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Point Mutation in Surveillance of Drug-Resistant Malaria

Sungano Mharakurwa^{1,2}

¹*Johns Hopkins Bloomberg School of Public Health, Baltimore,*

²*Malaria Institute at Macha,*

¹*USA*

²*Zambia*

1. Introduction

Simple point mutations can confer profound phenotypes on pathogens and parasites of major public health importance, such as malaria. In particular, single nucleotide polymorphisms (SNPs) are known to easily arise, where one base change occurs in the parasite genomic sequence. These SNPs may confer resistance to malaria therapeutic or preventive drugs, or even render other as yet uncharacterized phenotypes of public health concern, such as virulence. Modern advances in parasite molecular biology and genomics allow the typing of parasites with a range of levels of detail. Particular SNPs or combinations of these are identified which are associated with resistance, followed by confirmation through genetic cross experiments. Using simple polymerase chain reaction (PCR) techniques, the point mutations are now increasingly applied as molecular markers for tracking and containment of resistant malaria. These marker point mutations are particularly instrumental surveillance tools as they enable efficient detection of drug-resistant parasites or pathogens before escalation to a public health toll.

1.1 Drug resistance and malaria control

Malaria persists as a major global public health problem, affecting more than 100 countries in Africa, Asia, the South Pacific region, Latin America and the Indian sub-continent, as well as a vast assemblage of non-immune travellers continually visiting such areas. Notwithstanding substantial technological advances, the old scourge currently claims a global toll of 225 million cases and 781,000 deaths every year, mostly children in Africa [1,2]. This has been a relative improvement from 500 million cases and at least 2.3 million annual deaths that were occurring as recently as early-mid 2000s [3,4], with an escalating trend in overall burden of the disease. Thanks to the Roll Back Malaria programme, the President's Malaria Initiative and other public-private sector initiatives, and notably the Bill and Melinda Gates Foundation, endemic countries have scaled up vector control and artemisinin-based combination therapy (ACT) interventions against malaria [5,6], resulting in widespread reduction of burden [7,8]. A number of countries have presently been earmarked for possible local or regional malaria elimination [9,10], including those in southern Africa, which are located towards the natural fringes of transmission. However,

the resilient scourge prevails in significant segments of resident communities as asymptomatic[11] and often low-grade, sub-microscopic[12] infections.

Recent epidemiological studies have shown a striking link between availability of effective treatment drugs and historical successes in major reduction of the burden of malaria throughout the world[13,14]. Correspondingly, the emergence of antimalarial drug resistance, especially in *Plasmodium falciparum*, has proved a major obstacle for malaria control and elimination efforts since the eradication campaigns of the 1950s[15,16,17,18,19]. Drug resistance in *P. ovale* and *P. malariae* has not been documented, while in *P. vivax* the phenomenon, has been recently increasing[20,21,22].

The potential of the malaria parasite to generate genetic diversity through its complex genome and a proliferative life cycle involving sexual and asexual stages, means that drug resistance will remain a problem to contend with, escalating in the wake of wider introduction of each antimalarial. *P. falciparum* has repeatedly demonstrated this adaptation against virtually all antimalarial drugs[23,24,25], including the new regimens with or without artemisinin[26,27,28,29]. To minimize suffering and mortality due to use of drug regimens which are no longer effective, the epidemiology of drug resistance in malaria endemic regions needs continual monitoring[6].

1.2 Definition of drug resistance

Because of the immense amount of work that has gone into the subject, a number of related terms have evolved around “drug resistance”, with distinguishable applications, depending on the objectives of the characterization and methods used. These are summarized as follows, for clarity.

