Perspectives in the control of infectious diseases by transgenic mosquitoes in the post-genomic era – A Review

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Arthropod-borne diseases caused by a variety of microorganisms such as dengue virus and malaria parasites afflict billions of people worldwide imposing major economic and social burdens. Despite many efforts, vaccines against diseases transmitted by mosquitoes, with the exception of yellow fever, are not available. Control of such infectious pathogens is mainly performed by vector management and treatment of affected individuals with drugs. However, the numbers of insecticide-resistant insects and drug-resistant parasites are increasing. Therefore, inspired in recent years by a lot of new data produced by genomics and post-genomics research, several scientific groups have been working on different strategies to control infectious arthropod-borne diseases. This review focuses on recent advances and perspectives towards construction of transgenic mosquitoes refractory to malaria parasites and dengue virus transmission.

Key words: transgenic mosquitoes - post-genomic - effectors molecules - malaria - dengue virus - *Aedes aegypti* - *Anopheles*

Worldwide a variety of viruses, protozoans and helminths are transmitted to humans by mosquitoes, and these are responsible for more than 2 million deaths per year. Malaria transmitted by anopheline mosquitoes, leishmaniasis by phlebotomines, trypanosomiasis by triatomines, dengue and yellow fever transmitted mainly by *Aedes aegypti*, are examples of these arthropod-borne diseases, which impose major economic and social burdens on affected populations.

Today, management of human and veterinary arthropod-borne diseases is principally through the use of drugs and vector control. Despite many efforts, there still are no effective vaccines against malaria, dengue, leishmaniasis, etc. In recent years, genomics and post-genomics research have produced significant data that has been employed for the development of new tools to eliminate arthropod-borne infectious pathogens or to block their transmission. These studies performed by different scientific groups, globally-distributed, include investigation of new drugs to kill the parasite, development of vaccines capable to block infection in the host or inside the vector, searches for new insecticides to eliminate the arthropod, and genetic manipulation of vectors in order to abrogate its ability to be infected by parasites.

In this scenario research groups have been working on construction of transgenic mosquitoes refractory to malaria parasite and dengue virus transmission. Research on transgenic mosquitoes to control malaria and dengue fever will be the subject of this review.

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ANOPHELES SSP. AND *AEDES AEGYPTI* CONTROL: PRESENT SITUATION AND GENETIC MANIPULATION AS AN ALTERNATIVE STRATEGY

Vector control remains generally the most effective method to prevent malaria and dengue transmission. Insecticides were responsible in the past for malaria elimination from a variety of countries. *Ae. aegypti* was eradicated from most American countries in the 1950s decade with the use of insecticides during a continental campaign initiated in 1916 by the Rockefeller Foundation and followed up by the Pan American Health Organization in 1940-1960 (Vasconcelos et al. 1999). However, socioeconomic issues including population growth, increased urbanization without planning, lack of sanitation, deforestation, rapid movement of pathogens and vectors by jet travel, occurrence of drug-resistant parasites and insecticide-resistant mosquitoes, have been contributing to re-emergence and difficulties in controlling important arthropod-borne diseases.

Nowadays, insecticide spray use is an important strategy to control malaria transmission (WHO 2006), and the use of insecticide-impregnated bed nets is an effective method to decrease malaria transmission in Africa (Hawley et al. 2003). Re-emerging dengue fever is prevented only by vector control with insecticide and community-based strategies since no drugs or vaccine are available for this disease. Despite many efforts, malaria and dengue control is not effective, and the morbidity and mortality associated with these diseases are overwhelming: billions of people are now at risk for infection. Thus, the increasing public health importance of these pathogens, the failure of conventional approaches to control them, and the lack of alternatives in the face of pesticide-resistant vectors and drug-resistant pathogens, demonstrate the need of novel and efficacious control strategies for these diseases.

