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Comparing the efficacy of modified mosquitoes against malaria

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Abstract
Malaria is a serious disease caused by the Plasmodium parasite, which spreads to and among humans through vectors such as mosquitoes. Despite the numerous preventive measures implemented, the disease's prevalence has not significantly decreased, as is evident by the annual infection rate of 249 million people. This paper explores the potential of modified mosquitoes as a prevention or attenuation method against malaria. Three vector protein modifications are discussed, including antiplasmodial peptides, immune-related proteins and salivary gland proteins, as well as measures to control vector population, and bacteria introduction into the vector. Antiplasmodial peptides, including scorpine, melittin, and snake venom toxins, show promise in inhibiting Plasmodium parasite development in the vector and reducing malaria transmission rates. Immune-related proteins, particularly those targeting the IMD pathway and PAI-1, enhance mosquito immunity and reduce parasite survival in the vector. Salivary gland proteins, such as PAI-1 and Bax, interfere with parasite development, while population control proteins induce vector sterility and/or limit offspring viability. A modification introduces a genus (Wolbachia) of arthropod infecting bacteria into the vector which induces Cytoplasmic Incompatibility (CI), again resulting in vector sterility, reduction in Plasmodium transmissibility and/or limiting offspring viability. Although limitations to these modifications, such as off-target effects and scalability exist, bacteria (Wolbachia)-infected mosquitoes were found to be the most effective based on a Pugh matrix that compared a range of factors. These included off-target effects (and ethics), parasite numbers, population numbers, bite frequency (of mosquitoes to humans), cost, and possibility of the vector acquiring resistance, among others. Further research is recommended to assess the full effectiveness of immune-related proteins in field settings, not just in controlled laboratory environments. Modification of the vector using these methods offers a unique approach to malaria control with significant potential for addressing this global health issue.

Keywords
Malaria, Mosquito, Vector, Anopheles, Plasmodium, Antiplasmodial peptide, Genetically modified mosquito, Wolbachia, Cytoplasmic incompatibility, CRISPR

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Introduction

Malaria is a disease caused by parasites spread to humans through infected mosquitoes. It is a severe disease that is responsible for the death of 610,000 people out of 249 million people affected annually. The burden is especially heavy in Africa, where over 95% of global malaria cases occur. Among the cases, malaria is accountable for over 76% of deaths of vulnerable demographics like children under five and pregnant women.

Since the World Health Organization's initial declaration of malaria eradication in 1955, progress has been made, with more than 42 countries achieving malaria-free status by 2023. However, this falls short of the WHO ultimate goal of complete eradication, as malaria transmission still occurs in 84 countries. A more long-term efficacious way of preventing malaria would have to be developed to achieve this. For this reason, more careful consideration of malaria prevention is needed to eradicate malaria.

Conventional prevention methods include outdoor insecticide spraying of areas conducive to mosquito breeding and indoor residual spraying. Insecticides employed include the chemical classes of the pyrethroids, organochlorines, carbamates and organophosphates. The latter three categories carry significant toxic side effects for humans while vectors have started to become resistant to the first. The WHO highly recommends mosquito impermeable nets, especially the Long-Lasting Insecticidal nets (LLIN) treated with pyrethroids paired with (mosquito resistance impeding) synergistic compounds. The expense, inconvenience, inaccessibility and the development of vector resistance limit the effectiveness of these methods. Resistance has also developed against all classes of antimalarial drugs except the artemisinins, thereby making treatment of infected individuals increasingly ineffective, with the certainty of loss of potency of antimalarial drugs over time. Due to the various anatomical and physiological life-stages that the malarial parasite progresses through post-infection; vaccines developed against malaria are often ‘leaky’; preventing the development of disease symptoms but not against infection and onward transmission of the parasite. The recently approved recombinant vaccine, Mosquirix® is sub- immunogenic, and required the fusion of the *P. falciparum* circumsporozoite protein (CSP) with surface antigens from the hepatitis B virus to improve its antigenicity.

