Gene Drive System Based on CRISPR/Cas9 in Mosquito Control for Preventing Malaria

Zimeng Wang

Department of Biology, Northeastern University, Boston, the United States

wang.zime@northeastern.edu

Abstract. Malaria is a disease transmitted by mosquitoes to humans and has symptoms varying from mild such as fever and headache to severe such as loss of consciousness, bleeding, or even death. Although there are a few other pathways to get infected with malaria, female mosquitoes belonging to the genus *Anopheles* infected by pathogen parasites belonging to *Plasmodium* have been considered as the predominant media of transmission, and elimination of this pathway could greatly assist in controlling the spread of malaria among populations. CRISPR/Cas9, as an emerging genome editing technology, functions by designed guide RNA (gRNA) for targeting sequence and Cas9 for cleaving targeted double-stranded DNA. Due to the great power of CRISPR/Cas9 in gene editing, it has significantly facilitated the study of mosquito control. This review describes the potential and applicable properties of the CRISPR/Cas9 system in restricting the spread of malaria via genetic screening of mosquitoes and gene-drive population suppression by modifying female mosquito fertility to reduce pathogen infection rate by targeting alleles from mosquitoes and pathogen to prohibit mosquito infections or retard the parasite development process. Furthermore, this review discusses the urgency that adjusting CRISPR/Cas9 technique to be affordable by more performers with low off-target effect. In conclusion, CRISPR/Cas9 system is expected to affirm the applicability of the technique in nature to restrict the spread of malaria among populations.

Key words: Malaria, CRISPR/Cas9, Mosquito, Gene drive, Parasite.

1. Introduction

Malaria is an insect-borne disease transmitted to humans through female mosquitoes in the genus *Anopheles* infected by malaria pathogens belonging to the genus *Plasmodium* [1]. The disease is transmittable by female mosquito bites when the insects are in contact with human blood, however, malaria is not capable of passing between individuals through personal contact [1]. Among the five main human malaria pathogen species, which are *Plasmodium vivax, Plasmodium alciparum, Plasmodium malariae, Plasmodium ovale,* and *Plasmodium knowlesi,* the first two species in the list are the principal causes of malaria infection in Africa [1]. Depending on the severity of the infection, patients may experience various symptoms such as fever, headache, chills, fatigue, jaundice, and issue with consciousness, breathing, bleeding, and urine [1]. In addition to pathogen infection through direct parasitic behavior of *Plasmodium* parasites transmitted by mosquitoes, humans could also be infected through blood transfusions and placental contact between mother and unborn infants [2]. Even though malaria is curable, the high prevalence and high risk of this disease could lead to deaths within one day in the most severe circumstances and there are approximately 290 million infection cases each year [1,2]. Therefore, an efficient approach to control the spread of malaria is in urgent need.

CRISPR/Cas9 is a genome editing technology discovered from prokaryotes in nature with the word "CRISPR" referring to "Clustered Regularly Interspaced Short Palindromic Repeats" and "Cas9" abbreviated from "CRISPR-associated protein 9" [3,4]. CRISPR/Cas9 system functions as an immune strategy by protecting prokaryotes cells from virus invasion since viruses often use prokaryotes for their own DNA replication [3]. This adaptive immune system functions in prokaryotes by cleaving the externally injected virus DNA sequence for protection and editing CRISPR DNA loci from prokaryotes for memory restoring [3]. Nowadays, CRISPR/Cas9 has been engineered to be applicable to eukaryotes such as animals and human cells as a potential therapeutic direction [3]. When editing through CRISPR, the guide RNA (gRNA) is required for targeting the

sequence of interest, and the Cas9 endonuclease as an enzyme operates to cleave the DNA sequence, allowing genome edition afterward [4]. This gene editing technique has now been applied to various inherited genetic diseases or disorders such as sickle cell disease, hemophilia, and Duchenne muscular dystrophy [5]. Future development of the CRISPR/Cas9 technique has great potential in treating diseases through genetic perspective on editing animals and human genetic information.