A relatively new approach to combat malaria and dengue fever consists of genetic manipulation of mosquito vectors to impair their ability to be infected by and trans-

mit *Plasmodium* or dengue virus. At about 40 years ago, Knipling proposed the first initiative to use genetically-modified mosquitoes to combat a disease which consisted of the field release of sterile males to decrease arthropod-borne pathogens transmission or insect population (Knipling 1959, 1962). This approach, sterile insect technique (SIT), uses radiation-sterilized males released in the field to compete with wild ones. Females that have been mated with irradiated mosquito will not produce descendents. With SIT employment was possible to eliminate the New World screwworm, *Cochliomyia hominivorax* from South of US, Mexico, and Central America (Wyss 2000). However, when tested with *Anopheles*, the method showed promise in the laboratory (Andreasen & Curtis 2005), but was not efficient in field experiments (Benedict & Robinson 2003). Problems included difficulties in the separation of male insects by physical methods (Alphey & Adreasen 2002); poor mating competitiveness of the sterile male compared to the wild insect (Benedict & Robinson 2003); and the occurrence of emerging species with different ecological preferences and significant prezygotic reproductive isolation (Stump et al. 2005).

The approach to generate a transgenic mosquito unable to transmit pathogens emerges as an alternative strategy. Recently, bioinformatics analyses of the *An. gambiae* genome has enabled identification of sex-specific genes (Scali et al. 2005), which can be used to make transgenic mosquito lines expressing sex-controlled genes or sterile males by specific gene manipulation. How to generate efficient mosquitoes that are completely unable to transmit certain pathogens and how to spread and control this phenotype in natural populations are some of the difficult challenges to overcome in order to control dengue fever and malaria using pathogen refractory transgenic mosquitoes.

AVAILABILITY OF TECHNOLOGICAL TOOLS TO CONSTRUCT A TRANSGENIC MOSQUITO

To produce a transgenic vector refractory to a pathogen, several technological features are necessary: efficient transformation and driver transfection systems, suitable reporter genes, efficient tissue- and developmental-specific promoters, appropriate anti-parasite effectors genes and efficient microinjection techniques. Until now the most efficient method to stable transform a mosquito is the injection of the transgene construct into early embryos, a technique imported from *Drosophila* and that has low efficiency and is time consuming.

VECTOR TRANSFORMATION SYSTEMS

In the last decades, different stable and transient vector transformation systems to introduce a foreign gene into the mosquito have been developed: virus transducing systems, transposable elements, and endosymbionts.

Virus transducing systems in mosquitoes are mainly performed with the Sindbis virus (SIN). Sindbis virus from genus *Alphavirus* and *Togaviridae* family has a positive-sense RNA genome 11,703 nucleotides in length, and nonstructural and structural proteins encoded from two different promoters. The nonstructural proteins, including RNA-dependent RNA polymerase (RDRP), are encoded by and translated from the 5´-end two-thirds of the genome. SIN structural proteins are encoded in the 3´-end one-third of the virus RNA and are translated from a subgenomic mRNA transcribed intracellularly by the RDRP from an internal promoter (Strauss & Strauss 1994). The SIN natural cycle of infection involves birds and *Culex* mosquitoes (Taylor et al. 1955), but it is also able to infect *Aedes* and *Anopheles* mosquitoes and a broad range of vertebrate animal cells (Hurlbut & Thomas 1960, Xiong et al. 1989). Production of a genomic SIN complete cDNA allowed the use of SIN as a vector since infectious viral RNA can be generated by in vitro transcription (Rice et al. 1987). Two types of expression vectors derived from the cDNA SIN clone have been developed: the double-subgenomic SIN (dsSIN) expression vector TE/3´J and the SIN replicon (Olson 2000). TE/3´J has a duplicated internal promoter from which transgenes can be expressed. The SIN replicon has its structural protein genes deleted and this can be replaced by up to 6000 bp of heterologous genetic material. Infective virions are produced directly by injecting in vitro transcribed RNA of TE/3´J into competent eukaryotic cells. Producing the SIN replicon requires co-infection of cells with a helper virus expressing the viral structural genes.

Thus, insect transformation by SIN expression systems emerged as a tool to test genes that impaired pathogen transmission. Most of the experiments in mosquitoes have been done with *Ae. aegypti* and aimed to demonstrate the efficacy of effector molecules such as antisense RNAs for their ability to block dengue, yellow fever, and LaCross viruses transmission (Powers et al. 1996, Olson et al. 1996). The same strategy has been used to characterize single-chain recombinant antibodies as target molecules to block malaria transmission using the experimental model of avian malaria *Plasmodium gallinaceum* infecting *Ae. aegypti* mosquitoes (Capurro et al. 2000). SIN expression systems have also been used to silence expression of endogenous genes for rapid characterization of vector molecules in vivo (Johnson et al. 1999). Thus, SIN expression systems are contributing to the better understanding of the biology of important vectors of human diseases and to characterize target molecules potentially capable of blocking pathogen transmission.

