Gene mutations in parasitic diseases Part II: Parasite gene mutations

Review
Article

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ABSTRACT

Gene mutation may occur either in the parasite or in the host, which may be beneficial or harmful for each. As we previously discussed, part I covered causes and types of gene mutations as well as their relation(s) to or effect(s) on parasitic diseases; part II deals with parasite gene mutations. The most apparent manifestation is drug resistance especially with anti-malarial drugs for *falciparum* malaria. The majority of *P. falciparum* isolates are able to undergo gene mutations in genes encoding enzymes that control drug uptake. Parasite gene mutations are suggested to influence parasite virulence in toxoplasmosis and malignant malaria, and to have impact on the occurrence of malignancy in schistosomiasis and clonorchiasis. It is intended in the present review to present mechanism(s) of drug resistance due to parasite gene mutations, with special emphasis on anti-malarial drugs, albendazole, metronidazole, and other drugs used in treatment of African trypanosomiasis and toxoplasmosis. The review also presents other effects of parasite gene mutations on disease outcome and progress as well as occurrence of false diagnosis in *falciparum* malaria using ICTs utelizing histidine rich protein.

1. Drug resistance: Drug resistance is one of the main obstacles in eradication of endemic parasitic diseases including malaria, schistosomiasis, visceral leishmaniasis, lymphatic filariasis, onchocercosis and African as well as American trypanosomiasis (Chagas' disease). By definition, it is "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended but within the limits of the subject tolerance"[1]. Therefore, it is attributed to inability of the resistant strains to uptake the drug due to mutations in the parasite genes encoding the enzymes responsible for the uptake. A classification system into sensitive (S) and three degrees of resistance (RI, RII, RIII) was introduced to monitor results of *in vivo* drug resistance test, based on the parasitologic response[1].

1.1. Anti-malarial drugs: *P. falciparum* is a complex parasite with high plasticity in its genome. To survive, it is able to 1) delete certain portions of its genes that are unessential for its growth; 2) evade immune response by antigenic variations; and 3) undergo gene mutations in enzymes controlling drug uptake[2]. As a consequence of the strong selection force of anti-malarial drugs, resistance point mutations have spread through malaria parasite populations over the last 25 years[3]. The dynamics of

strong selection for parasite gene mutations and the obtained data indicated that the selection pressure on the drug-linked genes increased the spread of mutant *P. falciparum* isolates in the endemic areas. Frequent population-based studies were strongly recommended to evaluate the efficacy of anti-malarial drugs[4]. To identify gene mutations responsible for drug resistance in a locality, the following were recommended: 1) mapping a genetic data base for the known gene mutations; 2) identification of the putative drug-resistance loci in each gene; and 3) identification of the genetic basis (if present) to all novel drugs before clinical use[5]. In a meta-analysis study, 220 clinical, pharmacological, *in vitro* and molecular studies were analyzed. The reviewers claimed that these studies missed the limitations in standardization regarding different geographic locations and different methods for gene mutations detection. So, the worldwide anti-malarial resistance network (WWARN) designed a centralized resistance data network for these types of studies to present valuable resources to the health authorities for development of action plans that identify and combat anti-malarial drug resistance in their locality[6]. Another review article summarized mechanism(s) and genetic markers of chloroquine (CQ) resistance and their distribution frequencies in all endemic African, Asian and South American countries, with special emphasis in

