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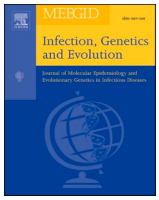

Review

CRISPR/Cas advancements for genome editing, diagnosis, therapeutics, and vaccine development for *Plasmodium* parasites, and genetic engineering of *Anopheles* mosquito vector

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ABSTRACT

Malaria as vector-borne disease remains important health concern with over 200 million cases globally. Novel antimalarial medicines and more effective vaccines must be developed to eliminate and eradicate malaria. Appraisal of preceding genome editing approaches confirmed the CRISPR/Cas nuclease system as a novel proficient genome editing system and a tool for species-specific diagnosis, and drug resistance researches for *Plasmodium* species, and gene drive to control *Anopheles* population. CRISPR/Cas technology, as a handy tool for genome editing can be justified for the production of transgenic malaria parasites like *Plasmodium* transgenic lines expressing Cas9, chimeric *Plasmodium* transgenic lines, knockdown and knockout transgenic parasites, and transgenic parasites expressing alternative alleles, and also mutant strains of *Anopheles* such as only male mosquito populations, generation of wingless mosquitoes, and creation of knock-out/ knock-in mutants. Though, the incorporation of traditional methods and novel molecular techniques could noticeably enhance the quality of results. The striking development of a CRISPR/Cas-based diagnostic kit that can specifically diagnose the *Plasmodium* species or drug resistance markers is highly required in malaria settings with affordable cost and high-speed detection. Furthermore, the advancement of genome modifications by CRISPR/Cas technologies resolves contemporary restrictions to culturing, maintaining, and analyzing these parasites, and the aptitude to investigate parasite genome functions opens up new vistas in the better understanding of pathogenesis.

1. Introduction

1.1. CRISPR-Cas strategy and previous gene editing methods

Plasmodium parasites cause malaria, and *P. falciparum* and *P. vivax* being the most clinically significant species. According to the WHO report published in December 2022, there have been estimated 247 million malaria cases and 619000 deaths in 84 malaria-endemic countries, in 2021. Due to having progress in determining interference aiming *Plasmodium* and *Anopheles* during past decades, an outstanding decline has happened in the malaria infection rate worldwide. Nevertheless, to eliminate and control malaria, new antimalarial medicines and more effective vaccines and interventions to block transmission in vectors to human must be developed to reduce the infection reservoirs and prevents antimalarial resistance (Olliaro and Wells, 2009; Rosenthal et al., 2019). One of these new technologies is the CRISPR/Cas system for the engineering of *Plasmodium* and *Anopheles* vector, and for detection of drug-resistant strains, vaccine development, more accurate diagnosis, and vector control.

The novel implement based on *Streptococcus pyogenes* clustered regularly interspaced short palindromic repeats-related protein-9 nuclease (CRISPR/Cas9) has revolutionized gene editing strategies in clinical and biological sciences in 2012 (Jinek et al., 2012). CRISPR/Cas9 can be effectively viewed as a three-component system due to *Plasmodium* species' homology-directed repair pathways (Behera et al., 2021). The most important difference between CRISPR, ZFP, and TALEN is the application of a short-length RNA aiding as the specificity-defining factor for double strand breaks. Furthermore, this new technique needs only the single guide RNA (gRNA) without requiring the expensive engineered site-specific nuclease. In this regard, the CRISPR nuclease Cas9 showed the capability to be recognized by a gRNA that characteristically resembles phage sequences and then establish natural

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immune machinery for CRISPR antiviral protection (Hsu et al., 2014). Using CRISPR/Cas9, the targeting DNA is identified and cleaved via Cas9/gRNA complex. Subsequently, the alteration of genome modification is accomplished by Watson-Crick base pairing. Whatever approach is used, the Cas9 nuclease, guide RNA(s), and a donor template are needed and must be delivered into the parasite (Ju et al., 2018).

Genome editing investigations in earlier times have relied on the application of site-specific nucleases to generate a double-strand break in the genome, comprising meganucleases, bacterial transportable genetic elements (Smith et al., 2006), zinc finger proteins (ZFP) established on eukaryotic transcription factors (Miller et al., 2007), and transcription activator-like effector nucleases (TALENs) recovered from *Xanthomonas* bacteria (Miller et al., 2011; Song et al., 2016). Some methods like Meganuclease, ZFP, and TALEN proteins can identify specific DNA sequences related to protein-DNA interactions in which ZFPs were efficacious to manipulate the malaria genome. In preceding research, the plasmids harboring the sequence homologous to the target sequence have been utilized for genome editing of *Plasmodium* parasites which this editing system has been performed by single or double crossover recombination (Wu et al., 1995). Until the mid-1990s, malaria parasites had not been successfully transfected. The first transfection of *Plasmodium falciparum* was reported in 1995 (Wu et al., 1995). Despite various improvements to make it specific and instantaneous, these early transfection and genome editing methods were relatively inefficient (Janse et al., 2006). Nevertheless, due to technical challenges and difficulties in introducing a plasmid vector through multiple layers of membrane, genetic modification of the malaria parasite remains challenging. Zinc finger nuclease proteins have been extensively applied to generate knockouts and allele replacements in the *Plasmodium* parasite (Straimer et al., 2012). Due to some restrictions in the association of functional ZFPs with the targeted DNA-binding proteins, new recognition methods would extensively facilitate the development of nucleases.

1.2. CRISPR-Cas strategy for malaria researches

The advancement of CRISPR/Cas9 for genome modifications in *P. falciparum* and *P. yoelii* has prominently improved the practical investigations in these parasites by augmenting the accuracy of editing and decreasing duration of experiments to fewer weeks (Zhang et al., 2017). By developing and applying new technologies and methods (such as high-throughput genome sequencing, genome editing, genome-wide linkage, and association analyses), progress has been made in the understanding parasite molecular biology, and mechanisms of disease pathology (de Koning-Ward et al., 2015; Su et al., 2007; Volkman et al., 2012). In this regard, the contemporary restrictions to culturing, maintaining, and analyzing these pathogenic parasites will be resolved and the aptitude to investigate parasite genome functions vigorously shed light on a better understanding of pathogenesis. Some recent investigations have confirmed the breakthroughs for genome editing of malaria parasites by using CRISPR/Cas9 (Ghorbal et al., 2014; Qian et al., 2018; Shinzawa et al., 2020; Wagner et al., 2014; Zhang et al., 2014). New antimalarial medicines and more effective vaccines must be developed to eliminate and eradicate malaria. Appraisal of preceding genome editing approaches confirmed the CRISPR/Cas9 nuclease

