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Antimalarial Drug Resistance: In the Past, Current Status and Future Perspectives

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Abstract: The aim of this study was to review the antimalarial drug treatment and its resistance during the course of therapy. Malaria affects the populations of tropical and subtropical areas world-wide, as well as an increasing number of travellers to these areas. Although malaria is found in over 100 countries, the major burden of disease is carried by the nations of Africa, where over 90% of all deaths from falciparum malaria are recorded and where the high levels of morbidity and transmission place considerable strains on public health services and economic infrastructure. In the absence of effective vaccines, management of the disease has depended largely upon chemotherapy and chemoprophylaxis. The number of available and effective antimalarial drugs is quickly dwindling due to different cause. This is mainly because a number of drug resistance-associated mutations in malaria parasite genes, such as *crt*, *mdr*1, *dhfr*/*dhps* and others, have led to widespread resistance to all known classes of antimalarial compounds. Unfortunately, malaria parasites have started to exhibit some level of resistance in Southeast Asia even to the most recently introduced class of drugs, artemisinins. Molecular evolutionary and population genetic approaches will greatly facilitate our understanding of the evolution and spread of parasite drug resistance and will contribute to developing strategies for better control of malaria. While there is much need, the antimalarial drug development pipeline remains woefully thin, with little chemical diversity and there is currently no alternative to the precious artemisinins. In general, four basic methods have been routinely used to study or measure antimalarial drug resistance: *in vivo*, *in vitro*, animal model studies and molecular characterization. This review endeavors to present the background facts and treatment and more on the current status of antimalarial drug resistance, what their causes, mechanisms, spread and management are and future perspective with antimalarial treatment. Furthermore, findings from this review will be useful to clinical researchers, health planners, policy makers and stakeholders and others concerned for malaria patients.

Keywords: Detection of resistance, drug resistance, malaria, mutation, *p. falciparum*, *p. vivax*

INTRODUCTION

Malaria is a systemic disease caused by infection of the red blood cells with intracellular protozoan parasites of the genus *Plasmodium* (Charles *et al*., 2000; Bosman *et al*., 2001). It is primarily caused by four species of the protozoan parasite *Plasmodium*: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, which are transmitted by over 70 species of Anopheles mosquitoes. These parasitic species occur sympatrically both in human populations and within infected individuals, with *P. falciparum* and *P. vivax* being the predominant species (Grimberg and Mehlotra, 2011).

Currently, a fifth, *P. knowlesi*, zoonosis, is diagnosed by microscopy and represents a small percentage of infections. It infects mainly nonhuman primates but was recently found also to infect humans, but it has not been established whether human-mosquito-human transmission can occur. It has shorter life cycle of replication than any of the other human malarias and can cause rapidly progressive and fatal disease if not treated promptly and other malaria species infect other mammals, reptiles and birds. Because it is responsible for most severe malaria disease and deaths, *P. falciparum* has been the target of most vaccine development efforts (Thera and Plowe, 2012).

P. falciparum is a virulent parasite. It has been estimated to cause approximately 500 million clinical cases (Snow *et al*., 2005) and kill approximately one million individuals (Murray *et al.*, 2012), every year, making it the leading cause of death in children under the age of five in Africa (Mathers *et al*., 2006). *P. vivax* has a considerably lower mortality rate but a far greater geographical distribution. It causes widespread malaria outside Africa, mainly affecting Asia and the Americas (Price *et al*., 2007).

Malaria is estimated to kill nearly one million people annually, with most of the deaths occurring in children under 5 years of age in sub-Saharan Africa. Additionally, pregnant women and newborns have reduced immunity and therefore are vulnerable to severe complications of malaria infection and disease (Bosman *et al*., 2001). Approximately half the world's

population lives in areas at risk of malaria transmission. WHO estimated that in 2010 malaria caused approximately 216 million clinical episodes and 655,000 deaths. Of these malaria deaths, 86% were children under 5 years of age and 91% occurred in sub-Saharan Africa (WHO, 2011).

In Ethiopia, it is estimated that three-fourths of the land below 2000 meters is malarious with two-thirds of the country's population at risk. This makes malaria the number one health problem in Ethiopia with an average of 5 million cases a year and 9.5 million cases per year between 2001 and 2005. The disease causes 70,000 deaths each year (Adugna, 2009).

The number of malaria-related deaths is increasing and one key factor linked to this, is widespread drug resistance to most of the commonly available antimalarial drugs (Trape *et al*., 1998; Eyasu *et al*., 2013). The emergence and spread of *Plasmodium falciparum* resistance to antimalarial drugs is now one of the greatest challenges facing the global effort to control malaria (Yusuf *et al*., 2010). The main strategy for the reduction of malaria-related morbidity and mortality in Africa is early diagnosis and institution of effective treatment. The evolution of drug-resistant pathogens is a major obstacle to this strategy in the fight to control infectious diseases. Malaria parasites are a prime example of this: resistance has evolved to nearly every antimalarial drug in use (WHO, 1993, 2010).

Most countries lack adequate and comprehensive information on antimalarial drug efficacy and this causes chemoprophylaxis and treatment of malaria to be more compromised in those resource poor-settings, resulting in sub-optimal antimalarial treatment policies (Plowe, 2003). This review provides an overview on the current drug management of malaria, the antimalarial drug resistance in the past, the current status and its future perspectives. Further, it also describes drug resistance detection methods, biomarkers, factors contributing to the emergence and spread of resistances and finally discusses strategies and preventive measures necessary to overcome the spread of drug resistant malaria.

MATERIALS AND METHODS

Search strategy and selection criteria: In brief, I searched for reports published in databases of PubMed, Medline, Embase, Web of Knowledge, Scopus and the World Health Organization's WHOLIS. I used search terms including "antimalarial drug treatment", "antimalarial drug resistance", "malaria", "*P. falciparum* drug resistance biomarkers", "*P. vivax* resistance biomarkers", "detection of resistance", "spread of resistance", "prevention strategy for emergence of resistance" and "antimalarial drug efficacy" to identify reports that included data for antimalarial drug resistance in the past, current status and future perspectives, diagnostic tools for drug resistance and the causes, mechanisms of drug resistance and how to prevent drug resistance. Full-text articles were read to evaluate how antimalarial drug resistance and related issues are ongoing. I selected important published literatures that add value to the title and categorized them according to the review components.