In order to obtain a transgenic mosquito, it is necessary to insert the transgene into the mosquito genome. Furthermore, to be used in the field, the transgene needs to be efficiently spread in the natural population. It is unlikely that reliance on Mendelian inheritance alone could accomplish this because it would require an enormous numbers of mosquitoes. Thus, drive mechanisms such as mobile DNA elements or other shuttles of genetic material that are spread in a non-Mendelian manner (e.g. symbiotic bacteria) have been the tool of choice to produce transgenic mosquitoes (Kidwell & Ribeiro 1992, Curtis & Sinkins 1998).

Transposable elements (TEs), or mobile genetic elements, are integral components of the eukaryotic genomes. Because they have the ability to replicate and

spread in the genome primarily as "selfish" genetic units, TEs can comprise significant proportions of the genome (Hedges & Batzer 2005). Genetic transformation of mosquitoes using transposable elements began with the modification of the naturally occurring transposable *P*-element from *D. melanogaster*. Initially, the *P*-element was tested in *Ae. aegypti* but the integration frequency was extremely low and did not result from the action of the *P*-element transposase (Morris et al. 1989). After that, others DNA transposable elements were searched and tested in mosquitoes and initially two elements were able to transform *Ae. aegypti*: *Hermes* from the *h*AT family of transposons derived from the housefly *Musca domestica* (Jasinskiene et al. 1998) and the *mariner* element *Mos1* from *D. mauritiana* (Coates et al. 1998). Two additional transposition elements have been employed to produce transgenic mosquitoes: *piggyBack* isolated initially from *Trichoplusia ni* and *minos* from *D. hydei*. *piggyBac* vectors transform efficiently *Ae. aegypti* (Kokoza et al. 2001), *An. stephensi* (Nolan et al. 2002), *An. albimanus* (Perera et al. 2002), *An. gambiae* (Grossman et al. 2001, Kim et al. 2004), and *An. fluvialitis* (Rodrigues et al. 2006). The *Minos* element has also been used to transform several insects including the mosquito *An. stephensi* (Catteruccia et al. 2000, Lycett et al. 2004, Yoshida & Watanabe 2006) and its transposition rate is similar to that found for other elements. An advantage for *Minos* is that non-canonical integration events have not been found in mosquitoes as reported for *mariner, Hermes and piggyBac* (O' Brochta et al. 2003). Additional research is needed to understand the behavior of these elements to verify the viability to use *Minos* transgenic insects in fieldwork.

An alternative potential mechanism to introduce an effective gene into a vector population involves the use of intracellular symbiotic bacterium *Wolbachia*, which infect a variety of insect tissues (Beard et al. 1998). An interesting characteristic of *Wolbachia* is its cytoplasmic incompatibility. Only when a male infected with the bacteria mates with an infected female are the eggs viable, and the bacteria are transmitted transovarially to the next generation. This effect leads to increased reproductive success and consequently to a fast dissemination of a transgene since genetically-modified *Wolbachia* could be used to transduce mosquitoes with parasite transmission-blocking genes (Sinkins et al. 1997). This interesting methodology still is not available to anopheline mosquitoes due to the lack of symbiotic bacteria that could be potentially used in these insects. Also, it is not yet possible to genetically transform *Wolbachia* (Christophides 2005).

REPORTER GENES

Earlier attempts to construct a stable transgenic mosquito were set up with insecticide and antibiotic resistance genes as markers (Miller et al. 1987, McGrane et al. 1988, Morris et al. 1989), a strategy imported from research in bacteria. After that, the *cinnabar* gene encoding the kynurenine hydroxylase of *Drosophila* was found to complement a white-eyed mutation in a strain of *Ae. aegypti* (Cornel et al. 1997). Fluorescent markers, such as the green fluorescent protein (GFP) from *Aequorea victoria* (Higgs et al. 1996), replaced the previous ones and have been extensively used. Fluorescence of GFP transformed individuals is observed even in early larval stages, saving time and effort of rearing the non-transgenic insects to adults. GFP constructs driven by the polyubiquitin (Handler & Harrell 1999) and PAX eye-specific promoters (Horn et al. 2000) were used first on *Ae. aegypti* germ line transformation (Pinkerton et al. 2000), and subsequently have been used to transform the germ line of others species of mosquitoes (Ito et al. 2002, Kokoza et al. 2001). More recently, the *Discosoma* sp red fluorescent protein (dsRED) (Matz et al. 1999) was also successfully employed in the transformation of *An. stephensi* (Nolan et al. 2002).