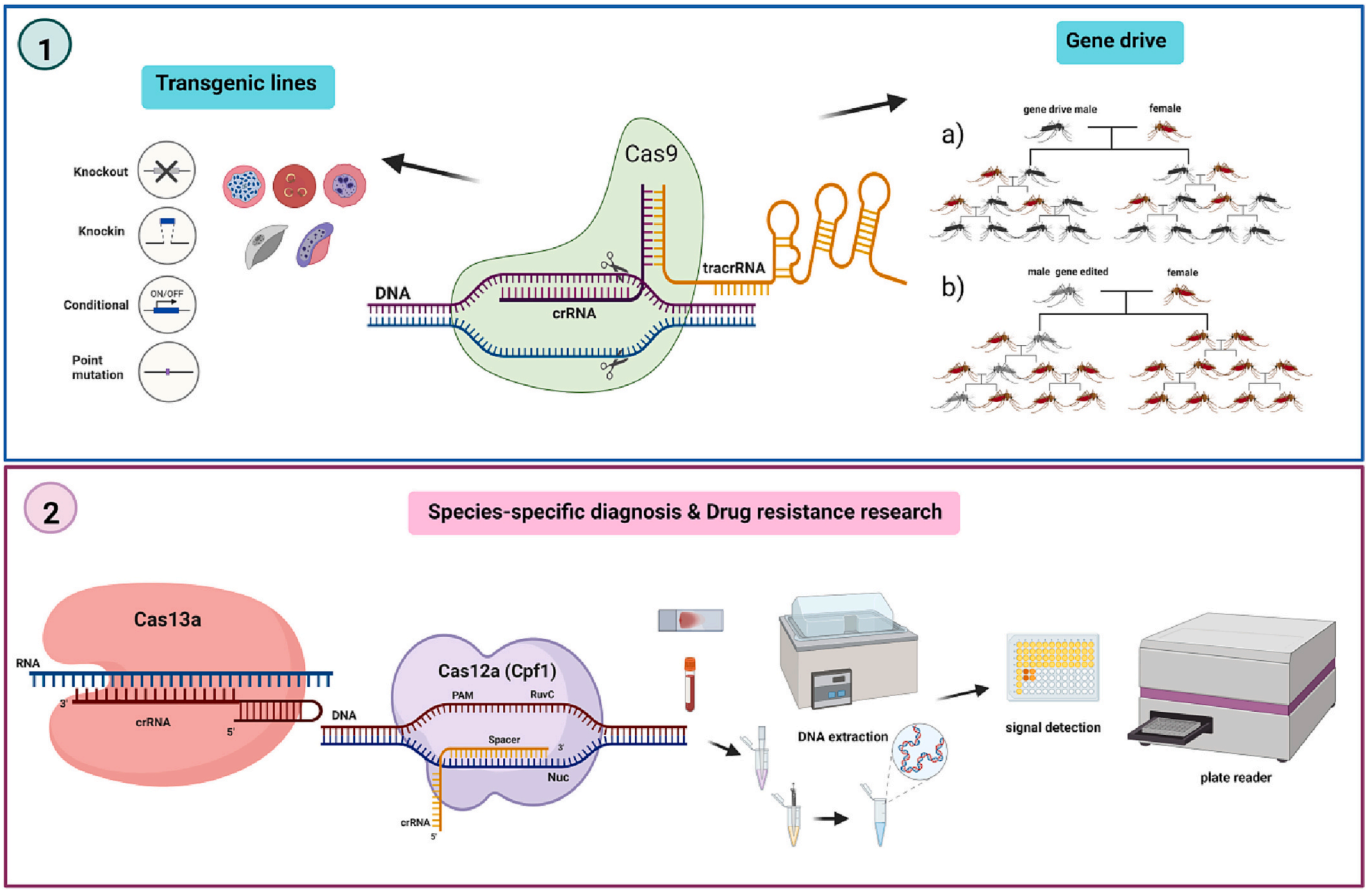

Fig. 1. The application of CRISPR-Cas systems in malaria and vector research. 1) Generation of transgenic lines such as knock out, knock in, conditional mutations, and point mutation lines can be facilitated by CRISPR/Cas9. Afterwards, these transgenic lines can be utilized for understanding parasite genome functions and pathogenesis which may facilitate drug and vaccine development research for *Plasmodium* species. Furthermore, CRISPR/Cas9 is a tool for genome editing for the creation of gene drive mosquitoes (a) or only male population (b). 2) Cas12a and Cas13a technologies enable the detection of parasites from different clinical specimens of whole blood, and sera to filter-papers and also for diagnosis of drug resistance variants in future.

system as a novel proficient genome editing system for producing transgenic lines, and also as a tool for species-specific diagnosis and drug resistance researches for *Plasmodium* species, and gene drive strategy to control *Anopheles* population (Fig. 1). In the present review, we highlighted the application of CRISPR-Cas strategy in malaria researches which opens up new vistas in the better understanding of *Plasmodium* and *Anopheles* to develop new strategies for malaria control and elimination.

In genome editing technologies, created double-strand breaks (DSBs) in DNA can be fixed through homologous recombination with donor DNA or nonhomologous end-joining (NHEJ) that can lead to mutations at the target genome. DNA repair investigations have been immensely developed by application of methods inducing site-specific DNA breaks in which endonuclease-based strategy that have been used before CRISPR development, demonstrated some benefits and drawbacks (Berzsenyi et al., 2021). Although, homing endonucleases can make restricted number of DSBs that are not sufficient for comprehensive experiments, faster generation of transgenic parasites due to a forced site specific integration rather than waiting for the parasites to integrate DNA on their own is of their advantage. Due to the deficiency of the canonical NHEJ (cNHEJ) pathway in *Plasmodium* parasites, the repairing cleavage is performed by homology-directed repair (HDR) in the attendance of the donor template. The lack of a cNHEJ pathway in malaria parasites leads to a break in the genome and results in fewer unintended mutations or off-target effects (Kirkman et al., 2014). However, to repair a double-strand break, *Plasmodium* can form microhomology-mediated end joining that uses tiny homology regions near the double-strand break to repair the lesion, creating potential indels; however it is not a very common process (Kirkman et al., 2014; Singer et al., 2015). Therefore, despite an alternative NHEJ being described in *P. falciparum*, homologous recombination is the only pathway observed when homologous sequences are present in *P. falciparum* (Kirkman et al., 2014).

CRISPR/Cas9 editing of *P. falciparum* is dependent on either one- or two-plasmids to supply the Cas9 nuclease, gRNA, and DNA repair template (Ju et al., 2018) while this system in rodent parasite *P. yoelii,* accomplished with one plasmid (Zhang et al., 2014). There have been many Cas proteins identified through bioinformatics analysis and experiments, but the most widely used in the genome editing of *Plasmodium* parasites was Cas9 and its variants from *Streptococcus pyogenes* (SpCas9) (Lee et al., 2019). The higher percentage of Adenine-Thymine (AT-rich) in the *P. falciparum* genome makes it difficult to recognize the gRNA binding site nearby the PAM sequence of SpCas9 (-NGG) and the cloning of repair templates. Accordingly, alternative Cas-nucleases with different PAM sequences, will resolve this challenge. One example is using Cas12a (originally called Cpf1) alongside the (TTTN-) PAM sequence. Other investigations have introduced other variants of Cas12a nuclease (e.g. LbCas12a and AsCas12a) as the appropriate system for *P. falciparum* (Chen et al., 2018; Zetsche et al., 2015). The dihydrofolate reductase (DHFR, a drug-selectable marker), the most commonly used marker utilized in *P. yoelii* and *P. berghei* parasites to select the modified parasites in CRISPR/Cas9 system. (Ghorbal et al., 2014; Knuepfer et al., 2017; Lu et al., 2016; Mogollon et al., 2016a). However, there are limited *Plasmodium*-specific drug-selectable markers are available to perform multiple modifications.

To synthetize gRNA, the application of RNA polymerase III U6 promoter, which transcribes the RNAs with certain 5' and 3' ends, is an indispensable factor required for the expression of gRNAs in the CRISPR/Cas9 system (Ghorbal et al., 2014; Zhang et al., 2014). Therefore, transcription is driven by RNA polymerase III in nearly all CRISPR systems (Di Cristina and Carruthers, 2018; Walker and Lindner, 2019). In another approach, the T7 phage snRNA promoters co-expressed with the T7 RNA polymerase to produce gRNAs with defined start (5') and end (3') in *P. falciparum* due to the use of appropriate promoter and terminator sequences (Lim et al., 2016; Spillman et al., 2017). However, the restrictions for utilization of the U6 promoter led to development of a novel technique of gRNA by using RNA polymerase II in which the final transcript took self-catalyzed cleavage to produce the target gRNA (Wagner et al., 2014). A recent study demonstrated that the RNA polymerase II promoters can be used to generate the CRISPR-RGR system (ribozyme-guide-ribozyme system) in *P. yoelii* with high efficiency for gene deletions or insertions (Walker and Lindner, 2019). To diminish the possibility of unintended genomic base mutations, the DNA repair template should be linear and then two gRNAs create two cut positions in the locus of interest or use a Cas9 cut position flanking the DNA repair template on the plasmid. It should be considered that the unanticipated concern of CRISPR/Cas9 genome manipulation in subtelomeric locations leads to removing DNA between the Cas9 cut position and the chromosome termination [25]. Whole-genome DNA sequencing will confirm the accuracy of the gene editing process. Although most research on *Plasmodium* genome editing has extensively used plasmids for Cas9 delivery, gRNA, and donor components, Cas9-gRNA ribonucleoproteins (RNPs) have also been applied due to its superior efficiency, limited off-target damage, and no need to selectable markers (Crawford et al., 2017; Nateghi Rostami, 2020).

1.3. CRISPR/Cas strategy for Anopheles mosquitos' researches

CRISPR/Cas9 technology, as a handy tool for genome editing can be justified for the production of mutant strains such as only male mosquito populations, generation of wingless mosquitoes, and creation of knock-out/ knock-in mutants of mosquitoes in near future approaches. Furthermore, transgenic lines of mosquitoes have been developed to arrest malaria transmission, overexpressing anti-Plasmodial effector genes or blocking activity of parasite host factor genes [reviewed in (Dong et al., 2022)]. CRISPR/Cas9 is a powerful genome editing tool targeting the mosquito vector to interrupt malaria transmission. Modification strategies to arrest the mosquito's capability to transmit malaria, by driving a genetic element into the mosquito population, will affect *Plasmodium* development but the host is unaffected. Modification drives can be applied for transgenic host factor gene inactivation, manipulation of miRNAs and lncRNAs, and expression of anti-parasitic effector genes in the aiming populations (Nolan, 2021). Then, these genetically modified mosquitoes can suppress or replace the population and/or disrupt malaria parasite transmission. Population suppression aims to drive mosquito populations toward local extinction and strategy uses nudging to target an essential gene or chromosome and disrupt its function (Bier, 2022; Nolan, 2021). The discovery of the bacterial CRISPR/Cas9 defense systems and the improvemnet of a simplified two-component system consisting of the Cas9 endonuclease (CRISPR-associated protein) and programmable guide RNA that binds Cas9, and directs the cleavage of DNA at desired sites, has resulted in the swift development of various insect gene drive strategies (Bier, 2022).

