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Genotyping for *Plasmodium* spp.: Diagnosis and Monitoring of Antimalarial Drug Resistance

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Abstract

Malaria is one the world's most widespread lethal diseases. *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* induce human pathology. These species could be differentially diagnosed using the genotyping of cytochrome b, *Pf*dhfr and RNA 18S. The persistence of *P. falciparum*, the most lethal parasite, is mainly due to antimalarial drug resistance. Indeed, a few years after the start of the ambitious malaria eradication program in 1960, chloroquine resistance emerged in Asia and spread widely in all the endemic areas. It was associated with genotypes in *P. falciparum* chloroquine resistance transporter (CVIET, SVMNT, CVMNT, CVIDT, SVIET and CVMET). The use of new drugs such as sulfadoxine-pyrimethamine (SP) leads quickly to SP-resistant parasites associated with genotypes on *P. falciparum* DiHydroFolate reductase (I51-R59-N108-I164) and *P. falciparum* DiHydroPteroate synthetase (436-437-580-613). Recently, the delay of parasite clearance has been described with artemisinine (the most efficacious antimalarial drug). This resistance was associated with the K13 propeller genotype. Since malaria species and antimalarial drug resistance markers could be characterized using nucleic acid sequences, genotyping is needed for malarial monitoring of species distribution and antimalarial drug resistance.

Keywords: *Plasmodium* parasites, drug resistance, diagnostic, genotyping

1. Introduction

Malaria remains a major public health problem. More than 40% of the world's population (3.3 billion) live malarial endemic areas in varying degrees. Despite tremendous efforts in the fight, and though this strategy or plan resulted in significantly decreasing the burden in the

last 20 years, Malaria still is persistent in nearly 91 countries (**Figure 1**). In 2016, the overall incidence was 216 million cases among these and 445,000 deaths were recorded [1]. Africa continues to account for 90% of malaria burden. African children under 5 years of age are the most affected. This infectious disease is due to the invasion of *Plasmodium* spp. Currently, the five species are known to infect and to induce significantly malaria in humans are *Plasmodium ovale, Plasmodium malariae, Plasmodium knowlesi, Plasmodium vivax* and *Plasmodium falciparum*.

P. falciparum is the most virulent species as it is responsible for more than 90% of malaria deaths [2, 3]. Its specific biology with antigenic variation, sequestration of infected blood cells and interactions with host cells leads to severe malaria [4, 5]. Also, it is the most recent human infection with limited adaptation in the host [6]. *P. knowlesi*, which is a specific Asian monkey parasite (*Macaca* genus), was recently transferred to humans, causing high mortality in the south of Asia [7–9]. One of its differences from other species is the time of its life cycle which is 24 h whereas 40–48 h for other human *Plasmodium* spp. *P. vivax* is the most prevalent in Asia and South America. Due to the Duffy-negative statue of Black people, it is the rarest in Black Africa. However, recent studies reported the presence of *P. vivax* in Blacks with Duffy-negative from some countries of central Africa such as Equatorial Guinea, Congo Republic *and so on* [10, 11]. *P. malariae* is less prevalent in Asia, while it is most common in sub-Saharan Africa and southwest Pacific [12]. It often finds minor prevalence compared to *P. falciparum*. This parasite is thought to be a zoonotic infection because is genetically close to *P. brasilianum* which infects monkeys of South America [12, 13]. *P. ovale* is prevalent in Sub-Saharan Africa, South-East Asia, India, Papua New Guinea, Timor and Indonesia [14]. It is the less-prevalent human malarial parasite. However, in most places where *P. ovale* is observed, it is relatively uncommon and its prevalence rarely exceeds 5% [12].
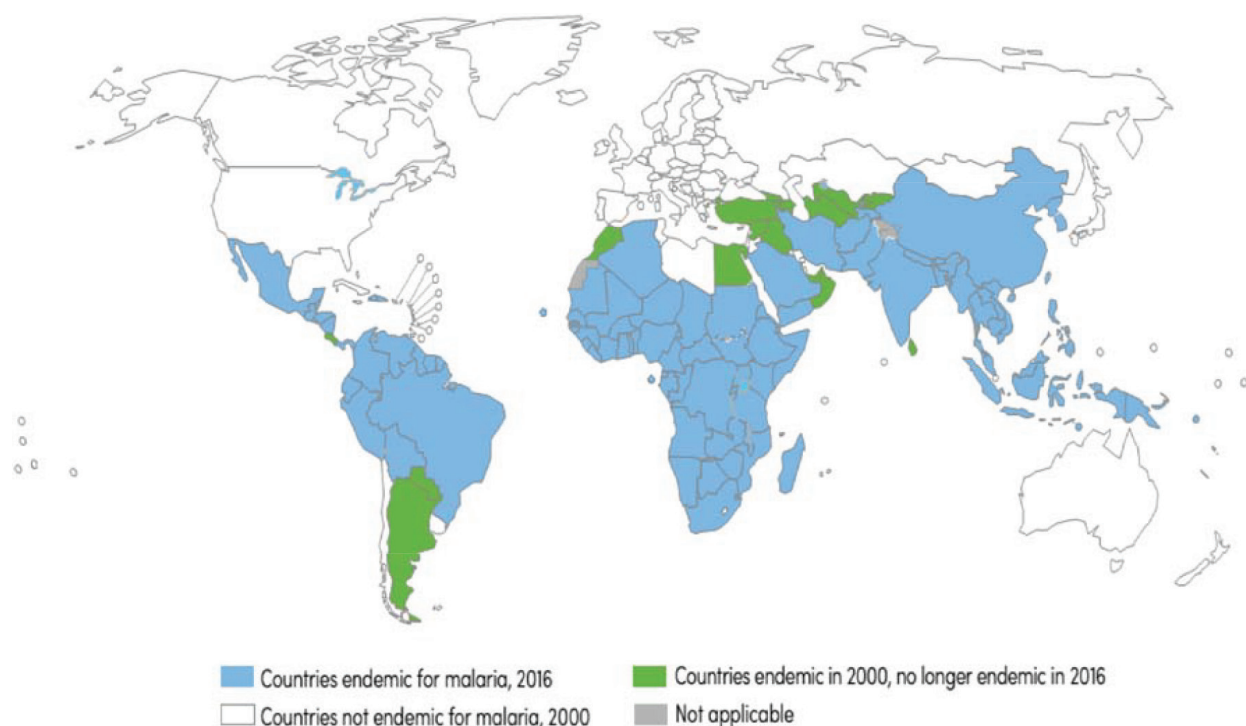
Figure 1. Countries endemic for malaria in 2000 and 2016. From WHO [1].