Genetic or non-genetic methods that target the mosquito vector (modified mosquitoes) are a relatively new development in the war against malaria. These methods prevent or attenuate the reproductive multiplication of the Plasmodium parasite in the vector, or render the offspring of modified mosquitoes sterile or incapable of causing disease and limit offspring viability. This innovative malaria prevention method can address multiple concerns with conventional prevention methods while decreasing infection rates. Alongside current methods like vaccines and chemoprophylaxis, modified mosquitoes may represent a potent tool for malaria eradication and/or control. However, due to the recency of this malaria prevention method, limited studies have been conducted. Although on the cusp, widespread implementation has not yet been approved by regulatory agencies.

By comparing the efficacy of different protein types of modified mosquitoes, this study aims to determine the most effective method of modification. By doing so, additional advancements can be made to the newly functioning mechanism and answer the research question: "What is the comparative efficacy of different types of modification methods, and which method shows the greatest potential for reducing malaria transmission?"

Background

Malaria is an acute febrile illness caused by Plasmodium parasites. It is spread to people through the bite of the infected female Anopheles mosquito. It is preventable and curable. Malaria is a blood borne disease and does not spread from person to person contact, even through bodily fluids. It can only be transferred through the introduction of the Plasmodium parasite into the blood circulation. The leading cause of transmission is the Anopheles mosquito. Only Anopheles mosquitoes can spread malaria, and a previous human host of Plasmodium parasites must have infected them. Both mosquitoes and humans act as biotic hosts for these types of parasites. Some exceptional cases of malaria transmission include blood transfusions.

Five Plasmodium parasite species cause malaria in humans, and two species—*P. falciparum* and *P. vivax*—pose the greatest threat. *P. falciparum* is the deadliest and most prevalent malaria parasite on the African continent. *P. vivax* is the dominant malaria parasite in countries outside of sub-Saharan Africa, such as Southeast Asia. The other malaria species that can infect humans are *P. malariae*, *P. ovale*, and *P. knowlesi* (1).

These parasites undergo a parasitic cycle; a period spent in a host organism divided into growth, reproduction, and transmission phases. The cycle is divided into two main harbors of malaria: humans and mosquitoes. The steps of the parasitic cycle in the human body are; 1] An infected mosquito injects sporozoites (an infective form of the parasite that can initiate an asexual cycle of reproduction in the host) into the skin, which travel to the liver and infect liver cells. 2] In the liver cells, sporozoites develop into schizonts (cells that divide by schizogony to form daughter cells), remaining dormant for about two weeks before rupturing. 3] Upon rupture, thousands of merozoites (capable of initiating sexual/asexual cycles of development) are released into the bloodstream, infecting red blood cells. Some merozoites transform into male and female gametocytes, continuing the cycle when another mosquito gets infected.

The steps of the parasitic cycle in mosquitoes are; 1] Mosquito ingests gametocytes during feeding. 2] Gametocytes transform into gametes. 3] Male and female gametes fuse to form a zygote. 4] Zygotes elongate into ookinetes and penetrate the stomach wall. 5] Ookinetes develop into oocysts. 6] Oocysts grow, rupture, and release sporozoites. 7] Sporozoites migrate to salivary glands, ready for injection to restart the cycle (2).

The CRISPR method is the most common method of genetically modifying (GM) mosquitoes. Two essential components in CRISPR are the Cas-9 protein and guide RNA (gRNA). Cas-9 proteins are large and are responsible for cleaving the target DNA to form a double-stranded break. The guide RNA

is used to target viral DNA, and in gene editing, it targets almost any gene sequence that is targeted for editing. Cas-9 acts as a genetic scissor, guided by sgRNA, and Guide RNA determines specificity. The method enables precise genetic modifications. The mechanism of CRISPR/Cas-9 genome editing can be generally divided into three steps: recognition, cleavage, and repair (3).

In addition, another phenomenon/mechanism; termed 'gene drive', along with CRISPR, can be used to rapidly spread a desired gene throughout a population of organisms. Unlike traditional inheritance patterns, where a gene has a 50% chance of being passed on, gene drive increases the likelihood of inheritance to nearly 100%, allowing it to spread through a population over multiple generations quickly. The key to gene drive is the ability to bias inheritance in favor of a particular gene variant, known as an allele. This is typically achieved by using molecular tools like CRISPR-Cas9 to edit the DNA of an organism in a way that promotes the spread of a desired allele. Gene drive mechanism is typically used in further possible studies in the wild environment, which is further discussed in the discussion section of this paper (4).