2. Engineering of transgenic lines of *Plasmodium* by CRISPR/Cas9 system

Transgenic lines of *Plasmodium* are used for many investigations, studying the function of genes, specifically to aid drug discovery or vaccine development against malaria. Some human malaria parasites, *P. falciparum* and *P. knowlesi,* have the capability of growth under *in vitro* continuous cultures, are used for genome editing and producing transgenic lines (Boltryk et al., 2021; Ghorbal et al., 2014; Mohring et al., 2020). Owing to the unavailability of animal models for human malaria parasites investigations, *in vivo* studies are performed using rodent malaria parasites, especially *P. yoelli* and *P. berghei.* Although many of the genes in rodent and human malaria parasites are orthologous, their encoded proteins are different in sequence and structure (Carlton et al., 2002; Kooij et al., 2006). Therefore, the establishment of new transgenic rodent malaria parasites enables the expression of the human malaria target proteins, which is necessary to design an exact *in vivo* experiment (Mlambo and Kumar, 2008; Qian et al., 2018; Salman et al., 2015;

Tewari et al., 2014).

Generation of transgenic lines of *Plasmodium* has been traditionally performed by using single or double crossover recombination without any targeted double strand break (Duraisingh et al., 2002; Wu et al., 1996). Later two techniques, zinc finger nuclease proteins (ZFP)-mediated technology (McNamara et al., 2013; Straimer et al., 2012) and Transcription activator-like effector nuclease (TALEN) which have the ability to identify specific points of DNA, were developed (Boch et al., 2009; Moscou and Bogdanove, 2009). Due to some restrictions such as limited choices of target sites useful for recognition by zinc finger, and some changes needed to distinguish the target site by the TALEN method, which has not been successfully adapted for gene editing in *Plasmodium* parasites, other novel methods were developed to address these restrictions. With the advances of the CRISPR/Cas9 system, gene editing in *Plasmodium* parasites was accelerated with a low cost and more efficient system than traditional techniques. Consequently, many transgenic malaria parasites were created with this new system as transgenic lines expressing reporter genes (Miyazaki et al., 2020a; Miyazaki et al., 2020b; Mogollon et al., 2016b; Zhang et al., 2014), *Plasmodium* transgenic lines expressing Cas9 (Nishi et al., 2021; Qian et al., 2018; Shinzawa et al., 2020), chimeric *Plasmodium* transgenic lines (Marin-Mogollon et al., 2018; Mizutani et al., 2016; Salman et al., 2017), knockdown and knockout transgenic parasites (Kudyba et al., 2018; Nasamu et al., 2021), and transgenic parasites expressing alternative alleles (Thiam et al., 2022; Uwimana et al., 2020).

2.1. *Plasmodium transgenic line expressing reporter genes*

CRISPR-based transgenic lines of *Plasmodium* which are able to express reporter genes like green fluorescent protein (GFP) or luciferase, applied for discovering malaria parasites by different growth inhibition methods throughout their life span (Dube et al., 2009; Prudêncio et al., 2011; Siciliano and Alano, 2015; Swann et al., 2016). The assays evaluate antimalarial drugs or vaccine efficacies in different stages of life-cycle. For the first time, the CRISPR/Cas9 system was used to create *P. yoelii* transgenic lines in 2014 via the insertion of GFP coding region in *py03652* locus in the genome of parasite and the results indicated the successful production of a transgenic line, and the subcellular localization of the tagged protein to GFP as punctate dots in the periphery of parasite (Zhang et al., 2014). In the mentioned system, a single pYC plasmid containing the cassettes of gRNA expression, Cas9 and hdhfr expression under regulation of *Pbeef1αa* promoter, and GFP as donor sequence was used (Zhang et al., 2014).

Some of the challenges in the application of CRISPR/Cas9 system for *Plasmodium* are the limited number of existing selectable markers and also the size of plasmid that limits the donor DNA size. The development of a marker-free system using reporter genes has addressed the issue (Lu et al., 2016; Mogollon et al., 2016a). Mogollan et al. showed that by using CRISPR/Cas9 system, the transgenic lines of *P. falciparum* can be produced using reporter genes under stage-specific promoters like *calmodulin*, *gapdh,* and *hsp70* promoters that have high transcription in blood stage (Mogollon et al., 2016a). The mentioned study confirmed the use of reporter genes instead of drug-resistant markers for generating the transgenic lines. Another study used two suicide/rescue plasmids for constructing *P. falciparum* transgenic lines in which the suicide vector expressing Cas9 nuclease and gRNA carries a *Plasmodium* specific selectable marker while the second plasmid (rescue) carries only donor DNA. Instead, the donor DNA is tagged to a reporter gene to identify the presence and expression of the target protein in the obtained transgenic *Plasmodium* (Lu et al., 2016). This system requires fewer selectable markers and exhibits potential for large gene cassette knockins (Lu et al., 2016). In 2020, transgenic lines of *P. falciparum* expressing mCherry-luciferase and GFP under housekeeping promoters ef1α, sui1, and 40s were manufactured by the CRISPR/Cas9 system that are considered an appropriate method for investigation of parasites in different stages (Miyazaki et al., 2020a). The mentioned investigation revealed that sui1 and 40s have strong expression of reporter proteins throughout the complete *P. falciparum* life cycle. While, 40s promoter showed the strongest expression in liver stage. Besides they found that the p47 gene locus is an appropriate losus for gene insertion in *P. falciparum* without any effect on the parasite fitness (Miyazaki et al., 2020a).

Several transgenic lines of *Plasmodium* expressing reporter genes have been developed to investigate the sexual stage of the parasite. To evaluate the ookinete motility-related genes (*pyctrp* and *pycdpk3*) in the development of oocyst within the mosquito mid-gut, a transgenic line of *P. yoelii* expressing mCherry in sexual stage (Pyp28- mCherry) was produced. The procedure was followed by knocking out of *pyctrp* and *pycdpk3* genes. The outcomes revealed the blockage of oocyst development which highlights the application of a transgenic line expressing the reporter gene in specific-stage examinations (Zhang et al., 2017). In other investigation, construction of a transgenic line of *P. falciparum* (NF54 strain) expressing a fusion of mCherry and luciferase under the *Pfetramp10.3* gene promoter, led to high expression of mCherry as well as luciferase in gametocytes, sporozoites, and liver-stages (Miyazaki et al., 2020a). These transgenic lines are recommended for investigating the transmission blocking assays and studies related to gametocyte and sporozoite biology (Miyazaki et al., 2020a). Due to the prominence of investigating the transmission of *Plasmodium*, transgenic line studies can also reveal valuable information about the conversion of the parasite to sexual form in the culture. To address this matter, recently, a transgenic line of *P. falciparum* was developed to accelerate fluorescent reporter *tdTomato* (tdTom) expression under *gexp02* promoter in the parasites' sexual stage (Portugaliza et al., 2019). Besides, one of the main challenges in sexual stage-related studies of *Plasmodium* is the difficulty to obtain the large number of gametocytes needed for investigations of gametocyte biology and transmission. To obtain large production of synchronous gametocytes, an inducible gametocyte producer (iGP) transgenic line of *P. falciparum* was engineered which manifested the sexual commitment rates of 75%. To create an iGP transgenic line, the essential nucleoprotein for sexual conversion, gametocyte development 1 (GDV1) in fusion with GFP was knocked in under an inducible promoter into a non-essentialcg6 locus. Consequently, the resultant iGP line of NF54 could produce a large number of gametocytes that can be traced by the GFP reporter protein (Boltryk et al., 2021).

