Transgenesis, Paratransgenesis and Transmission Blocking Vaccines to Prevent Insect-Borne Diseases

Marcelo Ramalho-Ortigão and Iliano Vieira Coutinho-Abreu

Kansas State University,
Department of Entomology
USA

1. Introduction

Insect-borne diseases are responsible for severely affecting human life around the world, causing significant morbidity and mortality. Malaria alone is responsible for 1-2 million deaths annually, and approximately 300 million are at risk of becoming infected. Insect-borne diseases also are responsible for an estimated 50% of all neglected tropical diseases (NTDs), which affect over 1 billion people – one sixth of the world population – and include such diseases as leishmaniasis, filariasis, Chagas’ disease, African trypanosomiases, onchocerciasis, schistosomiasis, trachoma and others. Such NTDs cause severe morbidity and are frequently referred to as “poverty causing diseases”. The lack of effective vaccines or drugs for many insect-borne diseases makes control mainly dependent on insecticides. However, the appearance of insecticide resistance makes control mainly dependent on insecticides. Moreover, the appearance of insecticide resistance requires the development of new strategies to reduce pathogen transmission in the field [1]. Among the research themes with potential to generate new tools to control vector borne diseases, major efforts have been carried out to establish transgenesis, paratransgenesis, and transmission-blocking vaccines (TBVs) as new weapons to reduce vector competence.

While vector competence encompasses the intrinsic genetic factors that define the ability of a vector to transmit a pathogen (and it is a component of vectorial capacity), vectorial capacity is a measurement of the efficiency of vector-borne disease transmission (i.e., total number of infective bites delivery to a single host in one day), and influenced by vector density and longevity [2]. Regarding vector competence, several molecular techniques, such as quantitative trait loci (QTL) mapping, and gene knock-down, can be used to identify the intrinsic genetic factors (i.e., molecules expressed by the vector) involved with the ability of vectors to transmit pathogens. Molecules involved in vector competence can be directly targeted by antibodies (as in the case of TBVs), or overexpressed in transgenic insects or paratransgenic symbionts in order to reduce pathogen development and transmission.

In the late 1990’s, the establishment of stable lines of genetically modified mosquitoes opened new avenues for studying molecules with potential to reduce vector competence [3]. Transgenic mosquitoes expressing dsRNAs (i.e., to induce RNAi pathways) targeting RNAs...
associated with mosquito immune-related proteins [4], or overexpressing microbial peptides [3, 5], or expressing a truncated transcription factor to generate a dominant-negative phenotype [6] were generated in order to investigate the role of these molecules in vector competence. Understanding such mechanisms is considered a pre-requisite for the development of molecular strategies to control vector-borne diseases.

For a heterologous protein (exogenous protein introduced into a disease vector) to be used to reduce vector competence, a gene drive mechanism is required to spread the gene encoding the protein throughout the targeted insect vector population. A gene drive system is spread within a population by increasing its frequency to ratios greater than those expected by traditional Mendelian rules. Thus, the combination of a given transgene (expressing a heterologous protein) with a gene drive system also can increase the frequency of the transgene in a population. Gene drive systems currently known include transposons, homing endonuclease, engineered under-dominance, meiotic drive, endosymbionts, and Medea element [7, 8]. Yet, only transposons are currently available to be used in the genetic transformation of insect disease vectors [8], and Wolbachia endosymbionts are thought to be a feasible way to spread paratransgenic symbionts in natural populations of these insect vectors [9].

The use of transposons to generate stably transformed insect germ lines, i.e., with exogenous DNA inserted into the genome and capable of being transferred into following generations (as depicted in Figure 1A), is well established [10] for a couple of insect vector species. Different species of mosquitoes, representing *Aedes* [11-14], *Anopheles* [15-24], and *Culex* [25] genera, have been genetically altered or transformed (Table 1), and, in some cases, the transformed mosquitoes expressed proteins targeting pathogen development [3, 20, 21, 26, 27]. Here, the common goal is to transform insect vectors with gene(s) whose protein(s) impair(s) pathogen development. As indicated above, genes that reduce pathogen development are to be associated with a gene drive system that increases the frequency of the transgenic vector when they are released into their natural habitats.

Paratransgenesis is an alternate approach to reduce vector competence via the genetic manipulating of symbionts commonly found in insect disease vectors (Figure 1B). The main characteristics of paratransgenesis are the simplicity with which symbionts are transformed (through viral or bacterial genetic transformation), the feasibility of the transformed symbiont to be spread across a population (maternally or via coprophagy), and the reduced fitness cost associated with the transformation of symbionts [28]. Symbionts currently targeted for paratransgenesis include bacteria that inhabit triatomine hindguts [28-30] and tsetse fly tissues [31], and densoviruses infecting *An. gambiae* and *Ae. aegypti* mosquitoes [32, 33]. To date, insect vector symbionts have been genetically modified to express antimicrobial peptides [28], single chain antibodies [29, 30, 34], and dsRNAs [35-38]. In all three approaches the expressed molecules proved harmful to the pathogens transmitted by each vector.

