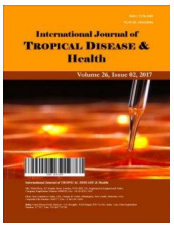

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Past and Current Findings in Antimalarial Drug Resistance Molecular Markers in Endemic Areas of Africa

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Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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ABSTRACT

Antimalarial drug resistance is the major challenge in the treatment of malaria all over the world. *Plasmodium* species are the parasite that causes malaria. *Plasmodium falciparum* is the most prevalent species found in sub-Saharan Africa that records the highest infections and death caused by malaria worldwide. Resistance to *P. falciparum* is caused by mutations in some target genes of the parasite, which includes *Plasmodium falciparum*: Na^+/ H^+ exchanger (*Pf*nhe-1), chloroquine resistance transporter (*Pf*crt), dihydropteroate synthase (*Pf*dhps), dihydrofolate reductase (*Pf*dhfr), multidrug resistance 1 gene (*Pf*mdr1), cytochrome b, multidrug resistance-associated protein 1 (*Pf*mrp1), cg2 (*Pf*cg2), Ca^{2+}–ATPase (*Pf*ATPase6) and kelch 13 gene**.** Most of these mutations are single nucleotide polymorphisms and has led to the decrease in susceptibility of some drugs like chloroquine, quinine, mefloquine, amodiaquine, sulphadoxine/pyrimethamine, lumefantrine and artemisinins in the treatment of malaria. The aim of this review was to survey on the existing antimalarial drug resistance in endemic areas of Africa and suggests a way forward in combating drug-resistant malaria in this region.

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1. INTRODUCTION

Malaria remains one of the most deadly diseases responsible for high rate of morbidity or mortality in tropical regions of the world. In 2010, the World Health Organisation (WHO) estimated that malaria causes over 240 million illness and 0.86 million deaths annually [1]. This disease is caused by the vector; Anopheline mosquito that transmits the protozoan parasite *Plasmodium* [2]. *Plasmodium falciparum* is the most deadly species of *Plasmodium* and it is the major cause of drug-resistant malaria in Sub-Saharan Africa, Southeast Asia and the Amazon region [3]. While the *Plasmodium vivax*, which causes about 25-40% of deaths, is mainly found in South America and Asian countries and it is responsible for relapsing malaria [1]. The emergence of drug-resistant parasites has threatened the efficacy of the available drugs in the treatment of malaria especially in sub-Saharan Africa where *P.falciparum* is responsible for 90% of deaths, thus increasing the morbidity and mortality rates of malaria [4]. Molecular markers can be used to monitor antimalarial drug-resistant parasites that are caused by genetic mutation in the target gene of the parasites [5]. This review describes the different molecular markers and state of drug-resistant malaria in Africa and the way forward to combat the issue of antimalarial drug resistant.

2. LITERATURE SEARCH

A comprehensive literature search was done about the prevalence of antimalarial drug resistance and molecular markers of antimalarial drug resistance of *P.falciparum* isolated from African patients and various studies done in Africa by using PubMed (http://www.ncbi.nlm.nih.gov) and Google Scholar (www.scholar.google.com).

3. MOLECULAR MARKERS OF ANTIMALARIAL DRUG RESISTANCE

Artemisinin-based combination therapy (ACT) is now recommended as the first-line drug for the treatment of uncomplicated malaria because of the prevalence of resistant parasites [6]. The replacements of ineffective failing treatments (chloroquine, sulphadoxine-pyrimethamine) with ACTs and also quinine with artesunate specifically for treating severe malaria have reduced the morbidity and mortality rate of malaria [7]. Although, ACTs is the new remedy for the treatment of malaria caused by *Plasmodium falciparum*, resistance to these drug has led to an increase in the rate of treatment failure and this is now prevalent and expanding in Southeast Asia [8]. Some genes have been identified and proven to be involved in *P. falciparum* antimalarial drug resistance which include: the genes encoding the *Plasmodium falciparum* Na^+/H^+ exchanger (*Pf*nhe-1), *Plasmodium falciparum* chloroquine resistance transporter (*Pf*crt), *Plasmodium falciparum* dihydropteroate synthase (*Pf*dhps), *Plasmodium falciparum* dihydrofolate reductase (*Pf*dhfr), *Plasmodium falciparum* multi drug resistance 1 gene (*Pf*mdr1), cytochrome b, [9] *Plasmodium falciparum* multidrug resistance-associated protein 1 (*Pf*mrp1), *Plasmodium falciparum* cg2 (*Pf*cg2), *Plasmodium falciparum* Ca^{2+} – ATPase (*Pf*ATP6) and kelch 13 gene**.** Single Nucleotide Polymorphisms (SNPs) in these markers are very important in monitoring or determining new drug-resistant patterns [10].

