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Eradication of malaria through genetic engineering: the current situation

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ABSTRACT

Malaria is an intra-cellular parasitic protozoon responsible for millions of deaths annually. Host and parasite genetic factors are crucial in affecting susceptibility to malaria and progression of the disease. Recent increased deployment of vector controls and new artemisinin combination therapies have dramatically reduced the mortality and morbidity of malaria worldwide. However, the gradual emergence of parasite and mosquito resistance has raised alarm regarding the effectiveness of current artemisinin-based therapies. In this review, mechanisms of anti-malarial drug resistance in the *Plasmodium* parasite and new genetically engineered tools of research priorities are discussed. The complexity of the parasite lifecycle demands novel interventions to achieve global eradication. However, turning laboratory discovered transgenic interventions into functional products entails multiple experimental phases in addition to ethical and safety hurdles. Uncertainty over the regulatory status and public acceptance further discourage the implementation of genetically modified organisms.

1. Introduction

The contest between malaria parasites and humans has continued since the evolution of mankind, with no resolution in sight[1]. With more than 200 million clinical cases and an estimated 700 000 deaths per annum, malaria is a major vector-borne disease that now rivals HIV/AIDS as the world most deadly infection[2,3]. Key contributors toward child death under age five were presented in Figure 1. To date, five *Plasmodium* protozoa have been identified, including *Plasmodium falciparum* (*P. falciparum*), *Plasmodium vivax* (*P. vivax*), *Plasmodium ovale* (*P. ovale*), *Plasmodium malariae* (*P. malariae*) and the recently discovered simian *Plasmodium knowlesi* (*P. knowlesi*), which infects human with malaria through transmission by the *Anopheles* mosquito[4-6]. Infections caused by *P. falciparum* and *P. vivax* have been reported extensively worldwide; until recently, *P. knowlesi* infection in humans occurred predominantly in Southeast Asia[7,8]. Infections caused by virulent *P. falciparum* and *P. knowlesi* are life threatening, as they might lead to fatal complications, while other species typically only cause milder symptoms[9].

Interestingly, the relationship between the host genetic profile and susceptibility to malaria is intricately intertwined. Conferred genetic resistance to malaria is not only enhanced by modifications to the immune system such as the major histo-compatibility complex gene, but also certain inherited haemoglobin disorders or erythrocyte polymorphisms[11]. Variations in the haemoglobin HBB gene that give rise to the HbS allele are higher in malaria-endemic areas where the heterozygous condition confers protection against severe malaria[12,13]. The Duffy-negative blood group resulting from the FY gene polymorphism is common in sub-Saharan Africa as it provides complete resistance to *P. vivax* infection, but it is not present in

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Asia or South America[3]. The Fulani ethnic group in Mali has comparatively higher resistance to malaria than other neighbouring ethnic groups, primarily due to their distinct genetic background and high levels of anti-malarial antibodies[14,15]. However, the host's genetic background is not the only key to determining susceptibility to malaria; the involvement of environmental factors, parasite genetic factors and multi-gene interactions also play pivotal roles[16]. As this review will described, we need to understand to a greater extent the complex parasite lifecycle, antigenic variation, human immunity and genetic resistance to malaria to maximally exploit new genetic control mechanisms for future implementation in malaria control programs.

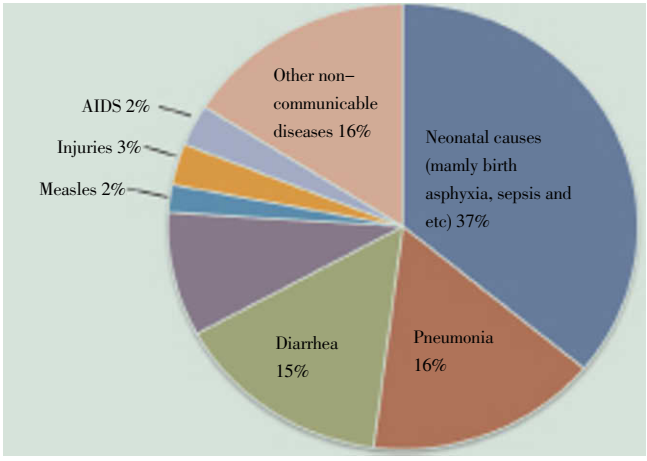

Figure 1. Global causes of death among children under age five between year 2008 and 2011.
Source: Black *et al* 2010[10]; and World Health Statistics 2011[98].