Transmission-blocking vaccines (TBVs) are intended to prevent the transmission of pathogens from infected to uninfected hosts (Figure 1C) by a disease vector. Such vaccines do not protect an individual from infection but rather can reduce transmission. TBVs target molecule(s) that are expressed on the surface of parasites during their developmental phase.
Fig. 1. Transgenesis, paratransgenesis, and transmission blocking vaccines (TBVs) (modified from (Coutinho-Abreu & Ramalho-Ortigao 2010; Coutinho-Abreu et al. 2010)). (A)
Transgenesis; the general technique for production of transgenic insects by germ line transformation is shown. (I) Insect eggs are microinjected with a donor plasmid [expressing a transgene (orange) and a reporter gene (green)], and with a helper plasmid, [expressing a transposase (purple)]. (II) After inoculation into the eggs, plasmids are taken up by some (or all) of the germ line cells. (III) transgenic and non-transgenic larvae can be separated by the expression of the reporter gene (green eye phenotype), controlled by an eye specific promoter. (IV) Transgenic insects are crossed with wild type to confirm that the transposon-carrying transgene was inserted into the chromosome. (V) Transgenic adult insect expressing the transgene (e.g., orange proteins in the insect midgut) is shown. (B) Paratransgenesis; the general technique used to obtain insect transformation via their symbionts is shown. Two insect orders are represented: Hemiptera/reduviids (left panel) and Diptera/mosquitoes (right panel). (I) Bacteria or viruses symbionts can be genetically modified to express a gene blocking parasite development in vectors’ tissues. (II) Symbiotic bacteria are transformed with plasmids (blue) expressing a gene (green) to inhibit parasite development in insect gut. Alternatively, viral genome (red) is inserted into a plasmid (blue) and manipulated to express a transgene (green). Viral particles can be generated by expression of such a plasmid in insect cells. (III) The transformed symbionts are acquired by insect hosts through larvae or nymph feeding, or through thoracic injection. (IV) Once insects acquire the transformed symbionts, these microorganisms can express proteins to inhibit pathogen development. (C) Transmission-blocking vaccines (TBVs). TBV is a strategy to prevent transmission of a pathogen by the bite of an infected vector. Frequently, TBVs rely on generating antibodies against vector molecules that are involved in pathogen development. (I) Healthy (blue) and infected (red) individuals are immunized with a TBV antigen; (II) Insect-vectors take an infected blood meal containing TBV antigen-specific antibodies; (III) Specific antibodies produced against the antigen inhibit pathogens development within the insect vector, (IV) preventing transmission to uninfected host(s).

within the insect vector (Table 2) [39-48], or that are expressed on the surface of vector tissues with which pathogens may be required to interact during their development within the vector (Table 3) [48-52]. The potential application of proteins (antigens) expressed on the surface of the malaria parasites Plasmodium falciparum and Plasmodium vivax as TBV has been tested [39-48]. Two of these proteins (Pfs25 and Pvs25) were deemed safe following phase-1 human trials [53, 54]. Specific antibodies against molecules expressed in midgut tissues of An. gambiae and Phlebotomus papatasi are also capable of reducing parasite loads in these vectors, pointing to their potential as TBVs candidates [48-52].

In the following pages, details of each of these three approaches are provided. Technical aspects of the strategy utilized as well as results from studies in vitro and in vivo, or depending on the case animal and human tests, as well as semi-field or field release of modified insects are also indicated. Due to the massive amount of information that has been generated in recent years regarding some of the topics (e.g., TBVs), we intend to limit our analyses to some specific points we find critical for the readers information.

2. Transgenesis in insect disease vectors

Generally, the goal for vector transgenesis is the interruption of pathogen transmission through introduction of exogenous DNA fragment (i.e., gene) into the genome of a disease
vector, followed by expression of the gene to inhibit pathogen development within the vector. Various mosquito species, vectors of different parasites and viruses, have been transformed (Table 1). Some of the transformed mosquitoes were shown capable of blocking pathogen development via tissue-specific expression of molecules that impair pathogen attachment to the midgut (Ito et al. 2002), or activate biochemical pathways detrimental to pathogen survival (Franz et al. 2006). However, vector transgenesis is a complex approach, highlighted by the fact that insect germ line transformation technique is only successfully performed by a handful of laboratories. Various issues related with transgenic vectors, including stability of the transgene in the genome, and fitness of the transformed insect in the field, need to be fully resolved prior to the successful application of transgenic insects fat body and hemocoel against insect-borne diseases.

A few aspects of development of transgenic vectors, how interference or blockage of parasite is achieved, and what may lie ahead for vector transgenesis are discussed below.

2.1 Germ line transformation

Stable genetic transformation of insects is accomplished by inserting an exogenous gene into the insect genome via the inoculation of plasmids containing the transgene (donor plasmids) into insect eggs (Figure 1). Donor plasmids are constructed to carry an engineered transposon element lacking the gene that encodes the transposase, the enzyme that mediates transposon activity by a cut and paste mechanism. Hermes, Mariner (Mos1), minus, and piggyBac, for the most part, have been the transposable elements of choice. Transposases are then supplied in trans (expressed in a separate plasmid) by the co-inoculation of a transposase-encoding helper plasmid along with the donor plasmid. Transposase expression is usually driven by a heat shock protein promoter that is activated upon raising the temperature, as it is frequently performed with injected mosquito eggs (Wimmer 2003).

Transformed offspring can be identified by a specific phenotype alteration, mediated by the expression of a reporter protein encoded by the donor vector, such as eye color (Wimmer 2003). However, this strategy is restricted to insect species displaying polymorphic eye phenotypes, such as *Ae. aegypti*. Alternatively, phenotypic markers such as firefly luciferase, or green (EGFP), red (dsRED) and cyan fluorescent (CFP) proteins can be used as transformation markers (Moreira et al. 2000; Kokoza et al. 2001; Nolan et al. 2002; Perera et al. 2002; Nirmala et al. 2006).