India[7]. Several benefits materialized from investigating the genetic markers of drug resistance that frequently occurred in malaria chemotherapy. First, mapping the genetic epidemiological data for drug resistance gives a mirror image of parasite migration patterns of drug resistance[8]. In other words, leakage of parasite anti-malarial drug resistance from country to another should be considered and neighbor countries should implement regional rather than national policies to prevent genetic drug resistance spread between them[9]. Secondly, a baseline record is built before changing national drug therapy in an endemic locality. In this context, a study conducted in Pakistan revealed the presence of high CQ resistance, with absence of Fansidar (FAN) resistance[10]. Thirdly, investigating genetic markers provides the proficiency to describe drug status in a malaria-endemic area. For example, a study conducted in Senegal investigating the prevalence of different genetic markers in *P. falciparum* isolates, concluded that there was: 1) a decreased level of CQ resistance; 2) an increased level of pyrimethamine (PYR) resistance; 3) a moderate resistance to amodiaquine; and 4) no resistance to FAN[11]. A study involving *P. vivax* isolates from China, also concluded relative susceptibility to CQ and FAN therapy[12]. Similarly, frequency distributions of the genetic markers linked to anti-malarial drug resistance in Haiti revealed normal CQ sensitivity[13]. Fourthly, the investigations showed that high prevalence of mutations in imported isolates indicated rapid development and spread of resistance against common anti-malarial drugs used nationally[14-16]. Lastly, several studies were conducted all over the world to investigate the efficacy of anti-malaria drugs and to regularly monitor their use. Mutations detected in certain *P. falciparum* genes were considered good predictors of potential FAN treatment failure in several countries[17-21]. In contrast, absence of mutations suggested use of FAN as first choice treatment in other areas[22,23]. Similarly, other investigators suggested that use of amodiaquine was no longer effective in Tanzania[24] while combined atovaquone and proguanil therapy was efficient in Thailand[25]. In addition, it was recommended that both CQ and FAN should not be used in the near future in China[26], and CQ was not effective in Malaysia[27]. Continuous investigation of *P. vivax* and *P. falciparum* molecular markers to monitor development of FAN resistance in Afghanistan isolates was recommended[28]. On the other hand, when CQ was replaced by artemisinin (ART) derivatives - combined therapy in treatment of uncomplicated *falciparum* malaria, the investigators detected known mutations frequently linked to anti-malaria drugs when combined with ART. Based on the obtained data, it was recommended to frequently monitor ART and the anti-malarial drugs in Zanzibar[29], China[30], Cameroon[31], India[32], Equatorial Guinea[33], Tanzania[34], Thailand[35], and Central African Republic[36]. In Yemen, which is a highly endemic area of *falciparum* malaria with high diversity, the investigators suggested that ART-combined therapy with FAN should be the first choice of malaria treatment[37]. Data obtained from

two studies conducted in Equatorial Guinea showed high CQ resistance, and suggested use of ART-FAN combined therapy[38] or lumefantrine and mefloquine[39] as alternative drugs.

•Dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes: Mutations in the *dhfr* and *dhps* genes of *P. falciparum* parasites have been associated with decreased parasite sensitivity to the anti-folate drugs. Pyrimethamine and the biguanides bind to and inhibit the bifunctional enzyme dihydrofolate reductase (DHFR)[40], and the sulfonamides and sulfones inhibit the enzyme dihydropteroate synthase (DHPS)[41]. On investigating the association between anti-folate (sulphadoxine) resistance and mutation of *dhps* gene in *P. falciparum* isolates from India *in vitro*, 5 mutations at codons 436, 437, 540, 581 and 613 were detected. The investigators detected quadruple mutant alleles in 36.7% of their isolates, and ~ 97% of them were highly sulphadoxine resistant[42].

On the other hand, *P. falciparum* isolates with mutation (serine→asparagine) at position 108 in *dhfr* gene are resistant to PYR, while those with serine→threonine at the same codon associated with an alanine→valine substitution at position 16, are resistant to cycloguanil (the active metabolite of proguanil). In isolates resistant to both drugs, an additional *dhfr* gene mutation (alanine→valine) at codon 164 is present[43]. Since then, several articles were published to estimate frequency distribution of gene mutation responsible for PYR resistance in several countries; Brazil[44], New Guinea[45], Tanzania[46], and for proguanil resistance in 3 African countries (Niger, Senegal and Kenya)[47]. Resistance to both drugs was reported in Cameroonian isolates where the investigators found *dhfr* 108 point mutation in 14 out of 15 isolates resistant to both drugs[48]. In addition, another 2 mutations in *dhfr* gene were found in *P. falciparum* strains isolated from Thailand with PYR resistance[49]. However, low level of PYR resistance was suggested in Haiti due to the presence of mutation in *pfdhfr* gene at codon 108 and the absence of other codons (51, 59 and 164) with no mutations in *dhps* gene[50]. In contrast, all *dhfr* mutations were rarely detected in *P. falciparum* strains from 5 countries in Africa (Kenya, Tanzania, Malawi, Gabon, and Nigeria) where PYR was intensively used[51].

Since the anti-malarial drug resistance for monotherapy has emerged, sulfadoxine is always provided in combination with PYR, known as SP (sulfadoxine-pyrimethamine combined therapy) or FAN (fansidar). In Pakistan, a single gene mutation (C59R) at codon 16 in *dhfr* gene was reported as the first field sample of a mutant *dhfr* allele where the 108 codon is unchanged[52]. One year later, Camerionian investigators found three *dhfr* gene point mutations at codons 51, 59 and 108 and assigned them as reliable genetic markers to predict FAN resistance

in Africa[53]. Since then, several studies were conducted for genetic mutations in FAN resistance. Simultaneous presence of mutations at 51 and 108 in *dhfr* gene and at 437 and 581 in *dhps* gene were associated with high FAN resistance in Uganda[54] and Venezuela[55]. It was concluded from several studies that triple *dhfr* gene mutations at codons 51, 59 and 108 with double *dhps* gene mutations were associated with mild and moderate FAN resistance in Kenya[56], Cameroon[57], Nigeria[58], Ethiopia[59], Ghana [60], Sri Lanka[61] and Tanzania[62]. In one of these studies, the investigators claimed that the degree of association between gene mutation and drug resistance might vary regionally in the same country[60]. However, in a retrospective study conducted on Kenyan *P. falciparum* isolates from 2008 to 2012, the investigators concluded that triple *dhfr* gene mutations with or without double *dhps* gene mutations were associated with FAN resistance[63].