RESULTS AND DISCUSSION

Antimalarial drugs: Important attributes for the successful implementation of antimalarial drugs are good tolerability and safety (especially in young children), affordability, availability in endemic countries and short course regimens (Petersen *et al*., 2011).

Treatment of sick individuals using correct dose of antimalarial drugs interrupted the life cycle of the parasite (Fig. 1). Early treatment of cases also reduces transmission by reducing the opportunities for mosquitoes to become infected. However, there are only a limited number of antimalarial drugs which can be used to treat or prevent malaria and *falciparum* malarial parasites develop resistance to almost all antimalarial drugs (Pillay, 2006).

Antimalarial drugs (Fig. 2) fall into groups. The first are quinoline based antimalarials, which includes quinine and its derivatives chloroquine (CQ), amodiaquine, primaquine and mefloquine. Quinine has been used for more than three centuries and was the only effective agent for the treatment of malaria until the 1930`s. Due to its undesirable side effect it is now mainly used as an intravenous injection to treat severe malaria (WHO, 2001).

Since the 1940s, mass-produced, inexpensive drugs have been available that could effectively treat individuals. However, the evolution of drug-resistance has repeatedly occurred, diminishing the therapeutic efficacy of drugs. The ability of the malaria parasite to quickly develop resistance to therapeutics is the result of its complex life-history (Klein, 2012).

The best compound chloroquine was discovered in the 1940s, is a synthetic 4- aminoquinoline produced as less toxic and well tolerable effective antimalarial drug structurally similar to quinine. Since this drug was cheap, non-toxic and effective against all strains of the parasite, became the mainstay of prevention, until resistance was developed by *P. falciparum* to an extent that chloroquine has been rendered virtually useless in most endemic areas (Talisuna *et al*., 2004). The second class of common antimalarial is the antifolate compounds that inhibit the synthesis of parasite pyrimidines and thus of parasitic Deoxyribonucleic Acid (DNA) (Robert *et al*., 2001).

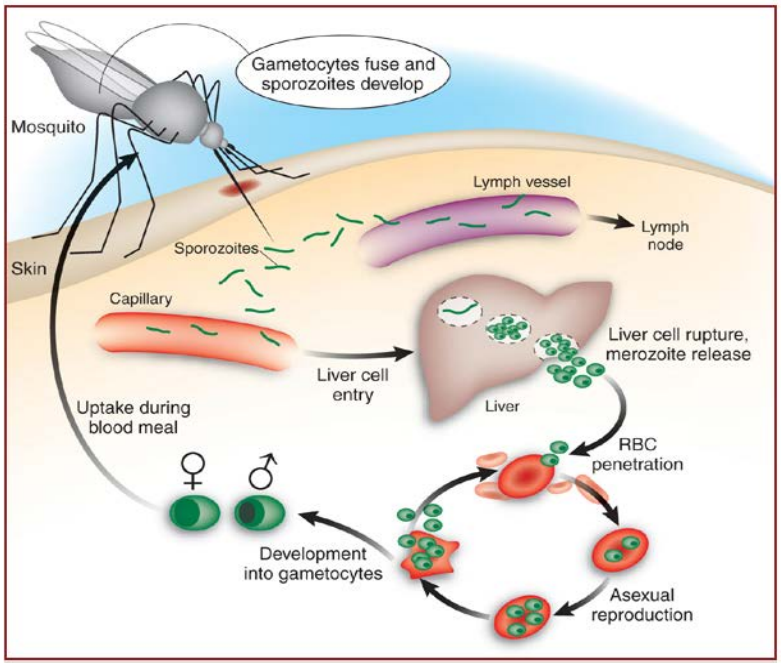

Fig. 1: Life cycle of human malaria parasite *Plasmodium falciparum* (Jones and Good, 2006)

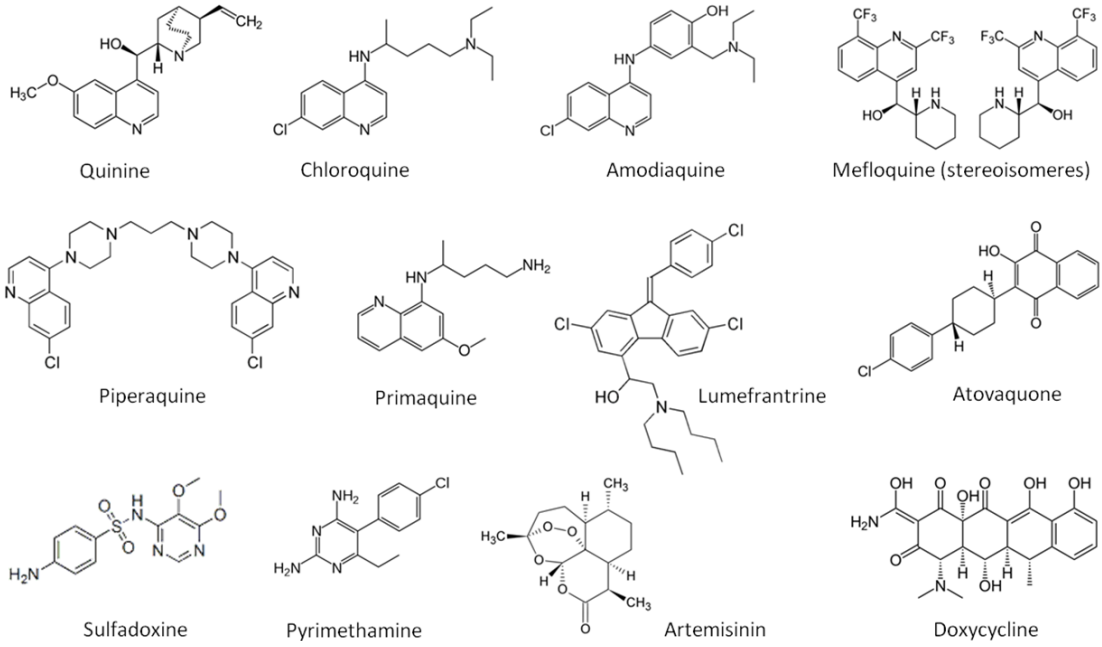

Fig. 2: Structural formulas of antimalarial drugs (Aminake and Pradel, 2013)

The third class of antimalarial is based on the natural endoperoxide artemisinin. Artemisinin and its semisynthetic derivatives (Dehydroartemisinin (DHA), artemether, arteether, artesunate, artelinic acid) are the most rapidly acting and effective against multi-drug resistant strains of the parasite. However, the poor solubility of artemisinin, coupled with its short plasma half life led to a high rate of parasite recrudescence (Pillay, 2006).