2.2. *Plasmodium transgenic lines expressing SpCas9 continuously*

The large size of SpCas9 used in all CRISPR/Cas9-mediated gene editing of *Plasmodium* has increased the plasmid size which negatively influences the construction of required plasmids and confines donor DNA size, and consequently decreases investigation efficiency (Jinek et al., 2012). Therefore, constitutive Cas9 expressing transgenic lines of *P. yoelii* (Qian et al., 2018), *P. berghei* (Shinzawa et al., 2020), and *P. falciparum* (Nishi et al., 2021) were developed. The produced transgenic parasites accomplished the entire life cycle and expressed the Cas9 protein during asexual blood stages (Qian et al., 2018; Shinzawa et al., 2020). Applying CRISPR experiments in the Cas9-expressing parasites leads to immediate cleavage of the target sequence after introducing the gRNA to the cell. Due to the continuous expression of Cas9 in these transgenic lines, a linear donor DNA can also be introduced to the cell to make the experiment easier, and also reduces the possibility of incorrect recombination (Shinzawa et al., 2020).

2.3. *Chimeric Plasmodium transgenic lines*

Chimeric *Plasmodium* parasites that express an orthologous gene from another species can serve to assess the *in vivo* studies of drug or vaccine protein targets of *Plasmodium* (Blume et al., 2011; Cockburn, 2013; Mlambo and Kumar, 2008; Salman et al., 2015; Tewari et al., 2014). In this regard, for vaccine investigations, mice were immunized with the target antigen(s) and then challenged with chimeric rodent parasites expressing the target antigen from *P. vivax* or *P. falciparum*.

Subsequently, the immunological responses and protection were measured in these mice that can be used before proceeding to clinical studies (Mlambo and Kumar, 2008). Additionally, chimeric *P. falciparum* parasites expressing *P. vivax* target genes can be utilized to define the efficiency of target antigens. The establishment of two chimeric *P. berghei* parasites expressing PvCSP-VK210 and PvCSP-VK247 antigens indicated the capability of the producing salivary gland sporozoites in *An. stephensi,* and the possibility to infect rodent hosts (Mizutani et al., 2016; Salman et al., 2017). However, *P. falciparum* chimeric parasites has been produced for replacing *pvcsp* alleles (VK210/VK247) with *pfcsp gene*, *in vitro* production of sporozoite in *An. stephensi* was unsuccessful. This failure strengthens the species-specific characteristics of CSP for full maturation and development of sporozoites in *An. stephensi* (Marin-Mogollon et al., 2018). Concerning the problem, *P. falciparum* chimeric parasites expressing PvCSP antigen of both VK210 and VK247 variants was introduced into the Pfp47 locus of the *P. falciparum* NF54 strain as an insertion so that the parasite could express PfCSP as well as both variants of PvCSP. The obtained transgenic parasite could produce viable sporozoites expressing both PfCSP and PvCSP antigens. The sporozoites could infect the human hepatocytes and induce antibodies against both antigens, which can be used in clinical Controlled Human Malaria Infection (CHMI) studies for PvCSP-based vaccines, and even as attenuated sporozoite vaccine to stimulate cross-protective immune responses against both human *Plasmodium* species (Miyazaki et al., 2020a). In another research, CRISPR/Cas9 genome editing in a *P. knowlesi* strain was established to generate a transgenic line expressing *P. vivax* Duffy binding protein (PvDBP). The results demonstrated the probable ability of produced antibodies to constrain proliferation of the strain but the inability of PvDBP in reticulocyte restriction (Mohring et al., 2019). The transgenic parasites, *P. falciparum* and *P. yoelii,* were also generated based on CRISPR-RGR, a SpCas9-based gene editing system. Their outcomes provide new information on well-organized genetic characterizations in *Plasmodium* species infecting both human and rodent (Walker and Lindner, 2019).

2.4. Knockdown and knockout transgenic parasites

In order to evaluate the function of genome, knockout or knockdown parasites are created. In this regard, CRISPR/Cas9 system has facilitated generating the knockout or knockdown parasites. For instance, in a research study, NF54::pCRISPRINT line (with *SpCas9 and T7 RNA polymerase* integrated at *cg6* locus) was transfected with a DSM-1 selectable *ydhodh* expression cassette harboring 3' and 5' sequence of *eba175* gene in two ends in order to knockout *eba175* gene and transgenic line with deletion in *eba175* gene was successfully achieved (Nasamu et al., 2021).

In an attempt to create a conditional knockdown *P. falciparum* transgenic line, a plasmid containing *glmS* (Glucosmine-6-phosphate synthetase) coding sequence and hemagglutinin (HA) tag flanked to the PfHsp70 sequence and also plasmids for expressing gRNA and Cas9 were used. The obtained transgenic line could conditionally knockdown the level of HSP70 protein in the presence of glocusamine (Kudyba et al., 2018). In another study conducted by Liu et al., 2020, conditional gene knockdown of actin-related protein nuclear 4 (*Arp4*) was generated by CRISPR/Cas9 system via introducing *glmS* into the 3' UTR of *Arp4*. They found that conditional knockdown of *PfArp4* in *P. falciparum* can inhibit blood stage development by impairing the cell cycle (Liu et al., 2020).

2.5. Transgenic parasites expressing alternative alleles

Genetic diversity and the presence of single nucleotide polymorphisms can impair vaccine development or creating drug-resistant parasites leading to challenges in malaria elimination and eradication. In this regard, the functional evaluation of the gene diversity and SNPs can assessed by creating transgenic lines expressing alternative SNPs or alleles of target genes (Thiam et al., 2022). For the first time, Zhang et al., 2014 showed a successful allelic replacement in *P. yoelii* (Zhang et al., 2014). Concerning the evaluation of SNPs in artemisinin drug resistant, various alleles of PfKelch13 with different SNPs (R561H, P574L) were replaced with laboratory Dd2 strain of *P. falciparum* using CRISPR/Cas9 system and the results showed increasing the survival rate of the transgenic parasite in the presence of drug (Uwimana et al., 2020). Crawford et al. (2017) proposed a new approach of CRISPR technology without the use of plasmids (transfection of Cas9-guide RNA ribonucleoprotein complex and a single-stranded oligodeoxynucleotide repair template) to introduce edits into *pfatp4* (a plasma membrane P-type ATPase). They confirmed that mutations in *pfatp4* are determinants of resistance to SJ733 (Crawford et al., 2017).

All in all, these transgenic *Plasmodium* lines can be utilized later in various areas like drug and vaccine development, analyzing the functionality of genome in *Plasmodium* life cycle, as well as in generating high copies of parasites in the desired stages. Although the creation of the transgenic lines were difficult and time-consuming in earlier times, the development of CRISPER/Cas9 system facilitates the procedures, and many transgenic *Plasmodium* lines have been produced or will be created in the future.

3. Malaria vaccine development using CRISPR/Cas9 system

For malaria control, developing a vaccine is one of the most cost-effective and promising strategies (Rappuoli et al., 2002). The ideal vaccine can be produced in large amounts suitable for the immunization of 3 billion people living in endemic areas. The establishment of efficacious malaria vaccines has been challenging, though there have also been significant achievements (Crompton et al., 2010; Cunningham et al., 2016). There are generally two types of vaccines; those containing attenuated or killed pathogens/microorganisms and those containing recombinant protein or conjugates (Hill, 2011); however, the routine approach for vaccine development is achieved subunit vaccines. Nevertheless, the immunogenicity of this type of vaccine is not as potent as whole vaccines, accordingly different strategies like the application of adjuvants (Mehrizi et al., 2018a; Mehrizi et al., 2018b; Nazeri et al., 2018; Pirahmadi et al., 2021a; Pirahmadi et al., 2021b; Pirahmadi et al., 2019; Shabani et al., 2019), various expression platforms (Pirahmadi et al., 2021a), nano delivery of target antigens such as SAPNs (Kaba et al., 2018; Seth et al., 2017; Zahedi et al., 2022), multi-species vaccines (Mehrizi et al., 2011), viral vector-based prime-boost immunization (reviewed in (Pirahmadi et al., 2021a)) (Ogwang et al., 2015; Sheehy et al., 2011; Tiono et al., 2018), and transmission blocking vaccines (Gholizadeh et al., 2010; Gholizadeh et al., 2009) are examined to achieve better immunogenicity. Moreover, another difficulty of the recombinant-antigens-based vaccine is the genetic diversity of candidate antigens which leads to the weak immunogenicity of target antigens versus different parasite strains. Hence to circumvent these challenges, the whole organisms' vaccine could be alternative cardinal strategy for malaria vaccine advancements.