Results

Antiplasmodial peptides

Antiplasmodial peptides purified from animal venoms, directly target the Plasmodium parasite and either inhibit its replication or kill it while it is undergoing development inside the mosquito. To test these specific peptides, mosquitoes were genetically modified to express them.

Scorpines are sourced from scorpion venom, and studies have demonstrated its high antiplasmodial activity against Plasmodium parasites. In 2002, researchers discovered its potential as an antiplasmodial peptide from observations that when tested in transgenic flies, it disrupted the sporogenic development of *P. berghei* (5). It was not until 2008 that a group could fully design and express scorpine in a mosquito by genetic modification. Mosquitoes expressing scorpine produced 98% mortality in the sexual stages of (*Plasmodium berghei*) at 15 μM and 100% reduction in (*Plasmodium falciparum*) parasitemia at 5 μM (6). A recent study in 2020 was able to follow up on the efficacy of this peptide. This group expressed five antiparasitic effectors: melittin, TP10 dimer, shiva1, four repeats of Plasmodium EPIP4, and scorpine (7). They also tested these peptides' efficacies in different locations of Anopheles mosquitoes and stages of Plasmodium parasites. The five antiparasitic effectors were expressed in *A. gambiae*. Compared to wild-type mosquitoes, the genetically modified mosquitoes showed a significant decrease in the median number of oocysts in their midgut and salivary glands. They next isolated the five different peptides, when the Plasmodium had reached the sporozoite stage. Compared to the control group, scorpine in this latter experiment showed a decrease of 41.2% in sporozoite prevalence and decrease of up to 84.6% when fed additional blood meals.

Melittin is an anti-malarial peptide that has shown promise in reducing Plasmodium parasites in genetically modified mosquitoes. It is a significant component of honeybee venom, comprising a large portion of its composition.

It is identified as the primary substance responsible for inducing pain in both humans and animals (8). Previous studies have demonstrated that different venoms strongly inhibit oocyst formation when administered to mosquitoes along with an infectious blood meal (9). The effectiveness of this peptide was first shown by a study that assessed the *in vivo* effects of several anti-microbial peptides (AMPs) against sporogenic stages of *P. berghei* when *A. stephensi* were fed on gametocyte-containing blood supplemented with 50 μM of each AMP separately. Among tested AMPs, melittin was shown to be the only effective peptide that significantly diminished parasite prevalence by an average of 10% and intensity of infection by 68% (10). Although the study did not directly express the peptide in the mosquito, it demonstrated its effectiveness against Plasmodium parasites. In another study, researchers genetically modified mosquitoes to express melittin. Both transgenic (with melittin expressed) and non-transgenic mosquitoes were fed on the same infected mouse to observe the results. Results showed a significant drop in the infection rate and oocyst formation in transgenic mosquitoes, and across five separate experiments, oocyst formation was reduced by 77% to 99%, with an average reduction of 87% (11). A more recent study in 2019 showed an especially efficient method of standard membrane feeding assay (SMFA), which involved culturing *A. coluzzii* mosquitoes and *P. falciparum* gametocytes together, allowing for the accurate assessment of transmission-blocking interventions. Using this method, the same group tested six AMPs previously shown to combat blood-stage *P. falciparum*. Melittin, at a concentration of 50 μM, significantly blocked transmission when added to blood-containing gametocytes. The result was highly significant compared with the wild-type mosquitoes (12). Although the method used six different peptides simultaneously, melittin was shown to be the most efficacious out of the six, making it an effective candidate peptide for consideration to be expressed in a mosquito.