2. Clinical symptoms

Malaria infections may lead to various symptoms ranging from very mild to severe complications and even death. The majority of the clinical signs of this disease are caused by the asexual multiplication of the parasite in red blood cells (RBCs)[17]. The release of infective merozoites from RBCs along with numerous waste substances, such as haemozoin pigment, glycosyl-phosphatidyl-inositol and several parasite-derived molecules, stimulates pro-inflammatory cytokine production that eventually causes fever and other complications associated with malaria[18].

The cyclic occurrence of malaria attack consists of cold and hot stages, during which host experiences a cold and shivering sensation, followed by fever, headache and convulsion, respectively. Added findings such as mild jaundice and an enlarged liver may be found in *P. falciparum* malaria. Anaemia is a common sign for all types of malaria[19]. Kakkilaya (2009) reported that the degree of malarial anaemia correlates with parasitaemia and schizontaemia. Pregnancy, secondary bacterial infections and bleeding disorders can also aggravate the anaemia. *P. vivax* and *P. ovale* have a strong preference for infecting only young RBCs, thereby limiting parasitaemia levels to approximately 1%-2%. *P. malariae* invades RBCs of all ages but at a much slower multiplication rate that results in limited parasitaemia and mild symptoms during infection, whereas *P. falciparum* and *P. knowlesi* invades RBCs of all ages and anaemia can therefore develop rapidly due to profound haemolysis[20,21].

Complicated malaria on the contrary normally occurs in areas of low endemicity or in individuals with low immunity due to certain health risks. Cerebral malaria is one of the clinical manifestations of severe malaria which induced changes in mental status and coma[22]. Metabolic acidosis, on the other hand, is an important cause of deaths in severe malaria as it can lead to acute respiratory distress syndrome[23]. Miller *et al* (2002) pointed out that numerous factors have contributed to metabolic acidosis. Cytokine stimulation and decreased clearance by the liver plays a vital role in the elevation of lactic acid production.

3. Control and treatment

The absolute capability for accurate malaria diagnosis before rational therapy deserves increasing attention in the face of rampant drug resistance. Correct and timely therapy of malaria is critical to delay the initiation of resistance and also to save cost on alternative drugs. Laboratory support and access to medical care are often out of reach in most malaria endemic areas of all affected countries. As a result, imprecise clinical diagnosis remains the exclusive basis of self-treatment for most of the febrile populations in malarious regions. Unfortunately, uncomplicated malaria symptoms mimic a large series of tropical diseases which impairs the diagnosis specificity, and this in turn encourages inappropriate use of anti-malarials and consequently increased the requirement of more expensive alternative drugs[24]. Although simple and inexpensive microscopy examination of Giemsa-stained thin or thick smears is regarded as the gold standard for malaria diagnosis, however, training and skills of microscopists, inadequate quality control and maintenance of laboratory equipments that lead to poor microscopy has greatly affected the sensitivity and specificity in routine diagnosis[2].

In the past few decades, alternative laboratory methods have been developed and became available for detection of *Plasmodium* parasites in human patients including enzyme-linked immunosorbent assay (ELISA), quantitative buffy coat centrifugal haematology system (QBC) and polymerase chain reaction (PCR)[2]. Generally, PCR is more repeatable and sensitive than standard microscopy especially in cases with low parasitemia levels or mixed infections. Accuracy of PCR varies with the selection of appropriate primers, collection and storage of samples as well as the extraction methods. Additionally, contaminations, high cost and complicated procedures hamper the regular use of these laboratory diagnosis methods[25-27]. Rapid diagnostic

test (RDT) on the other hand is a test strip or dipstick test that can be completed within 15 minutes with minimum training requirement. The principle of RDT relies on immunochromatography with monoclonal antibodies targeted against particular malarial antigens, histidine rich protein-2 specific for *P. falciparum* (*Pf*HRP-2) or parasite lactate dehydrogenase (pLDH). Today, most RDTs have achieved 95% sensitivity for falciparum malaria. Although easy to use and interpret, the high unit costs and low sensitivity of current RDTs to other *Plasmodium* infections in human will further determine the availability and implementation in resource-poor malarious areas[28,29]. Despite the obvious need for anti-malarial drug development or malaria vaccine development, a parallel commitment to advance diagnostic tools is also a pressing concern for malaria control.