The first evidence of sterile immunity by whole organism vaccination was done in the 1960s (Ivins and Welkos, 1988; Katz et al., 1960). To have a whole organism vaccine for malaria, implementing genetically attenuated parasites (GAPs) are mostly produced by deletions of essential genes leading to the arrest of parasites during the liver stage or the subsequent blood stage. The next generation of GAPs relies on the addition of genetic information that causes the parasites to seizure or dies in the defined time of the life cycle (Nagel et al., 2013; Singer et al., 2015). The lethal gene must be able to be expressed under a specific promoter at the desired stage of the parasite' life cycle. To this end, directed genetic engineering of *P. falciparum* has been accomplished via the application of the flippase (FLP) and Cre-loxP recombinases. These enzymes can omit the human dhfr selectable marker in GAPs by assisting drug recycling and guaranteeing the progression of these kinds of vaccine candidates in clinical experiments (Goswami et al., 2020; Mikolajczak et al., 2014; O'Neill et al., 2011; Vaughan and Kappe, 2017).

Such GAPs have been generated by introducing recombinases such as the flippase (FLP)/FLP recognition target system (Combe et al., 2009). FLP recognition site in the flanking regions of the *msp1* as the target gene has been used under the expression of the sporozoite-specific promoter. Hence, the *msp1* gene is inverted within the liver stage by FLP, thereupon, no *msp1* expression occurred in the blood stage (Combe et al., 2009). As an alternative, nucleases such as ZFNs can be used for this purpose (Singer et al., 2015).

The identification of additional target genes that can be knocked out to generate GAPs, that are homogeneously attenuated, more broadly immunogenic, and an appropriate choice for field application (Kappe et al., 2010), may lead to the generation of better vaccine candidates in the future (Gantz et al., 2015; Lee et al., 2019). Although a CRISPR/Cas9-based approach to produce GAPs has never been reported for malaria vaccination (Ramakrishnan et al., 2019), Miyazaki et al., 2020a introduced a chimeric *Pvcsp* gene into the Pfp47 locus of the *P. falciparum* NF54 genome by using CRISPR/Cas9. Their results showed that the Pf-PvCSP chimeric *P. falciparum* parasites (as a multi-species vaccine) produced viable, motile sporozoites expressed PfCSP and chimeric PvCSP on the sporozoite surface and were infectious to primary human hepatocytes. After immunization with these sporozoites, mice developed antibodies against the repeats of both PfCSP and chimeric PvCSP. The whole organism vaccine against malaria is the most worthwhile vaccine that has ever been delivered. Despite the challenges for the *Plasmodium* whole organism vaccine manufacturing and providing immunity against various strains, it can only be used for the cultivable species of malaria in endemic areas. Until now, most efforts toward developing this vaccine have used the preceding genome editing technologies. Now, the CRISPR system makes this path undemanding and this sort of vaccine will successfully be developed to prevent parasite growth at the end of the pre-erythrocytic stage.

4. Diagnosis of *Plasmodium* species-specific

Precise and accurate diagnosis of *Plasmodium* species is indispensable for malaria control and elimination, specifically in resource-limited settings. The absence of specific, sensitive, and reliable diagnostics leads to the insufficient prognosis of species and hindered treatment, which are the obstructions to achieving malaria elimination. Having the microscopy diagnosis as a gold standard method (Moody, 2002; Turki et al., 2012), the other techniques based on molecular detection or nucleic acid amplification; PCR (real-time, nested PCR, ultrasensitive PCR) (Nourani et al., 2022; Wang et al., 2014; Zakeri et al., 2002), loop-mediated isothermal amplification (LAMP) (Lucchi et al., 2016; Pöschl et al., 2010), recombinase polymerase amplification (RPA) (Kersting et al., 2014), next generation sequencing (NGS) (Lalremruata et al., 2017), and clustered regularly interspaced short palindromic repeats (CRISPR) (Cunningham et al., 2021; Lee et al., 2020), immunoassays; rapid diagnostic tests (RDTs) (Choi et al., 2019; Cunningham et al., 2019), ELISA (Noedl et al., 2006), Microarray (Yatsushiro et al., 2010), spectroscopy; Raman, NMR, Mass spectrometry (Kumar et al., 2020; Peng, 2021; Ray et al., 2017; Venkatesh et al., 2020) have also displayed hopeful outcomes. Besides, novel technologies such as smartphone-based diagnosis (Guo et al., 2021), biosensors/nanosensors (Cruz et al., 2021; Ragavan et al., 2018), soft computing applications (Toha and Ngah, 2007), and machine learning (Fuhad et al., 2020) revealed triumph in the detection of *Plasmodium* species. Considering the advantages and drawbacks of contemporary technologies, costs, simplicity, availability, and skilled technician interpretation as well as sensitivity and specificity are of main factors that influence their application for malaria treatment and disease management.

Morphological detection of malaria infections through light microscopy provides rapid diagnosis, as a labor-intensive method is dependent on trained personnel for staining and careful diagnosis. The conventional RDTs (PfHRP2 or pLDH, and aldolase) supply field portable detection instruments (Choi et al., 2019; Cunningham et al., 2019) which unable to discover low parasitemia of infections (specifically for non-*falciparum* species, Table 1) and are not stable in warmer places, as well as the longevity of histidine-rich protein 2 (pfHRP2) antigenemia in patient's blood stream leading to the false positive consequences (Mouatcho and Goldring, 2013) and gene deletion in *pfhrp2* and *pfhrp3* showing false negative results (Houzé et al., 2011). To overcome these limitations, molecular detection techniques like PCR, RPA, and LAMP presented reliable sensitive and specific outcomes for the identification of malaria pathogens. The adaptability, strength, and sensitivity of PCR have made nucleic acid-based detection of *Plasmodium* species DNA as a gold-standard method of molecular diagnostics. However, extensively adoption of isothermal amplification technology requires expensive reagents and skilled technicians to avoid lower detection specificity (Wang et al., 2014; Zakeri et al., 2002). RPA, another isothermal amplification approach to discover DNA and RNA targets, uses two targets, explicit primers to stick to the template with the cooperation of a recombinase through strand-displacement DNA synthesis (Kersting et al., 2014). The LAMP method, based on nucleic acid identification and target assortment, showed comparable sensitive and specific results to other molecular field-applicable diagnostics (Lucchi et al., 2016; Pöschl et al., 2010). "Ultrasensitive PCR" as a highly sensitive novel method, improved the detection standards to 0.02 parasite/μl. However, the expensive needed infrastructure, thermocyclers, and reagents restricted the general application of molecular detectors in deprived rural settings (Cunningham et al., 2021; Cunningham et al., 2019). CRISPR as a promising technology, have the potential to accompaniment the objective of prior methods for the precise point-of-care (POC) detection in routine clinical tests (Lee et al., 2020).

Despite the extensive application of Cas9/gRNA in gene editing of *Plasmodium* species, this method is infrequently administrated for distinguishing nucleic acids of malaria. Nevertheless, the specific DNA cleavage proficiency of other Cas proteins (Cas-12a and Cas13a) has made it more suitable for the detection of *Plasmodium*-infected specimens. Different range of clinical samples, laboratory strains, and pools of *Anopheles* mosquitoes from Thailand, Uganda, and Congo were analyzed by CRISPR-based SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) assays which are introduced as the sensitive and specific molecular method for detection of five human pathogenic malaria species (Cunningham et al., 2021). The coupled RPA reaction is associated with the amplification of dsDNA by the T7 promoter and conserved region of 18SrRNA gene primers. The collateral stimulation of LwCas13a RNase activity cleaves RNA reporter molecules which produce a target signal. Generated signals are detectable by lateral flow strips or colorimetric read-outs. Cas13a-based SHERLOCK assay with diagnosis capability of parasites DNA or RNA at attomolar concentrations compared with other molecular approaches for detection of *P. falciparum* species, amplicon-based deep sequencing and real-time PCR has shown 73% and 94% sensitivity, and 94% and 100% specificity, respectively (Cunningham et al., 2021). Lately, novel CRISPR-based SHERLOCK platform has revolutionized rapid point-of-care diagnostics of malaria species. The detection capability of two parasites per microliter of blood samples with 100% sensitivity and specificity for *P. falciparum* and *P. vivax* asymptomatic and symptomatic carriers significantly upgraded the standard of malaria field-deployable diagnosis. This isothermal, one-pot assay enables the detection of parasites from clinical specimens of whole blood, and sera to filter-paper dried samples through 10 minutes of specific extraction followed by a one-hour SHERLOCK assay. Stimulation of programmed Cas12a activity by identification of double-stranded target DNA is united with amplification of a reverse-transcriptase recombinase polymerase to upsurge the detection of the target fragment to reveal the endpoint results by using either handheld fluorescent or lateral flow strip (Lee et al., 2020).

Due to the objective of malaria elimination and a significant decline of 90% in morbidity and mortality in endemic countries, comprehensive surveillance and diagnosis systems are principles to achieve this goal by

Table 1
The comparative features of diagnostic methods for *Plasmodium* parasites.