Finally, snake venom toxins constitute of a mix of molecules and peptides with diverse biological effects, including those of neurotoxicity and cytotoxicity. One of these - Shiva toxins - hold promise for medical use, such as expression to prevent malaria transmission (13). It is currently known that several snake species present toxins mainly derived from proteins with antibacterial, antifungal, antiviral, and antiparasitic characteristics (14). Early testing in 2001 showed that Shiva toxins blocked malaria parasite development in the mosquito midgut by inhibiting ookinete association with the midgut surface. Although the study did not involve genetically modifying the mosquito, the inhibition was confirmed through various experiments and testing its effects on mosquito feeding (15). Another group was able to test the effectiveness *in vitro*. They were able to demonstrate that the venom extract of *Micrurus spixii* (a type of snake) strongly inhibited the development of *P. falciparum* within red blood cells, with an IC_{50} value of 0.78 μg/mL (16). More recently, in 2021, two groups investigated the effects of snake venom extracts on the parasites *P. falciparum* and *P. berghei*. In both studies, a small venom fraction derived from the crude venom extract of Naja naja oxiana was utilized for anti-Plasmodium tests, yielding promising inhibitory outcomes

(IC_{50} = 3.2 μg/mL) (17). Moreover, the study reported reduced parasitemia by 70%, 50%, and 30% at decreasing concentrations (18). Few studies have been performed to genetically modify mosquitoes to express snake venom toxins, but these peptides demonstrate strong anti-malarial effects, showcasing them as promising candidates.

Overall, antiplasmodial peptides, many of which originate from venom, can be expressed in mosquitoes using genetic engineering. The scorpine, melittin, and snake venoms showed a highly significant reduction of both ookinete prevalence and average parasite transmission in/from Plasmidium infected mosquitoes compared to the wild-type. Furthermore, most of the experiments were performed by genetically engineering mosquitoes to express these peptides, demonstrating the possible use of these peptides in decreasing malarial transmission.

Immune-related proteins

Another promising protein that can be genetically modified into vectors belongs to the category of immune-related proteins. These proteins enhance the mosquito's immune response to Plasmodium infection, thereby reducing the parasite load or preventing its development.

The immune deficiency (IMD) pathway is a signaling pathway found in insects, particularly in species like *Drosophila melanogaster* (fruit flies). It is a crucial component of the insect's innate immune system, which serves as the first line of defense against pathogens (19). The IMD pathway is also present in mosquitoes, and gene editing tools can be used to enhance the function of these pathways and increase the expression of a specific protein. Although there are multiple proteins in the IMD pathway, a study found that the Rel2 protein regulates the expression of AMPs and other immunity genes. Furthermore, Rel2 was found to be required for the antibacterial defense of most adult *A. gambiae* mosquitoes (20). Another study showed that over-activating the IMD pathway by silencing the gene encoding its negative regulator, Caspar, or over-expressing the gene encoding the Rel2 transcription factor conferred almost complete protection from cultured *P. falciparum* in laboratory-reared *A. gambiae*, *A. stephensi*, and *A. albimanus*. They found that the over-expression of Rel2 in transgenic mosquitoes in the midgut reduced oocyst infection by nearly 80%. In addition, the infection prevalence of the modified line was 28%, which was significantly lower than the 80% infection prevalence of the wild-type (21). In another experiment, the same group was also able to determine that the IMD pathway was the most potent against the parasite's ookinete stage yet also demonstrated reasonable activity against early oocysts and lesser activity against late oocysts. They further demonstrated that caspar silencing alone could induce a strong anti-*P. falciparum* response (22). A 2011 study showed very similar results with the addition of testing in different mosquito tissues. By over-expressing the Rel2 transcript in the IMD pathway, a ~ 77.1% reduction of infection rate was seen when expressed in the mosquito midgut, ~ 48.6% when expressed in the fat body tissues, and ~ 82.9% as a hybrid. The midgut-specific transgenic lines exhibited significantly greater resistance to *P. falciparum* infection than the fat body-specific transgenic lines. Overall, expressing the proteins,

especially the Rel2 protein, in the IMD pathway was a promising method for reducing the early stages of the parasite and the overall infection rate in mosquitoes.

