



CRISPR-Cas9 gene drive and conventional approaches for malaria control: A review of progress, challenges, and future prospects

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ABSTRACT

Malaria remains a global health challenge, having a steadily increasing incidence as well as death rate, particularly in sub-Saharan Africa. Modern methods of tackling malaria, which primarily include long-lasting insecticide-treated nets (LLINs), indoor residual spraying (IRS), and anti-malarial drugs, are faced with a serious threat emanating from drug and insecticide/pesticide resistance, thereby undermining eradication efforts. This work extensively reviewed the current literature on conventional means of controlling malaria to unveil the impediments to their use and the urgent need for a permanent solution through innovative strategies. It also provided a detailed evaluation of gene drive technology based on the CRISPR-Cas9 system as a crucial complementary tool for malaria control. Additionally, this present study carefully evaluated the ethical issues related to this technology, as well as the ecological concerns regarding the production and release of gene drive modified organisms. The CRISPR-Cas9 system employs a sequence-specific endonuclease mechanism to generate targeted double-strand DNA breaks, forming the basis for precise gene editing and drive development, and utilises two fundamental strategies for vector control: population suppression, primarily aimed at reducing the mosquito population, and population replacement, which aims to spread genes that make mosquitoes refractory to the *Plasmodium* parasite.

Introduction

Malaria remains one of the most significant parasitic challenges, with adverse effects on public health and well-being globally, especially in the sub-Saharan African region. This has resulted in high morbidity and mortality globally. Malaria was of Italian origin, being derived from the word 'mal'aria', because of the former notion that the disease was of an air pollution source [1,2]. It is a parasitic, vector-borne illness transmitted primarily by an infected female *Anopheles* mosquito, whose single bite into the dermis injects *Plasmodium* into its vertebrate host [3], thereby causing malaria [4]. Sub-Saharan Africa is the most suitable habitat for female

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Anopheles mosquitoes to flourish. This explains why malaria is most prevalent in tropical and subtropical areas [5]. Malaria incidence has been on the rise since 2014. In 2019, about 229 million cases were recorded worldwide [1,6], while in 2022, 249 million malaria cases were documented, along with an estimated 608,000 deaths, with 94% of these cases occurring within the WHO African Region [6]. In addition, 263 million malaria cases were estimated in 2023, with 569,000 deaths recorded in the WHO African region alone, highlighting the urgency required to address the burden of malaria globally [7]. The most vulnerable individuals include women with pregnancies, children below the age of 5, immigrants, and international travelers [8].

Considering the epidemiological profile of humans, malaria is noted to be primarily caused by five species: *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium vivax*, *Plasmodium knowlesi*, and *Plasmodium ovale* [6,9,10]. Amongst these species, *Plasmodium falciparum* is the deadliest [11], while *Plasmodium ovale* is the rarest form, which is commonly found in the West Africa region, possessing dormant capabilities in the host liver and may recur after several years (four years) from the initial infection [12]. On the other hand, *Plasmodium vivax* is widespread, accounting for >50% of the malaria cases reported in India [13]. Over the years, various vector control methods have been employed to manage mosquitoes, including insecticide-treated nets [14] and indoor residual spraying [15]. However, there has been an onset and spread of resistance to these primary malaria vector control methods [16]. Other significant challenges of this approach include its effects on non-target species and environmental persistence [17].

In addition, anti-malarial drugs such as quinolines, amodiaquine, primaquine, and piperaquine, are used in the treatment of malaria and prevention of disease transmission [18]. Such drugs perform their action at the asexual blood cycle of the *Plasmodium* parasite [19]. The threat posed by the growing resistance to anti-malarial drugs cannot be overemphasized. This is because resistance issues have been a major hindrance to successful interventions towards total eradication of malaria, as resistance often emerges soon after antimalarial drugs are deployed, thereby hindering efforts to control and ultimately eradicate malaria [20–21]. These resistance challenges and their devastating effects on human health necessitate the urgent need for newer techniques as complementary method to enhance existing interventions.

Advances in molecular biology have revealed that the CRISPR-Cas9 system can precisely manipulate genomic DNA of target organisms by introducing targeted double-strand breaks at specific genomic locations, directed by guide RNA sequences. The Cas9 protein itself consists of multidomain enzymatic regions, HNH and RuvC, that perform nicks on opposite DNA strands, utilizing a two-metal ion mechanism to catalyze phosphodiester bond cleavage. Repair of the derived DNA breaks is carried out by cellular mechanisms, such as homology-directed repair (HDR) or non-homologous end joining (NHEJ), whose effectiveness and error status greatly affect genome editing outcome [22–24]. New malaria control approaches increasingly focus on the biochemical manipulation of mosquito genomes via gene drive technologies powered by CRISPR-Cas9, enabling both population suppression and replacement approaches [25].

CRISPR-Cas9-based gene drive technology is a promising strategy in recent years, enabling targeted genetic modification via population suppression [26–28] and population replacement [29–31]. This technology has emerged as a widely recognized innovation across various subject areas, including medical science, agricultural science, where it has been applied to enhance crop resistance to parasitic weeds, evaluated in global field trials [32–33], and applied biotechnology [34–35]. This review discusses various vector control techniques, emphasizing the challenges posed by insecticide and antimalarial drug resistance. A critical investigation of the progress, promises, perils, and ethical concerns associated with CRISPR/Cas9 gene drive technology as a complementary vector control strategy is also addressed.

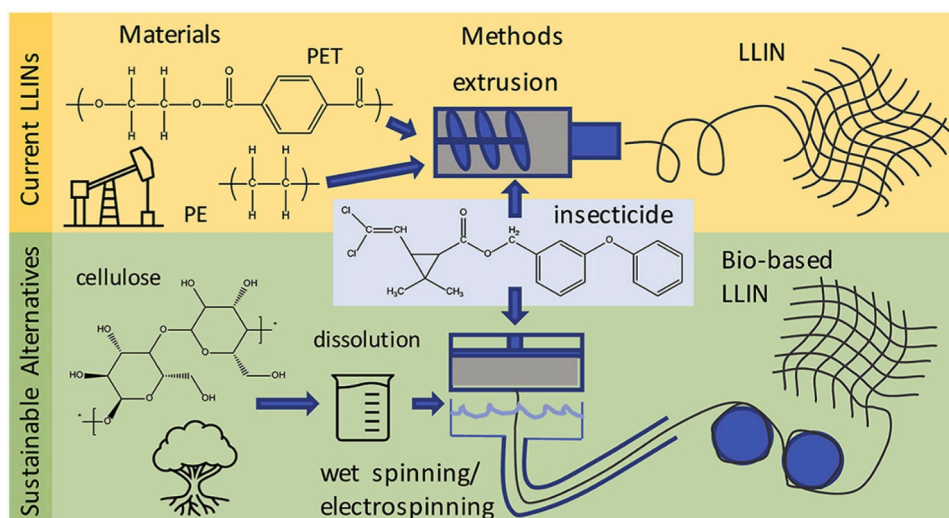


Fig. 1. Pictorial illustration of current LLINs and sustainable alternative [38].

