug[16]). In some cases, studies may measure patient responses to individual drugs, rather than the frontline artemisinin combination therapies (ACTs). Clinical failures are usually confirmed by PCR testing.[17] These studies are typically costly and can be performed only at selected well-equipped sites, and on a limited number of patients.
- **Genetic surveillance** allows the detection and monitoring of known variations associated with drug resistance. High-throughput technologies allow routine genotyping of entire panels of resistance markers for multiple drugs, which can be performed from samples collected routinely in public HCs (e.g. the GenRe-Mekong surveillance project[3]).
- **Genetic epidemiology surveys** may use high-throughput technologies to genetically profile sampled parasites, e.g. by reading several variants across the genome to form a representative genetic barcode, or by reading their entire genome. Using these data, statistical analyses may reveal signals related to drug resistance, such as clonal expansions,[13] extended haplotype homozygosity,[18] etc. which are not dependent on specific markers, and can provide early warning of drug failure.

Each type of evidence has important specific limitations:

- In the case of ACTs (commonly used first-line antimalarials), a clinical failure signifies a failure of the drug combination, i.e. failure of both component drugs. Therefore, clinical failures may not occur in the presence of resistance to a single component of the ACT. Nevertheless, resistance to a single drug may still be a considerable fitness advantage for parasites. As a result, selection may occur, allowing resistance to the component drug to spread undetected for considerable periods of time, increasing the risk of therapy failure.
- *In vitro* testing of ACT component drugs can detect resistance to individual components. However, since resistance to different drugs relies on different biological mechanisms, it is not always clear what *in vitro* test is most appropriate until resistance emerges. For example, IC50 measurements are not informative of artemisinin resistance, where a ring-stage assay is needed; likewise, piperaquine resistance is best detected using a specific assay.
- Genetic surveillance can be informative of component drugs separately, and less dependent on the mechanism of drug resistance. However, markers of drug resistance are generally not identified before resistance emerges, which imposes a time lag before large-scale monitoring can be put in place. Also, genetic markers may not account fully for the resistant phenotype (e.g. *pfmdr1* copy number in mefloquine resistance), and some may produce phenotype changes only in the presence of a specific genetic background.
- Genetic epidemiology analyses allow hypothesis-free genetic detection of drug resistance even in the absence of known markers. Such techniques include the detection of expanding subpopulations, or regions with long widespread haplotypes, or extensive inheritance by descent (IBD). The detection of these phenomena does not directly lead to markers of resistance, but can be an important “alarm bell” of emerging resistance, that requires further investigation by *in vivo* or *in vitro* studies.

Integration with Genetic Epidemiology Use Cases

The following Genetic Epidemiology Use Cases may inform the present use case:

- G01 “Monitor Genetic Markers” provides maps and site statistics of resistant allele frequency; it may cover a broad range of drugs besides the first-line therapy. Thus, these outputs can detect the emergence of resistance to first-line and alternative therapies alike. In addition, changes in allele frequency over time may be used to assess whether drug resistance is spreading in a given geography, and therefore use of the respective antimalarial should be avoided.

- G02 “Detect Treatment Failure” detects failures of the current first-line treatment at health centre level, with verification by genotyping. Thus, it can provide failure rates statistics (by health centre, district or province), as well as supporting evidence for individual case investigations. Failure rates data can be compared against a threshold, or over time to detect emergence of drug resistance; comparison between sites may also help confirm that the effect is not due to random failures.

- G03 “Detect Changes in Population Structure” can identify signatures of drug resistance, such as clonal population expansion, detection of founder populations and presence of extended haplotypes. Although these signals cannot directly be taken to reflect emerging drug resistance, they provide an indication to the NMCP of regions where efficacy monitoring should be prioritized.

- G05 “Identify Circulating Strains” provides information about the presence of specific strains carrying drug resistance mutations.

- G06 “Describe Gene Flow” provides reports on the spread of drug resistant strains, and thus is informative of the risk of future increases in failure rates. This use case provides maps predicting the routes of gene flow, and therefore informs the Control Programme about areas that are at risk even though they are currently resistance-free (because they are on a route of spread).

Current Gaps and Research Needs

- Reports of levels of failure, or of prevalence of resistance, must usually be tested against a threshold, which may be specified by MoH policy or by advisory bodies such as WHO, and may differ from country to country. Further research is needed to determine evidence-based thresholds that accounts for error.
- Epidemiological signals of selection (e.g. population expansion) are known to occur, and may be effective genetic markers, but their emergence and rise dynamics is not fully understood- in particular, they may occur in situations other than emerging drug resistance. Further work is needed to characterize and interpret these signals.

P03 - Monitor Efficacy of Diagnostics

Purpose

This use case captures the activities performed by Programmes in the monitoring of the efficacy of their malaria diagnostics. The use case applies to any type of diagnostics, the most common ones in malaria being lateral-flow rapid diagnostic tests (RDTs), and detection by microscopy. The use case may be conducted in any endemic region of the country.

Rationale

Diagnostics that confirm the presence of a *Plasmodium* infection are a critical part of the test and treatment process. Generally, antimalarials are only administered if the diagnostic test confirms the parasite infection. In high-transmission countries where malaria infections are very common (e.g. in some African countries) antimalarials may be given based on the symptoms, even if the diagnostic test fails to reveal the presence of parasites (e.g. when parasitemias are very low). In areas with lower endemicity, however, the efficacy of diagnostics is critical, because failure to detect results in failure to treat, which not only has repercussions on the health of the individual patient, but can also result in increased transmission if the diagnostic failure rate is high. It is therefore important to establish whether the current diagnostic methods are correctly identifying a satisfactory proportion of infections.

Remedial actions to diagnostic failures depend on the type of diagnostic. In the case of microscopy, retraining of microscopists and/or procurement of new equipment may be required. However, in most countries, microscopy has been largely superseded by the use of RDTs, which are currently under close scrutiny. The most commonly used RDTs detect HRP2 and/or HRP3 proteins in the patient's blood. In recent years, it has been reported that parasite populations in some countries carry deletions of their HRP2 and/or HRP3 genes, which affect the abundance of their protein products and thus the ability of RDTs to detect the infection.[19,20] In these cases, Programmes may switch to other RDT types (sensitive to different protein targets), which requires significant investment in procurement and logistics. Since many countries are not currently affected by this problem, it is important to periodically assess the parasite population to detect the emergence of HRP deletions. At the same time, testing for deletions in each country has to follow a sensible and parsimonious strategy until there is significant evidence of the problem.

Assessment of diagnostic efficacy may be conducted by comparing the detection performance of a diagnostic method to that of a reference method, using the same sample. PCR detection is often considered to have a higher sensitivity that both microscopy and RDT, and can be a useful reference method. However, comparisons between RDT and microscopy, or between HRP-based and non-HRP-based RDT can also be informative, in the absence of PCR testing facilities.

Programmatic Questions

- Are my current diagnostic methods detecting a satisfactory proportion of *P. falciparum infections*?
- If there are significant levels of detection failures, what is the cause?
- If there are significant levels of detection failures, what alternative diagnostics can be used?

It must be noted that, from a Programme perspective, the question is NOT "are there HRP deletions circulating?". The efficacy of diagnostics should be tested hypothesis-free; if it is demonstrated that there are failures, then the causes are investigated, e.g. by investigating HRP deletions.

Flow of Events

1. Use case takes place periodically, according to planned assessment of diagnostic efficacy.
2. Detection performance of a diagnostic method should be compared to that of a different method on a given samples of cases.

3. Programme officials review the results of these comparisons. If the proportion of missed infections is within a predetermined acceptable limit, use of the diagnostic can continue, and the use case ends.
4. Otherwise, the causes of the detection failures must be investigated (e.g. by testing for HRP deletions if RDTs are used), and remedial measures determined.
5. The use case ends.

Current Approaches

Several studies have estimated the efficacy of RDT detection, typically against microscopy or PCR; however, we are not clear how many countries routinely perform such studies.

The WHO published a standard protocol for HRP deletion detection studies.[21]

Integration with Genetic Epidemiology Use Cases

The following Genetic Epidemiology Use Cases may inform the present use case:

- G01 “Monitor Genetic Markers” provides statistics of allele frequency. This can include tests for the presence or absence of HRP2/3 deletions to confirm the cause of RDT failures.

Current Gaps and Research Needs

In some countries, different studies have provided discordant estimates of HRP2, sometimes contradictory.

P04 - Respond to Outbreaks

Purpose

This use case is triggered when the Programme becomes aware of an unusual (often rapid) increase in the number of cases in a specific endemic region, for causes unknown. The criteria for declaring an outbreak (see Glossary) are the prerogative of the Programme. The use case strives to:

- Classify the outbreak by its underlying cause: is it (a) a simple increase in the number of cases, whereby the parasite population grows but maintains its normal levels of diversity; or (b) the expansion of a particularly successful strains; and in this case, does it possess known genetic or phenotypic characteristics (e.g. resistance to a drug)?
- Determine whether there are changes in the efficacy of current treatment
- Identify the transmission dynamics of the outbreak

Rationale

A malaria outbreak is a sudden and rapid increase in the number of infections, often localized at particular sites or districts. There are no globally accepted rules to determine whether a given increase in case is considered to be an outbreak, and different authorities may use different criteria. Typical rules might compare the number of cases in a region during a given season against the average in that region over a previous period, e.g. 3-5 years. This use case is executed whenever the NMCP determines there has been an outbreak, no matter what the criteria were.

Outbreaks represent major departures from the normal epidemiological dynamics of malaria, and are therefore of great concern to Control Programmes. In order to intervene appropriately, the underlying cause of the outbreak needs to be understood. Malaria outbreaks are often ascribed to increased transmission due to factors related to ecology (e.g. vector activity or weather conditions) or to human behavioural (e.g. increased travel in forests, importation, etc.).[22] Typical responses aim interventions at interrupting transmission, according to the perceived cause of the outbreak (e.g. provide mosquito nets, or target forest workers).

However, malaria outbreaks may also be caused by the rapid expansion of a parasite population, driven by selection for genetic traits.[13] These traits may be responsible for an evolutionary advantage, e.g. resistance to the first-line therapy, adaptation to vectors, etc. A characterization of such outbreak by the genetic variants carried by the outbreak population and its geographical origin, may provide important information to guide intervention.