Human plasminogen activator inhibitor-1 (PAI-1) proteins are members of the serine protease inhibitor (serpin) superfamily and play a critical role in maintaining hemostatic balance by regulating fibrinolysis. Their dysregulation can have significant implications for thrombosis and hemostasis-related diseases (23). Human PAI-1 inhibits Plasmodium by blocking the activation of plasminogen. Plasminogen helps parasites move by breaking down proteins. However, PAI-1 stops this activation process, slowing the parasites' movement and limiting their growth (24). To test whether PAI-1 affects ookinete formation, one study fed mosquitoes with infected blood supplemented with PAI-1 and counted the number of ookinetes in the blood at 23 hours after feeding. The results showed that the PAI-1 treatment significantly reduced ookinete formation. The effect of PAI on ookinete formation *in vitro* was tested to examine the direct impact. It was found that the ookinete formation *in vitro* was not affected by PAI-1, showing that PAI-1 is not toxic to the parasite and that the inhibitory effect of PAI-1 only manifests in the mosquito blood (24). To add consistency to the presence of PAI-1 in the blood, another group genetically modified mosquitoes so that the mosquitoes secreted human PAI-1 into the midgut. The midgut modified mosquito reduced the number of *P. berghei* oocyst formations to negligible levels. Furthermore, the oocyst formation for *P. falciparum* and *P. vivax* in the midgut were completely inhibited as well. The same study also found that genetically modified mosquitoes could impede the transmission of parasites to other mammals by varying the number of mosquito bites on mice. With one mosquito bite, although 45% of the mice became infected with the WT mosquito, the transgenic mosquitoes showed significant prevention, ranging from 85% to 100%. With five mosquito bites, the genetically modified mosquitoes prevented around 70-100%. With ten mosquito bites, all mice became infected with WT present, while transgenic mosquitoes still showed prevention of around 50-93% (25). Both studies underscore PAI-1's efficacy in mosquito blood and demonstrate that it may take a significantly greater number of bites to transmit disease with modified mosquitoes. If genetically modified mosquitoes are developed as suggested, they hold significant promise in malaria prevention.

In summary, immune-related proteins, such as those in the IMD pathway and PAI-1, offer promise for genetically modifying mosquitoes to combat malaria. These studies found that they can lower parasite infection rates and transmission. However, future investigations must resolve challenges like long-term effectiveness, ecological consequences, and ethical concerns to ensure their responsible use as widespread malaria control measures.

Salivary gland proteins

A third way of genetically modifying mosquitoes is by modulating the expression of salivary gland proteins. These proteins interfere with developing or transmitting Plasmodium parasites within the mosquito's salivary glands, reducing the likelihood of transmission to humans. Inhibiting the salivary gland is crucial

in preventing malaria transmission because it is the site from where the parasite, such as Plasmodium, is injected into a host's bloodstream during a mosquito bite (26).

PAI-1; similar to its use to increase mosquito immunity, can specifically inhibit the parasites in the salivary gland. By genetically modifying mosquitoes to secrete human PAI-1 in their salivary glands, the mosquitoes effectively block the transmission of malaria parasites. By secreting PAI-1 into the saliva, the mosquitoes hinder the activation of plasminogen, which is essential for the parasites' movement and proliferation in the host's bloodstream. This inhibition prevents the parasites from establishing infection and spreading malaria to the bitten individual (24). One study discussed the efficacy of the PAI-1 proteins, tested the proteins in different areas, including the salivary gland, and compared their effectiveness in various measures. Out of the two areas tested, the midgut and salivary gland, the PAI-1 proteins inhibiting the salivary gland most effectively prevented oocyst formation with 100% reduction. Additionally, the inhibition of the salivary gland also exhibited the most exceptional result of malaria transmission by increasing the prevention rate to 100% when multiple mosquitoes were present (25). In conclusion, this targeted approach showed promising results, with PAI-1 proteins inhibiting the parasites in the salivary gland of the vector, demonstrating the highest efficacy in preventing oocyst formation and reducing malaria transmission rates. These findings highlight the potential of this strategy in innovative vector control measures to combat malaria transmission.