1.2.1 Classical definition of drug resistance

Antimalarial drug resistance has been defined as “the ability of a parasite strain to survive and (or) multiply despite administration and absorption of the usually recommended, or slightly higher doses of a drug, within limits of tolerance of the host”[30,31]. This original definition generally holds for most drugs, but in the light of the pharmacokinetic characteristics of some antimalarials, a qualification was subsequently added, that “the form of the active drug against the parasite must gain access to the parasite, or the infected erythrocyte, for the duration of time necessary for its normal action”[32]. The qualification was added to cater for drug bioavailability and narrow down the reason of drug failure to parasite resistance. This definition of drug resistance therefore, in the strict sense, attempts to centre on the response of a parasite to an antimalarial, excluding host factors. Resistance has been assayed by *in vitro* methods, primarily based on the system developed by Rieckman *et al*[33]. *In vivo* methods[19,31] have also been used, but discrepancies with *in vitro* findings have been common because host factors, especially immunity, cannot be entirely precluded *in vivo*.

1.2.2 Therapeutic failure concept

For public health purposes, the importance and relevance of *in vivo* resistance has been increasingly recognized in drug policy decisions[34,35] since it is net parasite response to

antimalarial medication in the human host that is paramount. However, *in vivo* resistance can, and indeed has, still occurred without much clinical relevance in the human population of interest, which may harbour resistant parasitaemias asymptotically, especially in endemic areas[36,37]. Over time *in vivo* approaches have therefore been progressively modified to assess the parasitological and clinical response to antimalarial drugs[38,39,40]. These approaches measure the therapeutic efficacy of antimalarials and the corresponding parasitological and clinical resistance, which is termed therapeutic failure. Drug resistance *per se* may or may not lead to therapeutic failure, depending on malaria endemicity and concomitant level of immunity in the resident populations.

1.2.3 Treatment failure

Under routine operational conditions in primary health care, resistance parasitologically and (or) clinically is frequently encountered. It is usually not feasible in these situations to completely supervise drug administration and confirm bioabsorption. The apparent resistance is termed treatment failure, and may be caused by true drug resistance, malabsorption or incomplete dose compliance by outpatients.

1.2.4 Summary

Albeit often confused and interchangeably used, the foregoing terms have distinct applications. In this chapter drug resistance refers to the original sense, based on parasite response as ascertained *in vivo* or *in vitro*. Where reference to the other conditions is made the appropriate terms shall be used.

2. Armamentarium of current antimalarials and mode of action

Despite a seemingly extensive list of potent compounds, the net armamentarium of effective, safe and usable antimalarials is limited, relative to the epidemiological magnitude and evolutionary potential of malaria parasites. Owing to the advent of drug resistance, there is a need to develop more antimalarials. The antimalarial drugs in current use can in effect be grouped into blood schizontocides and antimetabolites of the folate pathway.

2.1 Blood schizontocides

Blood schizontocides act directly on intraerythrocytic stages responsible for symptoms and tend to be fast acting compounds usually preferred for treatment of acute disease. They include: aryl amino alcohols; 4-aminopyridine analogues; and the more recently widely introduced artemisinin and derivatives. Compounds in the first two groups are often referred to as quinoline-containing drugs (QCD's).

The aryl amino alcohols include the old cinchona alkaloid drug, quinine; the quinolinemethanol, mefloquine; and the phenanthrenemethanol drug, halofantrine. Quinine has been used for treatment and, in some cases prophylaxis, since the 17th century, as crude cinchona (Peruvian) bark, and from the 19th century as the pure drug. In contrast, halofantrine is a relatively new compound, which was first registered in France and francophone African countries in 1988. The 4-aminopyridine analogues include amopyraquine, mepacrine and the 4-aminoquinolines: chloroquine and amodiaquine. The 4-

aminoquinolines were introduced among the first generation of synthetic antimalarials in the late 1940's, following the search for safer compounds, for use both as treatment and prophylaxis. Artemisinin and derivatives include the parent compound artemisinin, a sesquiterpene lactone which constitutes the active principle of the Chinese herb *qinghaosu*, and its derivatives. The Chinese herb was used for hundreds of years to cure malarial fevers, but only recently purified and introduced on a wider scale for treatment of multidrug-resistant malaria. Water and oil-soluble derivatives of artemisinin were subsequently developed which include the salt, sodium (or potassium) artesunate; the methyl ether derivative, artemether; and the ethyl ether derivative, arteether.