In Uganda, investigation of FAN resistance alone or in combination revealed that mutation in codon 540 of *dhps* gene is responsible for FAN resistance only, while the mutation in codon 59 of *dhfr* mediated resistance to the drug whether alone or in combination[64]. *P. falciparum* strains isolated from patients from Burkina Faso showed that mutations at codons 108 or 59 in *dhfr* gene would predict disease recrudescence after FAN treatment[65]. In other studies conducted in several African countries, the investigators suggested that the presence of triple mutations in both genes are genetic markers for FAN failure in pregnant women[66-68]. In Iran, mutations at codons 108 and 59 (*dhfr*) and 436 (*dhps*) were the commonest mutations associated with FAN resistance[69]. In the same country and after three years of FAN treatment against *falciparum* malaria, high frequency of double mutations at codons 59 and 108 of *pfdhfr* and single mutation at codon 437 of *pfdhps* genes was detected in ~96% of the examined isolates. The investigators claimed that the parasite would potentially develop quintuple mutations, and advised the health authorities to monitor FAN clinical resistance at regular intervals[70]. In India, triple mutations in *dhfr* gene (codons 51, 59 and 108) and *dhps* genes (436, 437 and 540) were associated with PYR and sulphadoxine resistance, respectively; while ~94% of the isolates with triple mutations in both genes were associated with high grade of FAN failure[71]. A novel mutation of isoleucine in *dhfr* gene (codon 108) was also recorded in India for *in vitro* resistance of *P. falciparum* isolates to FAN treatment. The investigators found double isoleucine mutations at codons 108 and 51 in more than three quarters of the resistant isolates. Together with the double mutations, one of 4 mutations in *dhps* gene was detected at codons 436 or 437 or 581 or 613[72]. Due to the high frequency of mutations and the appearance of novel mutations in *dhfr* and *dhps* genes, the investigators asked health authorities to stop FAN therapy in treatment of *falciparum* malaria in Malaysia[73], Burkina Faso[74] and Tanzania[75]. Moreover, two novel mutations of *dhps* gene at codons 540

and 588 were associated with FAN resistant *P. falciparum* isolates from Indonesia[76]. However, it was suggested that resistance to FAN does not occurr in the presence of mutation in *pfdhfr* gene at codon 108 and the absence of other codons (51, 59 and 164), with no mutations in *dhps* gene[50]. With ART-FAN combined therapy and shortly after its adoption in Afghanistan, the majority of *P. falciparum* isolates developed double mutation in *dhfr* gene at codons 59 and 108, while the minority developed triple mutations in *dhps* gene at codons 437, 540 and 581. The investigators claimed that all these mutations were previously linked to FAN resistance[77].

For *P. vivax*, three mutations at codons 57, 58, and 117 in *dhfr* gene were associated with high levels of FAN resistance in several strains isolated from widely separated countries from Asia and Africa[78]. The occurrence of three mutations at the same codons, but in *dhps* gene, in some *P. vivax* isolates suggested their association with increased resistance to sulfadoxine treatment[79], while increased frequency of mutations in both *dhfr* and *dhps* genes was associated with selection imposed by FAN therapy[80]. In contrast, frequency distribution of mutations of *pvdhfr* and *pvdhps* genes didn't exceed 50% in the studied isolates from China, a result suggesting relative susceptibility to FAN treatment[81]; while prevalence of these gene mutations for FAN resistance was low in Eastern and Central Sudan[82]. On the other hand, mutations at codons 58, 61 and 117 of *pvdhfr* gene were detected in PYR resistant isolates from China, and the most prevalent mutant allele was double mutations at 58 and 117[83]. A point mutation at codon 382 of *pvdhps* gene was detected associated with CQ resistance, but the investigators recommended further studies to confirm this association in larger samples from Brazil[84].