Decisions concerning malaria treatment depend on the identification of the species causing the disease. Traditionally, this diagnosis was based on the microscopic detection of *Plasmodium* parasites in Giemsa-stained blood slides. In recent decades, antigen detection assays and molecular detection assays were introduced as alternatives to microscopy [15]. These approaches were very useful; however, they are not very reliable. Indeed, the morphological features and life history traits of a parasite species can vary from one host species to another [16, 17]. For antigen detection assays are mainly aimed at the identification of *P. falciparum*. Indeed [18], only a few assays are able to identify infections caused by other human malaria parasites [15, 19]. However, the development of molecular tools for the identification of species in diagnosis and genotyping permits a better reading of plasmodial diversity circulating among the human population and allows best highlighting the phenomenon of resistance of *Plasmodium* than microscopic tools.

Plasmodium species like several other genera have specific genetic markers such as *18S rRNA*, *ITS, cytochrome b* and so on, used in studying speciation. These markers play an important role in molecular analysis of genotyping and monitoring antimalarial drug resistance. The persistence of malaria burden is partly due to the emerging and widespread nature of the antimalarial drug resistance. Indeed, in the 1950s, WHO launched the malaria eradication program, with chloroquine and *dichloro-diphenyl-trichloroethan* (DDT). But in the early 1960s, chloroquine resistance emerged [20, 21]. Antimalarial drug resistance is defined as the parasite's ability to survive after the absorption of drug doses is greater than patient-tolerated doses. This chapter described the methods of genotyping the malaria for species diagnosis that helps to monitor drug resistance.

2. Genotyping for *Plasmodium* spp. diagnosis

The genotyping of *Plasmodium* spp. infections allows for the characterization of distinct species and subpopulations present in hosts. The genotyping techniques presented in the following allow for the characterization of different *Plasmodium* species by sizing the polymerase chain reaction (PCR) product of the polymorphic marker gene merozoite surface protein.

2.1. PCR using the specificity of 18S RNA

rRNA is one of the ribosome components. Among rRNA, the *18S rRNA* gene is the most frequently cited marker for malaria detection. It is composed of highly conserved regions which can be targeted for a qualitative detection of *Plasmodium* spp. and of variable zones allowing species identification. Early in the 1990s, Snounou and colleagues reported high-sensitivity methods for the detection of plasmodium species using nested PCR [22]. This method was based on the conversion of 18S rRNA among human plasmodium and the specificity of 18S rRNA from each parasite. So, the method includes the first PCR with primers named rPLU1/5 and rPLU3/4 that match with human plasmodium. This step is followed by the amplification of the product of primary PCR separately with the four species-specific primer pairs: rFAL1 and rFAL2, rVIV1 and rVIV2, rMAL1 and rMAL2 and rOVA1 and rOVA2 to identify the

species *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, respectively. The primary PCR with rPLU1/5 and rPLU3/4 gives a 1100 bp band in a 2% agarose gel electrophoresis (**Figure 2**), in the presence of *Plasmodium*.

The nested PCR rFAL1 and rFAL2 generate a 205 bp in presence of the *P. falciparum* parasite. The nested PCR rVIV1 and rVIV2 generate a 120 bp in presence of the *P. vivax* parasite. The nested PCR rMAL1 and rMAL2 generate a 144 bp in presence of the *P. malaria* parasite. The nested PCR rOVA1 and rOVA2 generate an 800 bp in presence of the *P. ovale* parasite [22, 23].

2.2. RT-PCR NASBA 18S rRNA

Nucleic acid sequence-based amplification (NASBA) is a method in molecular biology, which is used to amplify RNA sequences. This novel approach of genotyping, based on the amplification of nucleic acid sequence (real-time QT-NASBA), was developed by Compton [24]. Immediately after its discovery, the NASBA method was used for the rapid diagnosis and quantification of HIV-1 in patients [25]. Some years later, Schooner et al. [26] developed a real-time quantitative nucleic acid sequence-based amplification (real time QT-NASBA) for the detection of *Plasmodium falciparum* 18S rRNA with a sensitivity of 10–50 parasites/ml [26, 27]. Thus, NASBA method that uses primers and probes were selected on the basis of the sequence of the small subunit 18S rRNA gene [26, 28], to characterize or identify the different human *Plasmodium* species [18]. For NASBA, Schooner et al. have defined primers Plas-1F (59-TCAGATACCGTCGTAATCTTA-39) and Plas-2R T7 (59-AATTCTAATACGACTCACTATAGGGAGAGAACTTTCTCGCTTGCGCGAA-39) which were used [26]. The RNAs from *P. falciparum*, *P. malariae*, *P. vivax* and *P. ovale* are specifics
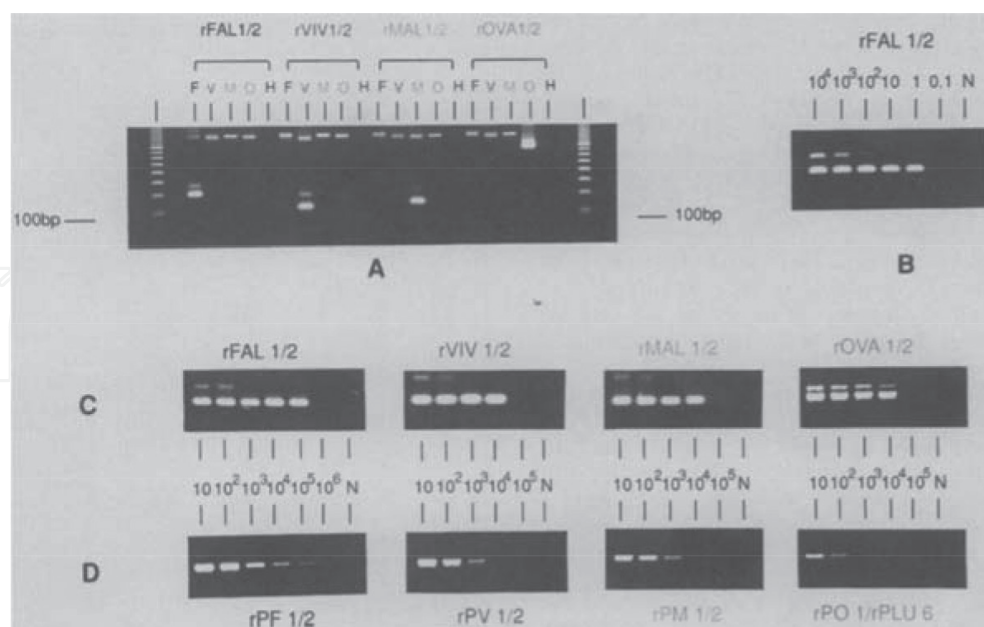
Figure 2. Specificity and sensitivity of the PCR detection assay. (a) Nested PCR amplification for the demonstration of the specificity of the primers employed. Control genomic DNA from *P. falciparum* (F), *P. vivax* (V), *P. malariae* (M), *P. ovale* (O) and human blood (H); (b) nested PCR assay for the detection of in vitro cultured *P. falciparum* ring-stage parasites; (c) nested PCR amplification using diluted control DNAs; (d) product of amplification of diluted control DNAs, using the PCR assay (Source: Snounou et al. [22]).