3.1 *Plasmodium falciparum* Na^+/H^+ Exchanger (*Pf*nhe-1)

*Pf*nhe is located on chromosome 13 fragments and has additional loci at chromosome 9 in the parasite. It is the second biggest eukaryotic Na^+/H^+ exchanger after that of the apicomplexan *T. gondii* [11]. *Pf*nhe-1 is a 226 kDa protein that is predicted to have 12 trans membrane segment with a signal peptide cleavage site and functions to intensify the cytosolic pH (CytpH) and to make up for acidosis that is caused by anaerobic glycolysis [12]. The genetic changes that are associated with changes in cytosolic pH, which is linked to chloroquine/quinine resistance, still remain unclear [11].

*Pf*nhe-1 has been described to have three-point polymorphisms at codon 790, 894 and 950 with three different repeat sequence of microsatellite variants MsR1, ms3580, and ms4760 that may be responsible for drug susceptibility or resistance [13]. The ms4760 is the most studied in Africa due to the strain selectivity in geographical areas and an increase/decrease in amino acid motif DNNND or/and an increase/decrease in that of NHNDNHNNDDD motifs have been linked to quinine susceptibility in some studies. A study done in Republic of Congo that involved 74 *Plasmodium falciparum* isolates discovered that the polymorphisms in the number of either the DNNND or NHNDNHNNDDD were not in

Table 1. Artemisinin combination therapies (ACTs) and examples of their trade names

Artemisinin combination therapies (ACTs)	Trade names
Amodiaquine + Artesunate*	Efonrex, Artesmodia, Camosunate
Artesunate* + Sulphadoxine/pyrimethamine	Amalar plus, Tesunate SP, CALSUNATE SP
Artesunate* + Mefloquine	Larinate-MF, Mefliamplus, Artequin
Pyronaridine + Artesunate*	Pyramax
Artemether* + Lumefantrine	Coartem, Lumartem, Lumapil
Dihydroartemisinin* + Piperaquine	Pymal, Pipart, Terocan
Artemisinin* + Piperaquine	Artequick

*Note the * ones are the artemisinin derivative drugs*

connection with quinine susceptibility while another study done with samples from different countries found out that an increase in the DNNND led to an increase in IC_{50} of quinine while an increase in the NHNDNHNNDDD repeats led to a decrease in IC_{50} of quinine [14,15]. In addition, some authors discovered that in Uganda and Kenya coast there was a decrease in quinine susceptibility in the presence of *Pf*mdr1 mutations combined with two DNNND repeats, while in the presence of 1 *Pf*mdr1 mutations with 3 DNNND repeats and 2 *Pf*mdr1 with 3 DNNND repeats led to the return of quinine susceptibility [16].

3.2 *Plasmodium falciparum* Chloroquine Resistance Transporter (*Pf*crt)

*Pf*crt is a 48KDa protein that is predicted to have 10 transmembrane spanning domains with 424 amino acids and it is found in the digestive vacuole membrane of the parasite [17]. It is found on chromosome 7 of the *P.falciparum*. When mutated, this transporter protein will lower the volume of accumulating drugs in the digestive vacuole of *P.falciparum* due to enhanced efflux of drugs [18].