•**Multi-drug resistance (*mdr*) gene:** It is also known as P-glycoprotein homolog 1 gene. There is much controversy regarding the point mutation (tyrosine→phenylalanine) at codon 86 in *mdr* gene of *P. falciparum* isolates and its link to CQ, mefloquine and halofantrine resistance *in vitro*. Some studies attributed this mutation to all mentioned drugs[85,86] and they considered it as a useful genetic marker to predict drug resistance levels if influenced by the history of drug selection of each population[87-89]. Moreover, double *pfmdr*1 mutation at codons 86 and 1246 was associated with the early CQ treatment failure *in vivo*[90]. In contrast, others were in incomplete agreement, and suggested that other genetic factors with *mdr* point mutation might be involved in CQ or mefloquine resistance in Africa[91,92], and Asia[93-95]. Also, no association was observed between *pfmdr*1 mutations and resistance to quinine, mefloquine and ART in Brazil[96]. However, a strong association was detected between *mdr* 86 mutation and increased sensitivity to mefloquine and halofantrine in Gambia[97]. For *P. vivax*, gene mutation at *pvmdr*1 gene was found inappropriate to monitor CQ resistance in Madagascar[98]. Furthermore, in spite of presence of mutation (codon 1076) in *pvmdr*1 gene

in the majority of isolates investigated *in vivo* and *in vitro* drug studies, still all the Indian isolates were susceptible to CQ[99].

•**Chloroquine resistance transporter (*crt* gene):** Several studies showed that mutation at codon 76 in *P. falciparum* CQ resistance transporter (*pfcrt*) gene encoding digestive-vacuole transmembrane proteins was linked with CQ resistance in Africa[100-102], and Asia[103-107]. However, discrepancy between resistant *P. falciparum* strains (14%) and the frequency of the mutant *crt* at codon 76 (92%), suggested that other factors might be involved in CQ resistance in Tanzania[108]. In a study conducted in Afghanistan, high prevalence of amodiaquine resistance *in vivo* was associated with mutation in *pfcrt* gene at codons 72-76 (SVMNT; i.e. Ser-Val-Met-Asn-Thr)[109]. Moreover, after 10 years of CQ withdrawal because of drug failure, single mutation (K76 CQ) and double mutations (K76T and C72S) in *pfcrt* were detected in 84% and 3% of Ethiopian *P. falciparum* isolates, respectively. As these mutations were more linked to isolates sensitive to CQ, the investigators anticipated the recurrence of CQ-sensitive isolates and that CQ should be considered as alternative therapy in CQ-based combination therapy[110]. On the other hand, the codon 76 mutation was detected in *pfcrt* gene of all of 96 samples isolated in Brazil in spite of CQ withdrawal from treatment since ~35 years, while it was still CQ-resistant[96]. Recently in Yemen, mutations of *crt* gene (codons 76, 271, 326 and 371) were highly associated with CQ resistance after 4 years of treatment shift to ART combinations, and the investigators asked Health authorities to forbid the use of CQ in *falciparum* malaria with its careful use in *vivax* malaria[111].

•**Combined genetic factors:** Detection of combined mutations in *pfcrt* (codon 76) and in *pfmdr*1 (codon 86) were used to monitor development and spread of amodiaquine resistance in Africa[112]. In Burkina Faso, mutation at *pfcrt* (76) or *pfmdr*1 (86 or 1246) predicted recrudescence after amodiaquine treatment[65]. Both mutations were associated with amodiaquine and desethylamodiaquine resistance in Colombia[113], while in Nigeria, they were associated with amodiaquine *in vitro* resistance[114]. It was also confirmed that high prevalence of the mutations at *pfcrt* SVMNT and *pfmdr*1 at codon 86 was associated with high levels of CQ resistance and the investigators suggested their association with reduced efficacy of amodiaquine in Angola[115]. Meanwhile, mutations at codons 76 and 86 in *pfcrt* and *pfmdr*1 genes respectively, were present together in CQ resistant isolates in India[116-118]. However, mutations at codons 97 and 184 in *pfcrt* and *pfmdr*1 genes, respectively influenced the level of CQ resistance in Thailand[119]. In contrast, results of a study conducted in Philippine, isolates showed that both mutations were not predictive of *in vitro* CQ resistance[120]. The mutation at codon 423 in *pfmdr*2 and 51 and 59 or 108 in *pfdhfr* were independently associated with *in vitro* PYR resistance in Thailand[121]. For

P. vivax, neither of *pvmdr*1 nor *pvcrt* genes mutations were associated with CQ resistance in Brazil[122]. High frequency distribution of mutations in *pvmdr*1 and *pvcrt* genes were associated with CQ resistance in the majority of *P. vivax* isolates from Ethiopia[123].