and they should all be amplified by the NASBA isolates with these primers. Therefore, detection is done by capture probe (59-ACCATAAACTATG CCGACTAGG-39) which is bound to magnetic beads. Finally, samples are hybridized separately to ruthenium-labeled WT (59-CCTTATGAGAAATCAAAGTC 39) and Q (59-AATAACTGCACCAGTGTATA-39) detection probes, followed by ECL detection in a NucliSens ECL reader [26, 29]. The NASBA method is very sensitive and specific. It can be used for the detection, identification and quantitative measurement of low parasitaemia of *Plasmodium species*, that is, the lower detection limit of the assay is 100–1000 molecules in vitro RNA for all human malaria parasites.

2.3. PCR sequencing cytochrome b

Cytochrome b (*Cyt*-b) is one of the respiratory chain systems present in mitochondria. It is highly conserved in *Plasmodium* species. But some of its region shows diversities and species specificities. It is also in multiple copies (20–100 copies) in the haploid genome, suggesting the increase of sensitivity. So, the alignment of *Cyt*-b sequences obtained after amplification by the nested PCR portion of mitochondrial DNA using couples of primers (**Table 1**) allows distinguishing the *Plasmodium* species present in the host (**Figure 3**).

The first round of PCR (PCR$_1$) and use of the primers DW2/F and DW4/R produce the fragments of 1253 bp. In the second round of PCR (PCR$_2$), primers *Cyt*-b1F and *Cyt*-b2R are used and this generates PCR fragments of 939 bp [30]. This approach was used more for the characterization of malaria parasites in primates (human and non-human) [31, 32] because it allows to identify all species known or unknown circulating in vertebrates [33].

2.4. PCR-RFLP from cytochrome b

Due to the specie specificity and diversity of Cytochrome b, it could be digested with the r estriction enzyme *Alu I* leading to species-specific patterns [34]. In this case, a nested PCR is used. Primers Plas 1 (5′-GAGAATTATGGAGTGGATGGTG-3′) and Plas 2 (5′-GTGGTAA TTGACATCCWATCC-3′) are used for primary PCR producing an 816 bp fragment, following by a nested with Plas 3 and Plas 4 primers. The n-PCR produces a 787 bp fragment. The digestion with *Alu I* gives 159 and 640 bp fragments; 187, 249 and 381 bp fragments; 111, 169, 187, 249 and 270 bp; and 224 and 584 bp fragments for *P. falciparum*, *P. malariae*, *P. vivax* and *P. ovale*, respectively, on agarose gel electrophoresis (**Figure 4**).

| Primer name	Sense	Sequence (5′➜ 3′)	Fragment size (bp)
DW2 (1st PCR)	Foward	TAATGCCTAGACGTATTCCTGATTATCCAG	1253
DW4 (1st PCR)	Reverse	TGTTTGCTTGGGAGCTCTAATCATAATGTG	1253
Cyt-b1 *(2nd PCR)*	Foward	CTCTATTAATTTAGTTAAAGCACA	939
Cyt-b2 *(2nd PCR)*	Reverse	ACAGAATAATCTCTAGCACC	939

Table 1. Amplification primers of the cytochrome b gene (*Cyt*-b). *Cyt*-b is amplified by nested PCR.
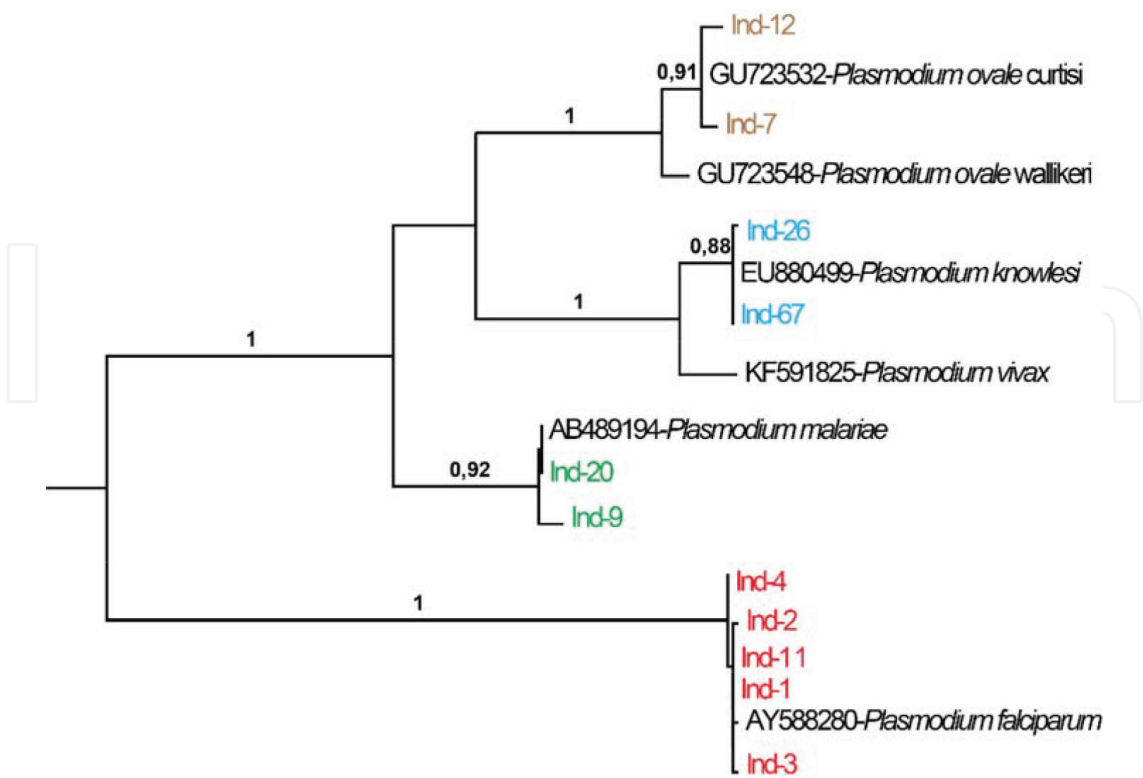
Figure 3. The tree of relationship of *Plasmodium* species. This tree was built with a portion of *Cyt*-b sequences characterized among some patients and other *Cyt*-b sequences from Genbank (accession numbers AB489194, EU880499, GU723548 and GU723532). The tree was built using maximum likelihood with Cyt-b sequences of 925pb sizes.