Artemisinin-based Combination Therapies (ACTs) are recommended as the first-line treatment for malaria caused by *P. falciparum*, the most dangerous of the *Plasmodium* parasites that infect humans. By 2011, 79 countries and territories had adopted ACTs as first-line treatment for *P. falciparum* malaria. *P. vivax* malaria should be treated with chloroquine where it is effective, or an appropriate ACT in areas where *P. vivax* is resistant to chloroquine. Treatment of *P. vivax* should

be combined with a 14- day course of primaquine to prevent relapse (WHO, 2012).

Malaria parasites have a complex life cycle and there are a number of key stages that are targeted by current antimalarial drugs and are potential targets for new drugs. The life cycle of malaria parasite is shown in Fig. 1. Infection in humans begins when an infected female *anopheles* mosquito feeds and injected sporozoites into the host's bloodstream. These sporozoites rapidly invade liver cells, where they multiply extensively, in all species of *Plasmodium* and form exoerythrocytic schizonts, each containing up to 30,000 merozoites. In *Plasmodium vivax* and *Plasmodium ovale* only, a proportion of the liver-stage parasites remain in the hepatocytes as a dormant form, or hypnozoite. This stage of the parasite can remain dormant for a few weeks or up to several years. These species of parasite can therefore start a cycle of asexual reproduction without the need for further mosquito bites, which is why *P. vivax* infection is also referred to as relapsing malaria (Wells *et al*., 2009). A total 6-16 days after infection (depending on the species), the schizont-infected hepatocytes rupture, releasing mature merozoites into the bloodstream. These merozoites invade Red Blood Cells (RBCs) and undergo a second round of multiplication that lasts 48-72 h and produces up to 32 merozoites (Gardiner *et al*., 2009). The released merozoites invade new RBCs and continue the asexual replication cycle. The asexual erythrocytic life cycle of *P. falciparum* is relatively synchronous in the natural host, lasting 48 h. In synchronous infections, rupture of the infected RBCs and merozoites release are associated with the characteristic fever and acute symptoms of malaria. Some merozoites also give rise to sexually differentiated forms (gametocytogenesis). The trigger for gametocytogenesis is unclear. When a female *anopheles* mosquito ingests the blood of a host containing malaria parasite, the RBCs and asexual stage parasites are digested, while the gametocytes undergo further development to form macrogametocytes (female) or microgametocytes (male). In the mosquito gut, the female and male gametes fuse to form a diploid ookinete (the parasite is haploid in the rest of the life cycle). As the oocyte matures, it divides to produce sporozoites, which travel to the salivary glands and are able to infect a new host when the mosquito next takes a blood meal (Lamar *et al*., 2007; Gardiner *et al*., 2009; Wells *et al*., 2009). Clinical manifestations occur at the erythrocytic stage and can include fever, chills and anemia, as well as delirium, metabolic acidosis, cerebral malaria and multi-organ system failure, which may be followed by coma and death (Batista *et al*., 2009).

Causes of resistance:

Definition of antimalarial drug resistance: WHO (1967) defined 'drug resistance' as the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended, but within the limits of tolerance of the subject (WHO, 1967; Bosman *et al*., 2001; Laufer, 2009).

Overview of antimalarial drug resistance: For decades, drug resistance has been one of the main obstacles in the fight against malaria. To date, drug resistance has been documented in three of the five malaria species known to affect humans in nature: *P. falciparum*, *P. vivax* and *P. malariae* (WHO, 2013b). Today when many mosquito vectors are resistant to insecticides and an effective vaccine is not yet available, chemoprophylaxis/chemotherapy remains the principal means of combating malaria. Over the past 60 to 70 years, since the introduction of synthetic antimalarials, only a small number of compounds, belonging to three broad classes, have been found suitable for clinical usage (Grimberg and Mehlotra, 2011).

This limited antimalarial armament is now severely compromised because of the parasite's remarkable ability to develop resistance to these compounds. In many different malaria-endemic areas, low to high-level resistance in the predominant malaria parasites, *P. falciparum* and *P. vivax*, have been observed for CQ, amodiaquine, mefloquine, primaquine and SP. *Plasmodium falciparum* has developed resistance to nearly all antimalarial drugs in current use (White, 2004), although the geographic distribution of resistance to any one particular drug varies greatly. In particular, Southeast Asia has a highly variable distribution of falciparum drug resistance; some areas have a high prevalence of complete resistance to multiple drugs, while elsewhere there is a spectrum of sensitivity to various drugs (Wernsdorfer and Payne, 1991).

Antimalarial drug resistance is mediated by two processes:

- The rate that de novo mutations conferring resistance appear and are selected through drug use within an individual
- The spread of those resistant alleles to other individuals

High mutation rates at the cellular level, which provide a means of continually evading the immune system, offer a mechanism for selection of resistance within a host, while interactions between other parasites and their hosts due to variation in transmission and host susceptibility and influence the probability of selection at the population level (Klein, 2012).

The artemisinins drugs are already an essential component of treatments for multidrug resistant *falciparum* malaria (White, 2004). Until 2009, no noticeable clinical resistance to artemisinin drugs was

reported. However, a number of recent studies have raised concerns about the efficacy of ACTs, particularly in Southeast Asia (Grimberg and Mehlotra, 2011).

Mechanisms of antimalarial drug resistance: *Plasmodium* parasite has extremely complex genome and case with which they can switch between the micro environments in different hosts and the metabolic changes they require illustrates the difficulty in studying the exact modes of action of the antimalarial drugs on parasite metabolism. In general, resistance appears to occur through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs (Mahajan and Umar, 2004).

CQ, SP and more recently the artemisinin class drugs have been widely adopted as first-line drugs because they are highly efficacious in eliminating *P. falciparum*-infected erythrocytes and they are well tolerated by almost all patients (Klein, 2013; Mehlotra and Zimmerman, 2006; Schlitzer, 2008). In addition, unlike other drugs such as atovaquone and pyrimethamine (when not combined with sulfadoxine), the rate at which de novo mutations conferring resistance occur is low.