Blood schizontocides are believed to target the haemoglobin digestion and excretion process in the parasite food vacuole. Chloroquine, one of the 4-aminoquinolines most extensively studied, is thought to act by inhibiting polymerization of the parasite's haemoglobin digestion by-product, haem (ferriprotoporphyrin IX), which is toxic to the parasite if allowed to accumulate[41]. The non-toxic polymer that the parasite generates in the absence of inhibiting drug is haemozoin, or malaria pigment. Artemisinin and its derivatives have a unique endoperoxide bridge that is thought to undergo reactive cleavage by ferriprotoporphyrin IX. This generates free carbon-centred radicals that alkylate biomolecules and kill the parasite cells.

2.2 Antimetabolites of the folate pathway

Antimetabolites of the folate pathway (often referred to as antifolates) tend to attack all growing stages of the malaria parasites, including the early growing stages in the liver (causal prophylactic effect) and developing infective stages in the mosquito (antispogonic effect). Like 4-aminoquinolines, the antimetabolites were also introduced in the late 1940's. They include the dihydrofolate reductase (DHFR) inhibitors (pyrimethamine, trimethoprim, cycloguanil and chlorcycloguanil) and dihydropteroate synthetase (DHPS) inhibitors or sulfa drugs (sulfadoxine, sulfalene, dapsone, sulfamethoxazole). Antifolates are usually used as combinations, e.g. sulfadoxine/pyrimethamine, sulfalene/pyrimethamine, or dapsone/pyrimethamine (prophylaxis).

The mode of action of antifolate drugs is among the most well understood. Malaria parasites synthesize folates during biosynthesis of pyrimidines which they cannot scavenge from the host. Antifolate drugs block two sequential steps within the folate synthesis pathway, eventually leading to (i) decreased pyrimidine synthesis and arrest of DNA replication; (ii) decreased methionine and serine production; and (iii) ultimate cell cycle arrest and death of the parasites. Dihydrofolate reductase inhibitors act by competing with substrate for the enzyme dihydrofolate reductase, while sulfa drugs are paraminobenzoic acid (PABA) analogues which competitively inhibit dihydropteroate synthetase in the preceding reaction.

3. Mechanisms of drug resistance

3.1 Quinoline-containing drugs

3.1.1 Group I blood schizontocides

Group I blood schizontocides are the dibasic QCD's, mainly the 4-aminoquinolines.

The mechanism of resistance to these drugs is due not to altered drug target, but to reduced drug accumulation in the parasite food vacuole[42]. Resistant parasites survive by accumulation of less drug than their sensitive counterparts. How the low drug levels are accumulated in resistant parasites is still not fully understood. A drug efflux mechanism has been proposed [43,44], and so has reduced drug uptake[45,46].

Enhanced Drug Efflux

Chloroquine-resistant isolates are known to expel intracellular chloroquine 40-50 times as rapidly as chloroquine-sensitive strains[44]. This process is energy-dependent and susceptible to ATP blockage[43]. Compelling evidence for a drug efflux mechanism has been the demonstration of reversal of chloroquine resistance by calcium channel blockers such as verapamil as well as some tricyclic compounds such as desipramine, *in vitro* and *in vivo*[47,48,49]. Reversal of chloroquine resistance by verapamil is independent of the weak base effect and is specific to resistant strains[50]. Although there were conflicting findings among earlier *in vivo* studies[51], subsequent work has abundantly illustrated chloroquine resistance reversal in malaria patients, with the antihistaminic agent chlorpheniramine[52,53], and with promethazine[54]. The observations on reversal of chloroquine resistance by calcium channel blockers have led to the theory that a similar system to the multidrug resistance (*mdr*) phenomenon in mammalian cancer cells[55] is responsible. The *mdr* phenomenon in mammalian cancer cells is mediated by an ATP-dependent transporter, P-glycoprotein, which resides on the surface of the cell and has affinity for a wide range of compounds, including anti-cancer drugs which it actively expels. The mammalian *mdr* phenotype is reversed by a broad range of compounds that compete for affinity for the P-glycoprotein, including calcium channel blockers like verapamil, calmodulin antagonists and other compounds. P-glycoprotein homologues were subsequently isolated in *Plasmodium falciparum*, pfPgh1 and pfPgh2[56] encoded by the genes *Pfmdr1* and *Pfmdr2*.