2. CRISPR/Cas9 in modifying and screening mosquito phenotype

Anopheles gambiae is one of the mosquito species in the genus *Anopheles* and is categorized as the principal vector for the malaria parasite, *Plasmodium falciparum*, to use as a host organism [1]. A comprehensive understanding of the genetic information that causes changes in mosquito phenotypes through phenotype screening would provide clues for potential targets for gene editing to minimize malaria spreading rate. Genetic sequences altering mosquito phenotypes relevant to infection by *P. falciparum* or transmission to humans would be valid resources for future study and research. CRISPR/Cas9 targets selected segments of the genetic sequence for knockout, capable of examining the functions and phenotypic impacts of the gene on the organism.

In a CRISPR/Cas9 screening, single guide RNA (sgRNA) for each gene was designed and simultaneously utilized in the pool for determining the necessity and responsibility of multiple genes at the same time [6]. The sgRNAs for *Anopheles* mosquitoes were generated based on an online library while the selective process for U6 promotors relied on previous studies in *Drosophila* screening [6]. CRISPR screening was first utilized to knockout *Rho1*, which was supposed to present an obvious phenotypic change in *Anopheles* size growth due to resulted deficiency in cytokines, using sgRNA AGAP005160 and the outcome confirmed the ability of CRISPR modification by successfully affirming the expectation [6]. After verifying the validation of CRISPR technology in phenotype screening, multiple orthologs were exerted into the pooled screening, and four orthologs for four *Anopheles* genes *FKBP12*, *EcR*, *usp*, and *PTP-ER* were successfully found, demonstrating the prospects of CRISPR in contributing to better acknowledgment in mosquitoes [6].

As for gene editing mosquito phenotypes, CRISPR/Cas9 was also applied to the insects by knocking out particular genes. Modification of the flight gene was demonstrated in *Aedes aegypti*, aiming to generate flightless females who would be disabled from transmitting diseases to human hosts [7]. Similar to *Anopheles*, female *Ae. aegypti* transmit arboviruses through contact with humans for blood meal [7]. Therefore, genome editing in the *Ae. aegypti* mosquito population is critical in controlling the spreading of infectious diseases transmitted by the species. Genes *AeAct-4*, *myo-fem*, and *Aeflightin* were associated with flight in mosquitoes and were knocked out via CRISPR. *AeAct-4* was haplosufficient in terms of flying ability while individual organisms with a single copy of *myo-fem* were disabled from flying, defining *myo-fem* as a haploinsufficient gene [7]. In addition, Aeflightin was also a haplosufficient gene as *AeAct-4* was [7]. Both *AeAct-4* and *myo-fem* predominately affected females, having knockout males remain flyable and capable of reproducing with the only difference appearing in *AeAct-4* knockout males being less competitive during mating activity [7]. The genes *AeAct-4* and *myo-fem* had a greater restriction on female mosquito flight by affecting flight muscle, while males experienced a partial impact on flight, mating, and reproducing but did not have the abilities entirely inhibited [7]. Phenotypes functioned with variance between sexes through gene editing and could be testified through CRISPR/Cas9. Noticeably, *Aedes aegypti* is not a vector for malaria but dengue, chikungunya, and Zika viruses [7]. Nevertheless, CRISPR-editing to alter flying phenotype has been presented, indicating possible application of CRISPR/Cas9 technology to *A. gambiae* for editing and changing undesired phenotypes.

Furthermore, phenotypic changes in species *Aedes albopictus*, another mosquito population act as a disease-transmitting vector, were achieved by CRISPR/Cas9 gene editing and were passed on to the next generations [8]. The gene *kynurenine hydroxylase* (*kh*) leads to a white eye color phenotype and the gene *yellow* relevant to melanin formation in the mosquitoes were mutated through CRISPR/Cas9 [8]. Both gene mutations generated G0 mosaic mutants with deficient phenotypes and

continuously inherited G1, the homozygous offspring of the population [8]. *Ae. albopictus* with *kh* knockout generated G0 mosaic mutants which later produced the G1 population having white eyes, the recessive phenotype [8]. The *yellow* knockout *Ae. Albopictus* expressed abnormal distribution of body color during formation in the G0 population and resulted in severe yellowing in color in the G1 population [8]. The observation of white eyes and light color in G1 population suggested the hardness of complementing introduced mutations and demonstrated the heritability of the CRISPR-induced mutations to exist across generations [8]. Therefore, genome editing using CRISPR/Cas9 is likely to resist changes over generations of reproduction, remaining in the genome to be inherited and impact the population.