Various vector control strategies

Long-lasting insecticide-treated nets (LLINs)

Long-lasting Insecticide-treated nets are regarded as a highly efficient approach for preventing malaria [14], designed based on the understanding that malaria vectors are usually very active at night, biting humans while they sleep indoors [36]. LLINs function by delivering a chemical barrier that either deters, immobilizes, or lethally affects mosquitoes upon contact with the insecticide-coated fibers of the netting material [37]. Their ability to kill mosquitoes is maintained for a longer duration compared to other methods of protection because they function as a knitted polymer coated with insecticide [38]. ITNs can generate a community-wide protective effect, offering indirect protection to individuals who do not use the net themselves. This occurs because insecticide reduces mosquito survival rates, thereby decreasing their ability to transmit malaria within the population.

Nevertheless, the efficiency of this vector control technique has been challenged due to its durability which affects the net longevity [38]; effects on non-target species [37]; insecticidal resistance by the malaria vector [16,39]; complaints of discomfort due to limited airflow; vector behavioral changes [40]; as well as the long-lasting environmental effect of some insecticides used remains a major issue of concern. The increase in outdoor biting and insecticide resistance in adult mosquitoes necessitates the need for enhanced larval control, which targets the vector before it matures into an adult [16]. The current LLINs are made from polymeric materials, which are derived from polyethylene (PE) or polyethylene terephthalate (PET) sources, with the technical incorporation of bioactive insecticides via the extrusion method [38]. However, to make the LLINs approach more eco-friendly, a sustainable alternative is being canvassed wherein the PE and PET raw materials are being replaced with cellulose (bio-based polymeric material) and the insecticides are incorporated via dissolution cum wet spinning/electrospinning as shown in Fig. 1.

Pyrethroid insecticides on nets function by disrupting sodium channels in the mosquito nervous system, causing sustained depolarization and paralysis. Resistance can arise from genetic mutations in sodium channel genes (kdr alleles) or the upregulation of metabolic enzymes that neutralize insecticide molecules, demonstrating the role of biochemistry in vector adaptation and the efficacy of control strategies [41,42–45].

There are two main classes of insecticide-treated nets as recommended by WHO [15,46], and they include the pyrethroid-only nets, including Long-Lasting Insecticidal Nets (LLINs), and the Pyrethroid-PBO nets, which were developed to address insecticide resistance in malaria vectors. PBO functions through effective inhibition of enzymes responsible for metabolism, such as mixed-function oxidases, which would otherwise break down or neutralize the insecticide before it can exert its toxic effect on the mosquito [46]. As a result, pyrethroid-PBO nets are expected to offer enhanced lethality against resistant mosquito populations compared to pyrethroid-only nets [37].

Despite the remarkable success achieved by the use of LLINs, several challenges persist; necessitating the need for a broader and inclusive strategy. This led to the adoption of the Indoor Residual Spraying, allowing broader coverage and the use of a broad spectrum of insecticides.

Indoor residual spraying

Indoor Residual Spraying involves spraying crucial areas where the malaria vector has high chances to rest, such as indoor walls, eaves [47], and the ceiling of the house. This kills the vector upon contact with insecticides [1,5], with the aim of controlling malaria transmission by reducing the vector population [47]. Its impact occurs after the mosquito takes a blood meal and rests on a sprayed surface, where it picks up a lethal dose of insecticide, thereby preventing it from transmitting the parasite to other individuals [48]. IRS is most effective against endophilic and endophagic vectors, it utilizes insecticides that interfere with the nervous system or enzymatic metabolic processes. For example, organophosphates inhibit acetylcholinesterase, disrupting neurotransmission, while pyrethroids act on voltage-gated sodium channels. These biochemical targets determine insecticide efficacy and explain the emergence of resistance. Resistance arises either through altered receptor structure or through increased expression of detoxification enzymes in mosquitoes, which eliminate the insecticide before it reaches its target [49–52].

Despite the success and promise attributed to the utilization of IRS as a malaria vector control method, it faces some setbacks, which include: exposure of residents and application team to chemical which differs in their potential to cause acute and chronic toxicity; the subsequent washing of spraying equipment poses a risk of contaminating surrounding aquatic ecosystems, such as streams, near areas where indoor residual spraying (IRS) is conducted [46]; the IRS campaign is sometimes challenged by refusal from some inhabitants which prevents access to spraying their structures and, this could limit the WHO recommendation of spraying 85% of the structures in the endemic areas [15,48]. While the IRS primarily targets adult mosquitoes, a more comprehensive intervention directed at the larval stage of the mosquito is required; thus, the need for Larval source management.

Larval source management

Vectorial density can be managed either at the adult or larval stage in an aquatic environment; larvicidal interventions target the habitat where mosquito larvae are developing or have the potential to do so [53]. Larval source management (LSM) entails effective modification of larval habitat, larviciding, as well as the use of larvivorous fish and other natural predators for biological control, like *Gambusia affinis* [54–56], with the sole aim of reducing the larval habitats and, as such, suppressing the population of the malaria vector [55]. In the early 1980s, *Bacillus thuringiensis* var. *israelensis* (Bti) and other *Bacillus* preparations were discovered to be potent biological control against the African *Anopheles* mosquito. A field trial was conducted in various ecological settings, revealing a

significant reduction in malaria incidence in Tanzania and the highlands of western Kenya. However, that was not the case in Gambia, where flooded landscape, made the sites inaccessible. Nonetheless, the use of drones to address this challenge is still being explored, which offers the benefit of identifying breeding sites and delivering biolarvicides to water bodies [56]. Larvicides and biological agents often act by interfering with developmental biochemical pathways, like chitin synthesis or mitochondrial respiration, rendering larvae nonviable. These molecular mechanisms are crucial for evaluating safety and resistance risks, as well as for designing novel interventions [57–60].

The larvae of *Anopheles* mosquitoes are relatively immobile; unlike the adult mosquitoes, which can fly when they sense danger, such as exposure to insecticide. This makes the larval stage a very easy target; thus, targeting the larvae helps eliminate a large number of them before they mature into adults. There is a wide range of larvicides for anopheline control, with different mechanisms of action; some preventing the larvae from developing into adults, some acting as toxins which directly interfere with the metabolic process of the insects, microbials that destroy the gut epithelium, and monolayers that result in larvae suffocation [61]. Despite the historical use of LSM in various eco-epidemiological settings, it has been underutilized as a contemporary technique for controlling malaria in Africa, particularly in sub-Saharan Africa [54–55]. In the 1950s, LSM was set aside, with more attention shifted towards the favourable consideration of IRS, which was believed to be cheaper, easier to implement during population awareness outreach, and requires less training, planning, or expertise in vector ecology [54]. Nonetheless, LSM is not without challenge, some of which include: (i) insufficient global technical guidance, policy support, and funding; (ii) limited operational experience; (iii) limited access to the full range of the World Health Organization (WHO) larvicide products.

Integrated vector control management

IVM advocates a holistic strategy that combines diverse vector control measures to achieve more effective outcomes than a single intervention; mathematical models of malaria transmission suggested the same [62]. This method encompasses IRS, which targets adult mosquitoes, as well as environmental management measures that eradicate mosquito breeding sites, such as LSM and LLINs, and personal protective measures [63]. Over the years, the IVM approach as a program has resulted in a significant reduction in mosquito population density, as well as a reduction in the malarial burden holistically [40].