PATHOGEN EFFECTOR MOLECULES

A remarkable number of effectors molecules capable of blocking pathogen transmission in mosquitoes have been investigated. For example, several mechanisms exist for interfering with different developmental stages of malaria parasites in mosquitoes (Nirmala & James 2003). Two groups of peptides have been tested against *Plasmodium* ssp and show interference on transmission. The first group corresponds to competitor peptides that bind to salivary gland receptors blocking sporozoite invasion (Ghosh et al. 2001, Myung et al. 2004). The second group consists of peptides that might have parasiticidal effects in mosquitoes such as cecropins, magainins and defensins or hybrid peptides such as the cecropin-like peptides and scorpine (Gwadz et al. 1989, Shahabuddin et al. 1998). Most of these natural peptides, at high concentrations, are capable of reducing the levels of sporogonic stages of malaria parasites. Arrighi and collaborators (2002) using natural and synthetic peptides showed that three short novel hybrid peptides can interfere with oocyst development. The authors also demonstrated that hybrid peptides consisting of random coils and turns were particularly active against the sporogonic stages of *P. berghei* and *P. yoelli nigeriensis*.

Using Sindbis expression system, several heterologous proteins and anti-sense RNAs with anti-parasite properties have been investigated in *Ae. aegypti* (Olson 2000, Sanchez-Vargas et al. 2004). Single-chain antibody fragments (scFv) comprising fused heavy-chain and light-chain variable regions of monoclonal antibodies expressed as the product of a single gene, have been used to block malaria parasite development. For example, a scFv specific to the malarial circumsporozoite protein (CSP), reduced by 99% the number of *P. gallinaceum* sporozoites in salivary glands of *Ae. aegypti* (Capurro et al. 2000). Further, Olson and collaborators (1996) showed inhibition of dengue 2 (DEN2) in mosquitoes *Ae. aegypti* transiently expressing an antisense RNA corresponding to a fragment of 567 bases of the premembrane protein from DEN2. Using the same strategy and the employment of different antisense RNAs, the same research group demonstrated the inhibition of DEN replication in mosquito and mosquito cells by a mechanism similar to the post-transcriptional silencing or RNA interference (RNAi) (Adelman et al. 2001, Travanty et al. 2004, Franz et al. 2006).

Microarray-based transcription analysis of the murine malaria model *An. stephensi* and *P. berghei* corresponded to an interesting and elegant approach to characterize molecules involved in the parasite-arthropod vector interactions. By this methodology, numerous novel genes were identified during malaria midgut invasion, oocyst differentiation and ookinete development from both parasite and mosquito origin (Abraham et al. 2004, Srinivasan et al. 2004). One novel molecule identified with this methodology corresponded to the gene encoding a *Plasmodium* surface protein WARP, with a von Willebrand factor A-like adhesive domain WARP that is expressed only in ookinetes and early oocysts (Yuda et al. 2001, Abraham et al. 2004). It was shown that anti-WARP polyclonal antibody inhibits strongly (70-92%) *Plasmodium* development in the mosquito, making it a candidate antigen for transmission blocking vaccines or for construction of refractory mosquitoes expressing recombinant antibodies rose against this protein (Abraham et al. 2004). The same approach has allowed the identification of transcription profiles of both host and parasite organisms with temporal correlation between processes such as *Plasmodium* invasion of the midgut epithelium, *Anopheles* immune responses to *Plasmodium* infection, and apoptosis and expulsion of invaded midgut cells from the epithelium (Xu et al. 2005). These data can be employed to help in the construction of a malaria refractory mosquito. Transcriptome experiments envisioning identification of specifically expressing transcripts from *Ae. aegypti* in response to dengue infection are in progress (pers. commun. of Margareth L Capurro).