Resistance also develops more quickly where a large population of parasites is exposed to drug pressure since it will remove sensitive parasites, while resistant parasite would survive. In order to appropriate the physical nature of resistance, it is necessary to look in more detail at the metabolism of the parasite and the mode of action of the antimalarial drugs. Intra-erythrocytic stage of malaria parasite ingests hemoglobin into its food vacuoles. Here exopeptidases and endopeptidases break-down hemoglobin into hemozoin pigment of which the cytotoxicity of ferriprotoporphyrin IX is a major component (Hyde, 2002). The heme binder protein (synthesised by the parasite) sequester the ferriprotoporphyrin IX into the inert hemaozoin complex to protect the plasmodium membranes from damage. It is now appropriate to discuss a number of antimalarials and apparent adaptation (Jain *et al*., 2014).

The biochemical mechanism of resistance has been well described for chloroquine, the antifolate combination drugs and atovaquone. Recently, there are also some reports on arthemisin and its derivatives.

Chloroquine resistance: *P. falciparum* resistance to chloroquine is thought to have arisen no more than ten times over the past half-century. Acquired resistance usually arises in low transmission settings in people with lots of parasites (hyperparasitaemic) who for reasons of drug quality, inadequate dose, adherence, absorption or distribution kinetics, or vomiting have inadequate blood concentration levels of the drug (Beith, 2008). Resistance mostly occurs through primary transmission of drug-resistant parasites.

Resistance in *P. Vivax* (a parasite which causes less severe disease than *P. falciparum* and generally does not result in death), has been less studied, although there are recent reports of CQ resistance emerging in Indonesia, Papau New Guinea and South America (Daily, 2006).

In general, due to mutations in ADR genes, some antimalarial drugs are not effective in certain patients. Through a literature survey, I identified eight ADR genes: *P. falciparum* ABC Transporter (PfABC), *P. falciparum* putative metabolite/drug transporter (PfMT), *P. falciparum* Dihydropteroate synthase (PfDHPS), *P. falciparum* multidrug resistance 1 (PfMDR1), *P. falciparum* Multidrug Resistance 2 (PfMDR2), *P. falciparum* sodium/hydrogen exchanger 1 (PfNHE1), *P. falciparum* dihydrofolate reductase-thymidylate synthase (PfDHFR-TS) and *P. falciparum* chloroquine-resistance transporter gene (PfCRT) (Briolant *et al*., 2012; Lee *et al*., 2013). Four genes (PfMDR1, PfMDR2, PfDHFR-TS and PfNHE1) among the eight *P. falciparum* ADR genes are inferred as *Plasmodium* SL genes.

Molecular markers of drug-resistant *P. vivax*, including mutations in multidrug resistance 1 gene (*pvmdr*1) and putative transporter protein CG10 gene (*pvcg*10 or *pvcrt-o*), which are orthologous to *pfmdr1* and *pfcrt-o* genes, respectively, have been identified as possible genetic markers of Chloroquine-Resistance (CQR) (Lu *et al*., 2012).

The molecular mechanism of the action of this conventional antimalarial drug, chloroquine, is becoming unveiled (Hyde, 2002). Chloroquine Resistance (CQR) in *P. falciparum* is now linked to point mutations in the chloroquine resistance transporter (PfCRT [encoded by *pfcrt*, located on chromosome 7]) (Van *et al*., 2011). *Pfcrt*-K76T mutation confers resistance *in vitro* and is the most reliable molecular marker for CQR (Sidhu *et al*., 2002). In April 2012, in five States of India, forty two isolates were genotyped for *pfcrt* K76T chloroquine resistant mutation; mutations were seen in 38 (90.47%) isolates (Anvikar *et al*., 2012). According to Sharma's (2012) study report on drug resistance done in India was proposed that detecting K76T mutation in the *pfcrt* gene would provide information on the CQR status of the parasite in an infected blood sample. However, there were exceptions where K76T mutation was present in the Pfcrt of the parasite isolates from patients who were otherwise responding to the CQ treatment.

In Yemen, treatment failure was detected in 61% of the 122 cases that completed the 14-day follow-up. The prevalence of mutant pfcrt T76 was 98% in 112 amplified pre-treatment samples. The presence of pfcrt T76 was poorly predictive of in vivo CQ resistance (PPV = 61.8%, 95% CI = 52.7-70.9). The prevalence of dhfr Arg-59 mutation in 99 amplified samples was 5%, while the dhps Glu-540 was not detected in any of 119 amplified samples. Sequencing the pfcrt gene confirmed that Yemeni CQ resistant *P. falciparum* carry the old world (Asian and African) CQ

resistant haplotype CVIETSESI at positions 72, 73, 74, 75, 76, 220, 271, 326 and 371 (Mubjer *et al*., 2011).

Polymorphisms, including copy number variation and point mutations, in another parasite transporter, multidrug resistance (PfMDR1 or Pgh1 [encoded by *pfmdr1*, located on chromosome 5]), contribute to the parasite's susceptibility to a variety of antimalarial drugs. Point mutations in *pfmdr*-1 play a modulatory role in CQR, which appears to be a parasite strain-dependent phenomenon (Valderramos and Fidock, 2006). There is a suggestion that *pfmdr*-1 mutation associated with chloroquine resistance may also account for reduced susceptibility to quinine.

Available evidence suggests the roles of the multidrugresistance gene 1 (pfmdr1) on the resistance of *P. falciparum* to several blood schizonticides including chloroquine, mefloquine, quinine and artemisinin. The presence of Asn86, Tyr184, Ser1034 and Asn1042 (wild-type) or Asn86, Phe184, Ser1034 and Asn1042 was found to be associated with increased resistance to mefloquine in Southeast Asia. These markers might play an important role in tracking mefloquine resistance in the region (Noedl *et al*., 2003).

As the malaria parasite digests hemoglobin, large amounts of a toxic by-product are formed. The parasite polymerizes this by-product in its food vacuole, producing non-toxic haemozoin (malaria pigment). It is believed that resistance of *P. falciparum* to chloroquine is related to an increased capacity for the parasite to expel chloroquine at a rate that does not allow chloroquine to reach levels required for inhibition of heme polymerization (Foley and Tilley, 1997). This chloroquine efflux occurs at a rate of 40 to 50 times faster among resistant parasites than sensitive ones (Krogstad, 1987).

Further evidence supporting this mechanism is provided by the fact that chloroquine resistance can be reversed by drugs which interfere with this efflux system (Martin *et al*., 1987). It is unclear whether parasite resistance to other quinoline antimalarials (amodiaquine, mefloquine, halofantrine and quinine) occurs via similar mechanisms (Foley and Tilley, 1997).