Eight point mutations M74I, N75E, K76T, A220S, Q271E, N326S, I356T and R371I have been identified in the chloroquine resistance transporter isolates from both Asia and Africa and in the chloroquine resistance Dd2 strain, however, the I356T was not found in some isolates [19]. Although there are eight-point mutations of chloroquine resistance transporter, K76T is the major mutation that is closely linked to increasing clinical and treatment failure in the use of chloroquine [5]. There are three haplotypes that are present in codon 72-76 of *Pf*crt, which are the wild-type CVMNK and the chloroquine-resistant type CVET and SVMNT. Among the three haplotypes present in codon 72-76 of the *Pf*crt, the CVET is the most important mutant haplotypes that are present in Africa [20]. Also, there have been reports that the mutation at codon A220S is associated to chloroquine resistance in African countries, while two specific mutations at codon A144T and 160Y is only linked to chloroquine resistance in Philippines [21].

Some studies done in Africa has linked the parasite clearance rate at *Pf*crt 76 to the age of the host thus suggesting that the age of the host is the best surrogate marker for immunity in resistance to chloroquine and amodiaquine. One of such studies done in Mali of K76T mutation confirmed that the infants had a slightly higher clearance rate than young children, which led to the belief that host immunity plays an important role in the clearance of parasites [22]. Another one done in southwestern part of Nigeria showed that amodiaquine selects the same marker as chloroquine (*Pf*crt 76 and Pfmdr1 86), probably due to their similar chemical structure and mode of action and also confirmed the age of the host as the best surrogate marker for immunity in the resistance to amodiaquine in West Africa [23].

3.3 *Plasmodium falciparum* Dihydropteroate Synthase (*Pf*dhps) and *Plasmodium falciparum* Dihydrofolate Reductase (*Pf*dhfr)

Sulpha drugs such as sulphadoxine bind *Plasmodium falciparum* dihydropteroate synthase (*Pf*dhps) which is an enzyme in the folate cycle that is important for DNA synthesis while drugs like pyrimethamine bind to *Plasmodium falciparum* dihydrofolate reductase (*Pf*dhfr) which is also an enzyme in the folic acid pathway [24]. The mechanisms for resistance for these two enzymes are that when a mutation occurs; it modifies the protein structure and thus reducing its binding affinity for the drugs [24].

WHO recommended sulphadoxine/pyrimethamine (SP) as the Intermittent Preventive treatment (IPTp) for pregnant women and children under

age 5 years [25]. *Plasmodium falciparum* resistance to the antifolate drugs is caused by mutations in the dihydropteroate synthase (*Pf*dhps) and dihydrofolate reductase (*Pf*dhfr) gene respectively [26].

A lot of studies have reported the prevalence of *Pf*dhps and *Pf*dhfr in Africa, this section highlighted on few of such studies. A study was done in Kombwa lowland and Kisii and Kakamega highlands in Kenya discovered that more than 80% of the samples had the quintuple mutants (51I, 59R, 108N for *Pf*dhfr and 437G/540E for *Pf*dhps) in this area [5]. Also, another study done on children under age 5years in southern Rwanda, 75% and 93% of the isolates had the *Pf*dhfr triple and the *Pf*dhps double/triple mutations, and 69% were found with the quintuple/sextuple *Pf*dhfr/*Pf*dhps mutations that have been linked to high level of sulphadoxine/pyrimethamine resistant, while LI64 mutation was absent in all isolates, but in a study done in Uganda from 2003 – 2012, five mutations (*Pf*dhfr N51I, C59R, S108N; and *Pf*dhps A437G, K540E) that connotes resistance to antifolate drugs were very prevalent, where as two additional allele (*Pf*dhfr 164L and *Pf*dhps 581G) linked to antifolates resistance showed moderate prevalence only from 2008-2011 while in Republic of Benin that involved 181 pregnant women, it was discovered that SP maintained efficacy in the treatment and prevention of placental malaria infection in pregnant women [27,28,25]. Then in Luanda, Angola, the ratio of the *Pf*dhfr wild-type C59 to the mutant type 59R was 60.6% to 20.6% with the presence of 18.8% mixed infections while that of *Pf*dhps 540E was 6.3% with mixed infections of 5.4% [26]. And in Tanzania, the clinical and molecular findings led to the conclusion that SP has a short useful therapeutic life in Tanzania after its introduction because of chloroquine resistance [29]

While some studies confirmed that the efficacy of SP is low due to the prevalent of the resistant parasite to the drug, other studies tried to check for the efficacy of chlorproguanil-dapsone; the new antifolate drug introduced after sulphadoxine/pyrimethamine. A study in Blantyre, Malawi, which involved aged 3months to 6 years children, revealed that resistance in SP did not impair the clinical efficacy of chlorproguanil-dapsone [30]. Two other works are done in Africa, one in Kenya and the other in Tanzania confirmed this and proposed that the chlorproguanil-dapsone may act as a salvage therapy for sulphadoxine/pyrimethamine resistance [31,32].