2.5. PCR-DHFR

Figure 5 shows the equation of transformation of dihydrofolate to tetrahydrofolate. The dihdrofolate reductase is one of the important malaria proteins involved in the plasmodium folate synthesis. This gene is coded in chromosome 4 and is highly conserved between
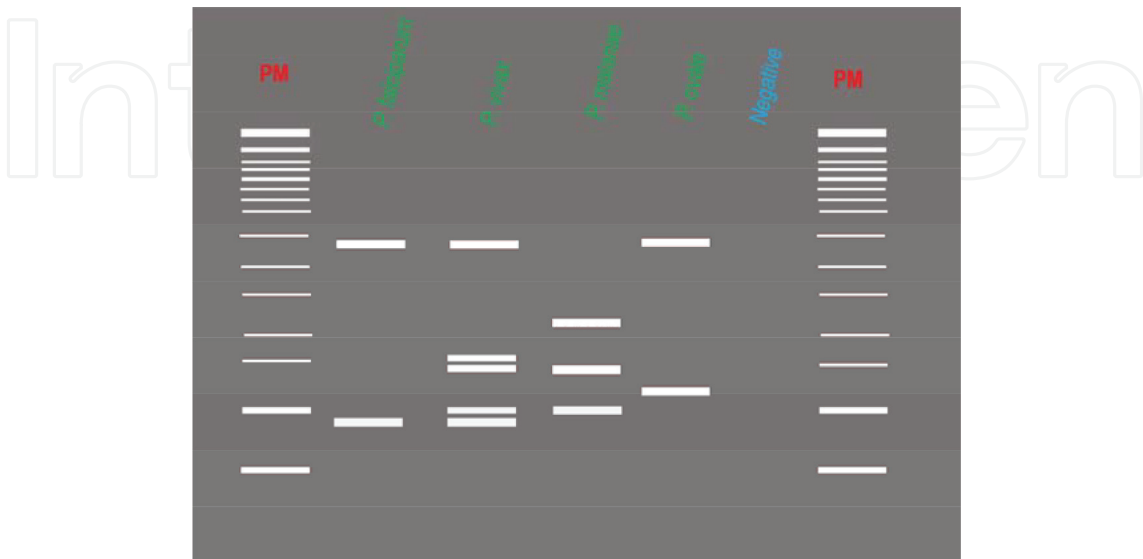
Figure 4. Illustration of a digestion cytochrome fragment using *Alu* enzyme.
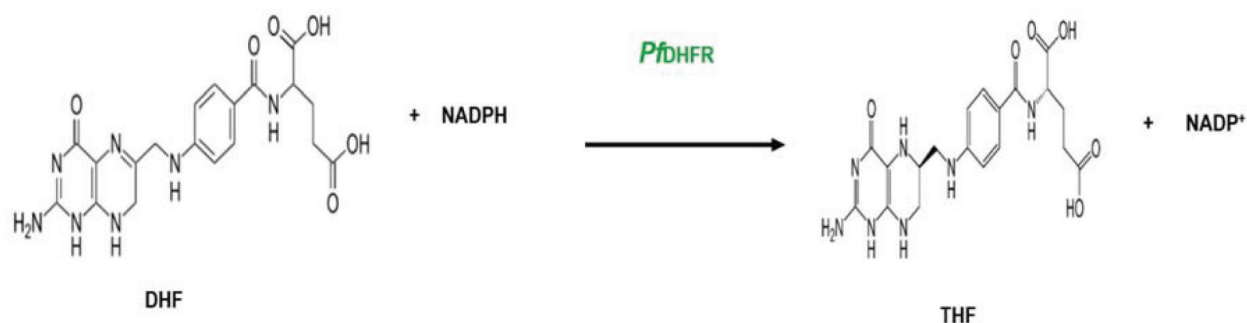
Figure 5. The equation of the DHF-THF reaction. Dihydrofolate reductase is an enzyme that catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. This reaction is essential for the de novo synthesis of purines and certain amino acids. This enzyme is essential for rapid growth and is the target for the action of the important antimalarial drugs pyrimethamine and proguanil.

distantly related species, like plasmodium species. Its linker region revealed significantly a higher sequence diversity than the relatively conserved enzymatic diversity. Different species of *Plasmodium* are characterized by a unique linker sequence. So, it has been used to identify human plasmodium species [35]. Nonsynonymous mutations on DHFR are associated with pyrimethanime resistance. Using primers ATGGARSAMSTYTSMGABGTWTTYGA and AAATATTGRTAYTCTGGRTG for primary PCR gives a 1000 bp fragment. The nested PCR species specific with primers shown in **Table 2** allow to amplify specifically 160, 177, 144, 231/237/243 and 134 bp fragments for *P. falciparum*, *P. malariae*, *P. vivax*, *P. ovale* and *P. knowlesi*.