3. CRISPR/Cas9 gene-driven system for mosquito population suppression

Gene drive system biasedly inherits desired alleles through reproduction, increasing the frequency of preferred alleles over generations [9]. For natural gene drive, alleles compete by fitness depending on the surrounding environment, while synthetic gene drive relies on biological technology to engineer and intervene in selective process [9]. As an insect-borne disease caused by parasites, mosquitos, specifically female mosquitos, belonging to the genus *Anopheles* are responsible for carrying and transmitting malaria pathogens to humans through their saliva [1]. Therefore, it is most effective to suppress the spread of the disease by controlling the number of female mosquitoes.

Usage of CRISPR/Cas9 in synthetic gene drive aiming to inhibit the mosquito population growth would prevent malaria spreading by controlling the surviving vectors. The gene *doublesex* (*Agdsx*) is a known sex-determination gene in *A. gambiae* which transmits malaria parasites to humans [10]. CRISPR/Cas9 designed with gRNA only targeting spliced transcripts of *dsx* in female, which is the *dsx-female* (*AgdsxF*), suppressed the population through eliminating female fertility (Kyrou et al., 2018). Splicing of the *dsx* gene region differs between female transcripts *dsx-female* (*AgdsxF*) and male transcripts *dsx-male* (*AgdsxM*) by retaining the exon 5 in the female genotype across *Anopheles* mosquitos [10]. Breeding of CRISPR-edited heterozygous mosquito with two separate groups of wild types in the cage resulted in a gradually rising frequency of the desired dysfunctional *dsxF* over generations of insects where the two groups of wild types reached 100% frequency for the mutation by generation 11 and generation 7 respectively with no egg production allowing further reproduction [10]. Besides generating sterile female mosquitoes, eradicating the female population contributes largely to population restriction. Synthetic gene drive through CRISPR targeting X-chromosomes leads to biased selection towards male insects compared to females *A. gambiae* [11]. This sex-distorter gene drive (SDGD) eliminates female mosquitos at a great rate by cleaving on X-chromosome, where males are favored by having Y-chromosome instead [11]. Since female *A. gambiae* are responsible for transmitting the parasite to humans through biting, the reduction in the female population would first lower the transmission rate by minimizing viable females that could be infected before the unbalanced ratio between the two sexes drops the reproduction rate and eventually leads to a suppressed population.

4. CRISPR-Cas9 gene-driven system for controlling pathogen infections of mosquitos

Controlling the parasite infection pathway in female *A. gambiae* mosquitos would regulate the spread of malaria in humans by diminishing the potential hosts or transmitters of the disease parasite from genus Plasmodium from mosquito vector populations.

Fibrinogen-related protein 1 (FREP1) in *A. gambiae* has been found as the gene allowing *Plasmodium* parasites to invade mosquitos through midgut epithelium and continues their life cycle within *A. gambiae* before being transmitted into a human host [12]. The gene sequence of FREP is conserved across the genus *Anopheles*, thus, was determined to be a probable gene to target for infection obstruction of the parasites in mosquitos [12]. CRISPR-Cas9 was designed with gRNA

targeting the FREP1 gene sequence to achieve knockout mosquitos since the gene is considered an agonist for parasite infection [13]. The gene edition on FREP1 in embryonic cells through CRISPR-editing generated homozygous mutants lacking FREP1 on both alleles and sufficiently resisting infection from the human malaria parasite, *P. falciparum*, when compared with wild types, having a 22.3% decrease in the total infected mosquito population and 39.2% decrease in the number of sporozoites in the salivary gland of mosquitoes [13]. Besides limiting the number of hosts and potential transmitters in *A. gambiae* population, the surviving skills, so-called fitness, as organisms also degenerated in the mutants where mosquitos had weaker blood-feeding, developing, and egg-producing abilities suggesting that FREP regulates multiple functions of the organisms [13]. Despite the considerable decrease in mosquito infectious rate, a portion of the insects would still become the transmitting vector.