3.4 *Plasmodium falciparum* Multi Drug Resistance 1 Gene (*Pf*mdr1)

*Pf*mdr1 gene is found in chromosome 7 of the parasite and it is an antigen binding-cassette transporter that is localised in the digestive vacuole membrane [24]. It is a gene that encodes the P-glycoprotein transporter belonging to the ATP-dependent transporter family, which is responsible for the multidrug resistance in some human tumours [33]. This gene is located in the *P. falciparum* food vacuole. At least 5 mutations on *Pf*mdr1 have been identified to be associated with resistance which includes: N86Y, Y184F, S1034C, N1042D and D1246Y [34]. *Pf*mdr1 N86Y, Y184F, and D1246Y are very common in Africa, where as S1034C and N1042D are rare in Africa but very common in Asia [28]. Mutations in Pfmdr1 N86Y and D1246Y that are common in Africa have been linked to decreased sensitivity to drugs like amodiaquine and chloroquine while mutations in the 1034C and 1042D that are common outside Africa have been associated with increased sensitivity to mefloquine, lumefantrine and artemisinins [35].

Point mutations in the *Pf*mdr1 N86Y codon have been linked to being associated with chloroquine, but the presence of the wild-type N86 and increased in the copy number of *Pf*mdr1 is linked to the resistance of mefloquine and quinine drugs [36,10]. Also, the increase in gene copy number is also linked to parasite resistance to lumefantrine, artemisinin and halofantrine [37]. In a study that involved 296 children with age range 0.5 – 10years that was done in Gabon (Lambaréné), Ghana (Kumasi) and Kenya (Kisumu), the prevalence of wild type to mixed variants of the N86Y allele was 40% to 12%, 73% to 17% and 82% to 8% respectively, while the prevalence of the wild type to the mixed genotype of Y184F allele was 25% to 2%, 30% to 79%, 44% to 7% respectively, and that of the double mutant N86Y/Y184F to D1246Y was 45% to 1%, 9% to 1% and 2% to 5% respectively, with only 1% of the samples having the triple mutant N86Y/ Y184F/ D1246Y allele in both Ghana and Kenya [38]. \ Also, it was reported that patients with an infected parasite that carried the N86 allele or increased *Pf*mdr1 copy number have a greater risk of treatment failure to Artemether-Lumefantrine than patients that have the 86Y allele or a single copy number of *Pf*mdr1 gene [39].

3.5 Cytochrome b

Atoquavone is the major inhibitor of cytochrome bc 1 (cytbc1) complex; that is the major respiratory enzyme found in the mitochondria membrane of *Plasmodium falciparum* [40]. *In vitro* resistance of *P.falciparum* to atoquavone have been linked to a mutation in the *Pf*cytb region with the codons ranging from 271-274 especially the Y268S [40]. Also, mutations at 268S have been linked to malarone treatment failure, although treatment failure can also be reported in the absence of this mutation [35].

3.6 *Plasmodium falciparum* Multidrug Resistance-associated Protein 1 (*Pf*mrp1)

*Pf*mrp1 is an ABC transporter family of transmembrane protein that is located on chromosome 1 with a mass of 214.44KDa encoding about 1822 amino acids and it is predicted to have two nucleotide binding domains and two membrane-spanning domains with each having a 6-helical transmembrane domain [41]. Its major function is to help in the transport of organic anionic substances such as gluconate, oxidized glutathione, sulphate conjugates and also in transports of the drug [41]. SNPs in *Pf*mrp1 Y191H and A437S have previously been reported to be associated with *in vitro* sensitivity of chloroquine and quinine [42]. SNP in 1466K of the *Pf*mrp1 gene was associated with SP resistance in children that have uncomplicated *Plasmodium falciparum* malaria in Tanzania [43]. It was proposed that *Pf*mrp1 might cause drug-resistant by efflux of drugs and by managing drug-driven oxidative stress [42].