•**Other genetic markers:** Three mutations were detected in cytochrome b (*cyt b*) gene which resulted in resistance of *P. berghei* to atovaquone *in vitro*[124]. *P. falciparum* showed a mutation at codon 268 (Tyr→Ser) of the *cyt b* gene after atovaquone-PYR combined treatment failure[125]. Another mutation at the same codon (Tyr→Asn) was detected in a patient with atovaquone/proguanil (Malarone) therapy failure [126]. Similarly, the highest atovaquone resistance in *P. falciparum* Indonesian isolates carried both mutations at codon 268[127]. In contrast, only one study denied involvement of cytochrome b in atovaquone-proguanil resistance in *P. falciparum* isolates from Angola[128].

Twelve point mutations in *cg*2 gene, which is a candidate for CQ resistance, was associated with *in vitro* resistance of *P. falciparum* strains to CQ[129]. The same investigators found that CQ resistant isolates had 16 repeat units in one of the polymorphic regions (omega region) of *cg*2 gene, while CQ-sensitive ones had either ≤ 15 or ≥ 17 repeat units, but they recommended further studies on a larger number of isolates to consider this mutation as a reliable genetic marker for CQ resistance[130].

P. falciparum sodium hydrogen exchanger (*pfnhe*) gene is another gene investigated with *pfcrt*-76 and *pfmdr*1-86 and their association with resistance against quinine, mefloquine and halofantrine *in vitro*. The results showed the importance of *pfnhe* with *pfmdr*1 (codon 86) mutations as indicators of reduced quinine susceptibility[131]. In studies conducted in China[132] and Viet Nam[133], the obtained results supported the association of *pfnhe* gene mutation in quinine resistance. In contrast, results from a study conducted in Thailand showed much doubt in the usefulness of *pfnhe*1 gene as genetic marker for quinine resistance *in vitro*[134].

On the other hand, no association was detected between mutation of sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPase (*pfatp*6) gene and *in vitro* susceptibility of *P. falciparum* isolates to CQ, mefloquine and quinine[135]. In 2014 came the first study investigating *P. falciparum* multidrug resistance protein-2 (*mrp*2) gene and its association with anti-malarial drugs in Thailand. The investigators found frequent and complex variations, including single nucleotide polymorphisms (SNPs) and polymorphic microindels (Mis) which were associated with quinolines (CQ, piperaquine and mefloquine) *in vitro*[136].

•**Resistance to ART derivatives:** Search for genetic markers associated with resistance to ART started in 2006. No mutation was detected in *mdr*1, *cg*10 and genes

controlling translational controlled tumor protein (*tctp*), and *atp*6 genes, when the investigators sequenced these genes in *P. chabaudi* grown in increasing ART concentrations[137]. The first report was published on a field isolate resistant to ART in the Cambodian population[138]. This was followed later by two publications; the first discussed ART resistance in Western borders of Thailand[139], while the second dealt with reduced susceptibility to ART in Southern Myanmar[140]. Negative data were obtained also on investigating *pfmdr*1, *pfatp*6, mitochondrial genome and the gene encoding deubiquitinating enzyme (*ubp*1)[141]. There was diverse genetic mutations in *atp*6 gene that enabled the investigators to conclude its negative association with ART resistance[142-147]. In addition, the frequency distribution of mutations of the genes *pfmdr*1 (6 codons), *pfcrt* (codon 76) and multidrug resistance protein 1 (*pfmrp*1) (5 codons) were significantly unchanged after 4 years of ART combined treatment in Iran[148]. However, 6 mutations at *P. falciparum atp*6 gene were detected as baseline data before starting ART treatment in Niger. Three of them were newly detected while the mutation at codon codon 569 was the most predominant[149]. After three years of administration of ART-FAN combination in treatment of *falciparum* malaria in Sudan (2004-2007), high frequency (72-75%) of point mutations at *pfcrt* (codon 76), *pfdhfr* (codons 51 and 108) and *pfdhps* (codon 436) was detected. Frequency of *pfmdr*1 gene mutation was ~55%, while almost the whole studied population harbored the wild type allele of *pfatp*6. The investigators advised the national health authorities to regularly monitor ART-based combination therapy especially in endemic areas of Sudan[150].

In the last decade, two genes were incriminated in ART resistance; cysteine protease falcipain-2 (*fp*2) and Kelch-domain 13 (*kelch*13). Only one article reported *fp*2 gene as genetic marker for resistance to ART; and the investigators found that this mutation was not prevalent in Uganda [151]. Kelch-domains are proteins whose interruption results in a wide variety of different organism phenotypes through protein degradation. It was found that some *P. falciparum* isolates from Southeast Asia and resistant to ART derivatives, have *kelch*13 mutations[152]. One of the main ART actions is to obligate ring stages to enter the dormant phase, facilitating their clearance by the host immune system and spleen; and mutations in *kelch*13 gene prevent ring stages from responding to oxidative stress clearing[153]. Investigating *kelch*13 gene in *P. falciparum* isolated from patients from 7 Asian and 3 African countries revealed its involvement in ART derivatives resistance. The investigators recommended prolonged ART-based combination courses in patients with standard 3-day treatments failures[154]. Several studies were conducted to confirm this link in *P. falciparum* strains isolated from several Asian countries; Bangladesh[155], China and Myanmar[156-159]. On the other hand, its mutation was not prevalent in several African countries; Uganda[151], Senegal[160] and Mali[161]. Neither of the mutations detected

in Cambodia nor in Southeast Asia were detected in isolates from Sub-Saharan African countries; instead the investigators detected several novel mutations, but in very low frequencies[162].