Bcl-2-associated X (Bax) proteins are a group of proteins involved in regulating cell death, particularly apoptosis. They belong to the Bcl-2 protein family, which plays a critical role in controlling the intrinsic or mitochondrial pathway of apoptosis. Bax proteins are primarily located in the cytoplasm of cells but can translocate to the outer mitochondrial membrane when cells receive signals to undergo apoptosis (27). Given the characteristics of Bax proteins, their expression in the salivary glands can disrupt saliva production in mosquitoes. Such transgenic strains of mosquitoes exhibited salivary gland cell death from the expressed Bax. This alteration in saliva production inhibited the mosquitoes' ability to feed on blood efficiently, thereby hindering the transmission of malaria parasites from hosts to the mosquito midgut. The diminished saliva production also impacted malaria transmission from mosquitoes to hosts (28). Despite similar initial parasite loads in the midgut, such transgenic mosquitoes showed significantly lower oocyst numbers than wild-type mosquitoes. Furthermore, the accumulation of sporozoites in the salivary glands, essential for malaria transmission, was inhibited in the genetically modified mosquitoes. Consequently, infectious blood-fed transgenic mosquitoes did not transmit the parasite to the mice tested.

Overall, the studies demonstrate the promising potential of genetic modification targeting Bax protein expression in mosquito salivary glands for malaria prevention. This approach offers a new strategy to reduce malaria transmission to humans by disrupting saliva production in the vector thus impeding parasite development and transmission. The observed inhibition of key

parasite life cycle stages showed the effectiveness of this approach, suggesting it as a valuable genetic modification tool in preventing malaria.

Population control

Mosquitoes transfected with Population control proteins, aim to reduce their populations or reproductive capacity and decrease the overall transmission of malaria parasites. The protein of interest is Bcl-2-associated X (Bax), a sterility-inducing protein that causes infertility or sterility in transfected organisms. In addition to Bax, a form of Sterile Insect Technique (SIT), termed Release of Insects carrying a Dominant Lethal (RIDL) is another mechanism to prevent malarial transmission.

Bax, also discussed in the previous section as a salivary gland protein modulator, also affects mosquito reproduction. When the Bax protein is expressed in the reproductive tissues of mosquitoes, such as in the testes or ovaries, it induces a process called apoptosis, which leads to programmed cell death. Specifically, Bax triggers the release of proteins from the mitochondria, which initiates a cascade of events culminating in cell death. In mosquitoes, this can destroy cells involved in spermatogenesis (sperm production) in males or oogenesis (egg development) in females, ultimately resulting in sterility (29). A study identified a functional promoter in *A. stephensi* that enables gene expression, specifically in the testis. Using this promoter, researchers created a transgenic mosquito line expressing Bax, which induced apoptosis in the testicular tissues. As a result, transgenic mosquito males developed aberrant testes, making them sterile. Females that mated with the genetically modified males laid eggs, but these eggs failed to hatch completely. Additionally, females had no sperm in their spermatheca after mating with the genetically modified males, further confirming male sterility. Moreover, females that mated with the transgenic males did not accept further mating with wild-type males, showing the effectiveness of this population control method (30).

RIDL, instead of causing cell death by expressing a particular protein, modifies the DNA of a target insect species to introduce a self-limiting gene. When these insects mate with other wild mosquitoes, this dominant gene is passed on to the offspring, making them unable to survive and reproduce (31). With regular releases of these genetically modified males, the number of damaging female offspring is reduced, resulting in a reduction in the pest insect population. The company Oxitec https://oxitec.com inserts the LA 513 transposon into the mosquito's DNA to produce offspring that die in the larval stage unless fed tetracycline. In the factory, the RIDL mosquitoes breed normally when fed a tetracycline supplement; in the wild, the genetic modification kills the offspring of the released males. It is important to note that the release of engineered female vectors may significantly reduce the effects of population decrease because of the nature of the kill mechanism.

Modification with bacteria

Lastly, modification with bacteria, specifically of the genus Wolbachia, offers a more natural way to combat malaria because of its natural presence in nature. Infection of Wolbachia is an injection of Wolbachia bacteria to either

repress the population of mosquitoes or prevent them from becoming infected with Plasmodium parasites. The method has met with considerable success in multiple areas where Wolbachia naturally infects mosquitoes.