Primer	Species	Sequence (5′–3′)	Annealing (°C)	No. of cycles	Product (bp)
Pla-DHFR-F	*Plasmodium sp.*	ATGGARSAMSTYTSMGABGTWTTYGA	50	30	1000
Pla-TS-R		AAATATTGRTAYTCTGGRTG			
Pla-DHFR-NF	*Plasmodium sp.*	AAATGYTTYATYATWGGDGG	55	35	509–587
		AAATATTGRTAYTCTGGRTG			
Pla-TS-R					
PF-Lin-F	*P. falciparum*	AAAAGGAGAAGAAAAAAATAA	50	35	160
PF-Lin-R		AAAATAAACAAAATCATC			
PM-Lin-F	*P. malariae*	GACCCAAGAATCCCTCCC	50	35	177
PM-Lin-R		CCCATGAAGTTATATTCC			
PV-Lin-F	*P. vivax*	CGGGAGCACTGCGGACAGCG	55	35	144
PV-Lin-R		CACGGGCACGCGGCGGGGC			
PO-Lin-F	*P. ovale*	GACACACAAAATGATGGGGA	55	35	231, 237 or 243
PO-Lin-R		ATTGTCCTTTCCTTGACTCG			
PK-Lin-F	*P. knowlesi*	CGATGGATATGGATAGTGG	58	35	134
PK-Lin-R		CGCGGGAGAGCATTTCCTC			

Table 2. Primer sequences and the PCR condition for detection of ***Plasmodium spp***. that infect humans.

3. Genotyping for the monitoring of antimalarial drug resistance

3.1. Antimalarial drug resistance

Antimalarial drug resistance is the ability of *P. falciparum* to survive after absorption of the drug at concentrations greater than concentrations tolerated by the patient. Drug resistance arises rarely because it is the result of some non-lethal mutations, but it spreads relatively quickly. The clinical failure treatment is the lasted event in the long way of changes in parasites genes. Antimalarial drug resistance markers are genes associated with antimalarial drug resistance. Among them, the most characterized are *Pf*crt (*P. falciparum chloroquine resistance transporter*), *Pf*mdr1 (*P. falciparum multidrug resistance 1*), *Pfdhfr* (*P. falciparum dihydrofolate reductase*), *Pfdhps* (*P. falciparum dihydropteroate synthase*), *Pfmrp* (*P. falciparum multidrug resistance protein*), *Pfatpase 6* (*P. falciparum atpase 6*) and *Pf*K13 (*P. falciparum kelch 13*).

3.2. The genotyping of markers associated with antimalarial drug resistance

3.2.1. P. falciparum chloroquine resistance transporter (Pfcrt)

*Pf*crt is an ATP binding cassette (ABC) protein, able to fixe and hydrolyze ATP. The general structure of the ABC transporter contains cytosolic nucleotide binding domains (NBDs), a nucleotide hydrolysis site and transmembrane segments. These transporters extrude effective drugs from the digestive vacuole and function as an efflux pump leading to the decrease of intracellular accumulation of drugs in the parasite. The genetic cross-experiment between chloroquine-sensitive and chloroquine-resistance strains allowed the identification of *P. falciparum* chloroquine-resistance transporter (*Pf*crt) in 2000 [20, 36]. It is a 45 kDa protein coding in chromosome 7, containing 10 predicted transmembrane domains located on the membrane of the digestive vacuole (**Figure 6**).

Several mutations have been identified in this transporter. The main mutation T76 allows for the abolition of accumulation of the drug chloroquine in the digestive vacuole. The association of mutations in codons 72, 73, 74, 75 and 76 defined different haplotypes. These haplotypes show a spatio-temporal specificity. CVMNK is the wild-type haplotype that is found in chloroquine-sensitive parasites. In Africa, the most prevalent chloroquine-resistance haplotype is the CVIET. It was also found with less prevalence in South America and in Southeast of Asia. Another *Pf*crt resistance haplotype, named South American haplotype, is the SVMNT. That is rarely found in Africa and Asia, has relatively little fitness cost and was associated with the emergence of amodiaquine resistance too [37, 38]. The other main *Pf*crt-resistance haplotypes were CVMNT, CVMET, SVIET and CVIDT. CVMNT is most prevalent in South America and in Asia but rarely found in Africa [39, 40]. CVMET is the rarest haplotype found in Asia whereas SVIET is the South American haplotype. CVIDT is the specific haplotype from Asia. Haplotypes CVIET and SVMNT were also associated with the plasmodial resistance against amodiaquine and lumefantrine [41]. In our recent study from Gabon and Congo we found new haplotypes. But the involvement of these in antimalarial resistance is needed yet (unpublished yet). The withdrawal of chloroquine has led to the decrease of the T76 genotype.
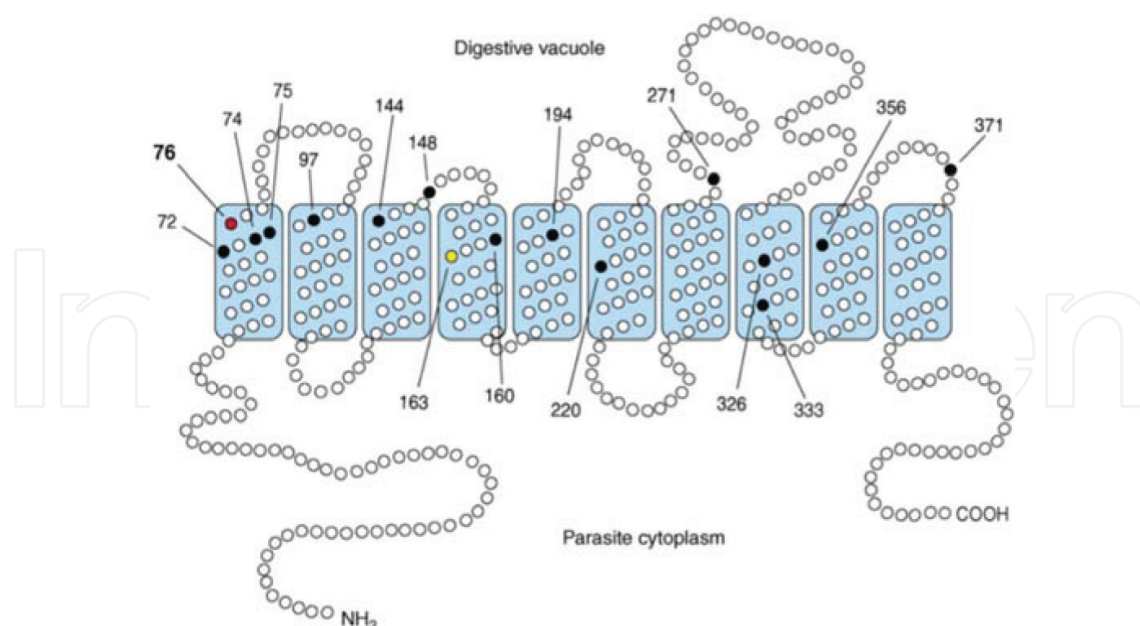
Figure 6. The predicted structure and representative haplotypes of *P. falciparum* chloroquine-resistance transporter. *Pf*CRT is predicted to have 10 transmembrane domains, with its N and C terminals located on the cytoplasmic side of the digestive vacuole membrane. Mutations identified in the *Pf*crt cDNA sequences from CQR lines (black circles), the crucial K76T mutation common to all CQR strains (red) and the S163R mutation identified in the amantadine- and halofantrine-resistant parasites (yellow circle) are indicated (this figure was pulled in [40]).