These genes have shown partial association with chloroquine resistance controversy, with some workers reporting no association between *Pfmdr1* and chloroquine resistance[57], although the chloroquine efflux trait genetically segregated as a single locus[58]. Others have reported amino acid changes in *Pfmdr1* linked with chloroquine resistance[59], including the single substitution N86Y (the K1 allele), and four substitutions Y184P, N1032D, S1034C and D1246Y (7G8 allele). However, the most convincing demonstration for the role of *Pfmdr1* has come from recent allelic exchange studies which show that replacement of the wild type allele in chloroquine-sensitive strains with resistant alleles resulted in decreased drug sensitivity[60]. There is evidence that more than one gene is involved in coding for the chloroquine resistance phenotype.

The *P. falciparum* Chloroquine Resistance Transporter Gene (*Pfcrt*)

Through transfection and allelic exchange experiments between resistant and sensitive parasites a chloroquine resistance transporter (*Pfcrt*) gene was identified, which exhibited polymorphisms that matched the chloroquine resistance phenotype without exception. An *in vivo* study in Mali demonstrated close association between *Pfcrt* and chloroquine therapeutic failure[61].

3.1.2 Group II blood schizontocides

These are monobasic quinoline containing drugs (QCD's), which include quinine, mefloquine and halofantrine. Although their mode of action is thought to be essentially similar to that of the chloroquine group in interfering with haemoglobin digestion[62], the mechanism of resistance to these antimalarials is not clearly understood.

Increased drug efflux as seen with chloroquine-resistant *P. falciparum*, has not been demonstrated with the group II blood schizontocides. Moreover, it is believed unlikely that group II schizontocides, which are monobasic, can accumulate sufficiently in food vacuoles to reach levels required to inhibit haem polymerization[42], yet at least some of these drugs are more potent inhibitors of parasite growth than chloroquine. In addition, resistance to mefloquine has been associated with *Pfmdr1* amplification and cross-resistance to quinine and halofantrine, but decreased resistance to chloroquine[63].

3.2 Resistance to antifolate drugs

Antifolates include dihydrofolate reductase (DHFR) inhibitors (pyrimethamine, trimethoprim, cycloguanil, chlorcycloguanil) and dihydropteroate synthetase (DHPS) inhibitors (sulfones and sulfonamides, e.g. sulfadoxine, sulfamethoxazole, dapsone). These drugs were also among the first generation of synthetic antimalarials introduced in late 1940's [64]. Combinations of pyrimethamine and sulfonamides have been used as second line medication to treat chloroquine-resistant *P. falciparum* infections since the mid-1960's. Prophylactic usage was terminated owing to high risk of adverse reactions, except with pyrimethamine alone or dapsone/pyrimethamine which remains an alternative prophylactic used in a few countries.

Resistance to pyrimethamine became widespread in large areas soon after introduction of the drug, but the potentiating combination with sulfonamides retained effectiveness for longer. Resistance to the antifolate combinations initially became a problem in Indochina and South America in early 1980's. Pyrimethamine/sulfonamide combinations lost therapeutic adequacy in wide areas of South East Asia, western Oceania and South America and although they are still relatively effective in Africa, resistance is on the increase. Like chloroquine, antifolates have remained useful for relatively longer in Africa compared to Asia owing to substantial host immunity reducing the levels of *in vivo* therapeutic failure.