Since the *Plasmodium* parasite was discovered as the cause of malaria in 1880, it has been a struggle to overcome and eradicate the disease. The parasite's complex lifecycle and high metabolic adaptability, selection pressure and the high proliferation rate of *Plasmodium* in humans, as well as social issues in endemic area such as malnutrition, ignorance and poor diagnosis of the disease, have hindered any significant progress[30,31]. The comparisons between three control and treatment strategies of malaria are summarised in Table 1.

Malaria control has always relied on vector control and chemotherapy medications. Routine indoor residual spraying of insecticides was a principal strategy in the prevention of malaria transmission. Yet, insecticides, especially dichlorodiphenyl-trichloroethane (DDT), cause environmental problems and mosquitoes become increasingly resistant[36,37]. To solve such a predicament, insecticide-treated nets or long-lasting insecticide-treated nets (LLINS), to which pyrethroids of low toxicity have been applied, have been rapidly deployed over the past 10 years. The implementation of these vector control measures in malaria-stratified areas, especially in Southeast Asia and Africa, effectively reduced the prevalence and mortality rate by 25%[4,38]. The failure to replace the nets or the improper use of LLINs in their 3-year lifespan has led to a resurgence of malaria cases in most malarious areas. Moreover, the widespread use of pyrethroids and the continuing absence of new chemical insecticides pose increase risk towards the development of pyrethroid-resistance in anopheline mosquitoes[39,40]. Given the remarkable importance of vector control in combating malaria, preserving the susceptibility of mosquitoes to pyrethroids and other currently available insecticides is of critical significance[38].

The promotion and emphasis of chemotherapy intervention, on the other hand, is to provide an efficacious treatment and cure. Highly effective and inexpensive chloroquine and sulfadoxine-pyrimethamine have been important anti-malarial drugs in recent decades. However, the misuse and overuse of these anti-malarial drugs has caused the devastating emergence and spread of *P. falciparum*-resistant strains from Asia to all areas with predominant *P. falciparum* infection[4,35,39]. Therefore, new anti-malarial interventions in the form of artemisinin combination therapies (ACTs) that have shown promising parasitologic clearance and excellent safety profiles were developed. ACTs combine naturally derived artemisinin, which is a fast-acting lactone endoperoxide, with a drug such as amodiaquine or lumefantrine to enhance its efficacy and longer-lasting therapeutic effects[39]. For replacement of existing ineffective anti-malarial drugs, ACTs are now the first-line treatment recommended by the World Health Organization. Despite their recent introduction, artemisinin therapies have actually been available and utilised as monotherapies in western Cambodia for more than 30 years. Extensive usage combined with the antigenic variation of the *Plasmodium* parasite had contributed to the development of artemisinin resistance in *P. falciparum* along the Cambodia-Thailand border[35,40]. The emergence of such resistance poses a dangerous threat for global malaria control, as there is no alternative class of anti-malarial drugs available for substitution. This increases the possibility that artemisinin-resistant strains of *P. falciparum* will soon strike the world and thwart the plan to globally eradicate malaria by 2015. Hence, novel concepts and rationally designed tools are

Table 1
Adoption of key malaria control strategies and its outcome.