Consolidating different control strategies has the potential to reduce the risk of resistance by disseminating selective pressures across various metabolic and neural pathways within mosquito populations [49,64–65]. For instance, the use of LLINs in combination with alternating insecticide categories regulates sodium channels. In contrast, IRS may affect acetylcholinesterase properties and mitochondrial function, thereby contributing to the evolutionary complexity of resistance. The report on the profile of metabolic genes suggests that resistance mutations lead to an upregulation of enzyme pathways responsible for detoxification (e.g. CYP450s, GSTs). This could be managed or mitigated by careful optimization of rotational schemes [65]. Guided incorporation of vector control helps ensure that both the target and off-target effects of insecticides and interventions are addressed, thereby reducing unwarranted ecological harm and enhancing the effectiveness of the adopted intervention. The administration of CRISPR/Cas9 gene drive for population suppression or replacement could be intentionally included in IVM templates, with careful consideration of molecular resistance mechanisms, fitness cost, and gene flow in target populations [43,64,66].

Personal protective measures

These are crucial measures that are taken to avoid or minimize personal contact with the malaria vector. The use of mosquito repellents is crucial to malaria prevention, as it can target both indoor and outdoor malaria transmission [40]. Insect repellents are synthetic or naturally derived compounds that create a sensory barrier within a few centimeters (4 cm) of the skin, effectively altering the local environment in a way that discourages insects from landing, biting, or feeding on bodily fluids [67]. Certain insect repellents, such as permethrin and other synthetic pyrethroids, also possess insecticidal properties [67]. Another personal protective measure is to avoid going to mosquito breeding sites during hours before sunrise or after sunset, which is the biting peak period for most mosquito species. Although *Aedes spp.* are diurnal biting mosquito species, many species still prefer the nocturnal biting period, which is between sunset and sunrise. Closing the doors and eaves during the evening hours is also a crucial step to limit the entry of the malaria vector into dwellings [2].

Environmental management

According to the definition by the WHO, environmental management (EM) is a vector control programme that plans, organizes, carries out, and monitors the activities needed to modify and/or manipulate the environmental factors or their interaction with humans so as to prevent or reduce vector spreading and minimize man-vector-pathogen contact [68]. Environmental management (EM) techniques are typically classified into three primary categories: Environmental modification, Environmental manipulation, and Adaptation of human dwellings and behaviors [69].

Environmental modification involves implementing long-term physical alterations to areas that could serve as breeding sites for mosquitoes, with the aim of preventing, reducing, or eliminating habitats suitable for vector development, achieved through construction of drainages, land grading and infilling [40]; Environmental manipulation involves the application of temporary alterations to aquatic habitats in order to disrupt or reduce the availability of convenient locations to breed mosquito larvae, nevertheless it is faced with challenge of heterogeneity because it depends on how well it matches the specific needs and behaviors of the mosquito species in a particular area [70]. In contrast, adaptation of human dwellings involves altering the location and structural design of

human dwellings, combined with changes in human behavior, which significantly reduces exposure to malaria vectors, thereby lowering transmission risk [2].

Since humans, vectors, and parasites coexist and interact within the environment, they are key to malaria control. In the fight against malaria, all of these factors must be given equal attention to achieve a high success rate and a sustainable outcome. Provision of fundamental amenities and infrastructures like water, proper sanitary hygiene, and effective waste disposal can help to reduce exposure to malaria vectors, although it depends on the characteristics of the malaria vector in the locality, also housing quality helps to put a barrier between human and the malaria vector, houses without screens, open eaves, windows and doors increase the risk of malaria incidence [71]. Environmentally managed malaria control, carried out on a community level in Dar es Salaam, Tanzania, from 2005 to 2007, achieved an 88% reduction in malaria incidence. Additionally, one of the treated drains showed a reduction in the presence of larvae. Nonetheless, community involvement, sensitization of community members, intersectoral collaboration, post-cleaning, and continued maintenance efforts are pivotal in achieving success in EM [55,62].

While vector control strategies have been instrumental in achieving tremendous progress in controlling malaria, antimalarial drugs have also been used for control; however, their effectiveness has been severely undermined by the surge in drug-resistant pathogens.

The crisis of antimalarial drug resistance

Managing and controlling malaria infection involves two main strategies: controlling the vector and managing infected individuals [72]. The type of antimalarial drug to be administered depends on several factors, which include the severity of the disease, the *Plasmodium* species, the region's drug resistance patterns, the individual's age, and their overall health status [5,18]. Nevertheless, most antimalarial drugs are stage- and species-selective; sadly, an effective antimalarial monotherapy with high efficacy against all species at both sexual and asexual stages of the parasite has not been developed [73]. Antimalarial drug resistance has been explained to mean the capacity of a parasitic strain to persist and/or reproduce despite the proper administering and absorption of a drug at standard doses or higher therapeutic doses that are still within the patient's tolerance limits, thereby reducing the drug's potency to inhibit pathogenic activities [72,74]. The spread of drug-resistant *Plasmodium falciparum* is one of the causes of the doubling and tripling of malaria incidence in Sub-Saharan African countries [75].

In the 17th century, quinine became the first commercially available antimalarial, obtained by extraction from the bark of the cinchona tree, native to South America, and was first introduced worldwide into Europe. Quinine and its derivatives were primarily utilized in combination with 8-aminoquinolines to achieve synergistic effects. In 1910, reports of quinine resistance began to emerge, but this combination therapy was abandoned in favour of chloroquine monotherapy during World War II due to its efficacy and cost [74,76]. Moreover, CQ-resistant *P. falciparum* was first reported to have emerged autonomously in Southeast Asia and South America between 1955 and 1969, which was the middle of the WHO intervention regarding the Global Malaria Eradication Programme [75–76]. Consequently, sulfadoxine–pyrimethamine (SP) gradually replaced CQ after the emergence of resistance [11], although not long after the introduction of SP, it also faced the arrival of resistance, which resulted in the resurgence of malaria worldwide mainly due to the delay in the replacement of CQ and SP with ACTs. Following the upsurge in resistance in SP, the introduction of artemisinin served as a lifesaver.

Artemisinin, along with some of its derivatives, is extracted from *Artemisia annua*, a member of the Asteraceae family [72], and has become a renowned and potent antimalarial drug in recent years, which is now commercially available as a marketed drug. It played a pivotal role in the effective treatment of acute and simple malaria [77]. It is an effective schizontocidal and gametocytocidal compound that helps cure infected individuals and prevents the transmission of the parasite. Reliable drugs derived from ART are artemether, arteether (AE), dihydroartemisinin (DHA), and artesunate (AS) [78]. Despite the efficacy of ART, its major limitation is its short

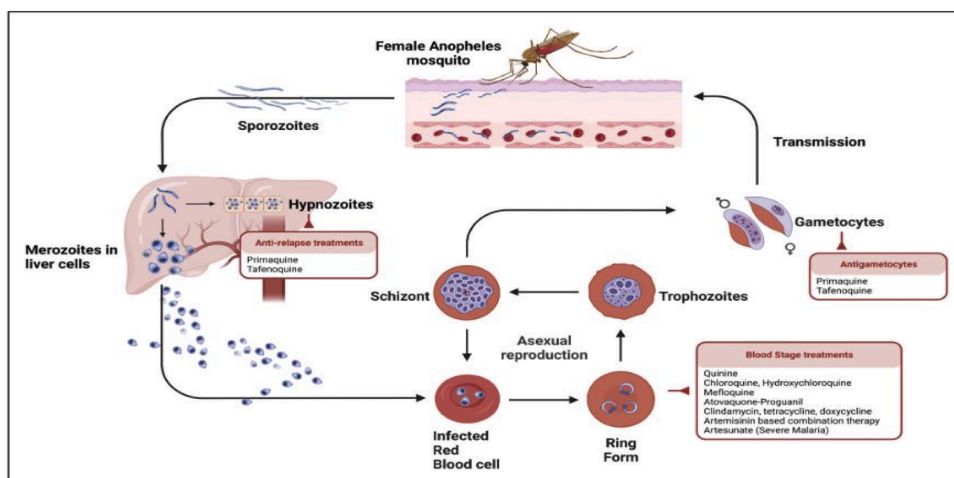


Fig. 2. Antimalarials that target different stages of the *Plasmodium* life cycle [73].

half-life (1hr), which leads to increased parasite recovery rate, and to address this, partner drugs with longer half-lives are co-administered as Artemisinin-based combination therapies (ACTs) [78], and quinine-zinc complex was also reported as an anti-plasmodial agent in another study [79]. Moreover, the emergence of ART resistance in the Greater Mekong subregion and several regions in Africa poses even greater concern for malaria eradication [80].