3.2.1 Mechanism of resistance to antifolate drugs

Unlike resistance to chloroquine and other quinoline containing drugs, the mechanism of antifolate drug resistance is directly related to the mode of drug action. The mechanism is also relatively well understood. Resistance to antifolates is known to be due to alterations in target enzymes which reduce drug binding affinity[65,66]. These structural alterations are caused by point mutations in the dihydrofolate/thymidylate synthetase (DHFR-TS) gene, for DHFR inhibitors[67,68] or in the dihydro-6-hydroxymethylpterin pyrophosphokinase-dihydropteroate synthetase (PPPK-DHPS) gene, for DHPS inhibitors[69,70].

Antifolate Resistance Point Mutations in the DHFR-TS gene

Point mutations conferring resistance to DHFR inhibitors have been well described for pyrimethamine and cycloguanil. A single point mutation (from wild type serine to asparagine) at amino acid position 108 in the DHFR domain of the DHFR-TS gene is associated with resistance to pyrimethamine, and only marginal decrease in susceptibility to cycloguanil. Additional mutations N51I and C59R have been associated with high levels of pyrimethamine resistance when they occur in combination with the S108N mutation. A mutation S108T, coupled with the mutation A16V, confers resistance to cycloguanil, with marginal effect on pyrimethamine sensitivity[71,72]. Cross-resistance to pyrimethamine and cycloguanil is conferred by a combination of mutations S108N and I164L, and is even heightened when the C59R mutation is also present.

Polymerase chain reaction (PCR) based assays have been developed, to detect pyrimethamine and cycloguanil resistant parasites and their correlation with standard *in vitro* or *in vivo* drug response profiles have been explored[73,74,75].

Antifolate Resistance Mutations in the PPPK-DHPS gene

Mutations conferring resistance to DHPS inhibitors have been also characterized, especially for sulfadoxine and sulfamethoxazole, frequently used in synergistic combinations with DHFR inhibitors. Point mutations at positions 436, 437, 540, 581 and 613 of the DHPS domain of the PPPK-DHPS gene have been implicated[69], with those at positions 436, 437, and 581 being reportedly the most common. As with the DHFR inhibitors, a corresponding PCR-based assay for detecting PPPK-DHPS mutations conferring resistance to sulfa drugs (sulfadoxine and sulfamethoxazole) has been developed.

4. Point mutations as molecular markers for drug-resistance

The gold standard approach for monitoring drug resistance is by performing *in vivo* antimalarial drug therapeutic efficacy assessments[35,40]. A complex protocol is implemented, where a sentinel group of malaria patients, mostly aged 5 years or less, is subjected to supervised drug treatment and followed up for periods usually lasting 28 days. Parasitological and clinical response to treatment is monitored during follow-up to ascertain whether clearance takes place within the stipulated time consistent with adequate drug efficacy. To distinguish persistent or recrudescence parasitaemia from reinfections, pre- and post-treatment samples must be subjected to genotyping using polymorphic markers, usually *P. falciparum* MSP1, MSP2 and GLURP genes. This approach is labour-intensive and costly, often with losses to follow-up, while some of the patients do not meet eligibility criteria at recruitment. Accrual of significant sample sizes on which to base incisive policy conclusions is therefore sometimes constrained, especially as malaria cases decrease.

Needless to say, the molecular detection of point mutations that confer drug resistance has proved a highly efficient approach for tracking and containment of drug resistant malaria. To date these molecular markers for drug resistance are best characterized for chloroquine and antifolate drugs (Table 1). Furthermore, the nature and combination of mutations that correlate most closely with clinical failure are characterized by standardizing against the *in vivo* assessment gold standard, making for highly instrumental surveillance tools. In antifolate resistance, combinations point mutations (double, triple, quadruple quintuple mutants) in the parasite DHFR and DHPS genes confer increasing levels of resistance. Research is under way to characterize markers for the other major antimalarials in use.