Control strategies	Regions adopted	Outcome
Insecticide residual spraying (IRS)	Africa, Americas, Europe, South-East Asia, Eastern Mediterranean andWestern Pacific[34]	– Mosquitoes killed and repelled[32] – Prevent seasonal increases in transmission[32] – Insecticide resistance developed[32-33] – Alleged safety and environmental hazards[34]
Insecticide treated nets (ITNs or LLINs)	Africa, Americas, South-East Asia, Western Pacific and Eastern Mediterranen[34]	– Regular usage reduced all-cause mortality rates in children[33,34] – Low toxicity to humans[38] – Rapid and durable effect[38] – Coverage and sustainability problems[34] – Emergence of pyrethroids resistance[33-,34, 38]
Chemotherapies with ACTs	Africa, Americas, Eastern Mediterranean, South-East Asia and Western Pacific[34]	– Treatment for chloroquine-resistant *P. falciparum* and *P. vivax*[34,35] – Improved treatment efficacy[35] – Oral artemisinin-based monotherapies increase risk of resistance development[34,35]

urgently needed to strengthen the arsenal to combat malaria as well achieve eradication.

4. Mechanisms of anti-malarial drug resistance

Chloroquine is an anti-malarial drug that once saved millions of lives, but its resistance has also created massive challenges towards malaria control. Single, non-synonymous nucleotide substitutions at approximately 16 positions on the *pfcrt* transporter gene with one significant amino acid change in codon 76, Lys→Thr (K76T), have been observed in chloroquine-resistant *Plasmodium* parasites[30,36]. The mutation in this gene that exhibits drug-resistant properties is accompanied by a set of at least three other changes in transmembrane domain 1, 4 and 9 to maintain the normal transporting function of CRT protein[30,41]. Meanwhile, polymorphisms in the energy-demanding glycoprotein pump *pfmdr1* were reported as a major determinant in increasing *Plasmodium* resistance to quinoline- or methanol-based drugs such as mefloquine and lumefantrine, especially in malaria-endemic Asia countries[42,43].

In countries where chloroquine-resistant malaria is prevalent, anti-folate drugs that interfere with the *Plasmodium* spp. folate pathway are commonly used as the first-line treatment. Both dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) are crucial catalysts for the conversion of dihydrofolate to fully reduced tetrahydrofolate, a co-factor essential in one-carbon transfer reactions in DNA, RNA and amino acid synthesis. Thus, the lethal effect of DHFR and DHPS inhibitors including pyrimethamine, cycloguanil and sulfonamides on *Plasmodium* spp. reaches its climax in the erythrocytic schizont stage during which DNA synthesis is arrested[44].

Mutations in the *dhfr*- and *dhps*-coding genes have also been determined to contribute to anti-folate resistance. Point mutations in *pfdhfr-ts* at codons 108 (S108N), 51 (N51I) and 59 (C59R) have been suggested to be allelic variants that confer significant pyrimethamine resistance[45,46]. Polymorphism I164L found in *dhfr* has also caused alarm in South America and East Africa recently. Ominously, the accumulation of all these mutations creates completely resistant mutants against anti-folate, intensifying the burden of selection pressure and the spread of resistance worldwide[47].

Similar mutation patterns were found in the highly conserved *dhps* region that compromise sulfa drug efficacy. Codon changes at S436A/F, A437G, A581G and K540E are common in Southeast Asia and sub-Saharan Africa. A similar situation exists for the mechanisms of *in vitro P. falciparum* DHFR and DHPS inhibitor resistance; the presence of multiple mutations is directly proportional to the degree of drug resistance[48]. Since artemisinin, which comes from an ancient Chinese herb called qinghaosu (*Artemisia annua*), was discovered, considerable debate on the mechanisms of its action has occurred. Several studies have proposed that artemisinin might possess more than one mode of action by targeting haemozoin, parasite mitochondria or translationally controlled tumour protein, but none of these mechanisms have been convincingly proven to be of functional relevance to the anti-malarial activity of artemisinin[49-51]. *Pf*ATP6, a parasite-encoded sarco/endoplasmic reticulum Ca^{2+}-dependent ATPase has recently been proposed to be a key player in artemisinin activity[52]. With respect to the expression of *Pf*ATP6 in oocytes of African clawed frog (*Xenopus laevis*) that commonly studied as model organism, mutations in L263 residue near the *Pf*ATP6 binding site exhibit reduced susceptibility to artemisinins[51,53].