PROMOTERS

Expression of a transgene in the mosquito also requires the use of an appropriate promoter. The ideal promoter should enable the expression of a foreign gene in the tissue of interest and at a proper time. According to the pathogen life cycle inside the arthropod vector the foreign gene would be expressed in salivary glands, hemolymph, midgut, Malpighian tubules or thoracic musculature.

Salivary gland promoters for the *maltase-like I* (MalI) (James et al. 1989) and *Apyrase* (*Apy*) (Smartt et al. 1995) genes have been able to direct expression of recombinant firefly luciferase in *Hermes*-transformed *Ae. aegyti* in the tissues and at time of expression of the corresponding endogenous genes (Coates et al. 1999). However, expression of the transgene driven by these promoters was too weak to be used in the construction of transgenic mosquitoes. In order to find a suitable promoter to express a transgene in the salivary gland of mosquitoes at a high concentration, Yoshida and Watanabe (2006) isolated the anopheline antiplatelet protein promoter (AAPP) from *An. stephensi*. This promoter is activated to a high level after a blood meal and was used to construct an *An. stephensi* transgenic mosquito with the *Minos* element, which expressed highly the DsRed fluorescent protein in the cytoplasm of the distal lateral lobes of the salivary gland (a region preferentially invaded by *Plasmodium* sporozoites). The authors suggest the possibility to use the AAPP not only to produce pathogen refractory mosquitoes but also to deliver vaccine antigens during a mosquito blood meal ("flying vaccinator").

With the aim of expressing a transgene in the gut of mosquitoes, the first site of interaction between the mosquito and pathogens, promoter sequences involved in the regulation of the gene encoding the digestive enzyme carboxypeptidase have been isolated and characterized from *An. gambiae* (Edwards et al. 1997) and *Ae. aegypti* (Edwards et al. 2000). Employing the transposable elements *Hermes* and *Mos1*, Moreira and collaborators (2000), generated transgenic *Ae. aegypti* mosquitoes capable of driving luciferase expression in the mosquito gut epithelium in a robust sex, tissue and stage-specific manner, under the control of both *Ae. aegypti* and *An. gambiae* carboxypeptidase promoters. Furthermore, the *Ae. aegypti* carboxypeptidase promoter was used to construct an *An. gambiae* transgenic mosquito expressing the immune peptide cecropin A in the posterior insect midgut (Kim et al. 2004). Both *Ae. aegypti* and *An. gambiae* carboxypeptidase promoters also have been successfully used to express anti-malarial peptides in *An. stephensi* (Moreira et al.2002, Ito et al. 2002). *P. berghei* oocyst formation was reduced by approximately 80% in transgenic *An. stephensi* mosquitoes expressing the phospholipase A2 (PLA2) protein under control of the *An. gambiae* adult peritrophic matrix protein 1 (AgAper1) regulatory elements (Abraham et al. 2005).

Expression of an anti-parasite transgene in the insect haemolymph can target *Plasmodium* sporozoites pathogen and with this purpose the vitellogenin regulatory sequence obtained from *Ae. aegypti* has been used to drive strong blood-inducible expression of the antibacterial peptide defensin (Kokoza et al. 2000). More recently, the vitellogenin promoter sequence has been obtained from *An. stephensi* and showed to have its expression regulated in a sex-, stage- and tissue specific manner, and therefore is a good candidate to express transgenes in anophelines (Nirmala et al. 2006).

Recently, a complete genome of *An. gambiae*, the most important human malaria vector, has been sequenced by an international consortium (Holt et al. 2002). In parallel, the first mosquito cDNA microarray was constructed with 4000 cDNA expressed sequence tags sequenced from a library prepared from hemocyte-like cultured cell line (Dimopoulos et al. 2000; 2002). These arrays were used to detect genes in the mosquito that are up-regulated during infection with parasites and bacteria (Dimopoulos et al. 2002). These authors showed specific expression of the mosquito genes encoding isocitrate dehydrogenase, dsRNA binding RNAse 3 and a mitochondrial phosphate carrier when RNA source was obtained from mosquitoes infected with malaria but not bacteria. Genomic analysis of these transcripts can in turn contribute to the characterization of promoter sequences that are activated only in the presence of malaria parasites, offering a tool to produce transgenic mosquitoes specifically refractory to malaria transmission.