2.2 Tissue-specific transgene expression

Within the insect vector, a pathogen may interact with specific tissues, such as the midgut (as is the case for *Trypanosoma cruzi* and *Leishmania sp.*), or midgut, hemocoel and salivary glands (as is the case for *Plasmodium sp.*). Frequently, molecules that can block pathogen development within its vector can be expressed in a tissue-specific manner to increase effectiveness. Tissue-specific expression of a transgene is accomplished by the use of a tissue-specific promoter (Table 1). Most promoters used in vector transgenesis drive protein expression specifically within the midgut, the hemocoel, or the salivary glands, as these are sites where pathogens are commonly found within an infected vector (Kokoza et al. 2000; Moreira et al. 2000; Abraham et al. 2005; Lombardo et al. 2005; Yoshida & Watanabe 2006; Chen et al. 2007b; Rodrigues et al. 2008).
In mosquitoes, promoters for carboxypeptidase and peritrophin have been widely used to drive midgut-specific expression of several transgenes. In *Ae. aegypti*, a vitellogenin promoter driving expression of transgenes in the fat body was used to express innate immune defense-related genes (Kokoza et al. 2000; Kokoza et al. 2001; Shin et al. 2003a; Bian et al. 2005), as well as dsRNA targeting REL1 transcripts (Bian et al. 2005). The same promoter was used to express CFP in *An. stephensi* (Nirmala et al. 2006). The robustness of the vitellogenin promoter was confirmed by its capacity to function following multiple gonotrophic cycles in transgenic *An. stephensi* (Chen et al. 2007b).

Salivary gland-specific promoters also have been used in germ-line transformation of mosquitoes. *D7* and *apyrase* promoters from *An. gambiae* and *antiplatelet* from *An. stephensi* were used to transform *An. stephensi* (Lombardo et al. 2005; Yoshida & Watanabe 2006). *Ae. aegypti* also was transformed and successfully expressed luciferase within salivary glands using *maltase-like 1* and *apyrase* promoters (Coates et al. 1999).

### 2.3 Transgenes targeting *Plasmodium* development

In spite of the many mosquito species successfully transformed, only a handful has been transformed with molecules that impair pathogen development (Ito et al. 2002; Moreira et al. 2002; Kim et al. 2004; Abraham et al. 2005; Franz et al. 2006; Rodrigues et al. 2006). A list of genetically modified mosquitoes obtained to date, including the transposon and reporter genes used, tissue-expression specificity and target pathogens, among others is seen on Table 1.

In one example of a transgene targeting *Plasmodium*, expression of phospholipase-2 (PLA2) in *An. stephensi* (Moreira et al. 2002) led to an 87% reduction of *P. berghei* oocyst intensity compared to non-PLA2-expressing controls. When a peritrophin promoter was used to drive the expression of PLA2 in *An. stephensi*, inhibition of *P. berghei* oocyst intensity ranged from 74% to 94% (Abraham et al. 2005). Nevertheless, expression of PLA2 in the mosquito midgut did not exert a direct effect on the parasite, but rather led to structural damage of the midgut epithelium (Moreira et al. 2002; Abraham et al. 2005).

Synthetic peptides that block *Plasmodium* development in mosquitoes also have been identified and tested. SM1, identified using a phage display library (Ghosh et al. 2001), blocked *P. berghei* invasion of *An. stephensi* midgut and salivary glands. In *An. stephensi* transformed with piggyBac expressing a four tandem repeat of SM1 under a carboxypeptidase promoter, *P. berghei* intensity was inhibited by 81.6%. Interestingly, these transgenic mosquitoes even with sporozoites in their salivary glands were unable to transmit *P. berghei* to mice (Ito et al. 2002).

Another molecule tested was the C-type lectin (CELIII) from sea cucumber. When expressed in *An. stephensi*, CELIII is was shown to be cytotoxic to *P. berghei* ookinetes, reducing prevalence and intensity by 84% and 90%, respectively (Yoshida et al. 2007). CELIII expression was driven by a midgut-specific *An. gambiae*-derived carboxypeptidase promoter.

Besides PLA2, SM1, and CELIII, mosquitoes also were transformed with antimicrobial peptides (Kokoza et al. 2000; Kim et al. 2004; Bian et al. 2005). *An. gambiae* transformed with a *cecropin A* (driven by a carboxypeptidase promoter from *Ae. aegypti*) inhibited *P. berghei*
intensity by 61% on average when compared with non-transformed controls (Kim et al. 2004). Recently, An. stephensi were transformed with the piggyBac transposon expressing single-chain antibodies (scFvs) targeting P. falciparum proteins. Some of these scFv genes have been attached to a Cecropin A gene so as to improve the effectiveness of the antibody against P. falciparum. In fact, the scFv-Cecropin A construct (m2A10) targeting the P. falciparum circumsporozoite protein reduced P. falciparum intensity by 97%, prevalence by 86%, and sporozoites load by 84% (Isaacs et al. 2011).

2.4 Transgenes inducing gene silencing and targeting viral transmission

Stable transformation of mosquitoes with two inverted repeats of the same gene to induce assembly of double-strand RNAs (dsRNAs) and activation of the RNAi pathway has also been obtained (Brown et al. 2003; Bian et al. 2005). This strategy takes advantage of the RNAi mechanism to block expression of insect molecules associated with vectorial competence (Bian et al. 2005), or it can directly target viral replication within insect tissues (Franz et al. 2006). In spite of the fact that both approaches are technically feasible, only the latter has led to substantial reduction in the development of any human pathogen (Franz et al. 2006).