Resistance mechanisms typically emerge following deployment of each new drug class. Mutation and amplification of target parasite genes (e.g. PfCRT, PfMDR1, PfKelch13) lead to altered drug transport, metabolism, and detoxification, rendering parasites less susceptible to traditional therapies [81,82–85]. In the case of artemisinin, resistance is linked to mutations in the Kelch13 propeller domain, which disrupts the protein's function in parasite cellular stress response and ubiquitin-mediated metabolic pathways [83,86]. Quinine and quinoline resistance mechanisms are also biochemical, involving changes in vacuolar pH, transporter activity, or enhanced activity of mitochondrial antioxidant systems [86–91]. Fig. 2.

Adequate knowledge of the molecular basis of antimalarial drug resistance is crucial for designing next-generation interventions. High-throughput biochemical studies have profiled the mutations underlying drug failure, and efforts are ongoing to target alternate metabolic pathways or genetically engineer resistance-breaking strategies such as CRISPR/Cas9-mediated gene disruption and molecular surveillance [37,85]. Considering the shortcomings of current chemical interventions, genetic strategies such as CRISPR/Cas9 gene drives are demonstrating highly promising attributes as complementary tools to control and eliminate the malaria burden.

Different drugs have been designed to target malaria at various stages of the parasite lifecycle, targeting its different forms at each stage. Nevertheless, diagnosing drug-resistant strains of this pathogen in a precise and timely manner through the experimental detection of molecular markers, such as PfCRT, Pfdhfr, Pfmdr1, Pfdhps, Pfk13, Pfnhe1, and Pfmpr (Fig. 3), is considered a significant factor in controlling and eliminating malaria. This is because they are primary protein targets associated with the drug resistance burden [92] (Fig. 3). *P. falciparum* MRPs (Pfmpr) are particularly responsible for drug response alteration, which might make them essential players in the emergence of multi-drug resistance phenotypes [93].

Introduction to the CRISPR/Cas9 gene drive technology

CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats, and Cas9 is the CRISPR-associated protein 9, a

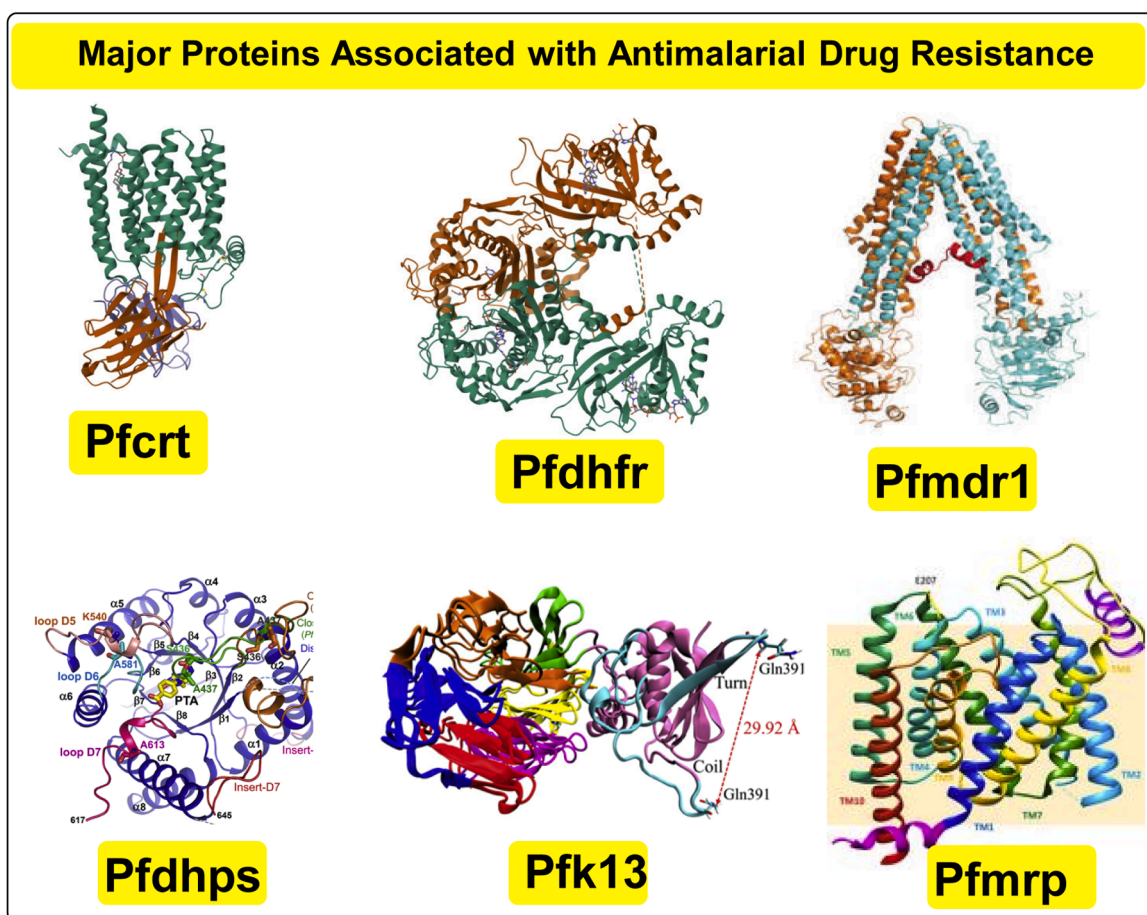


Fig. 3. *Plasmodium falciparum* protein commonly associated with drug resistance.

powerful genome-editing tool that enables the targeted modification of DNA sequences, including insertion, deletion, or alteration of genetic material. The Cas9 protein functions as a molecular "scissors," which can accurately cut specific regions of the DNA in order to facilitate targeted genetic changes [94]. The CRISPR/Cas9 system functions as a sequence-specific endonuclease, guided by a single-guide RNA, which is engineered to recognize a target DNA sequence (typically 20 bp) that must be adjacent to a PAM site for effective editing. The Cas9 protein utilizes two distinct catalytic domains (HNH and RuvC), each nicking one DNA strand, with cleavage driven by a two-metal ion mechanism critical to phosphodiester bond hydrolysis. Accurate recognition and editing rely on base complementarity between the sgRNA and the target DNA; while up to four mismatches may be tolerated in the PAM-distal regions, even one mismatch in the PAM-proximal region can completely abolish Cas9 cleavage activity. Once a Cas9-mediated double-strand break occurs, mosquito cells repair it via either non-homologous end joining (NHEJ), which is error-prone and may lead to the production of indel mutations, or homology-directed repair (HDR), which facilitates the accurate insertion of cassettes [26,84,95–96].