Substantial progress has been made in uncovering the causative mechanisms of anti-malarial resistance; it is not surprising, however, that no conclusion can yet be drawn. Point mutation and copy number variations for several key genes have been established as the vital factors that affect drug binding and transport. The host defence mechanism and variability in drug metabolism will also influence therapy outcomes[54]. Ideally, a broad spectrum of epidemiologically tailored combination therapies will offer protection against anti-malarial resistance and may well contain the spread of resistant alleles between endemic regions or even across continents.

5. Current status of malaria vaccine development

There is a general agreement that development of a safe, highly efficacious and affordable malaria vaccine would be a transformative tool that closes the gap left by other interventions[55]. Increased funding, greater awareness, and the discovery of *Plasmodium* parasite and *Anopheles* vector genome sequence have reinvigorated the development of new antigens and vaccine technologies. Considerable progress in the development of pre-erythrocytic and blood stage vaccines or even combination of multi-stage vaccines are on the move from laboratory to the real world. Depending on the forthcoming full trial results in 2014, RTS, S/AS01 vaccine is the first and only candidate vaccine that has managed to reach large-scale phase III clinical testing in seven African countries and WHO recommendation for licensing may be expected in 2015[56]. By targeting the pre-erythrocyte stage of the parasite, the sub-unit RTS, S vaccine is constituted of a fusion of recombinant circumsporozoite (CS) protein with antigen S of the hepatitis B virus formulated with a potent adjuvant. The protective efficacy and safety profile of RTS, S was reported to be consistent and promising by reducing the rate of all episodes of clinical malaria by 51% in Kenyan children aged 5-17 months[57].

Stimulation of a strong antibody response is a strength of the RTS,S vaccine, but Th1 cellular immunity has always been the comparatively weaker component. Th1 responses are the primary defence against intra-cellular pathogens through induced production of nitric oxide by IFN-γ and through cell-mediated cytotoxicity[58]. Underactive Th1 capability and overactive Th2 activity characterize Th-cell imbalance in vaccinated individual. With resulting failure to generate powerful Th1 response, individual is still

susceptible to the disease and the infected cells cannot be completely destroyed[59]. While RTS,S gives a better survival chance to the most vulnerable part of the population, it is clear that more has to be done in order to raise the bar for high expectations of a malaria vaccine. In support of this view, heterologous prime-boost vaccination is a rational approach for improving efficacy induced by RTS, S/AS01 alone[55,60,61]. Most recently, a pre-erythrocytic vaccine combination utilizing RTS,S/ AS01 and a non-replicative adenovirus serotype 35 vectored CS has been investigated extensively with interest to expand the possibility of developing a more efficacious second generation vaccines. This prime-boost regimen capitalizes on the priming role elicited by adenovirus 35 vector to enhance cell-mediated Th1 responses, followed by RTS,S booster that improve the magnitude and duration of both cellular and humoral arms of the immune system[60,62]. In preclinical trial the protective anti-CS immunoglobulin response remained potent with dramatically high levels of IFN-γ $CD8^+$ T cell responses[63,64]. Based upon these findings, a multicenter Phase 1/2a efficacy study has began in August 2011 to further examine this potential prime-boost combination in healthy malaria-naive humans[65]. Using the knowledge of DNA-based technology, an electroporation administered DNA vaccine EP1300 which contains polyepitopes with linker sequence of four various pre-erythrocytic antigens, CS, SSP2/TRAP (thrombospondin-related adhesion protein), liver-stage antigen 1 (LSA-1) and exported protein 1 (Exp-1) has started their Phase 1a clinical assessment in the United States from 2010. Few further details are available but the immunogenicity and durability for prophylactic vaccination in humans deserve attention. The progress of this vaccine also touches on the important question of how to ensure the safety and tolerability of DNA-based multiple epitopes approaches[66].