Gene silencing via dsRNA was first demonstrated with An. stephensi expressing sense and anti-sense RNAs targeting EGFP (Brown et al. 2003). EGFP dsRNA-expressing mosquitoes were crossed with a transgenic mosquito line that expresses EGFP. The double transgenic offspring displayed lower level of EGFP expression than the parental line expressing it, indicating the effect of the RNAi machinery reducing the expression of the EGFP transgene (Brown et al. 2003). Ae. aegypti expressing dsRNA targeting REL1, a gene involved in innate immune response, also inhibited expression of REL1 via RNAi (Bian et al. 2005).

As indicated, the activation of the RNAi pathway is intended to affect the replication of infecting RNA viruses transmitted by mosquitoes. Ae. aegypti expressing DEN2 sense and antisense RNAs reduced viral load by fivefold, confirming the effectiveness of the RNAi in controlling virus replication in disease vectors (Franz et al. 2006).

2.5 Future of vector transgenesis

Despite advances in the development of stable lines of genetically modified disease vectors (Moreira et al. 2000; Ito et al. 2002; Lobo et al. 2002; Perera et al. 2002), many challenges exist to the application of transgenesis to control vector-borne diseases outside the laboratory. Beyond issues dealing with social and environmental impact(s) that are inherent to the potential use of genetically modified organisms (not the scope of this chapter), of significance also is the fact that, for example, none of the most import human malaria vectors (i.e. An. gambiae s.l. and An. funestus) has been successfully transformed and displayed reduced vector competence. Moreover, only one strain of transgenic mosquitoes blocked the development of P. falciparum, the principal human malaria parasite (Yoshida et al. 2007). The only exceptions of transgenic insect lines robustly impairing development of a human pathogen in its human vector are the transgenic strain of Ae. aegypti capable of inhibiting DEN virus development (Franz et al. 2006; Mathur et al. 2010).
Fitness of transgenic mosquitoes in natural habitats is also an important issue. Laboratory tests demonstrated that in four lines of transgenic *An. stephensi* the frequency of transgenic individuals declined over time (Catteruccia et al. 2003). Although SM1-transgenic hemizygous *An. stephensi*, carrying a single transgene copy in the genome, exhibited higher fitness than wild type when fed on infected mice (Marrelli et al. 2007), transgenic homozygous *An. stephensi* (harboring two transgene copies), possibly advantageous for field releases, displayed lower fitness than non-transformed mosquitoes (Li et al. 2008). Transgenic lines of *Ae. aegypti* expressing either EGFP or a transposase also displayed lower fitness than wild type (Irvin et al. 2004). Such fitness load issue may be overcome by taking advantage of a site-specific recombination strategy, as shown for *An. stephensi* transgenic lines containing phi C31 attP 'docking' sites and expressing ECFP. (Amenya et al. 2011).

Although fitness in natural habitats is one of the main constraints of transgenic disease vectors, mathematical models suggest that a highly efficient transposon can spread through natural populations if it affects fitness by less than 50% (Hickey 1982; Ribeiro & Kidwell 1994). Nevertheless, pathogen refractoriness needs to be at or very close to 100% to substantially decrease disease prevalence in high endemic areas (Boete & Koella 2002). Future studies mimicking field conditions likely will uncover the importance of fitness to the establishment of transgenic mosquitoes in natural habitats.

Problems also are associated with transposons as genetic drive systems for transgenes. Transposons can remobilize in somatic tissues possibly causing damage in other regions of the genome (Atkinson 2004). Interestingly, none of the transposable elements (*Hermes, Mos1, minos,* and *piggyBac*) appears to remobilize in *Ae. aegypti* germ line, possibly reflecting a resistance mechanism, since the same elements can remobilize in *Drosophila* germ line tissues (O’Brochta et al. 2003). In addition, a mechanism to drive transposase expression and restrict gene drive system activity to germ-line tissues has been created using the regulatory sequence of *nanos*, a gene involved in early embryonic development (Adelman et al. 2007).

Further issues regarding the design and potential field release of transgenic disease vectors include: i) non-canonical transposition reactions, such as transgene insertion by a mechanism other than cut-and-paste, resulting in integration of donor plasmid fragments into the insect genome, as observed in transposition events accomplished by the transposons *Hermes, Mos1,* and *piggyBac* in *Ae. aegypti* (O’Brochta et al. 2003); ii) transgene size influencing transposon activity, as shown for *Mariner* (Lampe et al. 1998) and iii) inhibition of transgene expression after some generations due to unknown mechanisms, as observed with *Ae. aegypti* expressing a anti-Dengue virus dsRNA (Franz et al. 2009). Another issue is the possibility of horizontal transfer of the transgene between mosquito sibling species, as proposed for the introgression of the *P*-element between *Drosophila* lines (Engels 1997). Horizontal transfer also can be virus mediated, such as the case of *piggyBac*, initially identified in a *Tricoplusia ni* virus (Fraser et al. 1983). Technology to prevent the potential horizontal transposon transfer by viruses and to inhibit transposition activity mediated by endogenous transposases still needs to be developed.