1.2. Other anti-parasitic drugs

•**Drugs for schistosomiasis:** In 1989, American investigators found an association of BamHI fragment in the oxamniquine resistant *S. mansoni* strains[163]. The same researchers investigated the molecular characterization generating this fragment in resistant strains and they compared the genomic DNA from resistant and sensitive strains. They suggested that a drug induced parasite genomic alteration produced this fragment in resistant strains[164]. Two years later, a group of scientists from Italy analyzed the genetic behavior responsible for oxamniquine resistant strains. Their results showed that schistosomes sensitivity to the drug was strictly related to specific chromosome(s) in a dominant gene encoding the enzyme that converts the drug to reactive ester[165].

•**Albendazole:** Albendazole resistance in giardiasis was conducted *in vitro* and it was demonstrated that mutation of the β-tubulin gene was not involved in the resistance. An alternative mechanism of resistance involving the cytoskeletal structure, particularly the median body, was suggested. This was attributed to evident major chromosome rearrangements indicating differences in the cytoskeleton between sensitive and resistant lines[166]. On the other hand, Jiménez-Cardoso *et al.*[167] found several mutations in the β-giardin gene in both albendazole-resistant and recovered-sensitive *G. lamblia* strains indicating that resistance to albendazole is not necessarily caused by mutations in the β-giardin gene of *G. lamblia*.

•**Metronidazole:** Orozco *et al.*[168] reviewed multi-drug resistance in amoebiasis, and it was attributed to mutation of *Entamoeba histolytica* P-glycoprotein-like gene (*EhPgp*). However, several studies showed metronidazole resistance in intestinal giardiasis and amoebiasis as well as in cases of vaginal trichomoniasis[169-171]. In 1992, a study was conducted to investigate the role of ferredoxin gene mutation in *T. vaginalis* metronidazole-resistant isolates, and the investigators detected decreased ferredoxin levels in 50-65% of the resistant isolates. The investigators hypothesized that ferredoxin gene mutation could be implicated in metronidazole resistant clinical cases of trichomoniasis. They explained that ferredoxin gene mutation resulted in decrease of intracellular ferredoxin expression with a decrease in regulatory protein binding affinity; which would lead to inability of metronidazole activatation in its cytotoxic form[172]. In 2000, an American study was conducted to investigate metronidazole *T. vaginalis* resistant isolates. A point mutation at the position 66 of the internal transcribed spacer 1 (ITS1) fragment was detected in 16 out of 109 isolates (~15%), and *T. vaginalis* virus (TVV) was detected in ~50% of

their isolates. Correlating these data with metronidazole resistance, the investigators concluded that isolates with TVV and ITS1 fragment mutation are significantly more likely to be sensitive and resistant to metronidazole[173]. In 2009; it was shown that metronidazole-resistant isolates of *G. lamblia*, *Entamoeba* spp.and *T. vaginalis* have different combinations of gene mutations either similar to the metronidazole-resistant anaerobic bacteria *Helicobacter pylori* in nitroreductase (*ntr*) gene or to *Bacteroides fragilis* in nitroimidazole (*nim*) gene[174]. In two Iranian studies, the investigators detected point mutation at 209 of the ITS1 fragment in 3.9%[175], and at position 239 of ferredoxin gene in 8.7%[176] of their *T. vaginalis* isolates.