Wolbachia is a genus of vertically transmitted alphaproteobacteria that infect about 40% of arthropod species (33). They reach a high prevalence through the induction of cytoplasmic incompatibility (CI). CI is a mechanism by which the sperm of Wolbachia-infected male mosquitoes are unable to form viable offspring when eggs of uninfected females are fertilized, whereas the eggs of infected females are viable (34). This means that with the introduction of male mosquitoes with Wolbachia, there is a higher probability of vertical transmission since, with all the other possibilities, the offspring is not produced. Wolbachia's ability to induce CI helps in two main ways: population reduction and Wolbachia prevalence. The Wolbachia prevalence is shown to serve as a defense system against Plasmodium. One study observed wild mosquitoes carrying native Wolbachia infection in Mali. By collecting 131 total mosquitoes, 62 Plasmodium-positive and 69 Plasmodium-negative, the prevalence of Plasmodium infection in Wolbachia-positive females was significantly lower (29%) compared to Wolbachia-negative females (65%). The research also showed a negative correlation between the number of Wolbachia and the number of sporozoites (35), which displays further promise of this natural malaria control method. Another study discovered specific parts of mosquitoes that Wolbachia infected by observing the immune system and the number of Plasmodium parasites prevalent. A stable *A. stephensi* line infected with Wolbachia *in vitro* was partially protected against Plasmodium falciparum infections, resulting in a moderate decrease in oocyst numbers and a strong reduction in salivary gland sporozoites. Similar protection against Plasmodium was also observed when Wolbachia-infected *A. aegypti* were challenged with *P. gallinaceum* or when *A. gambiae* carrying somatic Wolbachia infections were infected with *P. falciparum* (36). Additionally, native Wolbachia has been shown to limit dengue virus infection in *Aedes albopictus* (37).

Many studies using Wolbachia have shown considerable promise in preventing malaria or reducing its transmission. It is also less controversial within the community because of the natural presence of Wolbachia in wild type mosquitoes in the environment. Furthermore, Wolbachia-infected mosquitoes involve no genetic modification or the use of extraneous chemicals. However, the release of engineered female vectors may reduce the effects of population decrease while still maintaining reduced transmissivity.

Discussion

Modified mosquitoes are a potential malaria prevention tool. Antiplasmodial peptides, immune-related proteins, salivary gland proteins, population control proteins/measures, and Wolbachia infection offer promising types of modifications, by inhibiting the parasite in the mosquito vector and/or making their offspring sterile or less transmissive.

Although all of these recent technologies regarding modification of mosquitoes

demonstrated significant effectiveness against the Plasmodium species, it is evident that most of the experiments were conducted in a laboratory environment, and may not represent real-life situations accurately. Other unique limitations existed for each type of modification as well. Among the five different methods of modifying mosquitoes, the most effective method was chosen by analyzing six main factors: off-target effects (and ethics), parasite numbers, population numbers, bite frequency (of mosquitoes to humans), cost, and possibility of resistance. A Pugh matrix (Table 1) was created to compare the five modifications.

Possible off-target effects on future generations exist for population control proteins and Wolbachia, as demonstrated by their modified offspring's shortened lifespan. Although it seems beneficial to decrease the population number, this could raise ethical concerns due to the impact on biodiversity and the ecosystem. As mosquitoes play a role in ecosystems as pollinators and as a food source for other animals, a significant reduction in their population could disrupt these ecosystems, potentially affecting other species and overall biodiversity. On the other hand, antiplasmodial peptides, immune-related proteins, and salivary gland prevention proteins did not cause observable changes in the mosquito population. However, further testing is needed to assess their potential off-target effects. Regarding the number of parasites within the mosquitoes, the antiplasmodial peptides showed the highest efficiency with the minimal amount, but all the other methods also showed a relatively high reduction (80 ~ 90%) of most stages of the parasites such as sporozoites and oocysts. Although antiplasmodial peptides showed one of the highest parasite mortality rate, they were coexpressed with other peptides in the study (7), hence their efficacy by themselves individually could not be determined. The other proteins coexpressed in the mosquitoes showed a significant decrease in oocyte numbers in various locations as well, further making it difficult to identify the true effect of the antiplasmodial peptides. The mosquito bite frequency to humans significantly decreased for population control proteins (specifically for the OXITEC's line) and for Wolbachia-treated mosquitoes. All genetically modified mosquitoes are likely to be a significant burden on public (taxpayer) finances. This leaves Wolbachia-treated mosquitoes as the most cost-effective method due to its natural presence. While the cost of developing and deploying genetically modified mosquitoes and gene drive technologies is substantial, the potential benefits, such as eliminating or significantly reducing malaria, are also significant. However, these decisions must be made carefully, considering the immediate costs, long-term implications, potential risks, and ethical considerations. Finally, Plasmodium parasites can acquire resistance to antiplasmodial peptides, immune-related proteins, and salivary gland proteins. The only modification that does not allow for mutation or resistance is population control proteins, while it is extremely unlikely that the Plasmodium parasite infecting the Wolbachia carrying mosquitoes will acquire resistance due to it having exerted selective pressure for a long time without such evidence.