Resistance in *P. vivax* is more serious as hypnozoites will cause relapse of resistant parasites and *P. vivax* is a mixture of various strains with respect to incubation period, relapsing pattern and response to primaquine (Borrmann and Matuschews, 2011).

Several countries have already abandoned Chloroquine (CQ) and Sulfadoxine-Pyrimethamine (SP) (Fansidar®) as monotherapy in favour of artemisinin combination therapy because of the emergence and worsening rise of CQ and SP resistance (Shretta *et al*., 2000). Chloroquine, to which resistance has emerged slowly and SP, to which resistance has developed apace (Peter *et al*., 2002). Because drug resistance is not an all-or-nothing phenomenon, chloroquine still retains adequate efficacy even in areas of known resistance for continued use to be justifiable for the time being (for instance, some areas of West Africa continue to use chloroquine successfully, although efficacy rates are declining) (WHO, 2001).

Antifolate combination drugs: Antifolate agents used for the treatment of malarial infection act on the folate metabolism of the parasite. With regard to the target enzyme they inhibit, the antifolates are subdivided into two classes: inhibitors of Dihydrofolate Reductase (DHFR), such as pyrimethamine, proguanil and chlorproguanil and inhibitors of Dihydropteroate Synthase (DHPS) such as sulfadoxine and dapsone. The combination of DHFR and DHPS inhibitors is synergistic, hence their use in combination in the treatment of malaria (Nzila, 2006). With rapidly growing Sulfadoxine-Pyrimethamine (SP) resistance, a new combination drug, Lapdap (chlorproguanil-dapsone), was tested in Africa in the early 2000s, but was withdrawn in 2008 because of hemolytic anemia in patients with Glucose-6-Phosphate Dehydrogenase enzyme (G6PD) deficiency (Luzzatto, 2010).

Antifolate combination drugs, such as sulfadoxine + pyrimethamine, act through sequential and synergistic blockade of two key enzymes involved with folate synthesis. Pyrimethamine and related compounds inhibit the step mediated by Dihydrofolate Reductase (DHFR) while sulfones and sulfonamides inhibit the step mediated by Dihydropteroate Synthase (DHPS) (Grimberg and Mehlotra, 2011). Specific gene mutations encoding for resistance to both DHPS and DHFR have been identified. Specific combinations of these mutations have been associated with varying degrees of resistance to antifolate combination drugs (Wang *et al*., 2005).

There are areas of the DHFR and DHPS genes with identified mutations that are found in isolates that fail to respond to pyrimethamine/sulfa treatment. These occur principally at codons 108, 51, 59, 164 and also occasionally 50, 140 and the ``Bolivian repeat'' of the DHFR gene and codons 436, 437, 540, 581 and 613 of the DHPS gene, reviewed in (Saifi *et al*., 2012). There is a broad correlation between increased frequency of such mutations and resistance to pyrimethamine/sulfa drugs across the world.

SP-resistant *P. Vivax* is more widespread (Beith, 2008). Pyrimethamine and sulphadoxine target the dhfr and dhps enzymes in *P. falciparum* and point mutations in these genes confer resistance to each drug (Foote *et al*., 1990). Mutations in codons I13L, P33L, F57L/I, S58R, T61M, S117N/T and I173L/F of the *P. vivax* dhfr enzyme have been proposed as conferring similar resistance to pyrimethamine in *P. vivax (*Khattak *et al*., 2013).

Point mutations in the *P. falciparum* DHPS enzyme (encoded by *pf-dhps*, located on chromosome 8) are involved in the mechanism of resistance to the sulfa class of antimalarials and accumulation of mutations in the *P. falciparum* DHFR domain (encoded by *pf-dhfr*, located on chromosome 4) defines the major mechanism of high-level pyrimethamine resistance. In field studies, a *pf-dhps* double mutant (437G with either 540E or 581G), combined with the *pf-dhfr* triple mutant (108N_51I_59R), was found to be frequently associated with SP treatment failure (Gregson and Plowe, 2005; Hyde, 2007; Costanzo and Hartl, 2011).

Atovaquone: Atovaquone is relatively new antimalarial drug that blocks the parasite's cytochrome electron transfer system (Shanks, 2006). It acts through inhibition of electron transport at the cytochrome bc1 complex. Although resistance to atovaquone develops very rapidly when used alone, when combined with a second drug, such as proguanil (the combination used in Malarone TM) or tetracycline, resistance develops more slowly (Fisher *et al*., 2012; WHO, 2001).

One proposed site for atovaquone's activity is Dihydroorotate Dehydrogenase (DHODase), a critical enzyme in electron transport. Inhibition of DHODase blocks pyrimidine synthesis. DHODase catalyses the reaction from dihydroorotate to orotate. It bridges pyrimidine de novo synthesis and the mitochondrial electron transport system and is also the major source of electrons for the mitochondrial electron transport chain (Olliaro, 2001).

Artemisinin resistance: In the current years, the most effective treatment for malaria are artemisinin-based combination therapies (ACTs) that combine a semi-synthetic derivative of artemisinin, a chemical compound isolated from the plant Artemisia annua, with a partner drug of a distinct chemical class. ACTs compensate for the poor pharmacokinetic properties of the artemisinins, increase treatment efficacy and are thought to reduce the emergence of drug-resistant parasites (Petersen *et al*., 2011) and are now central to the first-line treatment of *P. falciparum* malaria. The recent emergence of decreased sensitivity of the parasite to artemisinins in Cambodia is of grave concern and puts at risk the entire strategy for the treatment of malaria (Ward and Boulton, 2013).

A major advance in the search for effective treatment for drug-resistant malaria came with the discovery of artemisinin and its derivatives. These compounds show very rapid parasite clearance times and faster fever resolution than any other currently licensed antimalarial drug (White, 2008). Given the recent significant impact of ACTs on malaria morbidity and mortality, it is worrisome that higher recrudescence rates of *P. falciparum* malaria after artemisinin treatment are seen in some areas. Resistance mechanisms to artemisinin are poorly understood, although mutations in some parasite genes have been partially correlated with resistance (Vernet *et al*., 2014).

Recrudescence, the reappearance of an infection after a period of quiescence, occurs in up to 30% of patients on artemisinin monotherapy and in up to 10% of patients on ACTs (Meshnick *et al*., 1996). The underlying mechanism of recrudescence after artemisinin treatment is unclear. As illustrated by recent *in vitro* studies, the occurrence of parasite dormancy, where parasites enter a temporary growth-arrested state, may provide a plausible explanation for this phenomenon (Teuscher *et al*., 2010).