To further prevent the risk of malaria-spreading through the mosquito population, two antagonist genes of *P. falciparum* infection coexpressing magainin 2 and melittin in A. gambiae had their expression enhanced through CRISPR [14]. Magainin 2 and melittin are both antimicrobial peptides (AMPs), function to inhibit parasite development when invaded into mosquitoes via gut where viable oocytes of *P. falciparum* shrink and decrease in number within mosquito midgut and are therefore targeted to amplify the negative impact brought to parasites development inside mosquitoes CDPK1 [14]. In comparison to wild types, *A. gambiae* with CRISPR-editing had oocytes in the midgut that were both lower in prevalence and smaller in size. These restrictive effects were reflected through the decrease in a number of active oocytes measured post 7 days of the parasite infection and the shrinkage in size remained even until 15 days post infections [14]. Similar to FREP knockout, the fitness of homozygous mosquitos carrying two copies of CRISPR also reduced dramatically [14].

Despite inhibiting the infectious and developing pathway of malaria parasites through applying CRISPR/Cas9 technique in mosquitos, manipulation of the genome of *P. falciparum* would also prohibit mosquito infection by disabling the parasitic ability to infect at the beginning of the infectious pathway. For instance, CDPK1 as a member of the Calcium-dependent protein kinases (CDPK) family regulates important stages of parasitic growth, and kinase *Pf*CDPK1 is proposed to play an essential role in facilitating asexual parasite development in *P. falciparum* during their life cycle [15]. The successful knockout of *Pf*CDPK1 in parasite CDPK T145M (parasite with mutant CDPK1) with reduced transphosphorylation activity through CRISPR/Cas9 generated relatively slower asexual growth and inefficiency in proper gametes formation through gametogenesis in *P. falciparum* compared to wild types and the CDPK T145M mutants, where the gametes were disabled from exiting RBCs of the hosts by in absence of exflagellation [15]. Nevertheless, the extent of deficiency in the asexual growth process varies depending on species and requires more deliberate studies since *Plasmodium. falciparum* was not impacted by the knockout of *Pf*CDPK1 [15]. The *Pf*CDPK1 CRISOR-knockout parasites also experienced a lack of RBCs invading property and a dramatic reduction in their abilities to invade mosquitoes at midguts, which is a key concept of parasitizing mosquito hosts [15]. CRISPR/Cas9 here was utilized as a technique to discover the applicable site for the target for the purpose of eliminating parasitic invasion to mosquitoes by *P. falciparum*.

5. Other relevant CRISPR/Cas9 gene-driven system research

In addition to phenotype screening, population suppression, and control of pathogens, there are other discoveries relevant to CRISPR appropriate for enriching the field of preventing the spread of malaria the mosquito-borne disease.

In recent work, the Receptor-mediated Ovary Transduction of Cargo (ReMOT Control) technique was developed to provide an alternative delivery pathway for the Cas9 ribonucleoprotein complex rather than the current method of embryonic injection for gene editing [16]. ReMOT allows direct injection into mosquito ovaries in adults for CRISPR gene-editing in *Anopheles stephensi*, which is another species in the same genus as *A. gambiae*, requiring less than microinjection for embryos so that non-specialist laboratories could also demonstrate the adult injection process without pressure

[16]. CRISPR/Cas9 editing thus could be more ubiquitous, authorizing more laboratories to participate in the field to promote investigation in malaria studies for taking better actions at preventing infections by gene-editing mosquitoes [16]. Simplifying lab requirements to perform CRISPR/Cas9 gene editing would efficiently advocate studies in the field, moreover, evaluating risk factors relevant to the technique is also valuable for determining the feasibility and directing the future applications of results utilizing CRISPR.

