Chapter 12

Fighting malaria with engineered mosquito symbiotic bacteria

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Insecticides that kill the mosquito and drugs that kill the parasite are the only weapons presently available to fight the unbearably high human malaria toll. As mosquito and parasite resistance to these agents limits their effectiveness and there is currently no effective malaria vaccine available, clearly new means to fight the disease must be developed. This chapter explores the feasibility of an alternative strategy: rather than kill the vector mosquito, modify it to render it incapable of sustaining parasite development. This chapter investigates genetically modifying the symbiotic bacteria that naturally occur in the mosquito's midgut, by producing bacteria that carry the same anti-parasite genes. Major remaining challenges are to devise means to introduce the modified bacteria into mosquitoes in the field and to resolve regulatory and ethical issues related to the release of genetically modified organisms in nature.

Introduction

Malaria remains one of the world's most devastating diseases, afflicting close to 500 million people (nearly 1 in 12 humans) and causing over 1 million deaths every year. The available means to fight the disease are clearly insufficient and the development of new strategies is urgently needed.

Unlike AIDS and tuberculosis (the two other major infectious disease killers), which are transmitted directly from person to person, malaria is different in that the pathogen has to transit through a vector mosquito before it can be transmitted to another human. Consequently, eliminating the mosquito will stop transmission. As such, insecticides have been, and continue to be, the principal weapon to fight malaria. A major limitation in the use of insecticides is the rapid evolution of mosquito resistance to these agents (Maxmen, 2012) and another equally important, but rarely considered, limitation is that insecticides leave intact the biological niche where mosquitoes reproduce. In other words, mosquito breeding sites are unaffected by insecticide applications that occur mostly indoors, leading to two deleterious consequences: *i)* the cycle of continuous breeding (outdoors) and insecticide killing (indoors) constitutes a strong selection pressure for insecticide-resistant mosquitoes; *ii)* mosquito numbers that decline after insecticide use quickly revert to pre-treatment levels as soon as use is interrupted. Therefore, insecticide use can never stop, it would have to be used forever. In summary, insecticides are a very important weapon to fight malaria, but have significant limitations.

The other major weapon to fight malaria is drugs that kill the parasite in the infected human. However, in areas where the disease is endemic, drugs are used almost exclusively to treat, not to prevent, disease. A major limitation of this approach is the rapid evolution of parasite resistance, not unlike bacteria development of resistance to antibiotics. In the case of the malaria parasite, its exceptionally malleable genome exacerbates the problem, as drug resistance evolves quite rapidly, usually in the span of a few years. Another important limitation to the use of drugs is that the logistics needed to distribute the drugs to the people in need, largely in rural areas, is not easy to implement, as the countries with the highest malaria prevalence do not usually have adequate resources. The cost of the drugs and their distribution is also a limiting factor.

The third weapon under development is vaccines that either protect the vaccinated individual or prevent transmission (transmission-blocking vaccines). While extensive efforts have been invested in the last few decades into the development of vaccines, none is yet available. However, a partially effective vaccine is presently under phase III trial and will hopefully be added to the anti-malaria arsenal in a not too distant future (Agnandji et al., 2011).

Transgenic mosquitoes

Recent advances in mosquito molecular genetics and vector-parasite interactions suggest a new strategy to combat malaria, namely, rather than killing the mosquito, rendering it incapable of sustaining parasite development. Since the mosquito is essential for parasite transmission, hindering the mosquito's ability to sustain parasite development can be used to reduce or eliminate transmission. Considerable progress has been made toward this goal (Riehle et al., 2003). Indeed, mosquitoes can be genetically modified to substantially reduce their vectorial capacity (Ito et al., 2002). Despite this and other major advances made toward the generation of *Plasmodium*-resistant mosquitoes, important challenges still remain.

One crucial unresolved question is how to introduce effector genes (whose products interfere with parasite development in the mosquito) into wild mosquito populations. Several possible approaches have been proposed, such as the use of transposable elements or the bacterium *Wolbachia*, but each has serious limitations. In a recent major technological advance, cage experiments have shown that the MEDEA drive system can be used to introduce transgenes into *Drosophila* populations (Chen et al., 2007). While promising, this approach will take time to implement because the necessary tools (e.g. anopheline maternal effect genes, anopheline embryonic promoters) are not yet available.

Another limitation of this approach is that at least in the published cage experiments, a very high initial introduction rate (~25%) was necessary. Finally, this approach cannot overcome the reproductive barriers posed by reproductively isolated anopheline populations (cryptic species) which are common in malaria endemic areas (Powell et al., 1999). Another approach being explored for the spread of genes is the use of homing endonuclease genes originally derived from micro-organisms, but also synthetically assembled (Deredec et al., 2011). While this approach has promising features, there are technical obstacles to be solved, including the problem common to all genetic drive strategies of overcoming the barrier of reproductively isolated populations. It is not clear in what time frame these obstacles will be overcome.

Paratransgenesis

This section explores the use of an alternative strategy to render mosquitoes resistant to the parasite. It takes advantage of the fact that like the majority of higher organisms, including mammals and humans, the mosquito carries a significant microbiome (symbiotic bacteria) in its gut (Pumpuni et al., 1996; Straif et al., 1998). The idea is then to engineer these symbiotic bacteria to produce interfering products (effector molecules) that arrest parasite development. This approach is also referred to as paratransgenesis. An important strategic consideration is that the bacteria occur in the same compartment (the mosquito midgut), where the most vulnerable stages of the parasite cycle occur (Drexler et al., 2008). It is also important that midgut bacteria numbers increase dramatically (two to three orders of magnitude) after ingestion of a blood meal (Pumpuni et al., 1996), and therefore production of effector molecules can be expected to increase accordingly.