Programmatic Questions

- What are the driving forces for the outbreak? E.g. ecological factors, human behavior, genetic changes in parasite population.
- Is there any increase in the level of resistance to the first-line treatment in the area?
- Are known drug resistant parasite strains causing the outbreak?
- Is the outbreak primarily made up of cases from native parasites?
- If not, where do the outbreak cases seem to originate from?

Flow of Events

1. Use case is triggered when a sudden increase in the number of cases is detected in an area, and the NMCP declares an outbreak.
2. NMCP evaluates evidence about the cases, to determine their origins and underlying causes.
3. Programme officials classify the outbreak, and put in place appropriate interventions according to the type of outbreak.
4. Outbreak region continues to be monitored and interventions are evaluated (use case P06).
5. If the level of cases remains high the use case may return to step #2.
6. The use case ends.

Current Approaches

In GMS countries, current assessment of outbreaks uses primarily clinical and patient travel data reported by the health facilities. Additional task forces may be deployed to investigate the outbreak.

- In most cases, awareness of the outbreak is triggered by higher reported numbers of cases through normal channels (in some countries, smartphone- or tablet-based systems allow greater timeliness). Detection is typically through use of RDTs to confirm and to test, treat, and track.
- Travel and abode data may be analyzed to try to track the origin of the outbreak, and identify the earliest cases.
- In populations of forest workers, travel data accuracy is often undermined by patient reluctance to reveal details of activities that may be illegal. More generally, reporting biases are common, and can make it difficult to classify outbreaks through reports and surveys.
- In the GMS, the frequency of *kelch13* mutations may be estimated in an outbreak, to verify the contribution of artemisinin-resistant strains.

Integration with Genetic Epidemiology Use Cases

The following Genetic Epidemiology Use Cases may inform the present use case:

- G01 “Monitor Genetic Markers” provides site statistics of resistant allele frequency. These outputs can detect increases in the frequency of drug resistant mutations, and characterize the outbreak populations in terms of their predicted phenotypes. Changes in allele frequency in the course of the outbreak may also be used to assess whether drug resistance is spreading.

- G03 “Detect Changes in Population Structure” can detect whether the outbreak is caused by a clonal population expansion or, conversely, local population structure and parasite diversity remain essentially unchanged, indicating that elevated transmission is the cause.

- G04 “Identify Imported Cases” may associate outbreak cases to carry parasites that are unlikely to be autochthonous, suggesting that the outbreak could have been caused by imported parasites. In such cases, it may also be possible to reveal the likely population where these parasites have originated.

- G05 “Identify Circulating Strains” may provide information enabling the classification of the populations involved in the outbreak, and detailing their known phenotypic traits.

Current Gaps and Research Needs

- Timeliness is key in outbreak investigations, so any genetic testing has to be completed in a relative short period of time for the results to be useful in planning responsive interventions; longer timescales can only support retrospective analyses. More reliable processing timelines are needed, and the Programme expectations of these timelines must be informed.
- Even epidemiological measures such as the annual parasite index (API) are currently only estimated on a yearly basis, while outbreak management would benefit from more frequent estimates.
- Routine surveillance should be conducted to provide an epidemiological baseline of parasite populations against which outbreak populations can be compared.
- High-resolution genetic data (WGS) can provide additional evidence of the relationship between outbreak strains and previously characterized strains (e.g. by identity-by-descent analyses[13]).

P05 - Stratify interventions

Purpose

This use case aims to evaluate epidemiological evidence about a specific geographical region (or human population), to determine what type of intervention will be most effective. For example, it may seek to identify

- Malaria transmission or incidence hotspots, where interruption will have the highest impact.
- Routes of parasite and gene flow, where interruption could stop spread of parasites.
- Areas of drug resistance, that must be contained to prevent the spread of resistant strains.
- Areas of clonal expansion, where elimination of the expanding strain is desirable.

This use case is similar in intent to P04 “Respond to Outbreaks”, except in this case the purpose is a structured series of interventions to control and reduce malaria, rather than a reactive set of measure triggered by sudden epidemiological change. The present use case is therefore less time-sensitive.

Rationale

Programmes have at their disposal limited resources, and must take decisions about how to deploy them for maximum impact. Thus, it is necessary to evaluate epidemiological and clinical evidence in order to identify the locations where interventions are most effective, and the type of intervention to be used. A range of different classifications may apply, each with a different intervention approach; further classes may emerge as disease epidemiology becomes better understood, and new types of interventions are used.

Programmatic Questions

- Which sites can be classified as transmission hotspots?
- Are there identifiable routes of spread of malaria?
- Along the routes of gene flow, are there hubs where flow could be disrupted?
- Are there areas where drug resistance is rising in frequency and/or spreading?
- Are known drug resistant parasite strains spreading in the territory? What is their route?

Flow of Events

1. Use case is triggered periodically, when the programme is planning interventions- either routinely or in response to evidence.
2. Programme officials collect and evaluate evidence about malaria cases in different geographical contexts.
3. Programme officials classify geographical areas and put in place appropriate interventions according to the type of area.
4. The use case ends.

Current Approaches

In GMS countries, epidemiological assessments for the purpose of planning interventions currently use clinical and patient data reported by the health facilities, as well as prevalence and drug resistance frequencies supplied by research studies, TES, etc.

- Treatment failures rates may be estimated from clinical data, but are often not PCR-confirmed, except for targeted studies. In normal clinical settings, they may be classified based on Day 3 parasitemia (by microscopy), on time interval between first episode and recurrence, or on travel information
- Travel and abode data may be analyzed to try to designate routes of transmission, and identify hotspots where parasites are transmitted.

- In some cases, the frequency of drug resistance mutations, particularly those in *kelch13*, may have been estimated by targeted studies.
- Less commonly, genetic surveillance of drug resistance may use routine collections of DBS samples on filter paper for genotyping, providing information on allele frequencies.

Integration with Genetic Epidemiology Use Cases

The following Genetic Epidemiology Use Cases may inform the present use case:

- G01 “Monitor Genetic Markers” provides maps and site statistics of resistant allele frequency; it may cover a broad range of drugs besides the first-line therapy. Thus, these outputs can detect the emergence and increase of resistance. In addition, changes in allele frequency over time may be used to assess whether drug resistance is spreading in a given geography.

- G03 “Detect Changes in Population Structure” can identify clonal population expansion and founder population, suggesting areas where targeted intervention may be needed.

- G06 “Describe Gene Flow” provides reports on the spread of parasites, including known drug resistant strains, and provides maps predicting the routes of gene flow. From this information, officials could identify points of intervention where spread can be interrupted.

- G07 “Assess Transmission Intensity” can estimate transmission intensity at different sites, either in absolute terms, or relative to each other. This information may be used by Programme officials to identify hotspots where transmission can be interrupted.

Current Gaps and Research Needs

- Methods for reconstructing gene flow from genetic barcodes and whole-genome data are still under development, and will need refinement to produce reliable results.
- Similarly, methods for applying relationship between genetic diversity and transmission intensity, and consequently assess transmission intensity, needs further development before they can reliably identify transmission hotspots.
- Sampling frameworks need to be defined to optimize coverage with granularity.

P06 - Evaluate effects of interventions

Purpose

This use case aims to gather evidence to determine whether control and elimination interventions have been successful at eliminating, reducing malaria levels, removing specific strains, or interrupting transmission networks. These results allow Programme officials to evaluate specific interventions, whether they were appropriate, and whether the decision-making process leading to the intervention was effective.

Rationale

Control Programmes have to evaluate evidence, and take decisions on interventions to be enacted in order to control and/or eliminate malaria. Following interventions, the situation has to be reviewed in order to assess whether the interventions were successful. This assessment generally has to be carried out against the planned objectives of the intervention itself, which may be specific and use an intervention evaluation framework.

Here, we describe the general process, and a number of specific interventions and focused programmatic questions where genetic epidemiology can provide results that are useful for evaluation. However, the catalogue of such interventions may be expanded in future.

Programmatic Questions

- If new cases are observed after intervention produces local elimination, where do the cases originate from? Are they imported or similar to previous local strains?
- After intervening at a transmission hotspot, is there evidence that this has affected other neighbouring sites? Has transmission at this hotspot subsided?
- After targeting specific strains, is there evidence that their frequency has been reduced?
- After intervening at parasite flow hubs, is there evidence that flow has been interrupted and the route is no longer active?

Flow of Events

1. Use case is triggered when it is deemed appropriate by Programme officials to evaluate the effect of a specific intervention. The evaluation is typically planned and scheduled at the time when the intervention implementation is designed.
2. Programme officials gather evidence for evaluation.
3. The intervention is evaluated. If not deemed successful, a new intervention may be planned.
4. The use case ends.

Current Approaches

Specific approaches may vary depending on the type of intervention. Assessments performed by Control Programmes are often based on clinical data reported by the health facilities.

In the GMS, targetted elimination interventions (e.g. by mass drug administration or MDA) currently tend to take place in the context of controlled studies. These studies investigate the outcomes of the elimination exercises, and may collect blood samples to investigate the genetic epidemiology of new cases after MDA. Such elimination effort may be more commonly be undertaken by programmes in the future.

Integration with Genetic Epidemiology Use Cases

The following Genetic Epidemiology Use Cases may inform the present use case:

- G01 “Monitor Genetic Markers” provides maps and site statistics of resistant allele frequency, which can be used to detect changes in drug resistance frequency following intervention targeting resistant parasites. Conversely, this use case can also inform the Programme as to

whether the intervention has had adverse effects on drug resistance levels (e.g. in the case of massive deployment of antimalarial for elimination).

- G03 “Detect Changes in Population Structure” can characterize population structure and diversity, and inform Programmes on changes in population structure after intervention, as well as characterize the structure of parasite populations after re-emergence (e.g. in the case of an elimination intervention such as mass drug administration).

- G04 “Identify imported cases” may be able to assess whether new malaria cases following elimination interventions originate outside of the intervention region, or they are parasites that were missed by the intervention itself.

- G07 “Assess Transmission Intensity” can estimate transmission intensity at different sites, either in absolute terms, or relative to each other. This information may be used by Programme officials to observe the transmission levels changes at or around hotspots, to evaluate whether interventions have had the desired effect.