The underlying principle of gene drive drew inspiration from the natural attributes of biological processes, in which specific naturally occurring "selfish genetic elements" in sexually reproducing organisms promote their own transmission by copying themselves onto homologous chromosomes, thereby influencing inheritance in a non-Mendelian manner [97]. As a result, homing gene drives enable the efficient spread of engineered genetic elements across insect generations, even when introduced into a relatively small subset of the mosquito population [59]. Gene drive encompasses a diverse array of mechanisms by which genetic elements spread through a population at rates exceeding Mendelian expectations, including homing drive, split drive, daisy-chain drives, and sex distorter drives. The concept of using homing gene drives specifically as a biocontrol tool began around 2003, when the exploitation of selfish genetic elements known as homing endonuclease genes (HEGs) was proposed. The homing endonuclease genes (HEGs), actually encode an enzyme called endonucleases, which is responsible for the recognition as well as cleavage of DNA at specific sites on chromosomes lacking the HEG [98]. Following this cleavage, the broken chromosome is repaired using the HEG-containing chromosome as a template, which leads to the conversion of the gene from heterozygosity to homozygosity, enabling precise genome editing. The CRISPR/Cas9 gene editing technology needs two main components: Cas9 and a guide RNA [99]. Inheritance patterns have been evaluated by comparing Mendelian inheritance with homing gene drive inheritance. In each case, transgenic mosquitoes are intentionally introduced into the wild population [22]. Homing gene drive allows the target trait to spread through the population faster than Mendelian inheritance would permit, as shown in Fig. 4.

The molecular architecture of Cas9 and the sgRNA design principles described above collectively underpin the two main CRISPR-Cas9 gene drive strategies for malaria vector control discussed in this review: population suppression and population replacement. Key

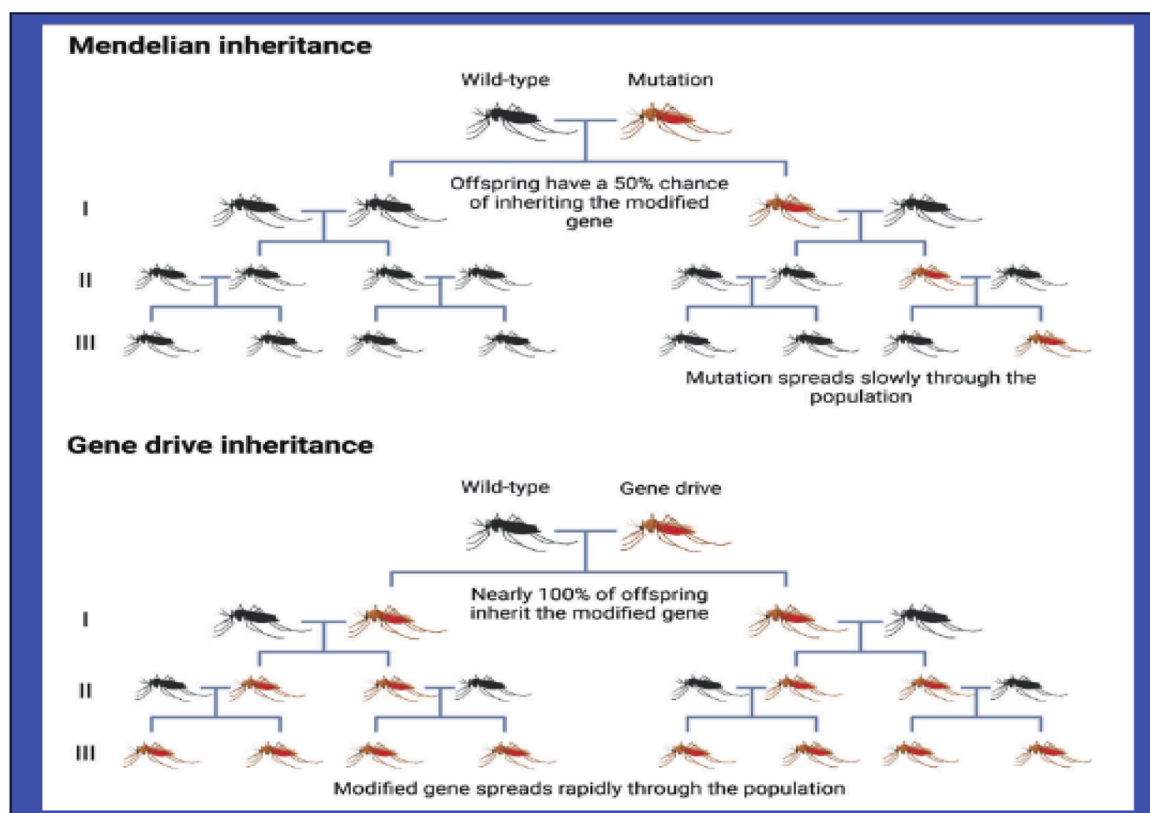


Fig. 4. Evaluation of inheritance mechanisms: Mendelian system vs. gene drive system [26].

biochemical advances in promoter selection, gRNA design, and molecular repair pathway targeting are now improving specificity and efficacy while limiting resistance. These successes highlight the value of Cas9 domain engineering, promoter optimization, and deep mechanistic understanding for real-world control applications [100–102]. Fig. 5.

Cryo-EM structural implication of Cas9–sgRNA–DNA complex in gene editing

Structural elucidation is crucial for understanding the molecular mechanism by which DNA binds to the Cas9–sgRNA binary complex, which is bilobe-like [103]. This is because it provides an understanding of how the CRISPR–Cas9 system activates the cleavage of DNA [104]. The cryo-EM structure of the Cas9–sgRNA–DNA ternary complex was identified, revealing that this complex is not in a conformation that activates DNA cleavage. The distance between the attacked phosphorus and the C-alpha atom of catalytic residue 840 is still greater than 19 Å, as seen in HNH-state 2 in Fig. 6(a). However, their study has recently established that there is another conformer wherein the location of the H–N–H domain is very near to the site of cleavage [11]. Moreover, sgRNA, RNA:DNA heteroduplex, PAM, and the PAM proximal terminal within 4UN3 have been documented to have clear resolution as shown in Fig. 6(b). The majority of tangential components around the structural framework, such as SpCas9 helices, the RNA:DNA heteroduplex, and PAM, are precisely resolved by adequate utilization of the density map denoted at the greater contour stage, as shown in Fig. 6.