| Gene | Molecular Marker (Point Mutation) | Antimalarial Drug |
|----------|--------------------------------------------|--------------------------------------------------|
| PfCRT | K76T | Chloroquine |
| DHFR-TS | S108N, S108T, N51I, C59R, A16V, I164L | DHFR inhibitors pyrimethamine, cycloguanil |
| PPK-DHPS | S436A(F/C), A437G, L540E, A581G, A613S(T) | DHPS inhibitors Sulfadoxine |

Table 1. Point mutations widely used to track drug-resistant malaria

With molecular markers, resistant parasites can now be monitored both in the human and vector hosts, enabling containment long before escalation to a public health toll (Figure 1).

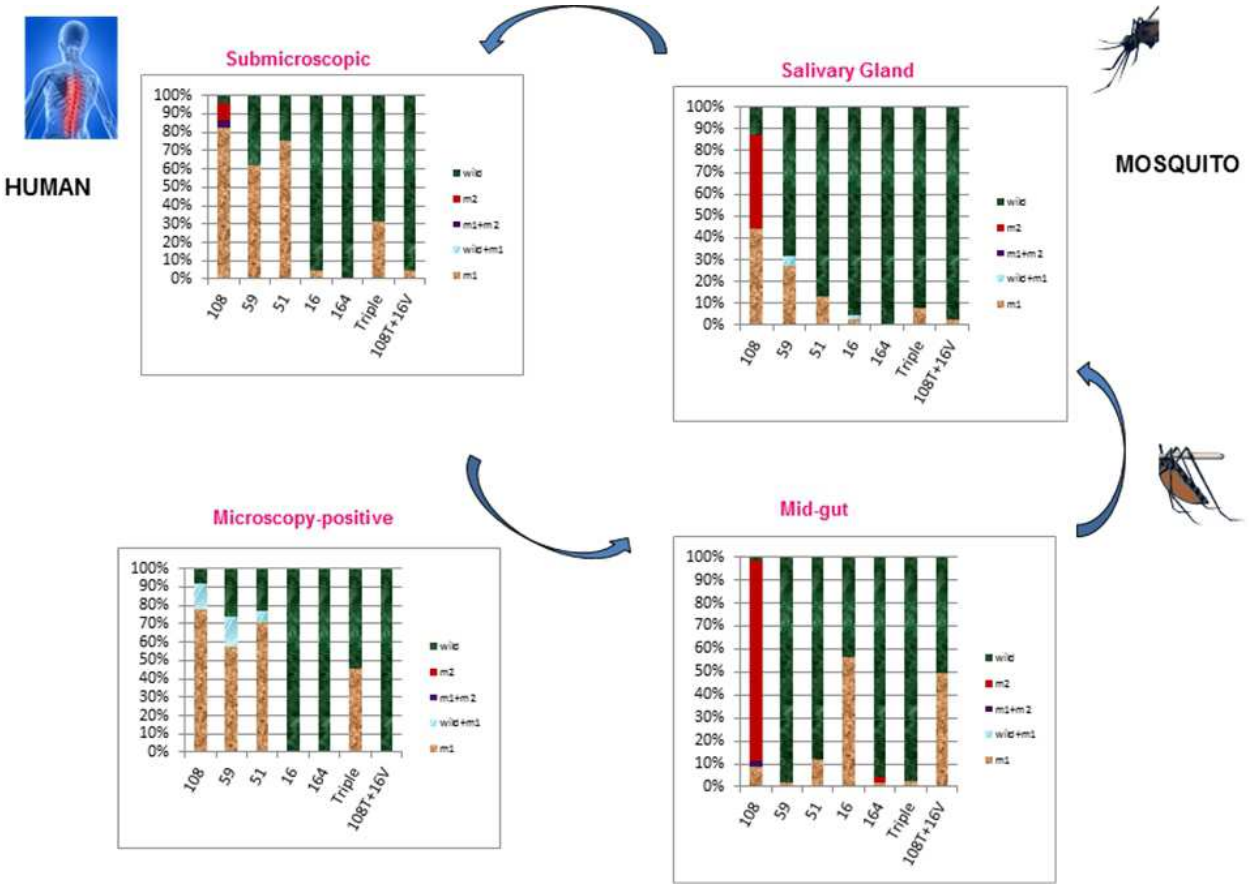


Fig. 1. Distribution of *P. falciparum* antifolate drug resistance alleles in human and mosquito hosts. Note major differences reflecting drug and immune selection. Using point mutations as molecular markers, drug resistance can be tracked in any phase of the parasite, enabling pre-emptive interventions before patient clinical failures set in.

4.1 Parasite bar-coding

Recently, SNPs located in numerous sections across the parasite genome have been applied as a potent tool to finger-print or bar-code malaria infections. This enables sensitive detection of changes in population structure or epidemiology of malaria in a given area in relation to interventions or re-invasion.

A typical bar-code[76] comprises 20-24 SNPs from across all the chromosomes of the malaria parasite, enabling medium-high resolution typing of malarial infections.

5. Conclusion

P. falciparum drug resistance persists as a key strategy by which malaria frustrates control and elimination efforts. The complex genomic blueprint of *P. falciparum* has repeatedly proven its ability to overcome any widely introduced antimalarial with relative ease. It is therefore imperative to develop and implement not only new antimalarial drugs, but also effective strategies for the surveillance and containment of drug resistance. Combination therapy is now stipulated as standard treatment for malaria by WHO, where constituent drugs protect each other from resistance development and the chances of resistance to both are less likely to occur. Artemisinin-based combination therapy (ACT) is preferred, containing artemisinin or its derivative as one of the partner drugs, since it is fast-acting and shows signs of relatively slow resistance development.