Diagnostic methods	Detection (parasite/μl)	Accuracy (reliability)	Speed	Cost	Expertise	Strength/Advantages	Weakness/Disadvantage
Microscopy	4-50	moderate	moderate	low	high	Easy and quick detection based on morphology and staining	Unable to differentiate strains, Expertise for detection and confirmation
Nested PCR	0.022–0.1	high	slow	high	high	Able to differentiate strains	Infrastructure setting is needed, very time-intensive
RDT	100-500	low	fast	low	low	Field-applicable	Not reliable results (due to gene deletion), not capable for species-specific distinguishing
NGS	7-40	high	slow	moderate	high	Information at SNP level, reliable and stable results, Used for better understanding of malaria transmission pattern, parasites movement, recognizing drug resistance genes	False negative results due to some mutations
LAMP	1-10	high	moderate	high	high	No need to thermocycler, need small amounts of blood on filter papers, detect sub -microscopic parasitemia	lacks sufficient accuracy, need cold storage for the reagent in field, complex sample preparation
CRISPR	0.36	high	fast	low	low	Rapid, reasonable and ultrasensitive, Field-applicable, capable for species-specific detection, used for blood, serum and dried blood samples	lack multiple detection competences of CRISPR/Cas12- and Cas13, need to optimization of detection assays (time and proficiency)

2030. Though, the incorporation of traditional methods and novel molecular techniques could noticeably enhance the quality of results. The striking development of a CRISPR/Cas-based diagnostic kit that can specifically diagnose the *Plasmodium* species (especially in submicroscopic carriers) or drug resistance markers is highly required in malaria settings with affordable cost and high-speed detection. Further improvements in CRISPR/Cas-SHERLOCK assay will expand our knowledge on *Plasmodium* genomic variety and progress the detection of simultaneous parasites and drug resistance single variations in one-pot reaction and lower parasitemia (Cunningham et al., 2021; Lee et al., 2020).

5. CRISPR/Cas9 technology in drug resistance research

The absence of an effective licensed vaccine against malaria highlights the importance of developing effective antimalarial chemotherapies. However, the occurrence of *Plasmodium* resistance to commonly used drugs poses a significant challenge to malaria control. The current therapeutics for most malaria cases caused by *P. falciparum, P. vivax, P. ovale,* and *P. malariae* infections are an Artemisinin derivate and a long-lasting partner drug (WHO guidelines for malaria, 2022). A molecular understanding of drug resistance mechanisms and identifying the genetic determinants of resistance enables surveillance of resistance markers and an understanding of the action of antimalarial drugs. The advent of CRISPR/Cas9 technology has provided many opportunities to manipulate the *Plasmodium* genome. In the case of *P. falciparum*, different mutations were introduced in *pfcronin* (*P. falciparum* coronin-like protein) (Demas et al., 2018) and *pfk13* (*kelch13* propeller domain) genes (Stokes et al., 2021; Uwimana et al., 2020) using CRISPR/Cas9 based genome editing and the role of target mutations in artemisinin resistance phenotype *in vitro* was confirmed. It is worth mentioning that a complex process involves artemisinin resistance with the contribution of different proteins and pathways as well as the genetic background of the parasite. These findings illustrate the power of CRISPR/Cas9 genome editing to identify and validate artemisinin resistance mechanisms.

With the emergence of resistance to artemisinin-based combination therapy, drug discovery efforts are underway to explore new compounds with antimalarial potential. Recently, different classes of drugs were developed with potent activity against different stages of *P. falciparum*. However, the exact mechanisms of action of these compounds remain poorly understood (Flannery et al., 2013; Kuhen et al., 2014; Leong et al., 2014; Meister et al., 2011). CRISPR technology could create insights into the mechanisms of actions of new antimalarial compounds by detection and confirmation of their resistance mechanisms that in some instances correspond to their actual mechanisms of action. By using *in vitro* drug resistance selections, individual mutations is identified by whole genome sequencing and their role in drug resistance is confirmed by introducing those mutations into parental lines using CRISPR system. Using this approach, is followed by the CRISPR/Cas9 tool, and different studies have confirmed the role of mutations in *pfcarl* (cyclic amine resistance locus) (LaMonte et al., 2016), *pfugt* (UDP-galactose transporter), *pfact* (acetyl-CoA transporter) (Lim et al., 2016) and *pfmdr1* (multidrug resistance-1) (Ng et al., 2016) in resistance against piperazine-containing compounds. Additionally, CRISPR-based introduction of mutations in *pfpare* (prodrug activation and resistance esterase), *puba2* (SUMO-activating enzyme subunit 2) (Sindhe et al., 2020), and *cpsf3* (a homologue of mammalian cleavage and polyadenylation specificity factor subunit 3) (Sonoiki et al., 2017) genes confer resistance to benzoxaboroles and mutations in *pfmdr1*gene confirmed parasite resistance to hexahydroquinolines (Vanaerschot et al., 2017). These findings can be used to explore the potential targets of these compounds and offer insight into their mechanisms of action and/or resistance.

Many of the essential genes for parasite invasion and growth during its life cycle represent potential targets for drug development. However, these essential genes are refractory to genetic manipulation and gene deletion. This proposes a challenge for identifying the mechanisms of action of new drugs targets. Conditional genome editing is a promising approach necessary for genetic manipulation resulted in knockdown of essential genes (Briquet et al., 2022). In this context, CRISPR system enables to create efficient conditional knockdown parasite lines to explore candidate targets of new antimalarial compounds targeting essential genes. In an effort to identify the main targets of MMV030084, a molecule with potent activity against liver, sexual and asexual blood stages of *P. falciparum*, Vanaerschot et al created knockdown parasite lines by using CRISPR system. They identified cGMP-dependent protein kinase (PKG) as the primary target of MMV030084 (Vanaerschot et al., 2020). Furthermore, CRISPR-based assays proposed a new generation of molecular tools for the detection of markers associated with drug resistance. FLASH-NGS (finding low abundance sequences by hybridization-next-generation sequencing) is a next-generation CRISPR/Cas9 diagnostic tool with a high multiplexing capacity. It demonstrated high efficiency in the detection of *P. falciparum* resistance genes (Quan et al., 2019). SHERLOCK is another CRISPR-based assay that demonstrates high potential for the detection of the A581G variant in the *pfdhps* (dihydropteroate synthase) gene for surveillance of *P. falciparum* resistance to sulfadoxine (Cunningham et al., 2021).

In the case of *P. vivax*, most of the potential molecular markers associated with drug resistance have been identified through genome-wide population genetic analysis. However, the lack of a well-established *in vitro* continuous culture system highlights the need to develop new techniques for experimentally studying potential molecular markers of drug resistance. CRISPR/Cas9 system has provided promising clues to this challenge. Verzier et al. (2019) used a model system to replace putative resistance genes to test their role in drug resistance using *P. knowlesi* adapted to *in vitro* culture in human erythrocytes. Despite, the candidate genes were not associated with drug resistance phenotypes, they established a promising model for *P. vivax* drug resistance studies (Verzier et al., 2019). CRISPR/Cas9 genome editing tool also makes it possible to use a rodent model of *P. berghei* infection to characterize resistance mechanisms to different antimalarials. To test artemisinin resistance-associated mutations in *k13* and *ubp-1* (ubiquitin hydrolase) genes in *P. berghei* sensitive lines, CRISPR could serve as an efficient genetic tool that provides experimental evidence on the role of target mutations in modulating sensitivity to artemisinin *in vivo* (Simwela et al., 2020a; Simwela et al., 2020b). In another study, the piperaquine resistance parasite was isolated from PbMut (*P. berghei* mutator) under drug pressure. Whole genome sequencing of mutant clones revealed a mutation in the *pbcrt* (chloroquine resistance transporter) gene that CRISPR-based introduction of the mutation confirmed its contribution to piperaquine resistance (Ikeda et al., 2021).