Population suppression

The population suppression gene drive strategy aims at reducing the population of the vector (female *Anopheles* mosquito), and this is achieved by releasing the male mosquitoes carrying a homing gene drive construct. The drive spreads through the population via

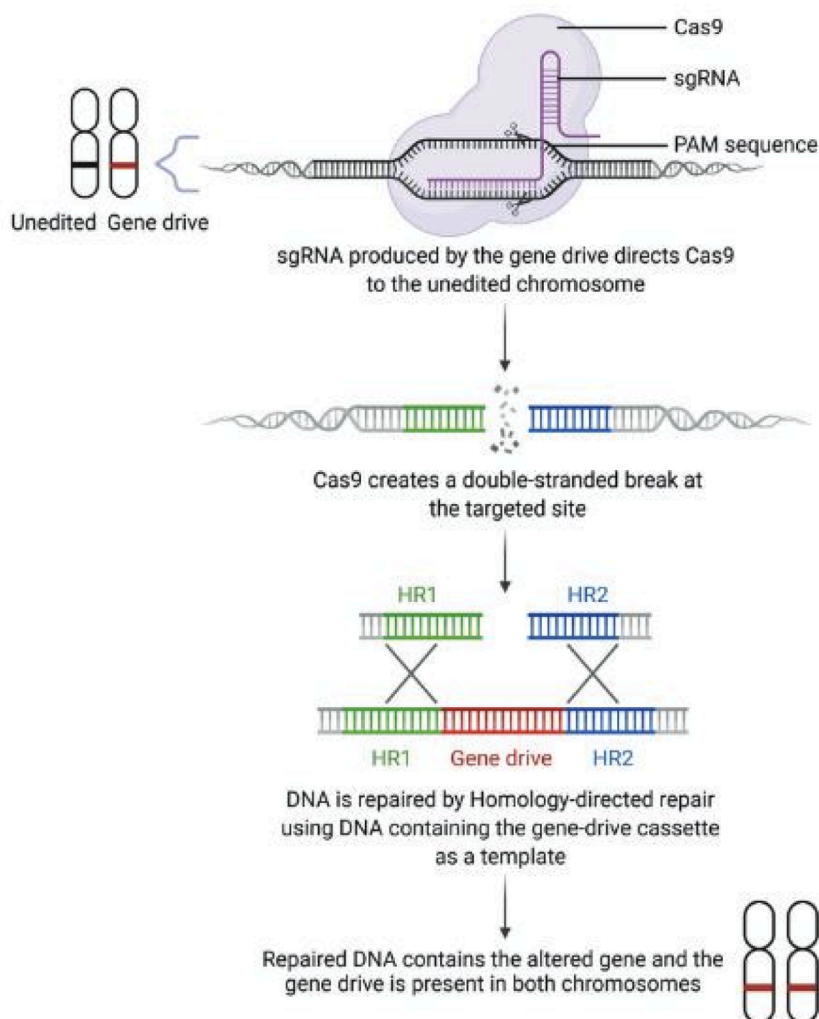


Fig. 5. Pictorial View of the mechanism of the CRISPR/Cas9 Gene editing [26].

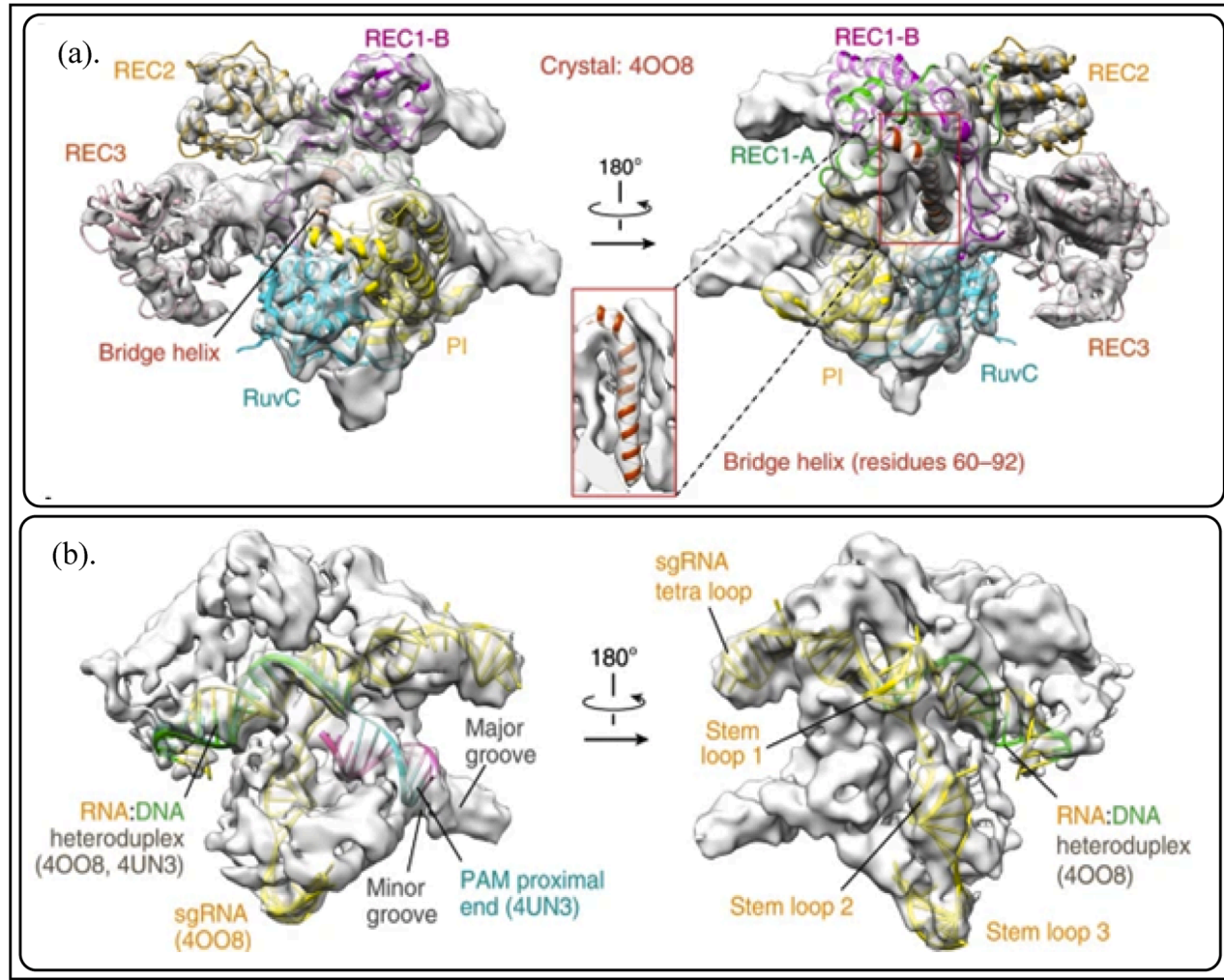


Fig. 6. Ternary complexes based on their cryo-EM structural diversity (a) Resolution of the longer bridge helix based on the EM density as shown by the central, small panel (b) Clearly resolution of sgRNA, the RNA:DNA heteroduplex, PAM, and the PAM proximal terminal by density map [20].

non-Mendelian inheritance, copying itself into offspring at rates far exceeding normal Mendelian expectations. The drive is designed to disrupt genes responsible for female fertility or viability [105], causing female mosquitoes that inherit it to become sterile, thereby reducing the population over successive generations. While male-only releases are often preferred for safety reasons, since females are the disease-transmitting sex, a small number of females may also be released in certain gene drive strategies, particularly population replacement approaches, as releasing both sexes can accelerate drive invasion into the population more effectively than male-only releases.

In a previous study by Hammond et al. [94], they demonstrated the use of a CRISPR/Cas9 endonuclease template that functioned within *Anopheles gambiae*, by employing a homing gene drive mechanism to achieve population suppression. They achieved this by identifying three pivotal genes (AGAP005958, AGAP011377, and AGAP007280), which were disrupted to confer a recessive female-sterility phenotype, and incorporated them into every locus of the CRISPR-Cas9 gene drive, which is designed to target and edit each gene. This achieved a robust homing gene drive at the molecular level with super-Mendelian transmission rates ranging from 91.4% to 99.6%. The study showed that the CRISPR^h alleles at AGAP007280 and AGAP011377 had a higher transmission rate in both sexes. In contrast, in progeny that lacked CRISPR homing alleles, a total of 13 indel mutations were observed in 32 offspring, suggesting that these mutations could have arisen from NHEJ or MMEJ repair mechanisms.