Presently, clinical development of second class malaria vaccine through identification of blood stage antigens that induce antibodies with similar specificities to immunoglobulin preparation during naturally acquired immunity has gained momentum[67,68]. Analysis of sera from immune adults from endemic regions as well as those from passive transfer experiments contributes to the discovery of merozoite surface protein 3 (MSP3) and glutamate rich protein (GLURP) as potent antigens that induce anti-malarial immunity. GMZ2 is a recombinant protein fused with parts of *P. falciparum* MSP3 and GLURP, expressed in gram positive *Lactococcus lactis* and adjuvanted with aluminium hydroxide. It remains unresolved how antibodies against MSP3 and GLURP might exert its anti-parasitic activity, however recent hypothesis centred on the association of antibody dependent cell-mediated inhibition (ADCI) as well as the interaction between the antibody, parasite and monocytes[69,70]. Recent clinical phase II b efficacy trial of other asexual blood stage vaccine candidates such as apical membrane antigen 1 (AMA-1) and FMP-AS02A has failed to protect naturally exposed individuals due to the strong selective pressure exerted by human immune system[71,72]. GMZ2 is the latest series of blood stage vaccine candidates that are now ready to enter phase II b trial. Previous phase I clinical trial in both German malaria naive adults and malaria-exposed Gabonese individuals exhibits fine safety, tolerability and immunogenicity. Furthermore, GMZ2 vaccination also induced potent antibodies and memory B-cells in response to both of its antigenic components. A multi-center phase II b studies are now underway to determine the feasibility and efficacy of GMZ2 targeting infants and children in endemic countries[67,68]. The malaria vaccination is progressing rapidly from the pre-clinical stage to clinical trials. The safety and efficacy issue, however, is an important consideration before its benefits can be realised.

6. Genetic engineering

Genetic manipulation is one of the greatest breakthroughs

Table 2
Advantages and disadvantages of genetic engineering technologies for malaria control.

Technologies	Advantages	Disadvantages
Genetically modified artemisinin	– Substitute conventional *A. annua* leaves extraction[76,77] – Enhanced artemisinin production through genetically modified microbes[77] – Less impact to the environment[76]	– Require multiple processes to obtain desired products[77] – Yield optimization and industrial scale-up are necessary[77]
Malaria vaccine	– Safety and tolerability[80] – Modifiability[79] – Reduced risk of malaria by provide partial protection[56]	– Relatively weak Th1 response[80] – Require prime-boost regimen to increase efficacy[60]
Malaria proof mosquito	– Reduced lifespan and reproduction rate of infected mosquitoes[81] – Reduced transmission of *P. falciparum* malaria[81]	– Resistance towards the anti-pathogen traits[83,84] – Occurrence of mutagenesis particularly mutant mosquitoes[83,84]
Transgenic fungi	– High specificity and efficacy[85] – Environmental friendly[85,86] – Wide applications[85,86] – Minimized development of insecticide-resistance[85]	– Restricted by regulatory and safety issues[90] – Require numerous laboratory trials and long-term observation[90]

in recent research history, along with the completion of Human Genome Project. The advances made possible by genetic modification have rekindled the hope for control and alleviation of numerous human diseases[73]. The potential for genetically modified organisms (GMOs) as a control strategy for vector transmission diseases such as malaria has been sustainably driven by the selection pressure of the parasites and the inadequacy of current control strategies[74]. The implementation of recombinant DNA technologies in malaria control is intended to promote human health while investigating the cause of recurring malaria attacks especially in sub-Saharan Africa. Table 2 summarises the advantages and disadvantages of various genetically engineered technique designed for malaria control.

7. Genetically engineered artemisinin

Artemisinin, a lactone endoperoxide isolated from glandular trichomes on the leaves of *Artemisia annua* L. is now in high demand as an anti-malarial drug. But limited extracts from *Artemisia annua* have led to a supply shortage, and its high cost hinders the global distribution of ACTs, especially in Africa where malaria is endemic[75]. The yield of artemisinin compounds greatly depend on the quantity and age of leaves. Young leaves tend to have very low quantity of artemisinin but gradually increased while leaf developed and senesced suggesting that artemisinin increases and accumulates as the glands reach physiological maturity. The sequestration of artemisinin in glandular trichomes further explains why artemisinin is not detected in roots and side stems of the plant that do not bear glands[76]. With the aid of genetic engineering, high titres of microbially sourced artemisinic acid were produced through cloning with increased expression of an artemisinin biosynthetic gene obtained in *Saccharomyces cerevisiae* and *Escherichia coli* recombinants. The oxidation of amorpha-4,11-diene performed by amorphadiene synthase enzyme available from *Artemisia annua* and a novel cytochrome P450 monooxygenase play roles in the engineered mevalonate pathway that produce artemisinic acid[77,78]. As the artemisinic acid is transported and remains outside the transgenic microbes, the desired product can thus be obtained subsequent to purification and biotransformational processes. However, yield optimisation and industrial scale-up will be essential in order to fulfil the global requirement for artemisinin and its derivatives, as well as to reduce the current cost burden of ACTs[77].