Off-target in CRISPR/Cas9 editing technology occasionally occurs when unwanted actions perform on the sequence untargeted by the gRNA. Four strains were studied with promoter SpCas9 that was nongermline restricted and gRNA with many sites that are related, examining the off-target events during gene drive mosquito research as a frequency lower than 1.42% with no ability to disseminate in the population over generations [17]. It is worth noticing that off-target has shown no harmfulness on strains of mosquitos nor their phenotypes and considerable design of the gRNA and target site selection could minimize the off-target event in a great scale that off-target could be barely detected [17]. The occurrence of off-target was able to be regulated by experimental processes and the impact of off-target in CRISPR editing was not as problematic as previously concerned with respect to population, inheritance, and phenotype [17]. However, variance in nature and fieldwork has more complexity compared to the lab environment [17]. Thus, although additional researchers would be required before establishing promised safety on the application of CRISPR/Cas9 to *A. gambiae* in the gene drive system, the feasibility of CRISPR in gene-editing in future applications to malaria vector study was supported.

6. Conclusions

Malaria has been a life-threatening disease transmitted via female mosquitoes from the genus *Anopheles* [1]. Malaria parasites from the genus *Plasmodium* infects mosquitoes, continues their life cycle in the insects, and infect human once entering the human body [1,2]. CRISPR/Cas9 as a rising genome editing technique has been active in the field of preventing the spread of malaria by showing its ability in screening mosquito phenotypes and modifying both mosquito and parasite genotypes. Drosophila screening based on CRISPR/Cas9 has found that *FKBP12*, *EcR*, *usp*, and *PTP-ER* were the orthologs for *Anopheles* genes [6]. *AeAct-4*, *myo-fem*, and *Aeflightin* were discovered as related to the flight ability in *Aedes aegypti*, which is a different genus from *Anopheles* [7]. Knockout of gene *kh* and *yellow* in mosquito *Aedes albopictus* suggested the inheritable characteristic of CRISPR/Cas9 gene editing [8]. In order to establish gene-drive population reduction in *Anopheles* mosquitoes, certain genes were knocked out using CRISPR/Cas9. Gene *dsx* as the sex-determining gene in *Anopheles* was used to generate sterile female mosquito by manipulating *dsxF* to eliminate future reproduction of the mosquito population [10]. X-chromosome targeting was also applied to perform SDGD, selecting males with a bias to avoid transmission and restrict population growth at the same time [11]. Additionally, CRISPR/Cas9 has been applied to knock FREP1, which prevented pathogens from invading [12,13]. Edited expression of magainin 2 and melittin affected parasite development within mosquitoes [14]. To expand from actions on mosquito genome, *Pf*CDPK1 knockout in parasites has been shown to slow down their development by the usage of CRISPR editing [15].

Since CRISPR system has shown great potential in controlling malaria infection by restricting the available transmission vector numbers and their reproduction ability to generate new generations. Gene editing on the particular trait to control the parasite life cycle extended the perspective to focus on the parasite rather than only the mosquitoes, expecting to eradicate the disease transmission by reducing the harm that could be brought by the pathogen. Further studies could focus more on bringing lab works into a bigger field to see the stability and accuracy of the laboratory results once stimulated in nature. Repetitive experiments in greater environmental space and field-work nature would also be preferable to move the use of CRISPR in mosquito editing for malaria forward. Multiple trials, larger spatial, and mimicking of the nature environment would examine the works further. The prevalence of utilizing such a technique could also be examined to evaluate the likelihood

of applying it in various regions affected by malaria. Besides, the requirements for presenting a CRISPR have been strict, and research on the topic is also developing to increase the applicable rate for scientists in the field [16]. Off-target as the concern of CRISPR results has also been studied, suggesting that the results won't be greatly impacted, indicating the reliability of the technique [17].

References

[1] World Health Organization. (2023). *Fact sheet about malaria*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/malaria

[2] Mayo Clinic. (2023). Malaria. Mayo Clinic. https://www.mayoclinic.org/diseases-conditions/malaria/symptoms-causes/syc-20351184

[3] Hossain, M. A. (2021). CRISPR-Cas9: A fascinating journey from bacterial immune system to human gene editing. Progress in Molecular Biology and Translational Science, 63–83.

[4] Redman, M., King, A., Watson, C., & King, D. (2016). What is CRISPR/cas9? Archives of Disease in Childhood - Education & Practice Edition, 101(4), 213–215.

[5] Liu, W., Li, L., Jiang, J., Wu, M., & Lin, P. (2021). Applications and challenges of CRISPR-cas gene-editing to disease treatment in clinics. Precision Clinical Medicine, 4(3), 179–191.