•**Drugs for African trypanosomiasis:** The link between mutations in *tbat*1 gene encoding P2 adenosine transporter (P2) in variant *T. gambiense* isolates from Uganda and melarsoprol resistance was investigated. It was found that 58% of the resistant isolates had *tbat*1 gene mutation. Although 58% of the resistant isolates had *tbat*1 gene mutation, several patients with relapse after melarsoprol therapy had the wild-type *tbat*1 gene. The investigators suggested that *tbat*1 gene mutations was not the only mechanism behind melarsoprol resistance or failure[177]. Two years later, another group of researchers investigated whether other factors contribute with *tbat1* gene in P2 activity responsible for mediation of melarsoprol uptake. Their hypothesis allowed them to identify and characterize two additional drug transport activities, a high-affinity and a low-affinity component of pentamidine transport (HAPT1 and LAPT1). Inhibition analysis of both factors confirmed the contribution of HAPT1 in P2 activity[178]. In contrast, a study conducted in South Sudan observed that all resistant isolates were sensitive to the drug *in vitro* and in experimentally infected mice. The investigators also did not detect any of the previously described point mutations in *tbat*1 gene in their isolates, and they concluded the irresponsibility of drug resistance to melarsoprol failures[179]. However, data obtained from a study conducted on animal trypanosomiasis resistant to diminazene aceturate revealed that mutations in genes controlling P2 transporter favors parasite survival (i.e. drug resistance)[180]. In 2013, aquaglyceroporin transporter (*Tb*AQP2) was identified as another transporter involved in melarsoprol/pentamidine resistance. It was found that mutations in the gene encoding *Tb*AQP2 significantly correlated with pentamidine and melarsoprol resistance in the clinical isolates[181]. On the other hand, isometamidium is a veterinary drug used against African trypanosomiasis, targeting the trypanosomes kinetoplast. British investigators screened mutations in 30 genes and 3 protein complexes associated with kinetoplast-dependent growth to investigate the link between gene mutation and isometamidium resistance. They observed that non-mitochondrial proteins and multi-subunit complexes were implicated in kinetoplast-independent growth, and mutations in their encoding genes might correlate with isometamidium resistance. The investigators also discussed

failure of other drugs used in some flagellated protozoa to attack their kinetoplast[182].

•**Drugs for toxoplasmosis:** Parasitic gene mutations incriminated in drug resistance included cytochrome b, *dhfr* and *dhps* genes for atovaquone[183], PYR[184] and sulfonamide[185]. In contrast, a French study investigated the previous drugs against 17 *T. gondii* isolates *in vitro*, and they observed some variability in the susceptibilities of these isolates to atovaquone and PYR, with neither clear evidence of drug resistance, nor defined mutations in the studied genes. On the other hand, they detected 3 strains resistant to sulfadiazine, with several identical mutations in *dhps* gene[186]. Recently, another group of French investigators denied the link between sulfadiazine resistance and polymorphisms or overexpression in *dhfr*, *dhps* and ABC transporter genes family. They recommended further studies to investigate mechanism of sulfadiazine resistance in toxoplasmosis and the genes associated with it[187].

•**Drugs for leishmaniasis:** Drug resistance and its relations to parasitic gene mutations in leishmaniasis is a complex phenomenon of distinct genetic diversities. Since the 1990s, several studies were conducted investigating this strange relationship. Mutations in genes encoding pteridine reductase 1 (*ptr*1 gene)[188], mitochondrial apocytochrome b (*cyb* gene)[189], P-glycoprotein gene (*pgpa* gene)[190], and topoisomerase I gene[191] were incriminated in resistance of *Leishmania* spp. to methotrexate, antimycin A, oxyanions and 3, 3'-Diindolylmethane (DIM), respectively. Two Brazilian studies detected mutations at the genes encoding heat shock protein 70[192], and aquaglyceroporin 1 (*aqp*1 gene)[193] contributed to resistance to antimonial drugs. However, three different mutations in the genes encoding the metabolic enzymes uracil phosphorybosyl transferase (UPRT), thymidine kinase (TK) and uridine phosphorylase (UP) were detected in *L. infantum* resistant to pyrimidine analogue 5-fluorouracil (5-FU)[194]. In contrast, no gene mutation was detected in paromomycin resistant *L. tropica* strains on nucleotide sequencing at both the DNA and RNA levels[195]. An Indian study denied the presence of point mutations in the miltefosine transporters (*ldmt* and *ldros*3 genes) previously reported in parasites with experimentally induced miltefosine resistance in their clinical isolates[196].

•**Other anti-parasitic drugs:** In a short report, Japanese investigators found cytochrome b (*cytb*) gene mutation in *Babesia gibsoni* strains isolated from infected dogs that resist treatment with atovaquone[197]. American investigators applied real time PCR assays to screen development of benzimidazoles resistance in *Ancylostoma caninum*-infected dogs. They found elevated levels of β-tubulin isotype-1 gene polymorphisms at codon positions 167, 198 and 200 in hookworm resistant strains[198]. β-tubulin gene isoform 2 was also incriminated in benzimidazole resistant *Echinococcus granulosus* strains in post-surgical treatment of hydatid cyst in a study conducted in India[199]. On the

other hand, only two studies were conducted to investigate parasite gene mutation and drug resistance in Chagas disease. While one study did not detect any association of drug resistance and P-glycoprotein (*pgp* gene)[200], another study found acquired mutations in the gene encoding mitochondrial *ntr* gene and *T. cruzi*-resistant strains to benznidazole[201].