The use of oral artemisinin-based monotherapies threatens the long-term usefulness of ACTs by fostering the emergence and/ or spread of resistance to artemisinin. To contain this risk and to ensure high cure rates for *P. falciparum* malaria, WHO recommends the withdrawal of oral artemisinin-based monotherapies from the market and their replacement by ACTs, as endorsed by the World Health Assembly in 2007 (Shillcutt *et al*., 2008). WHO also calls upon manufacturers to cease the marketing of oral artemisinin-based monotherapies.

However, the challenge now is that neither molecular markers nor in vitro assays for artemisinin resistance are well established (Imwong *et al*., 2010) For example, in vitro drug sensitivity tests of samples from Cambodia produced inconsistent results with respect to identification of the in vivo resistant phenotype and no molecular markers have been reported in the genes (*pfmdr*1, *pfcrt* and *pfserca*) thought to be associated with resistance to other antimalarials or putatively associated with artemisinin resistance. Furthermore, the relation between resistant genotypes and most drug resistant parasite phenotypes and clinical outcomes is not always straightforward (Talisuna *et al*., 2012).

Due to the high failure rate of ACTs in Pailin, a consensus meeting-held in November 2011 in Cambodia-recommended the use of atovaquone-proguanil delivered as directly observed therapy for Pailin province; stringent follow up of all treated patients was also recommended to detect any emergence of atovoquone resistance. To date, there have been no reports of delayed parasite clearance during routine therapeutic efficacy studies conducted in Africa (WHO, 2012).

Recent reports (Table 1) however suggest the association of mutations in the *pfatp*6 (*PfATP*6, is a target of artemisinins) and *pfmdr*1 genes might be the main contributor to artemisinins resistance (Cui and Su,

Table 1: A brief summary of the status of artemisinin resistance in the greater Mekong sub region

	Artemisinin resistance			AL		AS-MQ		DHA-PPQ	
Name of the sites	Suspect year of emergence	Detected	Containment activities started	D3+	TF	D3+	TF	D3+	TF
Cambodia	2001**	2006	2009	◆	◆	◆	◆	◆*	◆*
Laos	2013	2013	2014	◆*	-*				
Myanmar	2001**	2008	2011	◆*	-*	◆*	- *	◆*	-*
Thailand	2001**	2008	2009	◆	◆	◆*	◆*		
Viet Nam	2009	2009	2011					◆*	-*

Legend: *: First-line treatment; **: detected retrospectively using molecular marker or retrospective date;◆: observe to be >10%; -: observe to be <10%; blank: undetermined. AL: Arthemether-Lumefantrine; AM: Artesunate-mefloquine; DHA-PPQ: Dihydroartemisinin-Piperaquine; D3+: day 3 positive; TF: treatment failure (WHO, 2014)

2009; Ding *et al*., 2011). Recently, a molecular marker of artemisinin resistance was identified. Mutations in the Kelch 13 (K13)-propeller domain were shown to be associated with delayed parasite clearance in vitro and in vivo. This new tool will help to improve the global surveillance of artemisinin resistance (WHO, 2014).

Spread of resistance: Numerous factors contributing to the advent, spread and intensification of drug resistance exist, although their relative contribution to resistance is unknown. Factors that have been associated with antimalarial drug resistance include such disparate issues as human behavior, vector and parasite biology, pharmacokinetics and economics. As mentioned previously, conditions leading to malaria treatment failure may also contribute to the development of resistance (Austin and Anderson, 1999).

The emergence of antimalarial drug resistance in *Plasmodium* depends on multiple factors, including:

- The mutation rate of the parasite
- The fitness costs associated with the resistance mutations
- The overall parasite load
- The strength of drug selection
- The treatment compliance
- The spread of those resistant alleles to other individuals (Petersen *et al*., 2011)

Detection of resistance: In general, four basic methods have been routinely used to study or measure antimalarial drug resistance: *in vivo*, *in vitro*, animal model studies and molecular characterization. Additionally, less rigorous methods have been used, such as case reports, case series, or passive surveillance (WHO, 2001; Fairhurst *et al*., 2012; Mideo *et al*., 2013).

***In vitro* tests:** In vitro assays are based on cultivation of *P. falciparum* isolated parasites in the presence of a range of antimalarial drug concentrations for one life cycle or part of a life cycle. Drug efficacy is assessed by counting the number of parasites developing into schizonts (WHO *in vitro* test) or by measuring the quantity of radiolabelled hypoxanthine, a DNA precursor, incorporated into the parasites (isotopic microtest). The results of *in vitro* studies can complement the findings of therapeutic efficacy studies and can provide useful information on the epidemiology of drug-resistant malaria (Ringwald and Basco, 1999; WHO, 2013a).

The usefulness of in vitro study results for monitoring drug-resistant malaria is limited mainly because of the use of many different tests and methods, which are not always comparable. The tests include the WHO mark III test, the radioisotopic test, enzyme-linked immunosorbent assays with antibodies directed against *Plasmodium* lactate dehydrogenase or histidine-rich protein and the fluorometric assay with DNA-binding fluorescent dyes. As each test has a different end-point (such as the appearance of schizonts with at least three nuclei, a fixed incubation period, an optical density reading in control wells) and different measures of metabolism (incorporation of nucleotide precursor or fluorescent dye, production of parasite-specific enzyme, secretion of soluble antigen), interpretation of the data depends on which test was used (Basco, 2007).

From the point of view of a researcher interested in pure drug resistance, *in vitro* tests avoid many of the confounding factors which influence *in vivo* tests by removing parasites from the host and placing them into a controlled experimental environment. This test more accurately reflects "pure" antimalarial drug resistance. Multiple tests can be performed on isolates, several drugs can be assessed simultaneously and experimental drugs can be tested. However, the test has certain significant disadvantages. The correlation of *in vitro* response with clinical response in patients is neither clear nor consistent and the correlation appears to depend on the level of acquired immunity within the population being tested. Pro-drugs, such as proguanil, which require host conversion into active metabolites, cannot be tested. Neither can drugs that require some level of synergism with the host's immune system. Although adaptation of erythrocytic forms of *P. vivax* to continuous culture has been achieved, non-falciparum erythrocytic parasites generally cannot be evaluated *in vitro* (Golenda *et al*., 1997).