Current Gaps and Research Needs

- Expected outcomes and evaluation criteria for elimination interventions are still under development, and the selection of the best approach may still be problematic. Further research work is needed.
- Also, progress is needed in the estimation of transmission intensity from genetic data.

P07 - Establish Malaria-free Status

Purpose
This use case aims at providing evidence of malaria-free status in order to initiate the WHO malaria-free certification process.

Rationale
The World Health Organization grants Certification of malaria elimination to countries that have achieved "Interruption of local transmission (reduction to zero incidence of indigenous cases) of a specified malaria parasite species in a defined geographical area as a result of deliberate activities".[23]

In order to initiate the certification process, the Programme has to prove "beyond reasonable doubt, that the chain of local transmission of all human malaria parasites has been interrupted nationwide for at least the past 3 consecutive years, and that a fully functional surveillance and response system that can prevent re-establishment of indigenous transmission is in place." The certification process involves a verification and evidence review exercise (for details, see https://www.who.int/teams/global-malaria-programme/elimination/certification-process).

Hence, there is a three-year minimum period for which the Programme must provide evidence that zero incidence of local transmission is occurring. This means that any cases occurring need to be established if they are imported, introduced or indigenous. Currently, the key evidence is the travel history of a patient presenting with malaria in an elimination setting. Whilst this is an established epidemiological approach in public health, it is subject to reporting bias, either through poor patient recollection, or deliberate omission for fear of legal repercussions e.g. illegal activities in forested areas on country borders. Genetic analysis of cases, as part of surveillance efforts, can provide additional evidence, which may be helpful in assessing imported cases and their possible origin.

Programmatic Questions
- (In the three years prior to certification) Is this new case likely to be imported or the result of local transmission?

Flow of Events
1. Use case is triggered during the three years prior to certification, whenever a patient is diagnosed with malaria.
2. The Programme gathers evidence about the case.
3. The Programme reviews the evidence to establish likely origin of the parasites and take appropriate action.
4. The use case ends.

Current Approaches

Currently, the travel history of the patient is collected and analyzed to determine the likely place and circumstances of infection. This may involve interviewing the patient and collecting a travel questionnaire as evidence. Genetic evidence is not typically used.

Integration with Genetic Epidemiology Use Cases
The following Genetic Epidemiology Use Cases may inform the present use case:

- G04 "Identify imported cases" may be able to identify whether infecting parasites are likely to have originated locally, or to have been imported from another country.

Current Gaps and Research Needs
- Genetic evidence can give very clear results in cases where importation is from a parasite population highly differentiated from the local parasites. For example, if an infection is found in

Vietnam, in a patient that has recently worked as construction worker in Africa, genetic analyses are likely to be able to confirm whether the infection was contracted in Africa or locally in Vietnam.

- In the case of infections in patients living close to a country border, it may be difficult to answer the question of importation. Hence, the results must be clearly framed in terms of hypotheses and likelihoods, so that they can be interpreted by the Programme.

PART III - Genetic Epidemiology Use Cases

In this section, we described in detail the following Genetic Epidemiology Use Cases:

- **G01 - Monitor Genetic Markers**
- **G02 - Detect Treatment Failure**
- **G03 - Detect Changes in Population Structure**
- **G04 - Identify Imported Cases**
- **G05 - Identify Circulating Strains**
- **G06 - Describe Gene Flow**
- **G07 - Assess Transmission Intensity**

G01 - Monitor Genetic Markers

Purpose

This use case analyzes samples from malaria infections to detect the presence of mutations that have been associated to antimalarial drug resistance. The outcomes are typically aggregated temporally and geographically to report the frequencies of resistant alleles at different sites and detect variations over time, to inform public health authorities of the suitability of different antimalarial for treatment at those sites.

Rationale

Although clinical outcome remains the primary indicator of drug efficacy, monitoring genetic markers of drug resistance offers Control Programmes a different insight. In the case of ACTs, for example, the use of two drugs simultaneously means that clinical failure will result only when parasites are resistant to both drugs- in other words, emerging resistance to one drug could be masked by the efficacy of its partner drug. Monitoring resistance to each drug independently may avert combined resistance, and retain ACT efficacy.

Clinical monitoring of resistance to individual drugs independently cannot be performed except in some small carefully controlled studies. Instead, resistance to a specific drug can be monitored either by genotyping known genetic markers (if any have been confirmed) or *in vitro* if a reliable test of efficacy is available. Since *in vitro* testing is laborious and resource intensive, monitoring genetic markers is the most realistic high-throughput option for large-scale surveillance.

Wherever available, frequencies of genetic markers can be used by Control Programmes to predict the likelihood of drug failure, and to plan both intervention strategies and first-line therapies. Most current genetic surveys tend to target one specific drug (e.g. artemisinin) to confirm its efficacy, to reduce genotyping costs. However, monitoring genetic factors of resistance to multiple drugs, which is viable with high-throughput technologies, enables the evaluation of alternative treatment regimen options, and the monitoring of changes in resistance mutation frequency for different drug classes.

In general, the chosen genetic markers must be epidemiologically relevant: since there are a variety of mechanisms that cause parasites to become resistant, *in vitro* studies may uncover mutations that indeed confer resistance under laboratory conditions, but that are never observed in the field. While it is not harmful to test these variations, they are not an alternative to testing markers that are associated with drug resistance in a clinical setting.

Genetic Epidemiology Questions

- What proportion of parasites at a given site is likely to be resistant to a given drug?
- What drugs or combinations have the least probability of failure at a given site?
- Are there changes in frequency that suggest decline of efficacy or pending failure?

Flow of Events

1. Use case is triggered when blood samples collected from patients are submitted.
2. The samples are genotyped for the mutations of interest. Note that resistance to different drugs may be due to variations of different nature (SNPs, copy numbers, heteroallelic mutations), hence a variety of techniques may be employed.
3. The genotypes are reported, and wherever possible the system will interpret the result in terms of sensitivity to a given drug, such that the sample is classified as “Sensitive”, “Resistant”, “Mixed” or “Undetermined”.
4. Use case terminates and results are made available.

Related Use Cases

Programme Use Cases

The following Programme Use Cases may use analysis outputs from the present use case:

- P01 “Select Antimalarial Drug Policy” uses frequencies of drug resistant mutations in order to determine efficacy of current frontline treatment, and evaluate alternatives.
- P02 “Monitor Treatment Efficacy and Resistance” uses drug resistance marker frequencies to predict changes in efficacy and the emergence of resistance.
- P03 “Monitor efficacy of diagnostics” uses markers of antigen deletions to predict confirm causes of RDT failures to detect malaria.
- P04 “Respond to outbreaks” uses drug resistance marker frequencies to classify outbreaks whose probable cause is the expansion of a resistant parasite strain.
- P05 “Stratify Interventions” uses drug resistance marker frequencies in order to identify areas requiring targeted interventions to contain drug resistance.
- P06 “Evaluate Effects of Interventions” uses drug resistance marker frequencies in order to evaluate whether drug resistant strains are being selected as a result of intervention.

Current Approaches

There are several different approaches to genotyping known variations associated with drug resistance; in addition, there are different types of variations to be genotyped:

- **SNPs**: single point mutations where a mutant allele is known to confer resistance. Certain drugs (e.g. Sulfadoxine-Pyrimethamine) may be influenced by combinations of alleles (haplotypes) requiring multiple SNPs to be genotyped.
- **Heteroallelic genes**: resistance to artemisinin (ART-R) is associated with a variety of mutant alleles in the *pfkelch13* gene, and there are several loci within these genes where a mutation influences the parasite’s sensitivity to the drug. Therefore, complete genotyping for ART-R requires the sequencing of a large portion of the gene where these mutations can occur (BTB/POZ and propeller domains) rather than genotyping of single points.
- **Copy numbers**: resistance to certain drugs (e.g. mefloquine and piperaquine) is associated to the parasite developing additional copies of certain genes, rather than mutating at single points in its DNA sequence. Detection of these changes indicated by copy number variation (CNV), requires different types of analyses.

Here we summarize some of the main technologies that can be used to detect the above changes:

- **PCR**. This technology is described in Section “PCR SNP Genotyping”. This method is only suitable for typing SNP alleles.
- **qPCR**. This technology is described in Section “Quantitative PCR (qPCR)”. This method is generally used for typing copy number variations.
- **Capillary Sequencing.** This technology is described in Section “Capillary Sequencing”. It is a preferred method for typing heteroallelic genes, e.g. ART-R genotyping.
- **High-throughput Genotyping**. This technology is described in Section “High-throughput Genotyping”. The ability of genotyping multiple sites with technologies such as Agena means that experiments can be design to produce genotypes at many, if not all, SNPs associated with resistance to different drugs. Hence, samples can be broadly characterized not only for resistance to a specific drug, but also for their multi-drug resistance capability.
- **Whole Genome Sequencing**. This technology is described in Section “Whole-Genome Sequencing”. Since WGS data tends to cover most genome locations, it lends itself to genotyping all known SNPs associated to resistance to antimalarials. In addition, counts of sequencing reads can be used to estimate gene copy numbers, and mutations in heteroallelic genes can also be typed. In other words, WGS data has the potential to produce a complete drug resistance profile, without needing to use other technologies.

- **Amplicon Sequencing**. This technology is described in Section "Amplicon Sequencing". It allows the genotyping of the full drug resistance profile, as described for WGS above, by targeting all loci that are known to be associated with drug resistance. In addition, because it only covers a fraction of the genome, it allows massive multiplexing and is therefore much cheaper per sample than WGS. Furthermore, it can be implemented on small next-generation sequencers that are becoming available in endemic countries, obviating in some countries the problem of export licenses for DNA.