Also, the fertility of female heterozygous for a homing construct was significantly lessened; in contrast, heterozygous docking line females, where Cas9 activity was absent, retained normal fertility, indicating that the fertility reduction was because of the important expression of the somatic Cas9. Overall, the construct targeting AGAP007280 met the basic requirements for a gene drive designed to suppress female reproduction; consequently, the CRISPR/Cas9 system has significant potential to reduce the mosquito population, thereby markedly curtailing disease transmission.

CRISPR/Cas9-driven population suppression exploits the precise molecular targeting of essential genes, forcing inheritance patterns above normal Mendelian rates [26,94–95,100]. Fertility gene disruption drives population collapse by selectively impairing female gametogenesis, with Cas9/gRNA endonuclease activity directly responsible for sequence-specific double-strand breaks and subsequent gene disruption via indel formation [27,94–95,106]. The observed repair outcomes, NHEJ and MMEJ, highlight the importance of understanding DNA repair pathways in mosquitoes for drive effectiveness and for mitigating the production of resistant alleles.

A major limitation of the Hammond et al., [94] study was the generation of functional resistance alleles, which represent the most significant barrier to the efficacy of population suppression drives. When NHEJ repair generates mutations at the Cas9 target site, two outcomes are possible. In the first case, the mutation completely destroys the function of the targeted gene, producing a nonfunctional resistance allele; in this case, the female mosquito remains sterile, and the drive still achieves population suppression. In the second case, which is a more problematic outcome, the mutation is small enough to accidentally preserve the gene function, producing a functional resistance allele. Female mosquitoes carrying functional resistance alleles retain their fertility, are resistant to further Cas9 cleavage because the mutated sequence is no longer recognized by the sgRNA, and rapidly outcompete drive-carrying mosquitoes in the population. Over successive generations, these resistant mosquitoes take over, thereby causing the drive to fail completely. Furthermore, the use of the vasa promoter in that study resulted in Cas9 expression in somatic cells of early embryos where HDR is inefficient, leading to increased NHEJ-mediated functional resistance allele formation [57].

Kyrou et al. [27] addressed the limitations of the Hammond et al. [94], study through two key innovations. The first was the use of a zero-population growth (zpg) germline-specific promoter instead of the vasa promoter, which substantially reduced maternal deposition of Cas9 and somatic cleavage in early embryos. As such, fewer NHEJ repair events occurred overall, reducing the total number of resistance alleles generated. The second and most important innovation was the choice of target site, which was the female-specific exon 5 of the highly conserved doublesex gene. Because this exon is under extremely strong functional constraint, the vast majority of NHEJ-generated mutations resulted in nonfunctional rather than functional resistance alleles. Nonfunctional resistance alleles disrupt female fertility and are therefore less likely to compromise drive efficacy than functional resistance alleles, which preserve fertility while conferring resistance to Cas9 cleavage. Overall, the reduction in resistance allele formation and near-elimination of functional resistance alleles were more important than any fitness improvements in ultimately achieving population suppression. In Kyrou et al. [27] study, the CRISPR/Cas9 construct achieved a 100% spread across the 7th–11th generations of the caged *Anopheles gambiae* mosquito, as well as a reduction in egg production to the point where the population was effectively eliminated. The disruption of this gene had no phenotypic effect on males, whereas females homozygous for the disrupted allele displayed the sterile phenotype, demonstrating that the effect of the mutation is sex-specific.

Xu et al. [28] made a significant advancement by targeting the female-specific exon of double sex in *Anopheles stephensi*, where they constructed a homing suppression drive by the insertion of two gRNAs and a nanos promoter, in contrast to another study that used only one gRNA. The significance of this was that it helped achieve a low level of functional resistance allele formation. It also helped to guarantee Cas9 activity even when one target site is mutated, and the Cas9 promoter (nanos) is germline-specific, representing an improvement over the vasa promoter used by Hammond et al. [94], thus minimizing fitness costs. However, nanos in *A. stephensi* is not superior to the ZPG promoter in *A. gambiae* used by Kyrou et al. [27], as it has a comparatively lower cut rate. Female and male drive heterozygotes exhibited only moderate drive conversion. The study also revealed that the drive was recessive sterile in both males and females, which reduces overall drive performance.

On the other hand, upon the addition of homing suppression drive to a vasa-cas9 line, a 100% drive conversion rate was observed, effectively converting the construct into a self-limiting drive system. Self-limiting drives are considerably weaker than self-sustaining drives in terms of population-wide spread; however, they remain substantially more powerful than approaches such as Sterile Insect Technique (SIT). Critically, self-limiting drives do not spread beyond the release area, unlike self-sustaining drives, offering an important biosafety advantage, although at the cost of substantially higher release sizes than self-sustaining approaches [52]. These

studies demonstrate that promoter selection, target gene biochemical function, and repair pathway management are central to optimizing drive strategies that suppress vectors. Cas9-driven DNA breaks, germline-limited expression, and careful target choice collectively define the molecular efficacy, safety, and potential for resistance management of gene drive interventions [26,101].

Population replacement/modification

Population replacement aims to reduce vector competence by genetically engineering mosquitoes to carry anti-parasitic effector genes. These genetically modified mosquitoes are then introduced into the wild population to replace the susceptible wild population with a refractory one [107–108].

The Gantz et al. [29], study served as a landmark experimental proof of concept by using CRISPR/Cas9 homing gene drive as an effective tool to modify population in *Anopheles stephensi*, adapting the mutagenic chain reaction (MCR). Hence, the study demonstrated that the designed Cas9 system could carry a large, functional antiparasitic effector, and ~99.5% of the progeny following outcrosses of transgenic lines to wild-type mosquitoes carried the dual anti-*Plasmodium falciparum* effector gene, a marker gene, and the autonomous gene drive components. However, it was observed that due to the maternal deposition of Cas9 protein and guide RNA complexes into the eggs of the transgenic females, the maternally derived Cas9 activity targeted the paternally inherited wild-type chromosome at the early stage of zygote somatic cell development, resulting in NHEJ mutation. This led to reduced efficiency in transgenic females, underscoring the need for a male germline-specific promoter. This maternal deposition problem resulted in the generation of functional resistance alleles in somatic cells of early embryos; the same fundamental problem identified in suppression drives [94]. For population modification drives, functional resistance alleles are not as immediately disastrous as it is for suppression drives. Since modification alleles do not need to reach 100% frequency to be useful in reducing disease transmission [108]. Nonetheless, functional resistance alleles remain problematic even in modification drives, as they compete with and eventually displace mosquitoes carrying the drive within the population, thereby limiting long-term efficacy of the drive.

Cas9/gRNA systems are responsible for population replacement function by introducing double-stranded breaks at the targeted genomic locus, where homology-directed repair (HDR) mechanisms facilitate the insertion of an effector cassette. Precise molecular regulation ensures efficient gene integration and expression, while also minimizing off-target editing and fitness cost. Replacement strategies rely on drive element copying and the generation of homozygosity for refractory alleles within wild populations, which is a process underpinned by mosquito DNA repair biochemistry [27,29,95,109].