Testing the susceptibility of parasites to drugs in vitro is problematic because in vivo phenotypes do not necessarily correlate with in vitro performance (Dondorp *et al*., 2009), in addition to being technically challenging, laborious and expensive.

***In vivo* tests:** An *in vivo* test consists of the treatment of a group of symptomatic and parasitaemic individuals with known doses of drug and the subsequent monitoring of the parasitological and/or clinical response over time. One of the key characteristics of *in vivo* tests is the interplay between host and parasite. Diminished therapeutic efficacy of a drug can be masked by immune clearance of parasites among patients with a high degree of acquired immunity (WHO, 1973; White, 1997).

The simplified *in vivo* test is performed by a regular measurement of body temperature and microscopic examination of blood films. The standard or simplified *in vivo* test is the reference method to detect drug resistance. *In vivo* testing is an accurate and valid measure of therapeutic efficacy and is the most reliable means for detecting drug resistance (Ringwald and Basco, 1999).

Of the available tests, *in vivo* tests most closely reflect actual clinical or epidemiological situations (i.e. the therapeutic response of currently circulating parasites infecting the actual population in which the drug will be used). Because of the influence of external factors (host immunity, variations of drug absorption and metabolism and potential misclassification of reinfections as recrudescences), the results of *in vivo* tests do not necessarily reflect the true level of pure antimalarial drug resistance. However, this test offers the best information on the efficacy of antimalarial treatment under close to actual operational conditions-what can be expected to occur among clinic patients if provider and patient compliance is high (WHO, 2001).

Animal model studies: This type of test is, in essence, an in vivo test conducted in a non-human animal model and, therefore, is influenced by many of the same extrinsic factors as in vivo tests. The influence of host immunity is minimized by using lab-reared animals or animal-parasite combinations unlikely to occur in nature, although other host factors would still be present. These tests allow for the testing of parasites which cannot be adapted to in vitro environments (provided a suitable animal host is available) and the testing of experimental drugs not yet approved for use in humans. A significant disadvantage is that only parasites that can grow in, or are adaptable to, non-human primates can be investigated (WHO, 1973, 2001).

Molecular techniques: Apart from microscopy, nucleic acid techniques such as Polymerase Chain Reaction (PCR), nested PCR (Lee *et al*., 2012; Morassin *et al*., 2002) and Loop Mediated Isothermal Amplification **(**LAMP) have also been used for malaria detection. (Loop Mediated Isothermal Amplification) LAMP can be conducted under isothermal conditions and does not need expensive thermocyclers (Lee *et al*., 2012). However, this method may pose danger of cross-contamination during addition of dye for visualization of results. Also this method is not suitable for target DNA greater than 500bp, as this causes a hindrance to strand displacement (Lau *et al*., 2011).

These tests are in the process of being developed and validated, but offer promising advantages to the methods described above. Molecular tests use PCR to indicate the presence of mutations encoding biological resistance to antimalarial drugs. Molecular markers such as mutations in *dhfr*, *dhps*, *pfcrt* and *pfmdr*1 represent potential surveillance tools (Paul *et al*., 2005).

Case reports and passive detection of treatment failure: Additional methods for identifying or monitoring antimalarial drug resistance include the use of case reports or case series of spontaneously reported treatment failure. In general, these methods require far less investment in time, money and personnel and can be done on an ongoing basis by individual health care centres. They suffer, however, from presenting a potentially biased view of drug resistance primarily because denominators are typically unknown and rates of resistance cannot be calculated. Nonetheless, case reports can be useful and may indicate a problem that should be confirmed using one of the other methods. In the United States, for instance, case reports, especially when occurring in clusters, of prophylaxis failure have been used to help formulate recommendations for chemoprophylaxis of non-immune travellers to endemic areas (Lackritz, 1991).

Another method that has been used is passive detection of treatment failure. In this system, patients are treated following usual treatment guidelines and told to come back to the clinic or hospital if symptoms persist or return. Those cases which do return are considered to represent the population of treatment failures. Because this system does not ensure compliance with treatment regimens through directly observed therapy and does not attempt to locate and determine the outcome of patients who do not return on their own, data are seriously biased (Alene and Bennett, 1996).

Strategies to overcome drug resistance: Because of the constant battle with drug resistance, this began in the 1960s, WHO has established a strategy for dealing with antimalarial resistance, which has four key elements: preventing the emergence of antimalarial drug resistance, monitoring antimalarial drug efficacy and when necessary confirming drug resistance, ensuring a continuous pipeline of new antimalarial medicines and containing the spread of antimalarial drug resistance once it has emerged (WHO, 2009). The key elements of the strategy to prevent the emergence of drug resistance are:

Reducing overall drug pressure: Drug pressure is defined as the proportion of malaria infections that is

treated: the therapeutic treatment rate. The use of antimalarial drugs is the force that drives antimalarial drug resistance through a population; this force is often referred to as 'drug pressure'. These antimalarial drugs are used presumptively to treat all fevers, leads to drug resistance. The greatest decrease in antimalarial drug use could be achieved through improving the diagnosis of malaria (Hastings and Watkins, 2006).

Because overall drug pressure is thought to be the single most important factor in development of resistance, following more restrictive drug use and prescribing practices would be helpful, if not essential, for limiting the advent, spread and intensification of drug resistance. This approach has gained support in North America and Europe for fighting antibacterial drug resistance (Bauchner *et al*., 1999).

Improving the way drugs are used: Another approach that has not been widely adopted is the close follow-up and re-treatment, if necessary, of patients. The success of this approach is dependent on availability of reliable microscopy (to diagnose the illness initially as well as to confirm treatment failure) and either an infrastructure to locate patients in the community or a community willing to return on a given date, regardless of whether they feel ill or not. With this system, patients who fail initial treatment, for whatever reason, are identified quickly and re-treated until parasitologically cured, decreasing the potential for spread of resistant parasites (Wernsdorfer *et al.*, 1994).

Combination therapies: Use of the artemisinin (ART) group of drugs (artesunate, artemether, dihydroartemisinin, artemisinin and arteether) as monotherapy for uncomplicated malaria is dogged by their rapid elimination (Peter *et al*., 2002). A strategy that has received much attention recently is the combination of antimalarial drugs, such as mefloquine, SP, or amodiaquine, with an artemisinin derivative is improving access to affordable, quality-assured diagnostic testing and treatment with ACTs improves patient outcomes and limits the opportunities for emergence of resistance to both artemisinins and partner drugs. Programmes should have a multifaceted approach that improves access to both consistent, accurate diagnostic testing and quality-assured ACTs for confirmed cases, while removing oral artemisinin-based monotherapies and substandard or counterfeit drugs from the market (WHO, 2011).