Integration with Programme Use Cases

- Drug resistance genotypes, as well as imputed genotypes, derived phenotype predictions and other information on the infecting parasites, should be provided to NMCPs in tabulated form for all cases surveyed, so that case investigations may leverage on this information.
- In cases where the data is incomplete, it may be possible to impute some of the missing alleles, based on associations derived from statistical analyses of large genetic variation datasets.[3]
- Allele frequencies should be estimated by site, district and/or province by aggregating all samples within the geographical area and period of interest. Samples with missing genotype should be disregarded when computing the allele frequency of a specific variation. Samples with heterozygous genotypes should either contribute to the frequencies of both alleles, or be disregarded (depending on the resolution of available heterozygosity information).
- For several variations, it may be possible to use the genotyped allele, which consists of a single nucleotide change or a more complex haplotype, to predict a phenotype (typically a drug response phenotype). These predictions must be substantiated by peer-reviewed literature, which should be referenced in documentation accompanying the predictions. It is recommended that phenotypes be predicted for each sample individually, before data aggregation.
- Predicted phenotypes must be explicitly defined in the accompanying documentations, since critical terms such as "resistant" may be subject to interpretation in different contexts.
    - For example, in artemisinin resistant parasites the drug can still kill parasites, but clears them at a slower rate, undermining the efficacy of the therapy.
- It is recommended to define three-class phenotype predictions ("sensitive", "resistant" and "undetermined") wherever possible, since these are easier to evaluate by public health officials and straightforward to represent on maps. In all cases, the choice of prediction outcomes and terminology must aid understanding, but not oversimplify the prediction.
- Heterozygous genotypes (i.e. both resistant and sensitive alleles are present) should be regarded as "undetermined" unless there is evidence in the literature that one of the alleles is dominant in terms of the affected phenotype.
- Predictions may be aggregated by site, district or province, so that proportions of predicted resistant parasites can be derived. It is recommended that "undetermined" samples be disregarded in the proportion calculations. Proportions may be presented on maps, at the appropriate level, using simple colour range representations.
- Certain phenotypes, may require additional prediction classes (e.g. "sensitive", "partially resistant", "resistant" and "undetermined"). We recommend that complex distinctions should be introduced only where they are likely lead to public health officials taking significantly different decisions; thoroughness should not compromise clarity and intuitiveness.
- The aggregation of predictions with a higher number of classes is conducted in a similar way as three-class predictions, but different strategies may be needed for presenting the data to NMCPs- the method chosen should be that which maximizes clarity for the recipients. For example, multiple phenotypes may be collapsed into a single class, so as to make it possible to use the same representation as for three-class predictions (e.g. present two maps, one with simple colour shading to show the proportion of partially+fully resistant parasites, and one for fully resistant only). Alternatively, predicted phenotype proportions may be represented simultaneously, e.g. in pie charts or bar graphs.

Outputs Storage Requirements

A Drug Resistance Mutation Data Repository should include for each sample:

- the genotype at each tested locus;
- the predicted phenotype of the parasite for each drug for which this prediction can be made based on the tests performed;
- sampling location and date.

Sampling Requirements

There are no specific use case requirements. However, planning of surveillance operations will typically include a sampling strategy.

Prerequisite Data

No specific requirements, but data on common genomic variations around target loci are useful for the design of effective primers.

Outputs and Delivery Requirements

- At each site, the frequency of each tested allele, and of each resistance phenotype predicted, should be summarized and reported to the Programme.

Data Sharing Requirements

The data produced by this use case should be made available to all epidemiological analyses of drug resistance spread. This may include local, national and cross-border analyses. It is therefore recommended that the data be made public.

Limitations

Genotyping of drug resistance is only conducted at loci that have been identified, and prediction require published evidence of association. This is subject to change, and any system needs to build in the capability to extend.

Current Gaps and Research Needs

There are several areas where research can improve the methods used here:

- Cloud-based genotyping pipelines
- Development of primer kits and protocols for ease and consistency of implementation
- Methods for producing maps from the results of this use case
- Interactive visualization systems

Aspects not covered here

We have not considered specific regional needs, e.g. Africa vs. SE Asia; certain variants are relevant in one geographical context and not another.

G02 - Detect Treatment Failure

Purpose

This use case aims to analyze samples from recurrent malaria infections in a patient, to distinguish cases of likely treatment failures (recrudescences) from cases of re-infection following successful treatment. The outcome can inform public health authorities of the level of true treatment failures at sampled sites.

Rationale

Even in infections caused by drug resistant parasites, treatment typically causes symptoms to subside. However, infections with resistant parasites are often not fully cleared, which causes the within-patient parasite population to recover gradually, potentially giving rise to new symptoms post-treatment (recrudescence). This may take several weeks, during which the patient could be plausibly re-infected from a different mosquito bite, which could be an alternative cause of new symptoms (reinfection). Although recrudescences are symptomatically undistinguishable from reinfections, their correct identification is informative of treatment failure, and may provide evidence that resistant parasites are circulating, and treatment may be losing efficacy.

It is generally expected that recrudescent parasites are genetically identical to those in the first infection, while a re-infection would be caused by genetically different parasites. Hence, genetic approaches to detecting failures seek to compare the genetic make-up of the parasite in the first infection and those in the recurrent episode. The genetic resolution of this comparison determines the degree of confidence in the classification; the comparison may be rendered more challenging by various factors, such as complex infections (i.e. multiple parasite genomes in a single infection), and loss of diversity in the population.

Genetic Epidemiology Questions

- Given two blood samples from the same patient collected at different time points, do they contain identical parasites?
- At a given site, what is the proportion of malaria recurrences caused by recrudescence?

Flow of Events

1. Use case is triggered when a patient returns with a fever to a healthcare facility where they have recently been treated for malaria. Facility staff confirm malaria diagnosis.
2. Facility staff verify whether a blood sample was submitted for the previous episode (Day 0).
    - If no sample was collected, the use case ends and the outcome is “undetermined”.
    - If the sample was collected, the reference to Day-0 sample is retrieved.
3. A recurrence blood sample is collected and submitted to the system, marked as a recurrence (Day-Rec), with a reference to the Day-0 sample.
4. The Day-Rec sample is genotyped.
5. Day-Rec and Day-0 genotypes are compared, and the recurrence is classified.
6. Use case terminates and results are made available.

Related Use Cases

Programme Use Cases

The following Programme Use Cases may use analysis outputs from the present use case:

- P01 “Select Antimalarial Drug Policy” may use frequencies of treatment failure in order to determine efficacy of currently used and alternative treatments.
- P02 “Monitor Treatment Efficacy and Resistance” triggers responses when failures are detected at a given site.

Current Approaches

By genotyping genetic features that exhibit high variability in any parasite population, the number of tests applied is expected to be sufficient for a high degree of confidence that the two samples do or do not contain the same parasite.

MSP1/MSP2/GLURP genotyping. This is a commonly used method (also known as "PCR correction" of recrudescence frequencies), which requires the genotyping of DNA repeat regions in up to three highly variable *P. falciparum* genes: *msp1*, *msp2* and *glurp*.[24,25] The genotyping method is described in the section "Microsatellite genotyping". At each of the sites, the PCR products are compared in two samples by electrophoresis, and if no differences are detected at any of the three sites, the samples are deemed to carry identical parasites. Although only three sites are tested, each presents many repeat length alleles, providing crucial diversity. One disadvantage of this method, beside issue inherent to the genotyping method, is that the resulting genotypes are only useful for this particular purpose. A significant advantage is the low cost of this approach.

Low-resolution Barcodes. This method relies on comparisons of low-resolution barcodes, derived using one of the genotyping methods described in Section "High-throughput Genotyping." The barcodes are compared, and the number of differences is evaluated against the genotyping error rate to determine whether the samples contain the same genome. Ideally, comparison methods should take into account the allele frequencies in the local population. Compared to MSP1/MSP2/GLURP genotyping, this method can handle complex infections by excluding SNPs where multiple alleles are genotyped, and is more robust against missingness.

High-resolution Barcodes. Data from WGS can be used for sample comparison, essentially providing a high-resolution barcode, as described in Section "Whole-Genome Sequencing". The approach to sample comparisons are similar to those described above, with the additional advantage of having many more data points, and local error correction from multiple-read coverage.

Integration with Programme Use Cases

- To detect treatment failures, genetic surveillance systems require mechanisms for linking multiple samples that originate from the same patient regardless of the health facility attended, i.e. Day-0 and Day-Rec samples.
- When a Day-Rec sample is tested for identity against a Day-0 sample, depending on the testing method used, the outcome could either:
    - a classification into "Recrudescence", "Reinfection", or "Undetermined"; or
    - a probability estimate that the recurrence is due to a recrudescence (e.g. probability that the two samples contain at least one parasite genome in common).
- At each sampling site, the probabilities of recrudescence can be combined with the total number of samples to produce a crude estimate of failure rate, i.e. the probability of recrudescence. This will likely be an underestimate since many patients present at different health facilities when recurrences occur. However, these estimates may support comparisons between sites.
- The probabilities of recrudescence may be interpreted as a useful measure of efficacy, when the number of all cases are used as denominator.
- All proportions may be presented on maps, at site, district, province level, using simple colour range representations.

Outputs Storage Requirements

A Recurrence Data Repository may include the following for each case: location, Day-0 sample ID, Day-0 date, Day-Rec sample ID, Day-Rec date, test outcome (reinfection/recrudescence), test outcome details

Sampling Requirements

This use case can be applied at sites that conduct continued sample monitoring of parasite populations, and routinely collect blood samples for genetic analysis, recurrences cannot be predicted, and this use case relies on Day-0 samples being collected. Health facility staff must be able to connect the recurrence to the Day-0 case, and provide sample identifiers.

Prerequisite Data

- Frequencies of the genotyped alleles in the population should be used to determine the likelihood that two different parasites will yield the same genotype by chance. These data should be incorporated in comparison methods, such as barcode-based methods, to determine confidence (not applicable to the MSP1/MSP2/GLURP method)

Data Sharing Requirements

There are no specific data sharing requirements, other than information on recrudescences, specific to any site, should be made available to local as well as national authorities.

Limitations

- This section has been written specifically for *P. falciparum*, and may not apply to other malaria parasite species.
- This use case will produce a lower-bound estimate of the frequency of recrudescences, for various reasons, e.g.: patients returning to a different health facility; not seeking treatment if symptoms are mild; symptoms appearing when patients are at a different geographical location.
- The use case can only work at facilities that keep clear records of previous treatments, that use patient identifiers, and which conduct sample collections routinely.

Current Gaps and Research Needs

There are several areas where research can improve the methods used here, to the benefit of Control Programmes.

- Computational methods yielding confidence levels in result
- Computational methods incorporating corrections for local allelic frequencies
- Computational methods incorporating multiple allele genotypes in complex infections
- Development of standardized high-throughput methods to be implemented in endemic countries

The above are particularly important in the scenario where there has been a loss of diversity in the parasite population, increasing the likelihood that parasites from different infections are genetically similar.