[6] Viswanatha, R., Mameli, E., Rodiger, J., Merckaert, P., Feitosa-Suntheimer, F., Colpitts, T. M., Mohr, S. E., Hu, Y., & Perrimon, N. (2021). Bioinformatic and cell-based tools for pooled CRISPR knockout screening in mosquitos. Nature Communications, 12(1).

[7] O'Leary, S., & Adelman, Z. N. (2020). CRISPR/Cas9 knockout of female-biased genes AeAct-4 or myo-fem in AE. Aegypti results in a flightless phenotype in female, but not male mosquitoes. PLOS Neglected Tropical Diseases, 14(12).

[8] Liu, T., Yang, W., Xie, Y., Liu, P., Xie, L., Lin, F., Li, C., Gu, J., Wu, K., Yan, G., & Chen, X. (2018). Construction of an efficient genomic editing system with CRISPR/Cas9 in the vector mosquito Aedes albopictus. *Insect Science*, *26*(6), 1045–1054.

[9] Alphey, L. S., Crisanti, A., Randazzo, F. (Fil), & Akbari, O. S. (2020). Standardizing the definition of Gene Drive. Proceedings of the National Academy of Sciences, 117(49), 30864–30867.

[10] Kyrou, K., Hammond, A. M., Galizi, R., Kranjc, N., Burt, A., Beaghton, A. K., Nolan, T., & Crisanti, A. (2018). A CRISPR–cas9 gene drive targeting doublesex causes complete population suppression in caged anopheles gambiae mosquitoes. Nature Biotechnology, 36(11), 1062–1066

[11] Simoni, A., Hammond, A. M., Beaghton, A. K., Galizi, R., Taxiarchi, C., Kyrou, K., Meacci, D., Gribble, M., Morselli, G., Burt, A., Nolan, T., & Crisanti, A. (2020). A male-biased sex-distorter gene drive for the human malaria vector anopheles gambiae. Nature Biotechnology, 38(9), 1054–1060.

[12] Niu, G., França, C., Zhang, G., Roobsoong, W., Nguitragool, W., Wang, X., Prachumsri, J., Butler, N. S., & Li, J. (2017). The fibrinogen-like domain of FREP1 protein is a broad-spectrum malaria transmission-blocking vaccine antigen. Journal of Biological Chemistry, 292(28), 11960–11969.

[13] Dong, Y., Simões, M. L., Marois, E., & Dimopoulos, G. (2018). CRISPR/Cas9 -mediated gene knockout of anopheles gambiae FREP1 suppresses malaria parasite infection. PLOS Pathogens, 14(3).

[14] Hoermann, A., Habtewold, T., Selvaraj, P., Del Corsano, G., Capriotti, P., Inghilterra, M. G., Kebede, T. M., Christophides, G. K., & Windbichler, N. (2022). Gene drive mosquitoes can aid malaria elimination by retarding plasmodium sporogonic development. Science Advances, 8(38).

[15] Bansal, A., Molina-Cruz, A., Brzostowski, J., Liu, P., Luo, Y., Gunalan, K., Li, Y., Ribeiro, J. M., & Miller, L. H. (2018). PfCDPK1 is critical for malaria parasite gametogenesis and Mosquito infection. Proceedings of the National Academy of Sciences, 115(4), 774–779.

[16] Macias, V. M., McKeand, S., Chaverra-Rodriguez, D., Hughes, G. L., Fazekas, A., Pujhari, S., Jasinskiene, N., James, A. A., & Rasgon, J. L. (2020). Cas9-mediated gene-editing in the malaria mosquito anopheles stephensi by remot control. G3&Amp;#58; Genes|Genomes|Genetics, 10(4), 1353–1360.

[17] Garrood, W. T., Kranjc, N., Petri, K., Kim, D. Y., Guo, J. A., Hammond, A. M., Morianou, I., Pattanayak, V., Joung, J. K., Crisanti, A., & Simoni, A. (2021). Analysis of off-target effects in CRISPR-based gene drives in the human malaria mosquito. Proceedings of the National Academy of Sciences, 118(22).