Furthermore, recent data showed that the use of artemisinine-based combination therapies, mainly artemether-lumefantrine, selected the wild-type K76 genotype. Other polymorphisms in *Pf*crt were described including A144F, I194T, A220S, Q271E and N326S [40].

For the genotype haplotype 72–76 of *Pf*crt, PCR sequencing and PCR-RFLP are used. That is based on nested PCR with several primers (**Table 3**). The RFLP pattern indicates a specific genotype for each codon.

3.2.2. Plasmodium falciparum multidrug resistance 1 (Pfmdr1)

P. falciparum multidrug resistance 1 is a homologous of the bacterial multidrug resistance 1 protein. It is one of the main antimalarial drug resistance markers. Like *Pf*crt, *Pf*mdr1 is also an ABC transporter, located in the membrane of a digestive vacuole, coded in chromosome 5 by the 4758 bp gene. Its structure shows two homologous parts containing six transmembrane domains plus a nucleotide binding domain (**Figure 7**).

Some isolates exhibit multicopies of *Pfmdr1*. Polymorphisms in codons 82, 184, 1034, 1042 and 1246 (N86Y, Y184F, S1034C, N1042D and D1246Y) were associated with antimalarial resistance against mefloquine, lumefantrine, artemether, halofantrine, quinine and chloroquine [36, 42]. Copy number and polymorphisms of the *pfmdr1* gene have been investigated as molecular markers of mefloquine resistance. With the treatment of artemether-lumefantrine, a selection of N86 genotype was reported [43]. That was confirmed in areas where this ACT was implemented [44]. In same way, it was shown that ACT led to the selection of haplotypes NFD, NYD in codons 86, 184, 1246.

Genes, codons	Primer names	Primers	T°C	Restriction enzyme	Sizes (bp)*
Pfcrt, C72S	CRT72MS	TTTATATTTTAAGTATTATTTATTTAAGTGGA	55	*Mbo I*	55 + 38
	76-D2	CAAAACTATAGTTACCAATTTTG			
Pfcrt, M74I	CRT745MS	TAAGTATTATTTATTTAAGTGTATGTGTCAT	55	*Nla III*	53 + 31
	76-D2	CAAAACTATAGTTACCAATTTTG			
Pfcrt, N75M	CRT745MS	TAAGTATTATTTATTTAAGTGTATGTGTCAT	50	*BspHI*	53 + 31
	76-D2	CAAAACTATAGTTACCAATTTTG			
Pfcrt, 76	Pfcrt-76A	GCGCGCGCATGGCTCACGTTTAGGTGGAG	55	*Apo I*	136 + 56
	Pfcrt-76B	GGGCCCGGCGGATGTTACAAAACTATAGTTACC			

Sizes* indicate the sizes of fragments generated after restriction enzyme digestions. T°C= hybridization temperature during PCR program.

Table 3. Sequences of primer sets and restriction enzymes used to characterize polymorphisms.

To the genotype Pfmdr1, followed primers and restriction enzymes are used for PCR-RFLP or for PCR followed by sequencing gene. Digestion of PCR products gives fragments of 126 and 165 bp for mutant 86Y whereas wild type N86 is not digested by restriction enzyme *Afl III*. In codon 1246, wild-type D1246 is digested by restriction enzyme *Bgl II*, giving fragments of 113 and 90 bp, whereas the mutant is not digested (**Table 4**). For codon 184, the genotype is assessed using the PCR followed by sequencing.
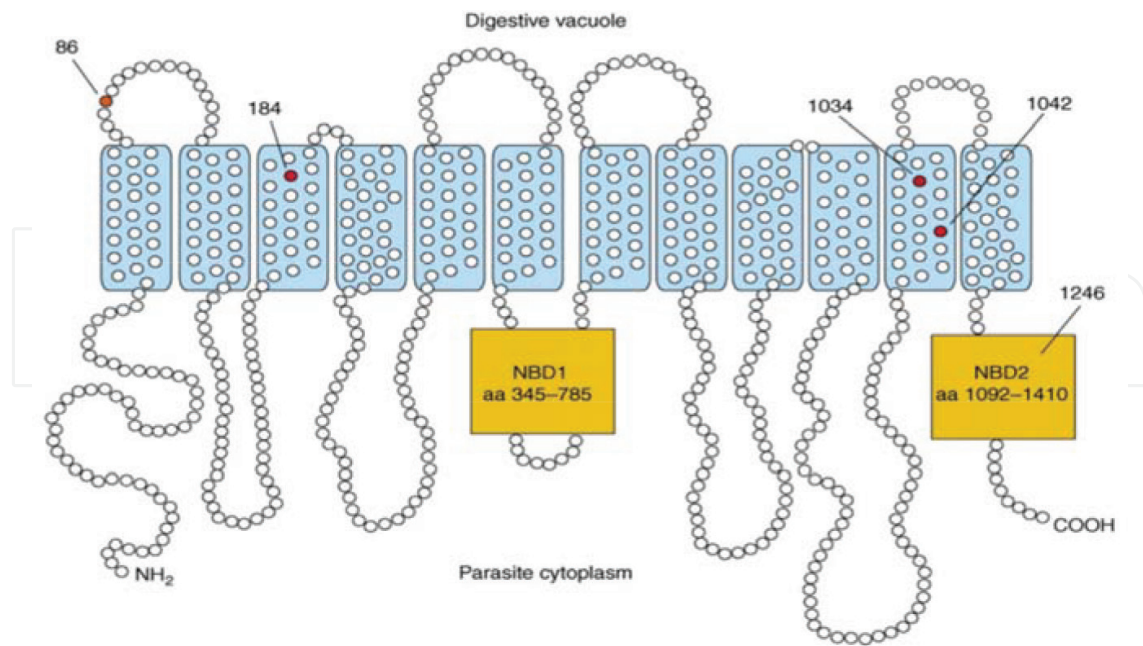
Figure 7. Genetic polymorphisms in *P. falciparum* multidrug resistance-1. *Pf*MDR1 has two homologous halves, each with six predicted transmembrane domains and a nucleotide-binding pocket. The nucleotide-binding domains (NBD1 and NBD2; orange boxes) are each formed by large cytoplasmic domains. Polymorphic amino acids found in the K1 allele (N86Y) and the 7G8 allele (Y184F, S1034C, N1042D and D1246Y) are indicated. The D1246Y mutation is located in the predicted NBD2 (this figure was pulled, from Ref. [40]).