Aspects not covered here

In this section we have only considered *P. falciparum* genetic epidemiology. We highlight that *P. vivax* has an additional level of complexity, in that symptoms may be caused by the activation (relapse) of dormant forms of the parasite (hypnozoites), left in the patient's liver from an earlier infection. Clearly, this complicates this use case further, and requires different test procedures.

G03 - Detect Changes in Population Structure

Purpose

This use case analyzes genomic similarity and variability in parasite populations, to identify patterns that can be associated with epidemiological phenomena, or changes in the structure of population, with the objective of detecting ongoing evolutionary selection. In particular, changes associated with the emergence and spread of drug resistance are of interest to Control Programmes, as are patterns that may be associated with high levels of inbreeding, or involving imported invading strains.

Rationale

Under normal circumstances, malaria parasites are expected to mate randomly ("panmixia"), undergoing sexual recombination in mosquitoes that produces genomic "shuffling" which control diversity within a population. Therefore, in a given *panmictic* population, patterns of parasite diversity are predictable, although they differ in different populations, depending on factors governing diversity, such as transmission intensity and effective population size.

Epidemiological events, such as the evolutionary selection of a particular parasite lineage, can cause a departure from expected patterns. Plainly, in a panmictic population we do not expect to be able to identify large groups of parasites that are more similar to each other than to the rest of the populations: differences between pairs of parasites should be more or less equal within a given population (analogous to, say, human population diversity in a busy metropolitan street). We observe *population structure* when groups of similar parasites can be identified (analogous to what one observes in metropolitan areas where one ethnicity is predominant and not panmictic).[13]

If significant population structure is detected, it can be inferred that the parasite population is undergoing some epidemiological process. For example, strong evolutionary selection of a drug resistant strain makes it more likely that members of this strain (subpopulation) mate with each other, producing an expansion of the subpopulation, a loss of diversity and detectable structure. These patterns may be detected without any knowledge of selection drivers; in other words, monitoring population structure could potentially give early warning of emerging drug resistant strains, even before clinical resistance is detected.[13,26]

There are several techniques for detecting and characterizing population structure, and various methods for leveraging on this knowledge. Amongst these, there are methods for detecting expanding populations; for detecting loss of diversity;[13] for detecting unusually long recombination segments associated with selected haplotypes;[18] for reconstructing ancestry of different parts of the genome.[27,28] These methods typically relate to genetic distance and variation across the genome. Therefore, they rely on the analysis of genome-wide genotypes, and tend to be most informative when working with high-resolution data, such as can be derived from WGS.

Genetic Epidemiology Questions

- Is the parasite population randomly mating, or are there groups that preferentially mate among themselves?
- Is there a rapidly expanding population that can be (or has been) characterized?
- Are there specific genome regions that are under selection in expanding subpopulations?

Flow of Events

1. Use case is triggered when blood samples collected from patients are submitted.
2. The samples are genotyped at a broad range of sites, genome-wide.
3. The genotypes are analyzed by methods supported by the available genotyping resolution.
4. Results are interpreted, and reported textually, or using network diagrams, trees, PCA plots, etc.
5. Use case terminates and results are made available.

Related Use Cases

Programme Use Cases

The following Programme Use Cases may use analysis outputs from the present use case:

- P02 “Monitor Treatment Efficacy and Resistance” can use information on signatures of drug resistance, such as clonal population expansion, detection of founder populations and presence of extended haplotypes, to provide early warning signals of genetic selection, such as those caused by emerging drug resistance.
- P04 “Respond to outbreaks” uses information on population structure change in order to classify the outbreak in terms of its diversity (clonal expansion vs increased transmission).[13]
- P05 “Stratify Interventions” uses information on expanding strains and “founder” populations[26] in order to identify areas where action is required to contain drug resistance.
- P06 “Evaluate Effects of Interventions” uses information on population structure in order to evaluate whether drug resistant strains are being selected as a result of intervention.

Current Approaches

To describe population structure, a range of analytical approaches can yield useful results. The following is a non-exhaustive list of key methods used:

- Frequency Analysis (of Barcodes/Haplotypes)
- Clustering methods, PCA/PCoA
- Phylogenetics
- Haplotype networks
- Ancestry Analysis (STRUCTURE)
- Identity by Descent (Chromosome Painting)
- Comparative analysis of Extended Haplotypes (XP-EHH)

Generally, these methods use genome-wide data, such as genotypes from WGS, although in specific cases it may be interesting to focus analyses to specific loci (e.g. *pfkelch13*). The genome-wide data may have different levels of resolution, e.g. low-resolution barcodes vs. WGS data; only a few of the methods are applicable to low-resolution data. Specifically (see Section “Genetic Barcoding”):

- Low-Resolution Genetic Barcodes. A relatively low number of SNPs allows low-accuracy frequency analysis and clustering, and can be used for the detection of expanding strains.[13] Such panels do not provide sufficient data points to reconstruct ancestry or extended haplotypes.
- High Resolution SNP panels. With thousands of genome-wide variations, sophisticated analyses of population structure can be performed- e.g. methods of ancestry reconstruction based on probabilistic frameworks require a high number of data points. Other methods, such as Chromosome Painting or IBD, analyze ancestry by processing recombination patterns inferred from local haplotypes, and therefore require a high density of SNPs to be genotyped.

Most methods require a representative sample of genomes from each sites (e.g. ~100 samples/site).

Integration with Programme Use Cases

- “Population structure” may not be a concept familiar to Programme officers. Furthermore, population structure analyses produce no simple measures that can be used in visual representations. As a result, results may need presenting in narrative forms, as reports comprising text, network diagrams, plots, trees, and any other useful evidence.
- Likewise, one cannot assume familiarity with population structure methods such as clustering, PCA, phylogeny, ancestry analysis, chromosome painting etc. Any such representation requires careful explanation (either in the form of documentation, or narrative if specific to the analysis).
- Since results might be difficult to interpret for Programme officials who may be epidemiologists but have no specific genetic epidemiology training, they must be accompanied by clear textual

narrative. In particular, all conclusions must be formulated in terms of the impact for control (e.g. “there has been a clonal expansion of a population… in the last 3 months… which may mean that… which requires the following confirmatory tests… “)

Outputs Storage Requirements

Currently, this use case relies upon analyses whose results cannot easily be reduced to single values, and should generally be evaluated qualitatively. Hence, outputs are expected to be delivered primarily as reports of qualitative evaluation of relevant analyses, presented textually, as network diagrams, plots, trees, or other methods of capturing population structure. This may be reviewed, if simpler measures should emerge.

Sampling Requirements

There are no specific requirements.

Prerequisite Data

No specific requirements; however, comparisons may be made with other sites from where compatible data (such as genetic barcode panels) have been genotyped and made available.

Data Sharing Requirements

The data produced by this use case should be made available to all epidemiological analyses. This may include local, national and cross-border analyses. It is therefore recommended that the data be made public.

Limitations

- This use case is only applicable to genome-wide data.
- In areas of high transmission, these analyses may not yield any interesting patterns because of extremely high levels of diversity.
- The methods described here are best applied to low to moderate transmission scenarios, as are common in the GMS.

Current Gaps and Research Needs

More research is needed to extend and improve the methods to the benefit of Control Programmes.

- Methods for evaluating population structure (e.g. can a given level of population structure be explained by normal mating processes, or is selection ongoing?)
- Methods for summarizing and codifying the results.
- Methods for visual display of results.

Aspects not covered here

Generally, in the use case we have mostly considered the case of low to moderate transmission.

G04 - Identify Imported Cases

Purpose

This use case compares an individual parasite sample with baseline population data- including genotypes from the sample's originating population, and from populations at other sites- to determine whether the parasites in the sample are likely to have originated locally, or imported from a different geographical population. Results may either label samples as "imported" or "local", or compute the probability that the parasites are imported; the latter may be more relevant when assessing border regions, where parasite populations on opposite sides of the border may be genetically similar. Programmes are interested in imported cases for multiple reasons: to understand the contribution of foreign migrants to local malaria, to describe gene flow patterns across borders, or for malaria-free certification purposes.

Rationale

Although there is expected to be gene flow between neighbouring populations, causing alleles to propagate through recombination, some parasites are directly imported from a remote location, typically by human movement. The identification of these imported cases is important to Control Programmes for a variety of reasons, especially when the origin of the parasites is across a national border:

- Before malaria-free certification, countries are required to report that all detected cases have a foreign origin, which is currently mostly done by reconstructing the patient's travel history and demonstrating that it is likely the infection was contracted abroad.
- Programmes have to assess whether in border regions there are significant contributions from abroad, e.g. seasonal migrant workers. As a result, interventions can be targetted to specific migrant groups.
- Programmes need to be aware of major gene flow routes, along which drug-resistant strains could be imported.

Genetic data can provide evidence of importation by analyzing genetic similarity with populations at multiple sites. Since local populations are genetically diverse and, under normal circumstances, genetic distance increases gradually as geographical distance increases, it can be difficult to ascertain with any accuracy whether a parasite has originated from a population that has substantial genetic connectedness to the population where it has been sampled. This is analogous to the difficulties in differentiating by genetics humans coming from Savannakhet (Laos) or from Mukdahan (Thailand) on the opposite shore of the Mekong. Still, given sufficient resolution at genomic level, and sufficient separation between parasite populations, it is possible to estimate the probability that the parasite comes from a given population.

Methods for determining the population of origin may include, among others, genetic distance estimation, clustering methods based on genotype similarity, probabilistic frameworks and/or machine learning classification algorithms. Generally, shorter genetic barcodes might only enable discrimination between highly differentiated populations, e.g. distinguish a parasite from Myanmar from a Cambodian one. This level of resolution may be helpful, but sometimes insufficient to answer questions relevant to Programmes. Larger panels of genotypes, such as those obtained from whole-genome sequencing, are more suitable for smaller geographical scales. One compromise is to design genetic barcodes that include SNPs that are differentiated between populations of interest- although such solutions are problem-specific, and may not reveal importation from other populations.

Genetic Epidemiology Questions

- How likely is it that the parasites in this sample originate from a given population?
- How likely is it that the parasites in this sample are not local?