The discovery of molecular markers associated with antimalarial drug resistance has greatly aided in the surveillance of the emergence and spread of resistance, such information is necessary for guiding malaria control strategies (Pirahmadi et al., 2014; Pirahmadi et al., 2013). To elucidate genetic determinant of antimalarial drug resistance, highly efficient *Plasmodium* genome editing tools are of particular importance. Traditionally, conventional allelic exchange strategies were used to confirm the role of drug resistance mediators shown by preceding studies (Reed et al., 2000; Sidhu et al., 2002; Triglia et al., 1998; Wu et al., 1996). However, the inherent inefficiency of this approach has inhibited any large-scale gene editing in *Plasmodium*. The advent of CRISPR/Cas9 gene editing tools enhances the feasibility of manipulating the parasite genome to identify molecular determinants of drug resistance and elucidate novel antimalarial targets. The continued advance and refinement of CRISPR/Cas9 technology for genome editing in *Plasmodium* can be a powerful approach for ongoing research into the antimalarial drug. As antimalarial drug resistance manifests the evolutionary selective pressure on the *Plasmodium* parasite, which is transmitted via the *Anopheles* vector to human hosts, subsequently the suppressive effect of these chemical drugs is shown in humans. Due to the possibility of resistance to ACTs as the most prescribed drugs for patients, novel techniques should be applied for symptomatic and asymptomatic infections treatment and prohibit the spread of resistance globally (Marshall et al., 2019). Nevertheless, due to the evolving resistance to drugs, as the natural characteristic of the parasite to survive, the combination of therapies may diminish the evolutionary impediment to the development of resistance. CRISPR/Cas9 technology may provide new drugs with reduced evolutionary pressure on parasites and vectors.

6. CRISPR/Cas9 mediated transgenic lines and gene drives in malaria vector

Vector control is essential in malaria elimination programs. The idea of controlling vector-borne diseases through the genetic manipulation of mosquitoes was proposed several decades ago (Wilson et al., 2020). Vector control is an indispensable measure being used with different methods of long-lasting insecticidal nets and indoor residual spraying, which resulted in the swift progress of insecticide-resistant mosquito populations (Caragata et al., 2020; Soma et al., 2021). However, technical hurdles, including the need for efficient germline transformation systems and effective pathogen transmission-blocking effectors, originally curbed progress in mosquito transgenesis. Therefore, other control methodologies such as spreading sterile mosquitoes and biological engagement has been utilized as alternative environmentally techniques (Benelli et al., 2016; Ickowicz et al., 2021). CRISPR/Cas9 strategy opened new avenues for effortlessly genome editing of mosquitoes, sexually reproducing organisms to spread genetic characteristics through non-Mendelian inheritance. Genetic engineering technologies using CRISPR-based gene drives have been used to create transgenic mosquitoes carrying anti-pathogen effector genes against human malaria parasites that disable vector populations from transmitting disease. Moreover, generating gene-drive systems is designed to negatively introduce specific genes throughout wild vector populations in order to disrupt an essential gene associated with viability or fertility, which eventually eliminates that population (Raban et al., 2020). Gene drive based on CRISPR strategy uses Cas9 endonuclease and guide RNAs to produce site-specific double-strand DNA breaks. Afterwards, non-homologous end-joining (NHEJ) or HDR can repair the DSB sites (Gantz and Akbari, 2018). NHEJ alleles mostly produce at zygotic phase, before germline allocation. If they do not have a detrimental fitness cost to the insect, at the time of allelic conversion in females, expansion of gene-drive system will be blocked. Due to the importance of Cas9 endonuclease expression/activity and efficacy of gene drive, more understanding about the mechanisms underlying the performance of copying versus producing of resistant alleles is required for the safe and successful distribution of gene-drive strategy (Terradas et al., 2022).

There are some similarities and differences in applying CRISPR/Cas9 methodology for malaria parasites and their invertebrate vector. CRISPR/Cas9 has been applied for insertion, deletion, and substitution of a gene of interest, and insertion or deletion in *Anopheles* species. The repair DNA mechanisms in *Plasmodium* and its vector do not follow similar procedures. The repair mechanisms are accomplished by HDR in the parasite, and HDR or NHEJ in mosquitoes. The repair template homology arm applicable for *Plasmodium* (~100-400 bp) is shorter than *Anopheles* (~1000-2000 bp). U6 snRNA is the gRNA promoter in *Plasmodium* and its vector, though the T7 RNAP can be utilized specifically for *Plasmodium.* There are various markers can be used for the selection of a transgenic line of *P. falciparum* including human dihydrofolate reductase, Blasticidin S deaminase, Neomycin phosphotransferase II (G-418), Puromycin-N-acetyltransferase (puromycin), yeast dihydroorotate dehydrogenase while the marker for *Anopheles* is a fluorescent protein (Bryant et al., 2019). Furthermore, the duration of experiments to achieve a transgenic line in the parasite is about 4 to 6 weeks when the microinjection of the embryo generates transgenic adult mosquitoes is short (Bryant et al., 2019). Due to the fact that *Plasmodium* parasite and its mosquitoes vector, *Anopheles* are haploid and diploid organisms, respectively, so the application of CRISPR-based gene editing to generate transgenic lines are different from each other. Although, the complexity of diploid multicellular organism makes the process more challenging, there is some restrictions for malarial parasites like being AT-rich that makes it difficult to identify the gRNA binding site nearby the PAM sequence and the lack of a cNHEJ pathway leads to eliminate off-target effects (Kirkman et al., 2014). Although, the inheritance between haplodiploids and diploid insects varies, this may influence the result of gene drive by using CRISPR strategy in wild populations (Li et al., 2020). The wild-type/driver allele heterozygotes transforming to driver allele homozygotes can happen in diploids. Furthermore, the recessive alleles in haploids can be target of selection while these alleles are protected in heterozygous diploids (Crowder and Carrière, 2009). Therefore, it has been hypothesized that deleterious alleles for CRISPR gene drive suppress harmful species populations resulting in less expansion haplodiploids than diploids (Rode et al., 2019). On the other hand, for CRISPR gene drive favorable alleles increase or improve valued species populations which leading to more extending of haplodiploids than diploids (Rode et al., 2019).

To the best of our knowledge, two methodological gene drives have been developed for *Anopheles* population manipulation. One of this

species is *An. gambiae*, the prominent malarial vector in Africa (Carballar-Lejarazú et al., 2020) and *An. stephensi*, (Adolfi et al., 2020). Their experiments in laboratory cage trials were victorious by substitution of gene drive insects with the reference populations. There are various molecular mechanisms to support the efficiency of gene drive systems. In the case of *An. gambiae* (Carballar-Lejarazú et al., 2020), the gene drive AgNosCd-1 was placed in the cardinal (cd) site to expresses the SpCas9 endonuclease along with nanos promoter. Their result demonstrated a high quality drive spread in both genders which extended into populations (Carballar-Lejarazú et al., 2020). The other species was *An. stephensi* which inserted into the kynurenine hydroxylase site to expresses Cas9 via vasa promoter. This gene drive produced a remarkable number NHEJ-induced drive-resistant when transmitted to male mosquitoes (Adolfi et al., 2020; Pham et al., 2019). These NHEJ alleles survive in male generation without function but the capability of being reproductive fitness costs in female mosquitoes (Pham et al., 2019). These investigations confirmed the unique drive properties which recommended that related variations in promoter-driven Cas9 expression patterns might be linked to the differential efficacy of these drives during oogenesis (Terradas et al., 2022).

Scientists have already acquired CRISPR/Cas9 for generating transgenic lines like sterile female phenotypes with interrupted genes or inactivating some part of the genome. For instance, this method was utilized to manipulate the genome of *Anopheles gambiae* with disrupted three genes (AGAP005958, AGAP011377, and AGAP007280) that confer a recessive female-sterility phenotype upon insertion of CRISPR/Cas9 gene drive constructs designed to target and edit each gene in the malaria mosquito vector. The results showed strong suppression at the molecular level in experiments in the cage as well as for further population modeling (Hammond et al., 2016). Hammond et al. developed a gene drive based on CRISPR nuclease to disrupt essential genes associated with the female fertility of malaria mosquito. Self-replication of these genes and inheritance of the following genetic changes from one homologous chromosome to another will cause long-lasting and sustainable population suppression. The long-term monitoring of the frequency of the gene drive in the subsequent 25 generations revealed a gradual decrease in frequency accompanied by the spread of small, nuclease-induced mutations at the target gene (Hammond et al., 2017). A modified CRISPR/Cas9 gene editing procedure for the malaria vector *An. gambiae* was established which inactivated the fibrinogen-related protein 1 (*FREP1*) gene. This experiment led to the suppression *Plasmodium* infection and significant detention at the oocyst and sporozoite stages (Dong et al., 2018). Another gene disruption was studied to generate an *An. stephensi* Leucine-Rich protein (LRIM1) knockout line enables inhibition of *Plasmodium* sporozoite development inside the mosquitoes. The line supporting melanization and phagocytosis of ookinetes using CRISPR/Cas9 mutant mosquitoes showed the decreased reproductive capacity in oviposition, fecundity, male fertility, and reduction of sperm transfer by males (Inbar et al., 2021).