Other gene-drive mechanisms have been developed to assist with problems associated with transposon elements in insect germ-line transformation (Sinkins & Gould 2006). Recently, a driving mechanism known as *Medea* (maternal-effect dominant embryonic arrest) was
shown capable of driving population replacement in *Drosophila* without an apparent fitness cost (Chen et al. 2007a). This gene drive system consists of a DNA segment encoding a protein lethal to insects and an antidote that neutralize the lethal protein. A heterozygous female (*Medea/*+) expresses the toxin within all oocytes, killing all the */+ offspring as they do not express the antidote to neutralize the maternal toxin. *Medea* can be designed to restrict transgene activity to the host species through the utilization of siRNAs-encoding genes as toxin genes (Chen et al. 2007a). Although *Medea* has been postulated to function in *An. gambiae* population replacement, it has yet to be developed for mosquitoes (Marshall & Taylor 2009). Transgenic insects also can be developed to express female dominant-lethal genes to reduce the number of females in an insect population (Thomas et al. 2000; Horn & Wimmer 2003). RIDL, or release of insects carrying a dominant lethal, was originally designed to overcome issues associated with SIT (sterile insect technique). Although SIT was successfully applied against the screwworm fly *Cochliomyia homonivorax* (Krafsur et al. 1987), the fruit fly *Ceratitis capitata* (Robinson et al. 1999), and the tsetse fly *G. austeni* (Vreysen et al. 2000), drawbacks such as reduced sterile male fitness and sterile female contamination, were detected (Thomas et al. 2000). RIDL consists of release of transgenic male insects expressing the female dominant-lethal genes, causing a reduction on the numbers of females in the following generations (Robinson et al. 1999). Robust transgenic vectors approaches could also be used with RIDL (Thomas et al. 2000), and a transgenic mosquito sexing-system has already been developed (Catteruccia et al. 2005). In fact, an *Ae. aegypti* RIDL line has been successful developed and tested in natural settings (Fu et al. ; James 2011). Taking advantage of the *Actin* gender-specific alternative splicing, female *Ae. aegypti* displays a flightless phenotype, reducing potential mating and consequently mosquito densities (Fu et al. 2010). Despite the substantial achievement, its release in field setting has been the subject of much criticism (James 2011).

In our view, hurdles to the establishment of an efficient transgenic vector approach include the lack of a transgene(s) that effectively reduce pathogen load, and the inefficiency of transposons as gene-drive mechanism(s). Further studies to identify traits associated with vector competence will likely pinpoint candidate genes that, when targeted, may effectively block pathogen development and transmission. The availability of gene drive mechanisms to overcome issues associated with the use of transposons, such as remobilization, fitness load, and the potential to introgress to closely related species, also is of interest. *Medea* was suggested as a gene drive that could overcome such issues, but it is yet to be developed for insect vectors. As for RIDL, the current absence of a gene drive mechanism also prevents its application against insect vectors.

The recent establishment of a binary Gal4/UAS system in *Ae. aegypti* (Kokoza & Raikhel) may also speed the establishment of other transgenic mosquito lines, as this system represents an invaluable tool for refinement of genetic tools in mosquitoes, and possibly for the discovery of new molecular targets for control of vector-borne diseases via transgenesis.