Flow of Events

1. Use case is triggered when a blood samples collected from a patient, whose origin is to be determined, is submitted.
2. The sample is genotyped by means of WGS or high-resolution barcodes.
3. The derived genotypes are analyzed against existing base data from multiple sites.
4. A probability that the sample is local, and/or that it is most likely to originate from a given population, are estimated.
5. Use case terminates and results are made available.

Related Use Cases

Programme Use Cases

The following Programme Use Cases may use analysis outputs from the present use case:

- P04 “Respond to outbreaks” uses results from this use cases in order to establish whether the causes of an outbreak are autochthonous parasites, or imported ones.
- P06 “Evaluate Effects of Interventions” uses information on gene flow in order to evaluate whether interventions have resulted in interrupted gene flow, or whether there has been reintroduction from a different area.
- P07 “Establish Malaria-free Status” uses results from this use case in order to establish possible origin of new parasites and probability that they are imported in a pre- or elimination setting.

Epidemiology Use Cases

This Use Case is dependent on the following use cases:

- G05 “Identify Circulating Strains” to catalogue parasite strains and define how to classify new members of the strain

Current Approaches

Most methods that can be used for this use case require genome-wide genotype data, and a representative sample of genomes from different site (e.g. ~100 samples/site). Although lower-resolution barcodes can be used for coarse resolution of geographical provenance,[29] high-resolution genotype data from WGS (see Section “Whole-Genome Sequencing”) usually provide stronger evidence to differentiate parasites to the level required by Programmes.

There are no “gold standard” methods to address this use case, and it currently requires more research. There is a rather wide range of analytical approaches that can yield useful results. Briefly, we list some of the possible methods:

- Allele Sharing Analysis,
- Genetic Distance Analysis,
- Clustering Analyses (PCA, NJ trees, etc.),
- Machine Learning Classifiers (Decision trees, PLS-DA, Neural Networks, Support Vector Machines).

Integration with Programme Use Cases

- The output from this use case may be nuanced (e.g. expressed in terms of probability rather than by labelling) and could be the subject of incorrect or unjustified interpretation, which may lead to important consequences.
- The sample provenance prediction should accompanied by an estimate of the uncertainty (e.g. “there is 39% probability that this parasite is local, 41% that it has originated from XYZ, and…”). If at all possible, results recipients should be provided guidelines for accepting or rejecting the hypothesis that the sample is imported (e.g. $p < 0.05$), accompanied by a plain text explanation.
- The recipients should be provided with clear information about the data which was used to determine the outcome (e.g. how many populations were compared, when sampled, etc.).

Outputs Storage Requirements

There is no standard output as yet for imported samples analyses. Once these analyses become feasible, a database could be created in which the following would be stored for each sample:

- The sample location and date.
- The probability that the sample is local.
- The foreign parasite population it is most likely to originate from, and the probability it comes from that population or geographical region.

Outputs may also include reports of relevant analyses, presented textually, plots, or other methods.

Sampling Requirements

The sites being compared must be chosen such that the distance between sites is compatible with the resolution of the method.

Prerequisite Data

Comparisons can be made between sites from where equivalent genomic level data has been genotyped and made available. The buildup of a large database of WGS data from multiple sites provides the building blocks for conducting provenance analyses at multiple sites.

Data Sharing Requirements

It is particularly important for this use case that genotype data is made available publicly, to enable cross-border analyses.

Limitations

It is probable that, at sites very close to the border, normal gene flow (e.g. spread by mosquitoes across forested areas) may be an important confounder in this analysis. Hence, realistic expectations for imported sample classification in these areas must be clearly stated.

Current Gaps and Research Needs

More research is needed to extend and improve the methods to the benefit of Control Programmes.

- Methods for incorporating strains and timeline information.
- Methods for classification, and for evaluating results.
- Methods for aggregating results from multiple tests and quantifying the outcome.
- Cloud-based genotyping pipelines.

Aspects not covered here

While this use case only considers *P. falciparum*, WHO malaria elimination certification can only be granted when there is neither *P. falciparum* nor *P. vivax* present.

G05 - Identify Circulating Strains

Purpose

This use case analyzes population structure in surveillance samples, and identifies populations (or "strains") that are deemed to be important for monitoring, e.g. because they are rising in frequency, because they are spreading geographically, or because they carry known drug-resistance mutations.

The identified strains are catalogued and labelled, and tests for assigning a sample to a strain are defined, enabling monitoring through genetic surveillance. The catalogue of strains allows Control Programmes to observe the spread of drug resistance in a methodical and focused fashion.

Rationale

Monitoring the frequency of drug resistance alleles at various sites (see use case G01) can provide important monitoring tools for detecting changes in frequency associated with the spread of resistant parasites. Drug resistance emergence can be multi-focal, i.e. different populations (or "strains" to use a commonly understood term) can develop drug resistance independently- as happened, for example, with the emergence of artemisinin-resistant *pfkelch13* mutants in South East Asia.[15,26,30] Some strains become particularly important, e.g. because they become dominant in a geographical area, or because they spread rapidly- current artemisinin/piperaquine resistant strains circulating around Cambodia are a good example.[31] Naming, cataloguing and monitoring these strains is useful for Programmes and researchers, since it shifts the focus to epidemiological, rather than genetic, entities. Monitoring the spread of specific strains may produce highly informative maps drug resistance.[32]

The word "strain" is used here for convenience, and for the purpose of this document, it is synonymous with "subpopulation"- a cluster of parasites sharing a highly similar common genetic background. The term "strain" has no universally accepted definition, but since it has been popularized by microbiology and virology, it has become a useful term for communicating information about populations of parasites with common genetic variants. We acknowledge that in recombinant organisms such as *Plasmodium* the relationships between "strains" may be more complex than in viruses and bacteria, and thus the analogy has limitations.

Testing for membership to a strain may require additional genotyping. Typical membership tests might detect a specific set of alleles, found to be associated with the strain, in a region of particular interest (e.g. flanking a known drug resistance locus);[33] or show that the region is IBD (identical by descent) with other parasites in that strain. Alternatively, it is also possible to assess whole-genome similarity, a more stringent criterion of membership: if a parasite exceeds a given similarity threshold, when compared across the genome to parasites belonging to a haplogroup/strain, it can be deemed to belong to the same haplogroup.

Genetic Epidemiology Questions

- Are there important strains of parasites whose spread should be monitored?
- What genetic tests must be performed in order to detect parasites that belong to a given strain?

Flow of Events

1. Use case is triggered periodically, when genetic data is analyzed in bulk; or whenever new important strains are characterized in the literature.
2. The genetic dataset is analyzed for the presence of important, emergent or spreading strains.
3. The strains identified are catalogued.
4. Genetic tests for the catalogued strain are specified, either through an in-depth genetic analysis of the results, or from the literature.
5. The master strain catalogue is updated, and the use case terminates.

Related Use Cases

Programme Use Cases

The following Programme Use Cases may use analysis outputs from the present use case:

- P02 “Monitor Treatment Efficacy and Resistance” uses the strain information generated by this use case to monitor resistant lineages known to be spreading in the region.
- P04 “Respond to outbreaks” uses strain information to define if it’s likely that there is a parasite epidemiological driver for the outbreak.

Epidemiology Use Cases

This Use Case is dependent on the following use cases:

- G01 “Monitor Genetic Markers” to estimate the allele frequencies of drug resistant mutations.

Current Approaches

Approaches for the study and detection of new strains generally require some form of population structure analyses. Typical methods include genetic distance analyses, PCA, phylogenetics, probabilistic ancestry analysis, IBD, chromosome painting, etc. In general, these methods require genome-wide genotype data and, although it is possible to perform some analyses with lower-resolution data, WGS data is expected to produce the most accurate results. It is also possible that the strain is identified by a published third-party study, and is catalogued from literature.

Once the strain and its members are identified, a test of membership should be specified. This may require sample genotyping at strain-specific loci. In some of these tests, a new sample is classified as a member of the strain if the genotypes at these sites are identical with those observed in the reference set of samples for the strain. The following technologies are in current usage:

- **SNP typing**: a panel comprising a number of characteristics SNPs is genotyped and analyzed for similarity to the strain’s set of alleles.
- **Microsatellites**: a number of microsatellite sites are identified (e.g. on both flanks of a drug resistance mutation), and are genotyped as described in Section “Microsatellite genotyping”.[30]
- **Genetic Barcodes**: these can be used to assign a sample to a strain by identification, i.e. a high proportion of SNPs in the barcoding panel carry identical alleles to the strain reference.[13]
- **Gene sequencing**: for relatively small haplotypes, it may be possible to perform capillary sequencing of the haplotype surrounding the core mutation (e.g. *pfcrt* 72-76 chloroquine resistant haplotype). The method is described in Section “Capillary Sequencing”.
- **Whole-genome sequencing**: data from WGS, as described in Section “Whole-Genome Sequencing”, can be used to characterize the haplotypes flanking the desired site, and identify loci proximal to the site that differ from other strains. This is the most effective technology for de novo characterization of the loci.

Classifying samples from complex infections presents difficulties because it may be difficult to phase (relate) alleles genotyped at different loci.

Integration with Programme Use Cases

- Important strains (e.g. those carrying resistant mutations) are known to spread across national borders. Therefore, information on notable strains should be made public, and available to all Programmes. This can be achieved with open-access online catalogues. Such catalogues should provide all relevant information about each strain, including genetic identification tests. They should also provide information about the strain’s current spread, in the form of text, or preferably map, e.g. shaded with affected areas, and/or in time series to show spread.
- Programmes should be informed about the presence of important strains in their geographical region, and the extent of their spread. This information, which is derived from the Strain Catalogue (see above) should be included in regular reports to the NMCPs.

Outputs Storage Requirements

A Strain Catalogue should include for each strain, the name, description, literature references, geographical extent, and details of the membership test.

Sampling Requirements

There are no specific requirements.

Prerequisite Data

A threshold can be defined for the number of members needed to identify and catalogue a strain (e.g. 10 samples in a given time period).

Data Sharing Requirements

The data produced by this use case should be made available to all epidemiological analyses of spread, including local, national and cross-border analyses. The Strain Catalogue should be public.

Limitations

The identification and characterization of important strains is a complex task, which may require consensus on parameters and thresholds. There is frequently lack of consensus over what constitute a strain, and what its characteristics are.

Current Gaps and Research Needs

There are several areas where research can improve the methods used here, to the benefit of Control Programmes.