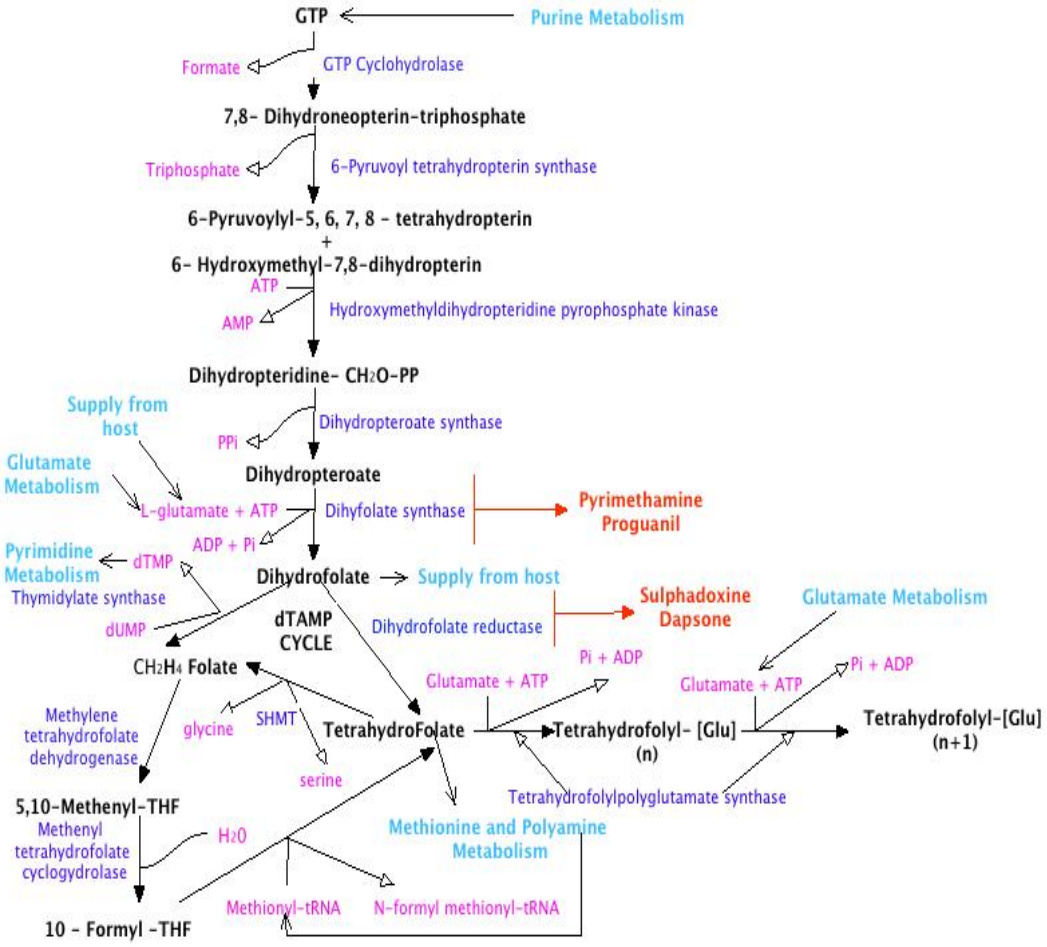

Fig. 1. Folate metabolism in *Plasmodium falciparum* showing the drugs that bind to the dhps and the dhfr enzymes