Initial experiments used a laboratory strain of *Escherichia coli* to produce a dimer of the salivary gland and midgut peptide 1 $(SM1)_2$ that interferes with ookinete invasion of the midgut (Ghosh et al., 2001) or a modified phospholipase A2 (Moreira et al., 2002). These experiments were promising as mosquitoes carrying these bacteria had a significantly decreased competence to sustain parasite development (Riehle et al., 2007). However, inhibition of parasite development was not robust for two main reasons: *i)* the *E. coli* used for these studies was an attenuated laboratory strain that did not survive well in the mosquito midgut; *ii)* the bacteria were engineered to display the recombinant proteins on their surface, therefore not allowing their diffusion to their intended targets on the parasite or the mosquito midgut.

In view of the promising results of the initial experiments (Riehle et al., 2007), the strategy was improved by focusing on four issues. First, a bacterial strain isolated from the mosquito gut was used instead of an attenuated laboratory bacterium. After isolation, this bacterium – *Pantoea agglomerans* – was further adapted to the mosquito midgut conditions by repeated passages through mosquitoes (Riehle et al., 2007). *P. agglomerans*

is commonly found in field anopheline mosquitoes, as well as in Africa (Pumpuni et al., 1996; Straif et al., 1998). Second, it was important to engineer the bacteria to secrete the effector proteins. While producing recombinant proteins in bacteria is straightforward, engineering Gram-negative bacteria to secrete recombinant proteins can be challenging. An efficient secretion of effector proteins by *P. agglomerans* was engineered making use of the *E. coli* hemolysin A (HlyA) secretion system (Tzschaschel et al., 1996). Third, to improve protein production by the bacteria, the genes encoding anti-malarial effectors were engineered by synthesising them with codon usage optimised for *P. agglomerans* (codon harmonisation). Fourth, several new effector peptides/proteins were developed and existing ones were adapted as follows:

- mPLA2: a mutant phospholipase A2 that inhibits ookinete invasion, possibly by modifying the properties of the midgut epithelial membrane (Moreira et al., 2002).

- Pro: a chitinase propeptide that inhibits chitinase activity, thus hindering ookinete traversal of the mosquito peritrophic matrix (PM; Bhatnagar et al., 2003). The PM is a chitin-based extracellular structure that surrounds the entire blood meal.

- Shiva1: a synthetic anti-parasitic lytic peptide (Jaynes et al., 1988).

- Scorpine: a scorpion (*Pandinus imperator*) anti-malaria lytic peptide, which has hybrid properties of the lytic peptides cecropin and defensin (Conde et al., 2000).

- EPIP$_4$: four copies of *Plasmodium* Enolase-Plasminogen Interaction Peptide (tetra-peptide), that inhibits mosquito midgut invasion by preventing plasminogen binding to the ookinete surface (Ghosh et al., 2011).

- Pro:EPIP: a fusion peptide composed of Pro and EPIP.

Bacteria that secrete these effector molecules were administered to mosquitoes followed one day later, by a *Plasmodium*-infected blood meal. Control mosquitoes were fed bacteria transformed with the HlyA parental plasmid and did not produce an effector protein. The recombinant bacteria strongly inhibited *Plasmodium* development in mosquitoes. Inhibition varied from 85% for mPLA2 to 98% for scorpine and (EPIP)$_4$ (Wang et al., 2012). Perhaps more importantly, the percentage of mosquitoes that had at least one parasite dropped from 90% in controls to 14~18% in mosquitoes carrying scorpine- or (EPIP)$_4$-expressing bacteria. This strong decrease in the proportion of infected mosquitoes should translate into an important reduction of transmission in the field. The use of multiple effector molecules, each acting by a different mechanism, should greatly reduce the probability of selecting resistant parasites. The inhibition of parasite development was equivalent when using an African mosquito (*Anopheles gambiae*) and an Asian mosquito (*An. stephensi*). Also, inhibition of *P. berghei* (a rodent parasite) and *P. falciparum* (a human parasite) was equivalent, suggesting that this approach may also work for other human parasites, such as *P. vivax*. Thus, the paratransgenesis strategy may well turn out to be "universal", being effective for multiple mosquito and parasite species.

Additional considerations in favour of transgenesis are that: *i)* genetic modification of bacteria is much easier to achieve than genetic modification of mosquitoes; *ii)* bacteria are easier to introduce into mosquito populations than transgenes and are unaffected by known genetic and reproductive barriers in wild mosquito populations; *iii)* bacteria can be produced easily and cheaply, also in disease endemic countries; *iv)* the paratransgenesis approach is compatible with, and could complement, other control strategies, such as insecticides, population suppression including transgenic mosquitoes.
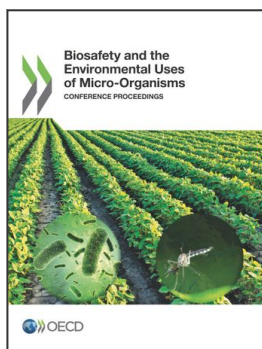
Challenges ahead

It is important to emphasise that while many technical aspects have been successfully addressed, several major issues need to be considered before paratransgenesis can be implemented. One key issue is to devise means to effectively introduce the engineered bacteria into mosquitoes in the field. One possible approach that is beginning to be explored is to place baiting stations (cotton balls soaked with sugar and bacteria and placed in clay jars) around villages where malaria is prevalent. Other major topics that need to be addressed are the resolution of regulatory, ethical and social issues related to the release of genetically modified bacteria in nature.

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