8. Malaria-proof mosquito

The complexity of the *Plasmodium* parasite lifecycle and the rapid reproduction of the mosquito vector greatly impact malaria eradication. The malaria-proof mosquito, a novel weapon resulting from genetic engineering to combat malaria infection in future, has been developed. Transmission of *P. falciparum* malaria was reduced by activating Akt signalling proteins in *Anopheles* mosquito. The Akt protein is important in the regulation of extensive cellular processes including glucose metabolism, apoptosis and antioxidant synthesis; to physiological mechanisms such as reproduction and insulin production in the mosquito mid-gut[81]. Over-expression of Akt in the mid-gut of *Anopheles stephensi* was reported to significantly reduce the intensity of *P. falciparum* by strengthening the mosquitoes' innate immunity. Therefore, the transmission of infective sporozoites through anopheline mosquitoes can be eliminated[81,82].

Despite the experimental success, there are two main hurdles obstructing the release of genetically modified mosquitoes for field trials. First, the malaria-proof gene must be given an evolutionary advantage over the natural population of the insects to compete with and displace them in the nature. Furthermore, the response of the *Plasmodium* parasite to the anti-pathogen traits varies. The *Plasmodium* parasite might negate the suppressive or killing effect of the anti-pathogen trait and develop resistance with its extensive surface antigen diversity[83].

Second, ethical and community challenges exist to the release of genetically modified insects. Uncertainties in terms of efficacy and safety of the genetic control intervention might provoke widespread discussion and serious public mistrust. Although public perception towards malaria-proof mosquitoes remains open and pragmatic, the safety and efficacy of the transgenic arthropods need to be proven through multiple trials, as well as long-term monitoring on the impact of malaria epidemiology[84].

9. Transgenic fungi

A specific, efficacious and environmentally friendly biopesticide employing genetically engineered fungus could be used to combat malaria transmission when pyrethroid-resistant mosquitoes began to threat the effectiveness of current pesticide. Transgenic anti-malarial fungus is engineered using the entomopathogenic fungus, *Metarhizium anisopliae* (*M. anisopliae*). The fungal spores infect mosquitoes directly via cuticle attachment and colonisation, eventually penetrating into the haemolymph for proliferation while producing a mixture of organic compounds that cause internal damage, nutrition depletion and eventually mosquito death[85,86].

M. anisopliae fungi have been manipulated to express four respective genes that could limit the transmission of *P. falciparum* in *Anopheles gambiae*[85]. Salivary gland and mid-gut peptide 1 (SM1) binds specifically to the mid-gut epithelium and surface of the salivary glands, thereby inhibiting the invasion of sporozoites by competing essential ligands with *Plasmodium*[87]. The insertion of a synthetic $[SM1]_8$ gene that expressed eight recurring SM1 peptides in *M. anisopliae* demonstrated a 71% sporozoite reduction in *Plasmodium*-infected mosquitoes. Subsequent to the

genetic manipulation, *Pf*NPNA-1, a recombinant human monoclonal antibody that binds to the *P. falciparum* surface circumsporozoite protein is capable of reducing sporozoite counts by 85% via sporozoite agglutination. In addition, the insertion of antibacterial peptide scorpine, which resembles hybridisation between defensin and cecropin, also expressed 90% sporozoite inhibition through its anti-plasmodial effect during the sporogonic stage. Co-inoculation of mosquitoes with spores containing the scorpine + $[SM1]_8$: scorpine transformed gene showed significant sporozoite density reductions in salivary glands by approximately 98%[88,85].