Scientists also considered inserting some effective genes into the genome by direct or indirection of Cas9 proteins. CRISPR/Cas9 mediated knock-in *An. sinensis* were generated by direct injections of Cas9 protein, double-stranded DNA donor, and Orco-gRNA to the most widely distributed species and the primary transmitter of *P. vivax* malaria in China. A similar success rate of the mutation rate of 3.77%, was gained (Wang et al., 2022). In other investigation, utilizing the non-injecting embryos approach targeted and heritable mutations of malaria vector *An. stephensi* were generated via delivering Cas9 ribonucleoprotein complex to adult mosquito ovaries using the ReMOT Control (Receptor-mediated Ovary Transduction of Cargo) technique, as efficient as standard embryo injection methods in laboratories not equipped with the required infrastructure (Macias et al., 2020). The study on the generation of indels in the germline and somatic cells in female gene-drive lineages using a series of selective crosses between a gene-drive line, AgNosCd-1, and wild-type mosquitoes, revealed the potential drive-resistant mutant alleles responsible for the increasing rates during embryonic development (Carballar-Lejarazú et al., 2022). Moreover, scientists have used some effective genes to modulate mosquito's fitness by CRISPR/Cas9 mediated knock-out. Inactivation of the fibrinogen-related protein 1 (FREP1) gene by application of CRISPR/Cas9 genome editing, demonstrated remarkable consequences on the mosquito's susceptibility to *Plasmodium* and also mosquito fitness. FREP1 mutants were inhibited transmission of rodent and human *Plasmodium* parasites and vectors lost blood feeding capability (Dong et al., 2018). In another investigation, gamma-interferon–inducible lysosomal thiol reductase (mosGILT) was knocked out in *An. gambiae* by CRISPR associated Cas9. The outcomes demonstrated that female mosaic mosGILT mutant mosquito's infection by *P. berghei* or *P. falciparum* had reduced number of oocyst (Yang et al., 2019). The generation of *An. stephensi* Leucin-Rich protein (LRIM1) knockout line (Δaslrim1) by CRISPR system, outstandingly improved bacterial load and microbiome composition in their mid-gut. These changes will lead to mosquito mortality and a decline in sporogony. The reproductive ability, oviposition, and fertility of male mosquitoes will decrease in (LRIM1) knockout mutants (Inbar et al., 2021). In other experiments, manipulation of mosquitoes' sex was the objective by using CRISPR/Cas9 strategy. Targeting male determining factors (M factors) and other intermediary genes in the sex determination pathway provide new ways resulting in female-to-male conversion or female killing, enabling efficient sex separation and effective reduction of target mosquito populations. CRISPR/Cas9 gene drive technology was used to target the gene doublesex (*Agdsx*) encoding two alternatively spliced transcripts, dsx-female (*AgdsxF*) and dsx-male (*AgdsxM*). The transcribed genes involved in controlling sex differentiation in *An. gambiae*. Female mosquitoes were homozygous for the disrupted allele and showed an intersex phenotype and complete sterility. Doublesex (*Agdsx*) spreads rapidly in caged mosquitoes, reaching 100% prevalence within 7-11 generations while progressively reducing egg production happened in mosquitoes population (Kyrou et al., 2018). Another research related to knockout of a male determining (Nix) gene has resulted in feminized genetic males with the successful application of the CRISPR/Cas9-mediated gene editing system. The ectopic expression of the Nix gene in females confirmed the Nix importance to initiate male development and converting female mosquitoes into harmless males (Hall et al., 2015).

By engineering some targeted genes, they will be spread inside the wild vector populations that could influence the viability or fertility of individuals or eradicate that population. Gantz et al. provided evidence from a highly efficient gene-drive system manifested a successful function of targeting anti-malarial genes, m1C3 and m2A10, contributing to binding the human malaria parasite *Plasmodium falciparum* ookinete protein Chitinase 1 and the circumsporozoite protein (CSP) in *An. stephensi* population. The inhibitory function of spreading gene drives for producing *P. falciparum* sporozoites confirmed the capability of this technique for the using in malaria vector control programs (Hammond et al., 2017). Hammond et al. challenged the suppressive activity of the gene drive in *An. gambiae* populations in large indoor cages that permit complex feeding and reproductive behaviors to bridge the gap between field and laboratory. The gene-drive construct spreads rapidly through the population fully suppresses them within one year, and with no selection for resistance to the gene drive (Hammond et al., 2021). Taxiarchi et al. have performed an inactivation process for CRISPR-based gene drives in which they restored inheritance to Mendelian rates in the malaria vector *An. gambiae* using the anti-CRISPR protein (AcrIIA4). They also predicted that a single release of male mosquitoes carrying the AcrIIA4 protein could block the spread of a highly effective suppressive gene drive preventing population collapse of caged malaria mosquitoes in a cage (Taxiarchi et al., 2021). In an investigation the induced X-chromosome-shredding I-PpoI nuclease inserted into a conserved sequence of the *doublesex* (*dsx*) gene by CRISPR-based gene drive system. The induced gene derive led to a male-only population by starting allelic frequency of 2.5% in 10-14 generations (Simoni et al., 2020). The

suppression potential of a drive allele with high fitness costs, can be enhanced by genome engineering tool (North et al., 2020). The collected data by suppression modeling these malaria vectors will be useful for field challenges such as highly seasonal regions.

Since eradication of mosquito populations is tremendously challenging and leads to some severe effects on ecosystem, the safety of CRISPR-based products and the probability of threatening the ecology are other steps that should be taken. To test their robustness and efficacy in larger population sizes under more realistic ecological settings, more investigation is also required to determine the target profile of each gene drive to meet specific performance and safety requirements. It is also needed to update guidance documents related to research, development, and testing of genetically engineered insects and arthropods (Adelman and Tu, 2016; Carballar-Lejarazú et al., 2022). Designing a release strategy is necessary for the deployment of gene drive mosquitoes and considerations of ecological confinement, and also the hazard of the establishment. Other measures should be taken to evaluate the possibility of accidental release of the gene drive construct anywhere in the environment months to years later. Regardless of the magnificent assistances of CRISPR as a novel genome editing and manipulation technology, the detrimental impressions on living organisms (especially non-aiming hosts), public health, environmental issues, and the social-economic assemblies should be highly considered. The likelihood of active gene fusion, the possibility of occurrence of mutations in non-target species, releasing transgenic or genetically modified creatures (e.g., mosquitoes) with the capability of inheritance through generations and disturbing effects on the ecosystem stability are major concerns related to ethics and application of CRISPR in the real world (Djekoun, 2021; Oye et al., 2014; Rodriguez, 2016). CRISPR is not immune to risks, like any other technology. Modified organisms may cause off-target effects that require careful evaluation. Tools in bioinformatics can be used to predict or minimize/avoid them. The concern, however, is horizontal gene transfer and the potential harm caused by cross-pollination to humans and the environment. In addition to the presence of functional nucleases, CRISPR does not appear to have the potential to cause unintended harm: gRNAs must match DNA target sites adjacent to protospacer motifs, RNAs degrade easily which cannot be used and targeted sequences may not match across species/organisms. Nevertheless, CRISPR is a new technology that is still not thoroughly understood, so objective scientific studies are needed to assess risks and harms. A better understanding of CRISPR persistence in organisms and the environment would also assist in risk assessment. A recall approach [46] or the use of controllable CRISPR nucleases in GDs and other applications would simplify their removal/stoppage (Gantz and Akbari, 2018).

7. Conclusion

Undoubtedly the advancements in revolutionizing genome engineering strategies will improve biomedical research through trial-and-error developments, especially for deadly diseases like malaria. Genome editing employing the Cas9 endonuclease facilitates applying targeted double-strand break for deletion, insertion, replacement, or mutation in the gene of interest without genetic scarring. Rising new drugs for treatment strategies versus protozoan parasites will be greatly influenced by the CRISPR system (Grzybek et al., 2018) and also to create immunogenic and non-pathogenic parasites mediated by CRISPR/Cas9 as upcoming vaccinations (Hollingdale and Sedegah, 2017). The remarkable advancements of CRISPR/Cas-oriented prognosis for specific-species identification and drug resistance markers will accelerate detection progress via single reaction and lower parasitemia. The gene-drive *Anopheles* strains could inhibit the wild population generation and spread out antimalarial genes. However, no larger-scale experiments have stated the achievements for malaria elimination. Likewise, CRISPR-based gene edited organisms will supply sustainable, efficacious, and profitable system, even combined with other methods for massive control of malaria.

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Authors' contribution

LN and AAM contributed to the conceptual framework of the review. All authors contributed to write the first draft of the manuscript. LN and AAM completed edits and revisions. All authors approved the submitted version.

Declaration of Competing Interest

Authors declare that they have no conflict of interest.

Data availability

No data was used for the research described in the article.

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