Simple point mutations can confer resistance to most antimalarial drugs. In the diverse and dynamic epidemiology of malaria, point mutations are increasingly adopted as efficient molecular markers for surveillance of drug resistant malaria in endemic countries. These molecular markers are readily standardizable and independent of host immune status, drug history or other confounding factors incorporated in the strict eligibility criteria for gold standard *in vivo* drug efficacy assessments. They are also less laborious and require much less time to perform once standardized against the gold standard *in vivo* assessments. Well characterized molecular markers for resistance to chloroquine and antifolate drugs have become standard surveillance tools, with global data being fed into world resistance monitoring programmes such as the World-wide Antimalarial Resistance Network (WWARN).

In recent times point mutations are also being utilized to identify malaria strains and detect changes in population structure during interventions or resurgences by bar-coding.

Furthermore, molecular markers for resistance to other major antimalarial drugs are being actively sought, placing the simple point mutation in good stead to be the future of surveillance in malaria and other diseases.

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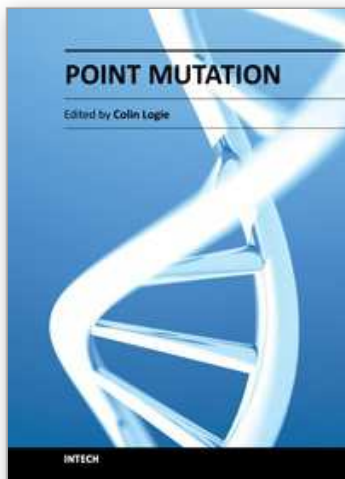
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This book concerns the signatures left behind in chromosomes by the forces that drive DNA code evolution in the form of DNA nucleotide substitutions. Since the genetic code predetermines the molecular basis of life, it could have been about any aspect of biology. As it happens, it is largely about recent adaptation of pathogens and their human host. Nine chapters are medically oriented, two are bioinformatics-oriented and one is technological, describing the state of the art in synthetic point mutagenesis. What stands out in this book is the increasing rate at which DNA data has been amassed in the course of the past decade and how knowledge in this vibrant research field is currently being translated in the medical world.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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