Clearly, much work remains before genetically modified insect vectors can be systematically released into natural habitats. When realized, transgenesis may provide a significant tool in the fight against vector-borne diseases.
<table>
<thead>
<tr>
<th>Insect species</th>
<th>Transposon</th>
<th>Promoter</th>
<th>Reporter gene</th>
<th>Transgene</th>
<th>Targeted pathogen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ae. aegypti</td>
<td>Hermes</td>
<td>D. melanogaster Cinnabar</td>
<td>D. melanogaster Cinnabar</td>
<td>Cinnabar</td>
<td>-</td>
<td>(Jasinskiene et al. 1998)</td>
</tr>
<tr>
<td>MoS1</td>
<td>D. melanogaster Cinnabar</td>
<td>D. melanogaster Cinnabar</td>
<td>Cinnabar</td>
<td>-</td>
<td>(Coates et al. 1998)</td>
<td></td>
</tr>
<tr>
<td>Hermes</td>
<td>Ae. aegypti malate-like 1/ aegyptase</td>
<td>Firefly luciferase</td>
<td>Firefly luciferase</td>
<td>-</td>
<td>(Coates et al. 1999)</td>
<td></td>
</tr>
<tr>
<td>Hermes</td>
<td>D. melanogaster Actin 5C</td>
<td>EGFP</td>
<td>EGFP</td>
<td>-</td>
<td>(Pinkerton et al. 2000)</td>
<td></td>
</tr>
<tr>
<td>Hermes</td>
<td>Ae. aegypti vitellogenin</td>
<td>D. melanogaster Cinnabar</td>
<td>Defensin A</td>
<td>Micrococcus luteus</td>
<td>(Kokoza et al. 2000)</td>
<td></td>
</tr>
<tr>
<td>Hermes</td>
<td>Ae. aegypti carboxypeptidase</td>
<td>Firefly luciferase</td>
<td>Firefly luciferase</td>
<td>-</td>
<td>(Moreira et al. 2000)</td>
<td></td>
</tr>
<tr>
<td>MoS1</td>
<td>An. gambiae carboxypeptidase</td>
<td>Firefly luciferase</td>
<td>Firefly luciferase</td>
<td>-</td>
<td>(Moreira et al. 2000)</td>
<td></td>
</tr>
<tr>
<td>piggyBac</td>
<td>Ae. aegypti vitellogenin</td>
<td>EGFP</td>
<td>Defensin A</td>
<td>Entrobacter cloacae/ P. gallinaceum</td>
<td>(Kokoza et al. 2001; Shin et al. 2003b)</td>
<td></td>
</tr>
<tr>
<td>Minos</td>
<td>D. melanogaster Actin 5C</td>
<td>dsRED</td>
<td>dsRED</td>
<td>-</td>
<td>(Nolan et al. 2002)</td>
<td></td>
</tr>
<tr>
<td>piggyBac</td>
<td>D. melanogaster Actin 5C</td>
<td>dsRED</td>
<td>dsRED</td>
<td>-</td>
<td>(Nolan et al. 2002)</td>
<td></td>
</tr>
<tr>
<td>piggyBac</td>
<td>D. melanogaster Cinnabar</td>
<td>D. melanogaster Cinnabar</td>
<td>Cinnabar</td>
<td>-</td>
<td>(Lobo et al. 2002)</td>
<td></td>
</tr>
<tr>
<td>piggyBac</td>
<td>D. pseudoobscura hsp82</td>
<td>EGFP</td>
<td>Mos1 transposase</td>
<td>-</td>
<td>(Wilson et al. 2003)</td>
<td></td>
</tr>
<tr>
<td>piggyBac</td>
<td>Ae. aegypti vitellogenin</td>
<td>EGFP</td>
<td>ARELI*</td>
<td>-</td>
<td>(Shin et al. 2003a)</td>
<td></td>
</tr>
<tr>
<td>piggyBac</td>
<td>Ae. aegypti vitellogenin</td>
<td>EGFP</td>
<td>Cecropin A</td>
<td>Enterobacter cloacae</td>
<td>(Shin et al. 2003a)</td>
<td></td>
</tr>
<tr>
<td>piggyBac</td>
<td>Ae. aegypti vitellogenin</td>
<td>EGFP</td>
<td>ΔRELI-A*</td>
<td>-</td>
<td>(Bian et al. 2005)</td>
<td></td>
</tr>
<tr>
<td>piggyBac</td>
<td>Ae. aegypti vitellogenin</td>
<td>EGFP</td>
<td>dsRNA against RELI transcripts</td>
<td>-</td>
<td>(Bian et al. 2005)</td>
<td></td>
</tr>
<tr>
<td>Insect species</td>
<td>Transposon</td>
<td>Promoter</td>
<td>Reporter gene</td>
<td>Transgene</td>
<td>Targeted pathogen</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------</td>
<td>------------</td>
<td>----------</td>
<td>---------------</td>
<td>-----------</td>
<td>------------------</td>
<td>-----------</td>
</tr>
<tr>
<td><em>Ae. aegypti</em></td>
<td>Mos1</td>
<td><em>An. gambiae</em> carboxypeptidase</td>
<td>EGFP</td>
<td>dsRNA against prM of DENV-2</td>
<td>Dengue-2 virus</td>
<td>(Franz et al. 2006; Franz et al. 2009)</td>
</tr>
<tr>
<td>Mos1</td>
<td>Mos1 transposase</td>
<td>Cal4</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mos1</td>
<td>Ae. aegypti</td>
<td>D7</td>
<td>dsRED</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mos1</td>
<td>Ae. aegypti</td>
<td>30K</td>
<td>EGFP</td>
<td>Mnp</td>
<td>Dengue-2 virus</td>
<td>(Mathur et al. 2010)</td>
</tr>
<tr>
<td><em>Ae. fluviatilis</em></td>
<td>piggyBac</td>
<td><em>An. gambiae</em> peritrophin</td>
<td>EGFP</td>
<td>mPLA2</td>
<td><em>P. gallinaceum</em></td>
<td>(Rodrigues et al. 2008)</td>
</tr>
<tr>
<td><em>An. albimanus</em></td>
<td>piggyBac</td>
<td><em>D. melanogaster</em> polyubiquitin</td>
<td>EGFP</td>
<td>EGFP</td>
<td>-</td>
<td>(Perera et al. 2002)</td>
</tr>
<tr>
<td><em>An. gambiae</em></td>
<td>piggyBac</td>
<td>Baculovirus hr5-sec1</td>
<td>EGFP</td>
<td>EGFP</td>
<td>-</td>
<td>(Grossman et al. 2001)</td>
</tr>
<tr>
<td>piggyBac</td>
<td><em>Ae. aegypti</em> carboxypeptidase</td>
<td>EGFP</td>
<td>Cecropin A</td>
<td><em>P. berghei</em></td>
<td>(Kim et al. 2004)</td>
<td></td>
</tr>
<tr>
<td><em>An. stephensi</em></td>
<td>minus</td>
<td><em>D. melanogaster</em> actin</td>
<td>EGFP</td>
<td>EGFP</td>
<td>-</td>
<td>(Catteruccia et al. 2000)</td>
</tr>
<tr>
<td>piggyBac</td>
<td><em>D. melanogaster</em> actin</td>
<td>dsRED</td>
<td>dsRED</td>
<td>-</td>
<td>(Nolan et al. 2002)</td>
<td></td>
</tr>
<tr>
<td>piggyBac</td>
<td>An. gambiae carboxypeptidase</td>
<td>EGFP</td>
<td>PLA2</td>
<td><em>P. berghei</em></td>
<td>(Moreira et al. 2002)</td>
<td></td>
</tr>
<tr>
<td>piggyBac</td>
<td>An. gambiae carboxypeptidase</td>
<td>EGFP</td>
<td>(SM1)4</td>
<td><em>P. berghei</em></td>
<td>(Ito et al. 2002)</td>
<td></td>
</tr>
<tr>
<td>minus</td>
<td><em>D. melanogaster</em> actin</td>
<td>dsRED</td>
<td>dsRNA against EGFP</td>
<td>-</td>
<td>(Brown et al. 2003)</td>
<td></td>
</tr>
<tr>
<td>minus</td>
<td><em>An. gambiae asparagine</em> <em>An. gambiae D7r4</em></td>
<td>EGFP</td>
<td>LacZ</td>
<td>-</td>
<td>(Lombardo et al. 2005)</td>
<td></td>
</tr>
<tr>
<td>piggyBac</td>
<td><em>An. gambiae</em> peritrophin</td>
<td>EGFP</td>
<td>PLA2</td>
<td><em>P. berghei</em></td>
<td>(Abraham et al. 2005)</td>
<td></td>
</tr>
</tbody>
</table>
Table 1. Germ line transformed mosquitoes (Modified from Coutinho-Abreu et al. 2010).