Genes, Codons	Primer names	Primers	T°C	Restriction enzyme	Sizes (bp)*
Pfmdr1, N86Y	mdr86D1	TTTACCGTTTAAATGTTTACCTGC	45	*Afl III*	126 + 165
	mdr86D2	CCATCTTGATAAAAAACACTTCTT			
Pfmdr1, D1246Y	mdr1246D1	AATGTAAATGAATTTTCAAACC	45	*Bgl II*	113 + 90
	mdr1246D2	CATCTTCTCTTCCAAATTTGATA			

Sizes* indicate the sizes of fragments generated after restriction enzyme digestions. T°C = hybridization temperature during PCR program.

Table 4. Sequences of primer sets and restriction enzymes used to characterize *Pf*mdr1 polymorphisms.

3.2.3. Plasmodium falciparum dihydrofolate reductase (PfDHFR)

P. falciparum dihydrofolate reductase is mainly involved in the synthesis of the thymidine base. This enzyme is the target of pyrimethamine which blocked the synthesis of DNA and lead to the death of the parasite. After implementation of this drug, isolates with resistance against it were described. This parasite carried the mutations in the *Pf*dhfr gene. Among these, the mutation of serine to asparagine in codon 108 is the main resistance mutation. Additional mutations in codons 16, 51, 59 and 164 contribute to the increase of the resistance level of the parasite against pyrimethamine. The double mutant 108N + 51I and 108N + 59R increased the IC50 of parasites from 2 to 16 times, compared to the simple mutant N108 [45, 46]. In this way the triple mutant 51I + 59R + 108N or 59R + 108N + 164R shows the highest resistance level compared to the double mutant. Quadruple mutants exhibit a higher level of resistance compared to the triple mutant [47]. In Africa, the AIRNI haplotype is the most prevalent. PCR-RFLP was developed to genotype *Pf*dhr codons associated with pyrimethamine resistance. For these, primers and restriction enzyme are contained in **Table 5.**.

Gènes	Types de PCR	Amorces	Séquences génétiques	T°C	Taille de l'amplican (pb)*	Mutation	Enzymes de restriction	Génotypes	Tailles de fragments (pb)
DHFR	PCR I	M1	5'TTTATGATGGAACAAGTCTGC3'	45	648				
		M5	5'AGTATATACATCGCTAACAGA3'						
	PCR II	M3	5'TTTATGATGGAACAAGTCTGCGACGTT3'	45	522	A16V	*Nla III*	16V	376+146
						N51I	*Tsp509I*	51I	218+120
		F/	5'AAATTCTTGATAAACAACGGAACCTtTA3'			S108N	*Alu I*	108N	522
						I164L	*Dra I*	164L	245+143+107
		F	5'GAAATGTAATTCCCTAGATATGgAATATT3'	45	325	C59R	*Xmn I*	59R	162+163
		M4	5'TTAATTTCCCAAGTAAAACTATTAGAgCTTC3'						
DHPS	PCR I	R2	5'AACCTAAACGTGCTGTTCAA3'	45	710				
		R/	5'AATTGTGTGATTTGTCCACAA3'						
	PCR II	K	5'TGCTAGTGTTATAGATATAGGatGAGcATC3'	45	437	A437G	*Ava II*	437G	404
		K/	5'CTATAACGAGGTATTgCATTTAATgCAAGAA3'			K540E	*Fok I*	540E	320+85
						S436A	*MnII*	436A	317+121
		L	5'ATAGGATACTATTTGATATTGGAccAGGATTcG3'	45	160	A613S	*Mwol*	613S	161
		L/	5'TATTACAACAATTTGATCATTCgcGCAAccGG3'						

Table 5. Primer sets and RFLP conditions to genotypes *Pf*DHFR and *Pf*DHPS.

3.2.4. Plasmodium falciparum dihydropteroate synthase (PfDHPS)

P. falciparum dihydropteroate synthase (*Pf*dhps) is one of the enzymes involved in the line of thymidine synthesis. It transforms pteridine to dihydropteroate in the presence of pABA. It is coded by the gene located in chromosome 8 of *P. falciparum*. The implementation of sulfadoxine to treat malaria led to *P. falciparum* isolates' resistance against this drug. Genotype analysis reported the association of *Pf*dhps mutations with sulfadoxine resistance [48]. So, mutations S436A, A437G, K540E, G581A and A613S were reported [49]. The mutation A437G increased the IC_{50} of isolates by five times [50]. Isolates with triple mutations S436A + A437G + K540E and S436A + A437G + A613S showed the level of sulfadoxine resistance around 9–24 times higher than the single mutant.

Triple mutant I51R59N108 in *Pf*dhfr and double mutants G437E540 and G437S581S in Pfdhps increase the risk of failure when treated with sulfadoxine-pyrimethamine [51]. The drug resistance against sulfadoxine and pyrimethamine could be monitored by genotyping *Pf*dhfr and *Pf*dhps according to the PCR-RFLP conditions shown in **Table 5**.

3.2.5. Plasmodium falciparum ATPase 6 (PfATPASE6)

The sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, ortholog of *P. falciparum* (*PfSERCA* or *PfATPase6*), is an active Ca^{2+} protein transporter. This is a part of P-type ATPases enzymes that transport ions across biological membranes with the energy provided by the ATP hydrolysis. Several P-type ATPases, that were reported in *P. falciparum* and *Pf*serca, correspond to type 6. The gene coding this protein is located in chromosome 1 and contains 3687 bp. The protein allows the trafficking of calcium though the sarcoplasmic-endoplasmic membrane. Several polymorphisms such as S769N, Y243H, K431E, G110A, A2694T or E623A were reported in *Pf*ATPase 6.