2. Virulence, pathogenesis and clinical manifestations

•**Toxoplasmosis:** In congenital toxoplasmosis, the relationship between *T. gondii* virulence measured by newborn clinical manifestations, and allelic polymorphism in *dhfr* gene was investigated. The French investigators found that mutated clones with allelic replacement at certain loci (36, 83 or 245) of the tested gene displayed different outcomes suggesting a clear difference between virulence of wild and mutant types[202]. Several studies were conducted to screen *T. gondii* mutants using modified signature-tagged mutagenesis. This approach allowed the investigators to monitor *in vitro* growth of the mutants as well as their virulence and pathogenesis *in vivo* (animal model). When they detected mutants with growth impairment or low virulence, genetic complementation was done to identify genes required for *T. gondii* growth, virulence and pathogenesis. The investigators identified genes encoding regulators of chromosome condensation 1 (RCC1), patatin-like protein, proteophosphoglycan and transmembrane pellicle protein 1 (TPP1) as essential genes for *T. gondii* growth and virulence[203], its survival and replication in activated macrophages[204], bradyzoite development[205], and virulence and invasion during acute toxoplasmosis[206].

•**Malaria:** On investigating the relation between mutations in *Pfcrt* and *Pfmdr1* genes and clinical status (severe or uncomplicated *falciparum* malaria), all isolates had *Pfcrt* (codon 76) and ~80% had *Pfmdr1* double mutations (codons 86 and 184), whatever their clinical status[207]. Similarly, gene mutations incriminated in CQ and antifolate drug resistance had no association with complicated malaria[208]. In contrast, there was significant association of *Pfcrt* (codon 76) mutation and malaria severity in both groups of patients (children 5 years or younger and above 5 years)[209].

3. Carcinogenesis

In a study conducted in Japan to evaluate the mutagenic activity of *S. japonicum* and *C. sinensis* crude extracts (adult and egg antigens) and their ability to induce tumor cells, the investigators found weak but significant activity reaction only to *S. japonicum* soluble egg extract suggesting its ability to promote induction of tumor cells[210]. Other Japanese investigators suggested low possibility of immediate gene mutation related to worm

and/or egg extracts of *S. haematobium* and *S. mansoni* leading to carcinogenesis[211].

4. False diagnosis

Deletion or mutation in the gene encoding histidine-rich protein 2 (HRP2) was attributed to false diagnosis of *falciparum* malaria cases using rapid diagnostic tests[212-217].

Concluding remarks

1. In drug resistance against anti-malaria drugs, molecular, genetic and biochemical analyses showed several criteria. 1) Impaired CQ uptake by the parasite vacuole is a common characteristic of resistant strains, and this phenotype is associated with mutations of the *Pfmdr*1, *Pfcg*2 and *Pfcrt* genes. 2) One to four point mutations of *dhfr* gene produce a moderate to high level of resistance to antifolates (PYR and proguanil). 3) Frequency of CQ resistant mutants varies among isolated parasite populations, while resistance to antifolates is highly prevalent in most malarial endemic countries. 4) The mechanism of resistance to sulfonamides and sulfones involves mutations of *dhps* gene. 5) Resistance to FAN is associated with *dhfr* gene mutations at codons 51, 59 and 108, and *dhps* gene mutations at codons 436, 437 and 540. 5) There is much controversy regarding gene mutations linked to resistance to quinine, mefloquine and halofantrine.

2. Regarding flagellated parasites, mutation in the gene encoding β-giardin may be involved in albendazole resistance in giardiasis, while two genes are suggested for metronidazole resistance in trichomoniasis, mutation in ferredoxin gene and ITS1 fragment. In African trypanosomiasis, there is still much controversy regarding the link between *tbat*1 gene (encoding P2 adenosine transporter) and melarsoprol, while aquaglyceroporin transporter involvement was recently identified in melarsoprol/pentamidine resistance. On the other hand, drug resistance and gene mutations in leishmaniasis showed some complexity due to distinct genetic diversities. However, mutations of the genes encoding heat shock protein 70, aquaglyceroporin transporter 1, and miltefosine transporters (*ldmt* and *ldros*3 genes) contribute to antimonial drugs and miltefosine resistance, respectively.

3. In congenital toxoplasmosis, mutation in *T. gondii* *dhfr* gene is suggested for strain virulence affecting the newborn outcome more than the wild strains. Certain genes encoding certain proteins and receptors were identified for *T. gondii* growth, virulence, survival and replication and bradyzoite development.

4. Mutation in the gene encoding histidine-rich protein 2 (HRP2) is incriminated in false diagnostic results

using immunochromatographic tests (ICTs) in *falciparum* malaria.

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