<table>
<thead>
<tr>
<th>Insect species</th>
<th>Transposon</th>
<th>Promoter</th>
<th>Reporter gene</th>
<th>Transgene</th>
<th>Targeted pathogen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. stephensi</td>
<td>piggyBac</td>
<td>D. melanogaster tubulin</td>
<td>dsRED</td>
<td>EGFP</td>
<td>-</td>
<td>(Catteruccia et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>piggyBac</td>
<td>An. stephensi vitelligenin</td>
<td>EGFP</td>
<td>CFP</td>
<td>-</td>
<td>(Nirmala et al. 2006)</td>
</tr>
<tr>
<td>minos</td>
<td>minos</td>
<td>An. gambiae carboxypeptidase</td>
<td>EGFP</td>
<td>CELJIR</td>
<td>P. berghei falciparum</td>
<td>(Yoshida et al. 2007)</td>
</tr>
<tr>
<td>minos</td>
<td>piggyBac</td>
<td>An. gambiae vitelligenin</td>
<td>dsRED</td>
<td>EGFP</td>
<td>-</td>
<td>(Yoshida &amp; Watanabe 2006)</td>
</tr>
<tr>
<td>minos</td>
<td>piggyBac</td>
<td>D. melanogaster actin</td>
<td>dsRED</td>
<td>minos transposase</td>
<td>EGFP</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>piggyBac</td>
<td>D. melanogaster 5C</td>
<td>EGFP</td>
<td>-</td>
<td>-</td>
<td>(Ameyia et al. 2010)</td>
</tr>
<tr>
<td>Cx. quinquefasciatus</td>
<td>piggyBac + aiitP docking site</td>
<td>3XP3</td>
<td>ECFP</td>
<td>-</td>
<td>-</td>
<td>(Allen et al. 2001)</td>
</tr>
<tr>
<td>Hermes</td>
<td>D. melanogaster 5C</td>
<td>actin</td>
<td>EGFP</td>
<td>EGFP</td>
<td>-</td>
<td>(Allen et al. 2001)</td>
</tr>
</tbody>
</table>
3. Paratransgenesis to reduce vector competence

Paratransgenesis usually refers to the use of genetically modified symbiotic organisms expressing molecules that can block pathogen development or transmission by vectors. Bacteria symbionts of blood sucking bugs (Durvasula et al. 1997; Durvasula et al. 1999; Durvasula et al. 2008), tsetse flies (Cheng & Aksoy 1999), and mosquitoes (Favia et al. 2007; Jin et al. 2009), and symbiotic viruses of An. gambiae (Ren et al. 2008) and Ae. aegypti (Carlson et al. 1995; Ward et al. 2001; Carlson et al. 2006), have been used (Figure 2). Recently, genetically modify entomopathogenic fungi strains have induced high levels of P. falciparum mortality in An. gambiae (Fang et al. 2011). Current data indicate that symbionts expressing molecules targeting pathogen development have the potential to reduce transmission in endemic regions, and appear unrelated to any fitness load (Durvasula et al. 1997; Cheng & Aksoy 1999). As with transgenesis, spread of transformed symbionts also would benefit from the availability of a gene drive system to replace non-transformed symbionts present in natural vector populations.

3.1 Transformation of reduviids, tsetse, and mosquitoes with bacterial symbionts

Paratransgenesis in disease vectors was demonstrated through the expression of cecropin A by Rhodococcus rhodnii within the midgut of the kissing bug (reduviid) Rhodnius prolixus (Durvasula et al. 1997). A 99% reduction in the intensity of Trypanosoma cruzi infection in the hindgut of R. prolixus was observed without interfering with insect fitness. Additionally, transformed symbionts were shown to be horizontally transmitted to R. prolixus carrying non-transformed symbionts via reduviid coprophagic habits (Durvasula et al. 1997). Subsequently, functionally active antibody fragments also were successfully expressed in the guts of R. prolixus (Durvasula et al. 1999) and Triatoma infestans (Durvasula et al. 2008) utilizing symbionts. Transformed symbionts were stably maintained within the gut of the insects without need for antibiotic selection (Durvasula et al. 1997; Durvasula et al. 1999; Durvasula et al. 2008).

Paratransgenesis seems to be a promising strategy to reduce African trypanosomes transmission by tsetse flies. Genetically transformed Sodalis, a symbiont of tsetse flies commonly found in the midgut and hemolymph of Glossina m. morsitans, Glossina p. palpalis, Glossina austeni, and Glossina brevipalpis, and the salivary glands of Gl. p. palpalis, is transmitted vertically via the female milk glands (Cheng & Aksoy 1999; Weiss et al. 2006; Aksoy et al. 2008). In addition, when Sodalis originally isolated from of Gl. m. morsitans and Gl. fuscipes was transformed with GFP, the recSodalis obtained colonized septic non-native tsetse host species at a density similar to a native colonization and without reducing host fitness (Weiss et al. 2006).