Moreover, functional genomic studies have enabled identification of diverse *An. gambiae* promoters specifically expressed in different mosquito tissues and

at different time in response to several biological conditions, including blood feeding and infection by *Plasmodium*. Vlachou and collaborators (2005) have shown that 7% of the *An. gambiae* midgut transcripts are differentially expressed during *P. berghei* oocyst invasion. The expression profile of *An. gambiae* females was assessed by microarray analysis in response to blood-feeding, resulting in the identification of three groups of developmentally expressed genes (early, middle, and late). These were classified according to physiological responses: blood feeding, blood digestion, peritrophic matrix formation, egg development and immunity (Dana et al. 2005).

The genome of *Ae. aegypti* mosquito, the major vector of dengue viruses, is almost completed and all sequence data obtained until now is available at the website www.vectorbase.org. *Ae. aegypti* midgut differential expression profile in response to blood feeding over a time course of 72 h also has been performed through the use of microarray technology (Sanders et al. 2003) and is available at the website http://www.etox.ucr.edu/gill/home.htm. More recently, the characterization of transcripts expressed in the fat body of *Ae. aegypti* at 24 h post blood meal was published. These data also provides a basic tool for understanding the processes occurring in the fat body and could identify putative new genes whose promoters can be used to specifically express transgenes in this organ of *Ae. aegypti* (Feitosa et al. 2006).

In addition to genomics, gene expression and DNA microarray technology, pathogen arthropod vectors have been investigated by proteomics, since many types of information cannot be obtained from genes alone. For example, exon-intron structure is difficult to predict with bioinformatics, mRNA is subject to pos-transcriptional regulation as alternative splicing and polyadenylation or can be regulated at the protein translation level. Proteins can also be regulated by proteolysis and compartmentalization. Thus, with proteomic approaches, several biochemical and physiological regulation features can be identified, such as signal peptides, which can be used as tools in the construction of pathogen refractory mosquitoes. Using mass spectrometry to characterize peptides from *An. gambiae*, it was possible to correct and confirm genome annotations and also, novel proteins were discovered (Kalume et al. 2005). Additional experiments seek the identification of differentially-expressed proteins by mass spectrometry of hemolymph from *Ae. aegypti* infected and not infected by the malaria parasite *P. gallinaceum* (pers. commun. of Margareth L Capurro).

SEVERAL TASKS NEED TO BE SOLVED BEFORE A TRANSGENIC MOSQUITO CAN BE RELEASED IN NATURE

Once a pathogen refractory transgenic mosquito is obtained in the laboratory, the next step is its introduction to the environment in order to substitute the specific pathogen susceptible vector population. To reach this objective several aspects have to be carefully investigated in either the wild target population and in the transgenic population. Recent research on the fitness of transgenic mosquitoes, including measurements of longevity and fertility and use of population cages, has been performed. Most of these studies (Riehle et al. 2003, Marrelli et al. 2006a), showed a reduced fitness load of transgenic mosquitoes, when compared to the wild population. Only one transgene, the peptide SM1 expressed in *An. stephensi* did not impose a detectable fitness load. The principal difference of this transgene was a specific promoter that restricted the foreign gene expression to posterior midgut cells for only a few hours after a blood meal and the protein was secreted from the cells, which might have minimized fitness load (Moreira et al. 2004). More recently, the same transgenic mosquito showed to be more fit than sibling nontransgenic mosquitoes when feeding on *Plasmodium*-infected with gametocyte producing parasites (strain ANKA 2.34) but not when maintained on mice infected with gametocyte-deficient parasites (strain ANKA 2.33). This was the first evidence for a selective advantage of transgenic malaria-resistant mosquitoes over nontransgenic mosquitoes (Marrelli et al. 2007). Catteruccia and collaborators (2003) reported a reduced fitness compared to wild type of four different transgenic mosquito lines expressing fluorescent reporter proteins from an actin promoter. Irvin and collaborators (2004) examined the impact of transgenesis on the fitness of *Ae. aegypti* transformed with enhanced GFP gene and two transposase genes derived from the *Hermes* and *Mos1* transposable elements. The authors found that life-table parameters were significantly diminished in transgenic mosquitoes relative to the untransformed laboratory strain.

Reduced fitness can occur due to different aspects of the manipulated vector: inbreeding depression, toxicity of a foreign protein expressed in abundance (Liu et al. 1999); random integration of transposable elements altering important genes; presence of a transposition repressor in the vector population which after several generations could inhibit transposition gradually. This latter aspect is of practical importance because in such cases the transgene(s) can be driven through a population only once (Riehle et al. 2003).