Building on this foundation, further study was conducted through the research effort of Carballar-Lejarazú et al., [30] which demonstrated and tested the potential for constructing a powerful gene drive system for population modification by creating a Cas9/guide RNA-based homing gene drive strain, AgNosCd-1. Although no antiparasitic molecule was integrated into *Anopheles gambiae*, the drive was highly efficient, achieving 98–100% efficacy in male and female mosquitoes, as well as total integration of little cage trials varying from 6 to 10 generations after the release of a single homing gene drive male. This suggests a significant population impact from a relatively small initial release, thereby reducing the cost associated with mass mosquito deployment.

Furthermore, there was a minimal fitness cost, a low rate of resistant allele formation, and a projected off-target site with exponential cleavability *in vitro*; however, infinitesimal removal/deletion was observed *in vivo*. Consequently, AgNosCd-1 met major performance requirements relating to target product profiling, thereby offering a field-ready strain for population modification and control of malaria transmission. In contrast to the findings by Gantz et al., [29] the use of the nos regulatory element by Carballar-Lejarazú et al., [30] provided more precise germline-restricted expression than that from *vasa*, thereby mitigating the high-frequency maternal effects and NHEJ drive-resistant alleles that were previously noticed. The same research group subsequently incorporated dual anti-parasite effector genes into *A. gambiae* and *A. coluzzii* homing gene drive strains. Carballar-Lejarazú et al., [110] developed two drive strains AgTP13 and AcTP13, each carrying dual anti-*Plasmodium falciparum* effector genes comprising single-chain variable fragment monoclonal antibodies targeting the ookinete and sporozoite stages of the malaria parasite. Both drive systems achieved complete spread within 3–6 months in small cage trials, and the effector molecules significantly reduced both the proportion of infected mosquitoes and the parasite loads within mosquitoes. These findings represent a critical advance from the proof-of-concept AgNosCd-1 drive, which lacked antiparasitic effectors, demonstrating that a single gene drive system can simultaneously achieve efficient population-wide drive spread and effective malaria parasite blocking, making them viable candidates for future field trials.

Additionally, Adolphi et al., [31] demonstrated the potential for developing a gene drive to render the malaria vector (*Anopheles stephensi*) refractory. They developed a recoded gene drive system for a rescue mission to modify the population of the malaria vector by incorporating the drive system into the kynurenine hydrolase gene (*kh*). The recoded gene drive is of high significance because it restores normal gene function, allowing mosquitoes with the gene drive to retain a functional *kh* gene while also reducing the fitness cost associated with the drive, thereby facilitating the efficient spread of the drive. In the small caged experiment carried out, ≥95% of mosquitoes carried the drive within 5–11 generations over a range of initial release ratios. Additionally, rare functional resistant alleles did not prevent drive-invasion, and nonfunctional resistant alleles were eliminated through a dominantly acting maternal effect combined with slower-acting standard negative selection. Although this study does not yet make mosquitoes refractory, it demonstrated a stable population modification system that could later carry anti-parasitic genes. The efficiency of population replacement depends on the specificity of Cas9-induced breaks, HDR rates, and the fitness effect of integrated genes. Success is monitored by tracking allele spread, parasite resistance, and transmission-blocking efficacy in field and lab populations [26,95,111,112].

Overall, this study demonstrated the potential of using the CRISPR/Cas9 gene drive technology to render malaria vectors refractory, thereby significantly contributing to the control of malaria transmission; nonetheless, this is not without challenges.

Ethical concern

Ethics is not opposed to science, and science is also not expected to operate in isolation; hence, as science continues to discover and develop new technologies for the benefit of society, ethics are central in ensuring that these developments have a fair and equitable impact on society at large [113]. The precautionary principle is one of the essential ethical principles that urges careful risk assessment, especially in research that could impact ecosystems, such as the release of gene drive modified organisms [114]. Similarly, the Intergenerational Equity (IE) in regard to gene drives and human disease eradication emphasizes that the well-being of present generations is made possible by the resources preserved by our predecessors; as such, we must safeguard these assets for future generations [115].

However, laboratory conditions are highly controlled and do not fully replicate the complexity of natural ecosystems. This makes predicting the full ecological impact of large-scale releases of genetically engineered organisms challenging, as well as assessing and balancing the risks and benefits [115–116]. Consequently, it is paramount that scientists agree and adopt an ethical code that assures the public and scientists themselves that moral values will be at the core of their actions [117].

Concerns regarding gene drive technology persist [22], for instance, some fear that gene drive may lead to the eradication of entire species, potentially violating IE's principle of "conservation of access and options" [115,118]. With due recognition of societal dispositions and ethical concerns, the World Health Organization (WHO) has reiterated the importance of engaging with and seeking public opinion in trials involving genetically modified mosquitoes, given that genetic modification often encounters strong resistance among local/indigenous populations. Hence, introducing gene drive as a new technology requires clarity, openness, and sincere awareness for the general public, who may lack familiarity with the technology. This is crucial for gaining societal acceptance and the trust of the masses, especially at the grassroots level [114].

Although CRISPR/Cas9 is a powerful and formidable gene-editing tool, it critically escalates several issues of ethical concerns, some of which are:

- i. **Germline Editing:** Editing genes in human embryos (germline cells) means changes can be passed to future generations, which might possess hidden long-term effects upon the pool of human genes.
- ii. **Unintended Consequences:** origination of new diseases and health challenges might result from off-target effects, which are a consequence of unwarranted alteration in the DNA.
- iii. **Community Consent:** unlike medical interventions that are directed at an individual, gene drive release affects the entire ecosystem and the individuals living within them. The concept of individual consent does not apply to insect genome editing; however, affected human populations must be consulted and agreement reached prior to field release.
- iv. **Dual Use:** This entails the possibility of misuse of this technology for dangerous intentions and detrimental motives, such as the creation of biological weapons of mass destruction.

Conclusion

The World Health Organization's goal to reduce the burden and death rate from malaria by up to 90% by 2030 is an ambitious projection that requires an innovative and outstanding strategy to be accomplished. CRISPR/Cas9 has been described herein as a promising and powerful gene editing tool, used to modify the genome of the malaria parasite, making it a supplementary technique for the eradication/reduction of malaria incidence. Nevertheless, it was also highlighted herein that CRISPR/Cas9 technology utilization was not without a bottleneck, which serves as a barrier towards its full implementation, ranging from ethical concerns about its use, fitness cost, and the potential public resistance, which affect the acceptance and deployment of the technology. Hence, addressing all of these challenges is crucial to the safe and effective implementation of this technology in malaria control efforts.

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The authors declare that no generative-AI was used in the writing or preparation of this manuscript. Authors also take responsibility for the full content of the manuscript

Data availability

The data used for this study are available from the corresponding author on reasonable request.

CRedit authorship contribution statement

Ayibanuah F. Robinson: Conceptualization, Formal analysis, Writing – original draft. **Esther O. Okafor:** Resources, Writing – original draft, Writing – review & editing. **Gbolahan O. Odusel:** Writing – review & editing, Formal analysis, Validation, Writing – original draft. **Oluwabukayo T. Opebiyi:** Resources, Validation, Writing – review & editing. **Natasha October:** Supervision,

Resources, Writing – original draft. **Olayinka O. Ajani**: Supervision, Validation, Writing – original draft, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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