Notably, the toxins and transgenes expressed by the fungi were suggested to undermine the mosquito resistance mechanism, which consequently improved the efficacy of the pesticide towards resistant mosquitoes. The slow speed at which the genetically engineered fungus kills allowed the *Anopheles* mosquitoes to undergo several reproduction processes before death, enabling the susceptible-related genes to dilute the resistance gene in the next generation. The development of selection pressure for insecticide-resistance can hence be minimised, and there is the possibility of evolution-proof transgenic fungi[86,89-94]. *Metarhizium* has wide applications in arthropods and various mosquito strains, and this, along with the multiple transgene and antimicrobial expression, further facilitates its usage in integrated vector management.

No field trial has been reported for this novel technique so far, but extensive laboratory testing is still in progress. The release of transgenic pathogens in the field is not only challenged by related safety issues and the tangible health benefits it could cause, but equally important are the regulatory and ethical issues. Generally, *Metarhizium* only affects its target and several closely related species; they are considered low risk and have shown no adverse effects in laboratory testing[95]. In accordance with genetic modification, it is less clear whether the malaria parasites will evolve to become 'super resistant'. Therefore, as with any other genetically modified organisms, the implementation of transgenic fungi as an anti-malaria tool remains uncertain.

10. Conclusion

Over the past few decades, the deployment of chloroquine and DDT were once the key tools to moving towards malaria eradication. However, the decline in political and financial support for malaria control strategy along with the spread of resistant strains of the *Plasmodium* parasite and *Anopheles* mosquito have resulted in a resurgence of malaria globally. Polymorphisms of the gene encoding important proteins for drug binding and transport activity in *Plasmodium* spp. have been recognised as the cornerstones that induce anti-malarial drug resistance. Gene amplification and point mutations have been established as key players in the relationship of *pfcrt* and *pfmdr1* genes to chloroquine resistance. Moreover, several field studies have suggested that reduced anti-folate susceptibility in *P. falciparum* is conferred by point mutations of the gene encoding DHFR and DHPS enzymes involved in the folate biosynthesis pathway. The principal mechanism for artemisinin and related compounds remain poorly understood; more important, however, is the emergence of artemisinin resistance, which has undermined the control of malaria. Assessment and identification of polymorphisms in relevant sequences for early detection of resistance, along with the translation of essential research into effective, clinically-proven drug formulations, has rekindled the hope of delaying the emergence of drug-resistant parasites[96-98].

Today, efforts and resources to roll back malaria are moving forward. Prevention is always better than a cure and vaccines that are capable of preventing and blocking the transmission of malaria infection would have a major impact on malaria eradication[4]. The first malaria vaccine RTS, S is expected be on the market by 2015; with less than 50% efficacy, it is clear that this new tool will have hard time accomplishing the ambitious goal of global eradication as proposed by WHO[99]. Extensive research has been focused on designing a rational and effective human vaccine against malaria. Various optimised approaches have undoubtedly contribute to the development of an optimal malaria vaccine that causes strong and long-lasting immune responses. Prime-boost vaccines have shown promise and are currently progressing through the pipeline of clinical testing. Other enhancement strategies such as genetic adjuvants and multi-valent plasmids are underway to amplify the efficacy of malaria vaccines in conjunction with renewed efforts for anti-malarial drug development[82]. Hence, the deployment of an efficacious malaria vaccine still requires some time to be realised. On the other hand, novel genetic control mechanisms may be employed to achieve eradication. Potential public resistance and ethical issue against these genetic control trials is the main obstacle towards the implementation of new interventions that could have impeded malaria transmission and save millions of lives. Open-field releases of malaria-proof mosquitoes and transgenic fungi will encounter multiple challenges in the coming years.

Current technologies have advanced us to the identification of the genome of the *Plasmodium* parasite and *Anopheles* vector, but there is still a big gap in understanding the complex biology of the parasite, its correlation with the human immune system and its diverse antigenic variation[100,101]. The future of malaria control therefore relies on the discovery of new interventions while prolonging the lifespan of existing tools.

Conflict of interest statement

We declare that we have no conflict of interest.

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