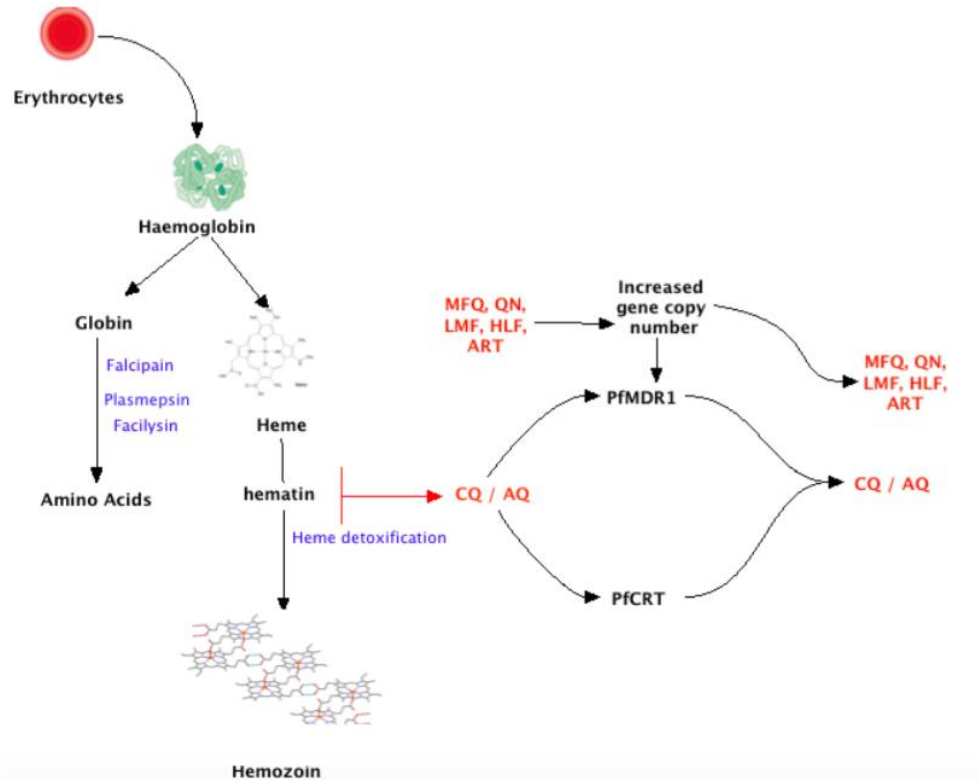

Fig. 2. Heme detoxification in *Plasmodium falciparum* and how antimalarial drugs interacts with *Plasmodium falciparum* multidrug resistance 1 and chloroquine resistance transporters. CQ – chloroquine, AQ – amodiaquine, MFQ – mefloquine, QN – quinine, LMF – lumefantrine, HLF – halofantrine, ART- artemisinin

3.7 *Plasmodium falciparum* cg2 (Pfcg2)

*Pf*cg2 is a 320-330KDa gene that is found in the electron-dense patches at the periphery of the parasitophorous vacuole in the cytoplasm and in the digestive vacuole of the *Plasmodium falciparum* trophozoites and schizonts [17]. It is made up of four peptide repeats that include kappa (κ), gamma (Y), psi (ϕ) and omega (ω), the kappa and the omega domain and some point mutations of cg2 have been linked to chloroquine resistance in some field isolates from Africa, thus suggesting them as molecular markers to monitor drug resistant parasite [21]. Also, a study done in Nigeria linked the cg2 Ala-281, Ddk2 repeats and *Pf*mdr1 Tyr 86 genotype to chloroquine resistant parasite [44].

3.8 *Plasmodium falciparum* Ca^{2+}–ATPase (PfATP6)

The *Pf*ATP6 is an ATP-dependent calcium pump that is almost identical to the mammalian sarcoplasmic/endoplasmic reticulum type calcium ATPase (SERCA) pumps and acts as a receptor for artemisinin antimalarial drugs [33]. It was hypothesized that artemisinin interacts and selectively inhibits *Pf*ATPase6 and the SNPs that have been linked to resistance of artemisinins are S769N, A623E and E431K [45]. A report from Tanzania that involved assessing *Pf*ATPase6 mutant S769N and A623E with 615 asymptomatic *Plasmodium falciparum* infections showed that there was no mutant genotype detected [45]. Also Krishna *et al*, 2006 reported that there has been no association between the mutant genotype S769N *Pf*ATPase6 and antimalarial drugs like quinine and chloroquine [46].