Inbreeding depression is characteristic of the transgenic laboratory mosquito strains since each transgenic line arises from a single fertilized zygote containing transgenic gametes and this characteristic can also reduce mosquito fitness (Taylor et al. 2001).

Ribeiro and Kidwell (1994) and Boete and Koella (2003) developed a theoretical modeling suggesting that absolute absence of fitness load may not be essential for introducing genes into wild population. The same authors also suggested that any released mosquito would need to be nearly 100% refractory to have any impact on malaria transmission. Thus, to obtain such a high blocking capability it will be necessary to construct a transgenic mosquito with multiple refractory genes that, probably, may incur greater fitness costs to the mosquito. However, there is considerable lack of experimental data to corroborate or disprove these models.

The target wild population has also to be analyzed. Locations where releases of transgenic mosquitoes are to occur also need to be well studied so that the transgenic mosquito population can be introduced without disrup-

tion of the surrounding natural environment. Thus, research has to be done to define the populations in the wild that will be the targets of genetic intervention, including size, ecological features, breeding structure, migration characteristics, and number and distribution of genetically distinct vector populations. It is important to stress that in malaria transmission, the ecology of the vectors is complex in most locales. For example, the main African vectors, *An. gambiae* and *An. funestus*, are complexes of seven and nine (at least) species, respectively, with different behaviors and ecology. Furthermore, *An. gambiae sensu stricto* consists of at least two sibling species revealing another level of complexity that is likely to be common within anophelines (Taylor et al. 2001). Additionally, employing the ITS2 sequence as a marker, Marrelli and collaborators (2006b) have analyzed a broad range of Anopheline species from Latin America, showing also a high genetic diversity of malaria vectors from the Neotropical countries, including Brazil. The data obtained by these authors demonstrate the need for research on Anopheline mosquitoes in order to understand its ecology before it can be used to control malaria.

Mosquito mating behavior is another task to be investigated since in the past the low success to genetic control arthropod vectors through the sterile insect technique (SIT) was because of the low mating competence of released sterile males (Benedict & Robinson, 2003).

Another important aspect to be investigated is the biology of the pathogens to be eliminated, principally with respect to the genomic plasticity of malaria parasites and viruses such as dengue. Thus, molecular epidemiology studies should be performed on the pathogen population also, principally if the effector gene is based on parasite gene targets. These problems can be solved in part by transforming mosquitoes with multiple effector molecules but even so, it is important to stress the potential escape from the block and the development of resistant parasites.

A number of other aspects of the eventual release of genetically-modified arthropod vectors of human pathogens must be considered. These include potential environmental hazards, public health risks, and public perceptions. Some governmental organizations developed standards and protocols to guide manipulation of important biological resources involved in human diseases. The NIH and CDC have developed standards for the classification of risk and conditions for work with human disease agents. The US Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS) has established guidelines and an approval process for the release of transgenic arthropod plant pests. While none of these addresses directly the release of genetically-modified arthropod vectors of human disease, they establish precedents for risk assessment and regulation (Hollander 1991, Hoy 1995, Simonsen & Levin 1988). Individual and public health risks must be assessed in both laboratory and pilot field studies, and accurate information must be provided to the public at the earliest possible time (Asner 1990).

FUTURE PERSPECTIVES

A number of technological breakthroughs such as the identification of efficient transposable elements, the finding of suitable transformation markers, the characterization of efficient tissue-specific promoters, the discovery of anti-pathogen effector genes candidates, have been extensively explored by genomics, microarray techniques, proteomics and bioinformatic analyses. Now, scientific efforts have to be performed in order to test all new molecules characterized in a short period of time. Effector transgenes that confer full resistance against *Plasmodium* or dengue virus have yet to be developed. Difficulties in obtaining such molecules may result from the high genomic plasticity of parasites, which may escape from human and arthropod immune systems. The biology of an ideal effector trait must be fully understood before it is put in use, as unintended phenotypes leading to irreversible alterations of the mosquito biology and potentially harmful ecological effects could be disastrous. Thus, several tasks have to be solved before we can expect to release transgenic insects to the environment to block human diseases.

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