Artemisinin drugs are highly efficacious, rapidly active and have action against a broader range of parasite developmental stages. This action apparently yields two notable results. First, artemisinin compounds, used in combination with a longer acting antimalarial, can rapidly reduce parasite densities to very low levels at a time when drug levels of the longer acting antimalarial drug are still maximal. This greatly reduces both the likelihood of parasites surviving initial treatment and the likelihood that parasites will be exposed to suboptimal levels of the longer acting drug (White, 1999). Second, the use of artemisinins has been shown to reduce gametocytegenesis by 8- to 18-fold (Price, 1996).

Monitoring antimalarial drug efficacy: Clinical evaluation of therapeutic efficacy is based on the determination of the proportion of treatment failure in a patient population at a particular study site (Ringwald and Basco, 1999). Routine monitoring of therapeutic efficacy of first and second-line medicines as an integral component of malaria control makes possible to detect drug resistance early and to be able to rapidly change drug policy when efficacy decreases (total treatment failure at day 28>10%) in order to avoid the further selection and spread of multidrug resistance. A standard element of these studies is the measurement of parasitemia on day 3 after enrolment. Failure to clear parasites by day 3 is an early indication of artemisinin resistance. Current guidance is to conduct additional confirmatory drug efficacy studies when the proportion of patients failing to clear parasitemia by day 3 exceeds 10% (WHO, 2011).

Ensuring a continuous pipeline of new antimalarial medicines: It is clear from the size of the antimalarial drug development pipeline that the pipeline has not reached critical mass, which is of concern particularly when we consider the recent emergence of artemisinin resistance and the apparent decrease in time to resistance to each new drug/drug combination. The challenges for now and for the future are:

- How to ensure that we have compounds to combat emerging and future antimalarial drug resistance?
- How to initiate and expedite the development of compounds that are as innovative as possible with respect to their chemical scaffold and molecular target (Grimberg and Mehlotra, 2011)?

Future perspectives of antimalarial drugs: The goal of President's Malaria Initiative (PMI) is to reduce malaria-related deaths by 50% in PMI-focus countries and regions with a high burden of malaria by expanding coverage of four highly effective malaria prevention and treatment measures: Insecticide Treated Bed Nets (ITNs), Indoor Residual Spraying (IRS), Intermittent Preventive Treatment During Pregnancy (IPTp) and Artemisinin Combination Therapy (ACT). CDC-led science has established the efficacy and potential impact of each of these recommended interventions (CDC, 2014).

In January 2011, WHO released the Global Plan for Artemisinin Resistance Containment (GPARC), which puts forward four main goals and recommendations: to stop the spread of resistant parasites to increase monitoring and surveillance to evaluate the artemisinin resistance threat; to improve

access to diagnostics and rational treatment with ACTs; to invest in artemisinin resistance-related research (Malaria Consortium, 2014).

Routine monitoring of the therapeutic efficacy of ACTs is essential for timely changes to treatment policy and can help to detect early changes in *P. falciparum* sensitivity to antimalarial drugs. WHO currently recommends monitoring the efficacy of first-line and second-line ACTs every two years in all endemic countries. The results of the therapeutic efficacy studies allow researchers to determine: the proportion of patients who are parasitemic on day 3, which is currently the indicator of choice for routine monitoring to identify suspected artemisinin resistance in *P. falciparum*; and the proportion of treatment failure after 28- or 42-day follow-up (depending on the specific ACT). A treatment failure rate exceeding 10% should prompt a change in the national antimalarial treatment policy (WHO, 2014).

In April 2013, WHO launched the Emergency response to artemisinin resistance (ERAR) in the Greater Mekong subregion, Regional framework for action 2013-15. The framework urges malaria partners to work in a coordinated manner to provide malaria interventions to all at-risk groups; to achieve tighter coordination and management of field operations; to obtain better information for artemisinin resistance containment; and to strengthen regional oversight and support (WHO, 2013c).

Olliaro and Wells, 2009 reviewed the global pipeline of new antimalarial combinations and chemical entities, in various stages of development till February 2009. Overall, the pipeline consists of 22 projects, either completed (artemether- lumefantrine and artesunate- amodiaquine), in various clinical stages (phase III/registration stage, n = 4; phase II, n = 8; phase I, n = 5), or in preclinical translational stage (n = 3). Chemical novelty is relatively low among the products in clinical stages of development; the prevailing families are of artemisinin-type and quinoline compounds, mostly in combination with each other. Among the non-artemisinin and/or non-quinoline combinations and single compounds are: Azithromycin-CQ (phase III), fosmidomycin-clindamycin (phase II), methylene blue-amodiaquine (phase II), SAR 97276 (a choline uptake antagonist, phase II) and tinidazole (an anti-parasitic nitroimidazole compound, phase II).

Drug discovery in general is a very challenging process, but it is especially challenging for antimalarials for several reasons (Nwaka and Hudson, 2006). The slow development of immunity in people living in areas where malaria is endemic is consistent with the hypothesis that effective immunity only develops after exposure to a large number of genetically different parasite strains. The clinical trials of malaria vaccines (based on different stage specific antigens) seen to be encouraging (Mahajan and Umar, 2004).

CONCLUSION

Almost all antimalarial drugs faced malaria parasite resistance and it became a serious challenge for malaria control. Since the drug development pipeline remains unproductive, there is currently no alternative to precious artemisinin. Based on the present review findings, extensive studies were done on *P. falciparum* drug resistance along with different diagnostic methods, causes, biomarkers and mechanisms of resistance than *P. vivax.* Antimalarial drug resistance is mediated either by de novo mutation conferring resistance or spread of those resistance alleles. The key elements of strategy to prevent the emerging and spread of drug resistance were not found to reduce drug resistance to much extent as drug efficacy is still continue to decrease.

Addressing these issues allow for better understanding in designing and implementing the antimalarial therapeutic formulations in malaria control programmes and call international and local institutions working on malaria to work jointly and urgently so as to confined the spread of drug resistance. Furthermore, findings from this review will also be useful to clinical researchers, health planners, policy makers and stakeholders and others concerned for malaria patients. The future solution for antimalarial drug resistance is to conduct an extensive artemisinin resistance-related research in addition to malaria infection prevention.

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