Table 1. Comparison of effectiveness of different vector control and/or eradication methods

Type of modification	Off-target effects	Decreased number of parasites in vector	Decreased vector population	Decreased mosquito bite frequency	Genetic modification of vector required	Need sorting system at scale to prevent release of engineered female vectors	Cost	Vector can acquire resistance
Antiplasmodial peptides	Not known	Yes*	No	No	Yes	Not known	Expensive	Yes
Immune-related peptides	Not known	Yes*	No	No	Yes	Not known	Expensive	Yes
Salivary gland peptides	Not known	Yes*	No	No	Yes	Not known	Expensive	Yes
Population control (RIDL)	Not known	Yes	Yes	Yes	Yes	Yes	Expensive	No
Wolbachia	Yes	Yes	Yes	Yes	No	Yes**	Cheap	No

* Coexpression with other peptides makes it difficult to determine true effect
** Not an absolute necessity but non-conformity will decrease the potential to reduce population

After careful analysis, Wolbachia bacteria emerged as the most promising method. It harnesses the benefit of multiple factors, such as decreasing the mosquito bites to humans and its low cost. Additionally, Wolbachia's natural presence in arthropods enhances public acceptance compared to the other genetic modification methods. Community acceptance is crucial for the success of any modification method, as it aids with faster implementation and spread. Wolbachia also protects against other viruses, such as dengue and yellow fever, which shows a potential multi-purpose modification (37). However, as this method was tested in a laboratory environment, a field study with Wolbachia-infected mosquitoes is necessary. If CI were to be induced, the eggs would not hatch, leading to a reduction in the mosquito population. If CI were not to be induced, the offspring would still exhibit an anti-Plasmodial effect (36). Ethical concerns regarding population reduction and the introduction of Wolbachia need to be addressed. It is possible that introducing Wolbachia could cause multiple unintended ecosystem impacts in areas where it is not naturally present.

Moving forward, continued research and development with Wolbachia bacteria, with careful consideration of their effects on mosquitoes and the ecosystem, is essential to utilize the full potential of this modification method for malaria prevention. The company Mosquitomate (http://mosquitomate.com) has already started using this modification method to spread Wolbachia-infected male Tiger mosquitoes into the wild. Since male mosquitoes do not bite and less off-target effects are evidenced with Wolbachia present in the mosquitoes, these controlled releases (in backyards or on private property) present an excellent opportunity to observe the effects of these bacteria on the population and transmission rates in the wild. The company successfully obtained approval to bring their

ZAP® mosquitoes to market in 2017. Their efforts included obtaining EPA approval, conducting extensive field trials, particularly in Fresno and the Florida Keys, and engaging in public outreach. The product has significantly reduced mosquito populations and is now available for broader use (38). The next research iteration would require discovering cost-effective ways to spread these modified mosquitoes worldwide.

In summary, exploring antiplasmodial, immune-related, salivary gland prevention, population control proteins, and bacteria-infected mosquitoes for malaria control offers a range of promising strategies. While each modification category presents distinct advantages and limitations, bacteria-infected mosquitoes stand out as particularly promising due to their high efficacy against Plasmodium parasites while having minimal environmental, ecological and cost concerns. More research efforts will further advance the development of these modified mosquitoes as a viable tool for malaria control, potentially establishing it as a safe and effective option for the decrease of malarial transmission, and the eventual eradication of malaria.

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