- Development of models and guidelines for characterizing epidemiologically relevant strains.
- Development of visualization of strain extent and spread.

G06 - Describe Gene Flow

Purpose

This use case analyzes samples from sites distributed across the territory, and identifies likely routes of gene flow. This may include the routes of spread of drug resistance mutations, travel paths of specific parasite strains, or more generally, connectedness network representing geographical routes of genetic exchange. The outcome may typically be in the form of maps and/or reports, which may be used by the Programmes to identify gene flow hubs where interventions are desirable, or to evaluate the risk of introduction of resistant strains in given geographical areas.

Rationale

Monitoring allele frequencies at multiple sites can provide important information about the genetic exchange between these sites, and monitoring tools for detecting changes in frequency associated with the spread of resistant parasites. It is known that drug resistance emergence can be multi-focal, i.e. different strains (see use case G05) can develop drug resistance independently- for example, this was the case for the emergence of artemisinin-resistant *pfkelch13* mutants in SE Asia.[26] Once emerged, resistant mutations spread from one site to another, through mosquito and human movement, recombination with local populations, and so on. Monitoring allele frequencies and distributions at multiple sites across a geographical territory may reveal the routes, or at least the directions, in which genes flow, and drug resistance spreads.

In its simplest form, gene flow of parasites can be monitored by observing allele frequency gradients across the territory and their change. Another useful analysis is to estimate genetic differentiation between pairs of sites, based on the assumption that sites well connected by gene flow, will be least differentiated from each other.

Mapping the routes of spread of known parasite strains may add value to connectedness analyses. Populations of selected parasites haven been known to spread, as has happened in and around Cambodia.[31] In these cases, it is desirable to be able to identify parasites belonging to these strains, and map the spread of the strain (see use case G05).

Genetic Epidemiology Questions

- Can sites with the greatest genetic similarity be connected to form gene flow routes?
- Where are the points where interventions are likely to stop the flow of resistant strains?
- What are the directions in which changes in drug resistance frequency are propagating?
- What are the directions in which known parasite strains are propagating?
- Where did strains come from?

Flow of Events

1. Use case is triggered when blood samples collected from patients are submitted to the system.
2. The system evaluates allele frequencies at different sites (see use case G01), producing maps of frequency gradients and/or genetic connectedness.
3. If important circulating strains are identified, strain prevalence at each site can be estimated, and gradient/connectedness maps produced.
4. Use case terminates and results are made available.

Related Use Cases

Programme Use Cases

The following Programme Use Cases may use analysis outputs from the present use case:

- P01 “Select Antimalarial Drug Policy” can use information on gene flow routes to evaluate risks of future increases in failure rates, even in areas that are currently resistance-free (if they are on a route where drug resistance is advancing).

- P02 “Monitor Treatment Efficacy and Resistance” uses strain spread information to map the geographic spread of the resistance strains.
- P05 “Stratify Interventions” uses gene flow route information and maps generated by this use case to identify areas where targeted interventions could interrupt parasite spread.

Epidemiology Use Cases

This Use Case is dependent on the following use cases:

- G01 “Monitor Genetic Markers” estimates allele frequencies of drug resistant mutations.
- G05 “Identify Circulating Strains” to catalogue parasite strains and define how to classify new members of the strain

Current Approaches

Allele Gradients Analysis

Approaches for the estimations of drug resistant allele frequencies are detailed in use case G01. Estimated frequencies can be compared between sites and at different time points.

Site Connectedness Maps

To identify gene flow routes, it is common to compare genetic profiles of populations at different sites, and join sites that exhibit the greatest genetic similarity, based on the assumptions that highly connected sites frequently exchange parasites and therefore have a low degree of differentiation.

A range of analytical approaches can yield useful results, often somewhat different from each other if they analyze different properties to estimate genetic similarity. The following is a non-exhaustive list of key methods used:

- Allele Sharing Analysis
- Comparative Frequency Analysis (F_{ST})
- Genetic Distance Analysis
- Identity by Descent or Chromosome Painting

Generally, connectedness methods analyze genome-wide genotypes, although in specific cases it may be interesting to map allele sharing at specific loci (e.g. *pfkelch13* which is responsible for artemisinin resistance). Data may have different levels of resolution, e.g. low-resolution barcodes vs. WGS data; however, only some of the methods are applicable to low-resolution data (see Section “Genetic Barcoding”):

- Low-Resolution Genetic Barcodes. A relatively low number of SNPs allows some degree of allele sharing estimation, and low-accuracy estimation of genetic distance and differentiation; these may be biased by the choice of SNPs (e.g. the allele frequency of the chosen variations). Such panels do not provide sufficient data points to reconstruct ancestry.
- High-Resolution SNP panels. With thousands of genome-wide variations, sophisticated analyses of population structure can be performed- e.g. methods of ancestry reconstruction based on probabilistic frameworks require a high number of data points. Other methods, such as Chromosome Painting, analyze ancestry by processing recombination patterns inferred from local haplotypes, and therefore require a high density of SNPs to be genotyped.

Most methods require a representative sample of genomes from each site (e.g. ~100 samples/site).

Drug Resistant Strain Tracking

Approaches for the classification of parasite samples into strains are detailed in use case G05.

Principal Component Analysis

One of the pioneering methods for mapping gene flow, rarely used nowadays, is to reduce genetic variance by Principal Component Analysis (PCA) and map in two dimensions the gradients of the most important components.[34]

Integration with Programme Use Cases

This use case can produce multiple outputs that can be interpreted and used by Programmes. There are no universally accepted standards for visualizing these results, which can be depicted in textual or graphical narrative. The following are guidelines capturing useful characteristics of these outputs.

- In analyses of connectedness, it may be appropriate to use maps to show links between sites as networks of lines, fashioned to show relationship strength between sites. For example, lines may be thicker when they join sites that are thought to be linked by strong gene flow. Such visual clues must be clearly explained, and the methods used to rank connectedness made clear.
- Gene flow can be intuitively represented on maps by means of arrows, e.g. lines joining sampling sites may be given an arrowhead. Arrows are appropriate only if analysis results suggest a direction of spread (e.g. differential frequencies and increases over time), or sink/source relationship. The significance of the arrows must be clearly stated, and the methods by which they are derived should be made clear.
- Wherever possible, estimates of timescale should be attached to lines indicating the spread of a specific lineage or mutation. The Programme needs to know how rapidly spread is occurring.
- If sufficient data are available, map gradients may be suitable representations, e.g. as colour shades on a map, contour lines, etc. Colour codes/levels must be clearly explained, and the limitations of methods used (e.g. sparse data, interpolation, etc.) should be made clear.
- In the case of complex relationships between sites, or tracking of specific strains, relatedness networks may benefit from being split into multiple diagrams, or using different base colours to distinguish different “circuits” of connectedness.

Outputs Storage Requirements

This use case produces maps and reports that describe gene flow routes. No specific standard has been identified for these. The maps and reports may focus on

- Differentiation and connectedness between sites
- Prevalence (or change of prevalence) of specific mutations
- Prevalence (or change of prevalence) of parasite strains (see use case G05)

Sampling Requirements

There are no specific requirements.

Prerequisite Data

Strains must be catalogued before strain detection is implemented.

Data Sharing Requirements

Data on strain spread should be made available to all epidemiological analyses of drug resistance spread. This may include local, national and cross-border analyses. It is therefore recommended that the result be made public. Analyses of connectedness between sites may be shared at a more local level; however, cross border connectedness is a critical information for sharing between countries.

Limitations

Tracking of specific strains is only meaningful in regions where these strains are catalogued because they have been found to be relevant (see use case G05).

Current Gaps and Research Needs

There are several areas where research can improve methods used here, to benefit programmes:

- Methods for reliably inferring connectedness
- Methods for producing maps from the results of this use case

G07 - Assess Transmission Intensity

Purpose

This use case analyzes the genetic variability in parasite populations at given locations, in order to characterize transmission intensity at those sites. Transmission can be characterized either quantitatively, by estimating some epidemiological parameters, or qualitatively by comparing variability patterns and inferring underlying transmission dynamics. Identifying locations where transmission intensity is higher (hotspots) can help programmes intervene at sites where interrupting transmission breaks up transmission routes.

Rationale

Analysis of transmission intensity usually relies on the notion that different levels of transmission intensity give rise to different epidemiological patterns. Specifically, in the absence of selection, high transmission is usually characterized by high levels of diversity, since frequent mosquito inoculations produce a high probability of different parasites recombining.

Quantitative analyses of transmission intensity are rather complex, and tends to estimate biological or epidemiological parameters for which accurate standard measurements are rarely available. While this is an area where further research is needed, in the short to medium term Programmes can benefit from more qualitative analyses, such as comparisons of genetic diversity levels. This can be done using medium- and large-scale genotyping platforms, using variant panels covering a representative set of genome-wide sites.

Genetic Epidemiology Questions

- How do different locations compare in terms of transmission intensity?
- Can transmission hotspots be identified, where elimination and/or intervention may interrupt malaria spread?
- Are there seasonal variations in transmission that can be leveraged upon for interventions?

Flow of Events

1. Use case is triggered when blood samples collected from patients are submitted to the system.
2. The samples are genotyped or sequenced across the genome.
3. Analyses of diversity are conducted on parasites from the same site; such analyses may be stratified by season, if enough samples are available.
4. Levels of diversity are identified and described, and inferences on transmission intensity made. This typically will require the comparison of multiple sites.
5. Use case terminates and results are made available.

Related Use Cases

Programme Use Cases

The following Programme Use Cases may use analysis outputs from the present use case:

- P05 “Stratify Interventions” uses information on transmission hotspots in order to identify areas where interventions are likely to interrupt transmission most effectively.
- P06“Evaluate Effects of Interventions” uses information on transmission intensity in order to evaluate whether interventions have resulted in transmission patterns changes.

Current Approaches

Current methods use genome-wide genotype data, which can be at varying levels of resolution, e.g. genetic barcodes vs. whole-genome sequencing. The data are processed by population-level diversity analyses; different approaches can be applied, e.g.:

- Analyses of haplotype diversity and frequency distribution, possibly comparative over time or between sites;[11]
- Analyses of heterozygosity at variable sites, which is expected to be higher when transmission levels are also high, which may also be compared between sites or timepoints.