3.9 Kelch 13 Gene (K13)

The *Plasmodium falciparum* K13 (PF3D7_1343700) is located on chromosome 13 of the parasite and it is a 1-exon gene that codes for the putative Kelch protein, which comprises of three domains: a *Plasmodium*-specific domain, a BTB/POZ and a C-terminal six-blade propeller [6]. The kelch motif containing gene known as Kelch

13 is the major molecular marker used in determining artemisinin resistance [8]. Ariey *et al*, 2014 reported that mutations at Y493H, I543T, R539T and C580Y were associated with prolonged parasite survival *ex vivo* while mutations at Y493H, R539T and C580Y were associated with *in vivo* delayed parasite clearance and the M476I mutations were associated with artemisinin tolerance *in vitro* [47].

In an epidemiology study done in sub-Saharan Africa, none of the major five mutations previously described to cause resistance to artemisinins was found in the >1000 African parasite samples, although the P553L that was previously reported in Cambodia was found in the African samples [48]. This result is similar to another study that was conducted in Uganda where none of the 5 major mutations was detected in any of the samples [49]. While another reported that the 2 African non-synonymous mutations A578S and V566I SNPs were greater than 1% prevalent in at least 1 location among the 12 countries surveyed, and this is of interest because these two SNPs are found near to the C580Y that is the major genetic determinant for artemisinin resistance in Southeast Asia [50].

Table 2. Polymorphisms in molecular markers of antimalarial drug resistance and the various drugs of interest

Molecular markers	Gene polymorphism	Drugs (Decrease in susceptibility)
*Pf*nhe-1	Ms4760 DNNND and or NHDNHNNDDD	Quinine [14], [15] [16]
*Pf*crt	76T	Chloroquine, Amodiaquine [22] [23]
*Pf*dhfr	51I, 59R, 108N, 164L	Pyrimethamine [5], [27], [28], [25]
*Pf*dhps	437G, 540E	Sulphadoxine [5], [27], [28], 25]
*Pf*mdr1	N86	Mefloquine, Quinine, lumefantrine [36], [10]
	86Y, 1246Y	Chloroquine, Amodiaquine [35]
	Increased copy number	Mefloquine, Quinine, Lumefantrine, Halofantrine, Artemisinin [36], [10], [37]
Cytochrome b	268S	Atoquavone, Malarone [40], [35]
*Pf*mrp1	191H, 437S	Chloroquine, Quinine [42]
	1466K	Sulphadoxine/Pyrimethamine [43]
*Pf*cg2	Ala-281, Ddk2	Chloroquine [44]
*Pf*ATP6	769N, 623E, 431K	Artemisinin [45]
K13	476I, 439H, 543T, 539T, 580Y	Artemisinin [47]

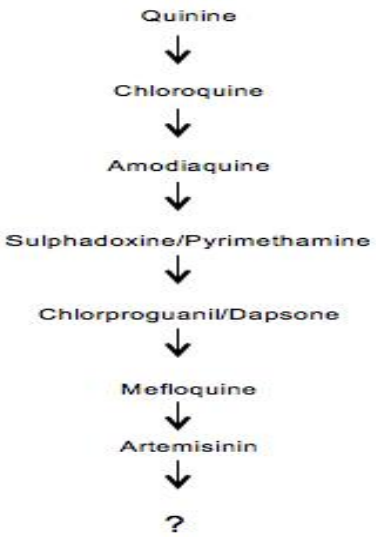

Fig. 3. The thread of antimalarial drug resistance in endemic areas in Africa

4. CONCLUSION

Plasmodium falciparum is the most virulent species of *Plasmodium* and the most prevalent in sub-Saharan Africa, one of the most endemic regions of malaria in the world. Antimalarial drug-resistant plays a major role in determining the therapy that will be used to eradicate this disease. For some time now, there have been records of antimalarial drug resistant to drugs like quinine, chloroquine, mefloquine, and sulphadoxine-pyrimethamine. This is as a result of single nucleotide polymorphisms in some target genes of the parasite. From this review done, it was confirmed that most parasite isolates from this endemic regions are already resistant to most of the available drug except artemisinin. No paper reviewed documented any of the five key mutations (476I, 439H, 543T, 539T, 580Y) of the k13 gene responsible for artemisinin-resistant. But almost all paper reviewed confirmed resistance to ACTs partner drugs. Resistance to the partner drugs can lead to clinical failure for the treatment of this tropical disease. Therefore, more work has to be done to find a solution for this deadly disease before it becomes a sore in this endemic region.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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