*Pf*ATPase 6 has been associated with antimalarial drug resistance [52]. The most important paper on this protein was the report of its involvement in artemisinin resistance [53]. But, the role of *Pf*ATPase 6 in artemisinin resistance was strongly disputed. S769 N was associated with artemether resistance in French Guinea [53]. However, several articles investigating *Pf*ATAPase 6 in artemisinin drug resistance failed to confirm this point [54–56]. Genotyping using PCR followed by sequencing is usually used to monitor antimalarial drug resistance. Primers used are previously described by Tahar et al. [57].

3.2.5.1. P. falciparum multidrug resistance protein (PfMRP)

P. falciparum multidrug resistance protein is another marker of antimalarial drug resistance. It belongs to the C subfamily of ABC transporters containing two NBDs, two membrane-spanning domains and six transmembrane domains. *Pf*mrp can transport glutathione, glucuronate, as well as glucuronide and sulfate-conjugated compounds that increased susceptibility to several antimalarial drugs like chloroquine, quinine or artemisinin [58]. *Pf*mrp1 and *Pf*mrp2 are expressed in the plasma membrane in all asexual stages of the parasite. Their expression is upregulated by mefloquine and chloroquine. *Pf*mrp1 is a 215 kDa protein coding on chromosome 1. Mutations on codons H191Y and S437A were associated with quinoline resistance [59]. But no association was found between these polymorphisms and resistance against

pyronaridine [60]. The hypothetic mechanism of involving of *Pf*mrp on antimalarial drug resistance is the pump efflux mechanism associated with the extrusion of glutathione. The genotyping of *Pf*mrp 1 and Pfmrp2 is achieved using PCR followed by sequencing. The primers used for amplification and sequencing are pfmrp-501F 5'-TTT CAA AGT ATT CAG TGG GT-3' and pfmrp-1409R 5'-GGC ATA ATA ATT GAT GTA AA-3'.

3.2.5.2. P. falciparum Kelch 13 (PfK13)

Chinese populations used *Artemisia annua* to treat malaria for a long time. Over the years, artemisinin was extracted from this plant. That is a sesquiterpene lactone. Now, artemisinin is one of the best antimalarial drugs. So, WHO recommends the use of this drug in association with other antimalarial drugs, artemisinin combination therapy (ACT) for the treatment of uncomplicated malaria. Since the 1990s, the use of artemisinin was highly intensified in Asia. Consequently, the delay of *P. falciparum* clearance was reported after treatment with artemisinin which translates to artemisinin resistance [56]. These slow clearance rates are associated with enhanced survival rates of ring-stage parasites briefly exposed in vitro to dihydroartemisinin. Recently, a large-scale study identified molecular markers of this resistance: polymorphisms on propeller kelch 13 (K13) [61]. That is a region on chromosome 13 (**Figure 8**) [62].

Several investigations on K13 genotyping reported that mutations M476I, Y493H, R539T, I543T, P553L and C580Y conferred a greater artemisinin resistance [63]. Other mutations F446I and A578S were described in PfK13. A578S, widespread in Africa, is not associated with artemisinin resistance. These genotypes are investigated by PCR sequencing.
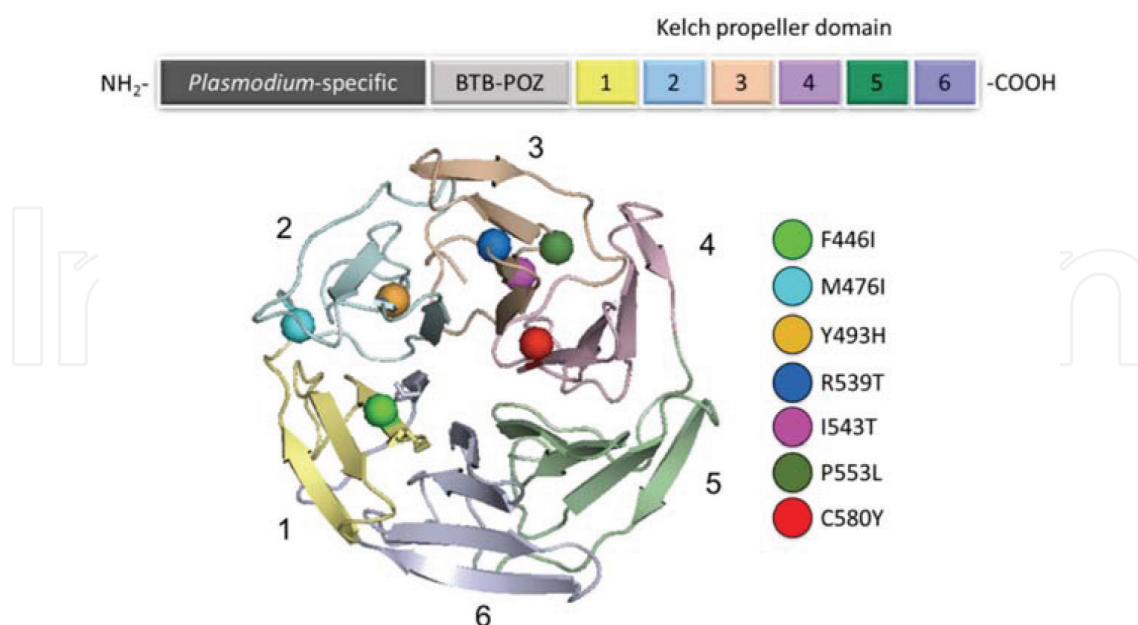
Figure 8. *P. falciparum* kelch13 (K13) protein. The parasite K13 protein consists of plasmodium-specific sequences, a BTB-POZ domain and six kelch domains that are predicted to form a six-blade propeller. In the structural model, the original M476I mutation discovered by Ariey et al. [61] and six other mutations associated with artemisinin resistance (all details of this figure was pulled of Fairhurst and Dondrop [62]) are shown.

4. Conclusions

Due to the nucleotide specificities of each *Plasmodium* species and the molecular changes associated with antimalarial drug resistance, genotyping is used for *Plasmodium* species diagnosis and monitoring antimalarial drug resistance. This genotyping could be achieved by specific PCR, PCR-RFLP, PCR-sequencing or RT-PCR of some molecular markers.

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