Methods may trade off resolution for cost and complexity: some use low-resolution barcoding, others high-resolution genotyping from WGS data or SNP microarrays. Generally, the requirement is to have a representative set of whole genomes from a given site (e.g. >100 samples).

Integration with Programme Use Cases

- Although there is a common understanding of what "transmission intensity" means, and there are accepted measures of transmission intensity, such as entomological inoculation rate (EIR) or the more pragmatic annual parasite incidence (API), measures deriving from genetic data do not currently translate directly to these standard measures. Hence, it is possible that programmes may be unfamiliar with the measures reported by genetic analysis methods. It is therefore essential that these measures be carefully explained and contextualized.
- Generally, it is desirable to reduce transmission intensity information to a single value that can be directly compared between sites or over time (e.g. heterozygosity, haplotype entropy etc.).
- Single-value predictions may be presented on maps, per site, using simple colour range representations.
- If the available genetic data produces multiple different measures, these can be presented on separate charts/maps to be compared and contrasted. Clear statements about the implications of differences between these maps could be critical to Programme officers' understanding.
- If diversity analyses yield complex answers (e.g. count of unique haplotypes, plus their frequency distribution), different types of representations may be needed which may be difficult to depict in a map.[11]

Outputs Storage Requirements

This use case produces estimates of measures related to transmission. There are different approaches and measures that may apply, and there may not necessarily be a universal scale against which they can be translated. Thus, they may have different storage requirements, which in general will entail storing a value for each site/timepoint pair; values can then be used in comparative analyses, but typically not across different measures.

Sampling Requirements

Sites are chosen based on the hypothesis that their transmission levels are of interest (e.g. suspected hotspots of transmission). Collections need be continual if seasonal comparisons are needed. Sites are best compared if collections take place in the same period of the year.

Prerequisite Data

No specific requirements; however, comparisons may be made with other sites from where equivalent genomic level data (such as barcodes) has been genotyped and made available.

Data Sharing Requirements

The data produced by this use case should be made available to all analyses of malaria prevalence and transmission intensity. This may include local, national and cross-border analyses. It is therefore recommended that the data be made public.

Limitations

There are still relatively few example of these analyses, and standardized methods have not emerged. As a result, this use case should still be considered a research area.

In areas of high transmission, a relatively small sample size may not yield any meaningful result because of the extremely high levels of diversity. The methods described here are best applied to low to moderate transmission scenarios, as are common in the GMS.

Current Gaps and Research Needs

More research is needed to extend and improve the methods to benefit Programmes.

- Methods for estimating transmission intensity parameters.
- Methods for translating transmission intensity parameters to standard measures (e.g. EIR).
- Methods for producing maps from the results of this use case.
- Criteria for qualitative reporting / evaluation.

Aspects not covered here

Generally, we have only considered the case of low to moderate transmission here. This use case does not cater for the identification of “foci” in areas of interrupted transmission as defined in the WHO framework.

References

1. Manske M, Miotto O, Campino S, et al. Analysis of Plasmodium falciparum diversity in natural infections by deep sequencing. *Nature* 2012; **487**(7407): 375-9.
2. MalariaGEN. Pf7: an open dataset of Plasmodium falciparum genome variation in 20,000 worldwide samples. *Wellcome Open Research* 2022.
3. Jacob CG, Thuy-Nhien N, Mayxay M, et al. Genetic surveillance in the Greater Mekong subregion and South Asia to support malaria control and elimination. *Elife* 2021; **10**.
4. Dalmat R, Naughton B, Kwan-Gett TS, Slyker J, Stuckey EM. Use cases for genetic epidemiology in malaria elimination. *Malar J* 2019; **18**(1): 163.
5. World Health Organization. Strategy for Malaria Elimination in the Greater Mekong Subregion Geneva: World Health Organization, 2015.
6. Miotto O, Imwong M. Genotyping Technologies for Genetic Surveillance of Drug-Resistant Malaria. *International Journal of the Royal Society of Thailand* 2018; **X**: 65-82.
7. Jacobson I, Christerson M, Jonsson P, Overgaard G. Object-Oriented Software Engineering: A Use Case Driven Approach. Boston: Addison-Wesley; 1992.
8. Auburn S, Campino S, Clark TG, et al. An effective method to purify Plasmodium falciparum DNA directly from clinical blood samples for whole genome high-throughput sequencing. *PLoS One* 2011; **6**(7): e22213.
9. Oyola SO, Ariani CV, Hamilton WL, et al. Whole genome sequencing of Plasmodium falciparum from dried blood spots using selective whole genome amplification. *Malar J* 2016; **15**(1): 597.
10. Morris U, Aydin-Schmidt B, Shakely D, et al. Rapid diagnostic tests for molecular surveillance of Plasmodium falciparum malaria -assessment of DNA extraction methods and field applicability. *Malar J* 2013; **12**: 106.
11. Daniels RF, Schaffner SF, Wenger EA, et al. Modeling malaria genomics reveals transmission decline and rebound in Senegal. *Proceedings of the National Academy of Sciences of the United States of America* 2015; **112**(22): 7067-72.
12. Wong W, Volkman S, Daniels R, et al. R (H): a genetic metric for measuring intrahost Plasmodium falciparum relatedness and distinguishing cotransmission from superinfection. *PNAS Nexus* 2022; **1**(4): pgac187.
13. Wasakul V, Disratthakit A, Mayxay M, et al. Malaria outbreak in Laos driven by a selective sweep for Plasmodium falciparum kelch13 R539T mutants: a genetic epidemiology analysis. *Lancet Infect Dis* 2022.
14. Kattenberg JH, Nguyen HV, Nguyen HL, et al. Novel highly-multiplexed AmpliSeq targeted assay for Plasmodium vivax genetic surveillance use cases at multiple geographical scales. *Front Cell Infect Microbiol* 2022; **12**: 953187.
15. Ashley EA, Dhorda M, Fairhurst RM, et al. Spread of artemisinin resistance in Plasmodium falciparum malaria. *N Engl J Med* 2014; **371**(5): 411-23.
16. Lim P, Dek D, Try V, et al. Ex vivo susceptibility of Plasmodium falciparum to antimalarial drugs in western, northern, and eastern Cambodia, 2011-2012: association with molecular markers. *Antimicrob Agents Chemother* 2013; **57**(11): 5277-83.
17. Felger I, Snounou G, Hastings I, Moehrle JJ, Beck H-P. PCR correction strategies for malaria drug trials: updates and clarifications. *The Lancet Infectious Diseases* 2019.
18. Amambua-Ngwa A, Jeffries D, Amato R, et al. Consistent signatures of selection from genomic analysis of pairs of temporal and spatial Plasmodium falciparum populations from The Gambia. *Sci Rep* 2018; **8**(1): 9687.

19. Menegon M, L'Episcopia M, Nurahmed AM, Talha AA, Nour BYM, Severini C. Identification of Plasmodium falciparum isolates lacking histidine-rich protein 2 and 3 in Eritrea. *Infect Genet Evol* 2017; **55**: 131-4.
20. Gamboa D, Ho MF, Bendezu J, et al. A large proportion of P. falciparum isolates in the Amazon region of Peru lack pfhrp2 and pfhrp3: implications for malaria rapid diagnostic tests. *PLoS One* 2010; **5**(1): e8091.
21. World Health Organization. Master protocol for surveillance of pfhrp2/3 deletions and biobanking to support future research. Geneva: World Health Organization; 2020.
22. Lu G, Zhou S, Horstick O, Wang X, Liu Y, Muller O. Malaria outbreaks in China (1990-2013): a systematic review. *Malar J* 2014; **13**: 269.
23. World Health Organization. WHO malaria terminology. 2021 update ed. Geneva: World Health Organization; 2021.
24. Snounou G, Beck HP. The use of PCR genotyping in the assessment of recrudescence or reinfection after antimalarial drug treatment. *Parasitol Today* 1998; **14**(11): 462-7.
25. Medicines for Malaria Venture, World Health Organization. Methods and techniques for clinical trials on antimalarial drug efficacy : genotyping to identify parasite populations : informal consultation organized by the Medicines for Malaria Venture and cosponsored by the World Health Organization, 29-31 May 2007, Amsterdam, The Netherlands. Geneva: World Health Organization; 2008.
26. Miotto O, Almagro-Garcia J, Manske M, et al. Multiple populations of artemisinin-resistant Plasmodium falciparum in Cambodia. *Nat Genet* 2013; **45**(6): 648-55.
27. Kelleher J, Wong Y, Wohns AW, Fadil C, Albers PK, McVean G. Inferring whole-genome histories in large population datasets. *Nat Genet* 2019; **51**(9): 1330-8.
28. Speidel L, Forest M, Shi S, Myers SR. A method for genome-wide genealogy estimation for thousands of samples. *Nat Genet* 2019; **51**(9): 1321-9.
29. Tessema S, Wesolowski A, Chen A, et al. Using parasite genetic and human mobility data to infer local and cross-border malaria connectivity in Southern Africa. *Elife* 2019; **8**.
30. Miotto O, Sekihara M, Tachibana SI, et al. Emergence of artemisinin-resistant Plasmodium falciparum with kelch13 C580Y mutations on the island of New Guinea. *PLoS Pathog* 2020; **16**(12): e1009133.
31. Hamilton WL, Amato R, van der Pluijm RW, et al. Evolution and expansion of multidrug-resistant malaria in southeast Asia: a genomic epidemiology study. *Lancet Infect Dis* 2019; **19**(9): 943-51.
32. Imwong M, Dhorda M, Myo Tun K, et al. Molecular epidemiology of resistance to antimalarial drugs in the Greater Mekong subregion: an observational study. *Lancet Infect Dis* 2020.
33. Amato R, Pearson RD, Almagro-Garcia J, et al. Origins of the current outbreak of multidrug-resistant malaria in southeast Asia: a retrospective genetic study. *Lancet Infect Dis* 2018; **18**(3): 337-45.
34. Menozzi P, Piazza A, Cavalli-Sforza L. Synthetic maps of human gene frequencies in Europeans. *Science* 1978; **201**(4358): 786-92.
35. Green MS, Swartz T, Mayshar E, et al. When is an epidemic an epidemic? *Isr Med Assoc J* 2002; **4**(1): 3-6.
36. Last JM, Abramson JH, International Epidemiological Association. A dictionary of epidemiology. 3rd ed. New York: Oxford University Press; 1995.