Symbiotic bacteria also have been isolated from An. stephensi (Favia et al. 2007; Riehle et al. 2007). One such symbiont, Asaia sp., was successfully transformed with plasmids expressing GFP (Damiani et al. 2008) or with dsRED gene cassette inserted into bacterium genome (Damiani et al. 2008). Asaia was found in mosquito tissues, such as midgut and salivary gland, which are sites for pathogen development, as well as in male and female reproductive tracts, supporting bacteria transovarial and venereal transmission (Riehle et al. 2007; Damiani et al. 2008). Additionally, larval stages can acquire such bacteria strain from the environment (Riehle et al. 2007).
3.2 Transformation of viral symbionts

Symbiotic densovirus also can be genetically manipulated to express molecules to reduce vector competence. Densoviruses are linear single-stranded DNA viruses with the genome packaged in a non-enveloped particle. These viruses are suitable vectors for expression of foreign genes in mosquitoes because they are highly specific, environmentally stable, kill mosquito larvae in a dose-dependent manner, decrease lifespan of surviving adults, and are transmitted vertically (Carlson et al. 1995; Carlson et al. 2006). In *Ae. aegypti*, densoviruses can spread to fat body, muscles, and nerves (Ward et al. 2001) following infection through the anal papillae. Densoviruses infecting *Ae. aegypti* (AeDNV) and *An. gambiae* (AgDNV) were isolated and modified to express GFP (Ward et al. 2001; Ren et al. 2008). The green phenotype obtained by the expression of GFP in recombinant AgDNV-infecting *An. gambiae* was observed in 20% of F2 and F3 generations, suggesting that transformed densoviruses may be used to express molecules targeting pathogen development in mosquitoes (Ren et al. 2008).

3.3 Transformation of entomopathogenic fungi

Several studies demonstrated that entomopathogenic fungi are capable of reducing mosquito life span as well as vector competence (Carlson et al. 2006; Thomas & Read 2007; Cook et al. 2008; Read et al. 2009). Blanford et al. (2005) observed a high mortality rate (55-80%) in mosquitoes 7-14 days following infection with the fungus *Beauveria bassiana*, which was suggestive of the ability of entomopathogenic fungi to drastically reduce pathogen transmission in endemic areas. However, fungus-mediated killing is a slower process compared to chemical approaches, and critics have suggested that the use of entomopathogenis fungi alone is incapable of controlling mosquitoes in malaria-endemic areas.

More recently, genetically modified entomopathogenic fungus *Metarhizium anisopliae* expressing molecules that affect the development of *P. falciparum* in *An. gambiae* were generated (Fang et al. 2011). Specifically, *M. anisopliae* expressing the SMI peptide ([SM]₈), the anti-microbial peptide scorpion, and a single chain antibody targeting a plasmodium surface protein were shown to reduce sporozoite load by 90% without affecting mosquito fitness. Moreover, co-expression of a [SM]₈-scorpion fusion protein along with scorpion led to nearly elimination of sporozoite infection in the salivary glands of the infected mosquitoes (Fang et al, 2011). The results using the transgenic *Metarhizium* suggest that this could be a powerful approach to control malaria transmission.

3.4 Future of vector paratransgenesis

Although a low number of bacteria symbionts have been transformed to date, a potential advantage of this approach over transgenesis is lack of fitness load (Durvasula et al. 1997; Weiss et al. 2006). Also, the alternate use of genetically modified symbiotic viruses (instead of bacterial symbionts) may provide additional tools against pathogen development. Densoviruses efficiently expresses heterologous proteins in *An. gambiae* and *Ae. aegypti* and are transmitted vertically (Carlson et al. 2006; Ren et al. 2008). Viral symbionts can be engineered to express single chain antibodies (scFv), blocking pathogen
Transgenesis, Paratransgenesis and Transmission Blocking Vaccines to Prevent Insect-Borne Diseases

595

... the mosquito immune system (particularly antimicrobial peptides), leading to reduction of...

development (de Lara Capurro et al. 2000). Recombinant Sindbis expressing transcripts from an infecting virus genome can reduce viral load (of the infecting virus) in mosquitoes (Powers et al. 1996; Travanty et al. 2004). The results from Sindbis expressing transcripts from LaCrosse (LAC), dengue (DEN), or yellow fever (YF) viruses indicated a substantial interference with the replication of these viruses in *Aedes triseriatus* (LAC) and in *Ae. aegypti* (DEN and YF) (Olson et al. 1996; Powers et al. 1996; Higgs et al. 1998; Adelman et al. 2001). Such viral replication inhibition is accomplished by the mosquito RNAi machinery (Cirimotich et al. 2009).

Despite early successes with transformation of insect vector symbionts, it is not known if transformed symbionts can replace non-transformed in natural insect populations, and potentially affect pathogen development and transmission in natural habitats. Symbionts seem to have no fitness load on insect hosts and are capable of being transmitted vertically (via trans-ovarian transmission) or laterally (due to feeding habits). Thus, a strong gene drive system can potentiate the effectiveness of paratransgenesis. *Wolbachia* endosymbionts have been proposed as such gene drive system (Aksoy et al. 2008).

*Wolbachia* are intracellular, maternally inherited bacteria that manipulate reproduction of insects via cytoplasmatic incompatibility (CI) (Sinkins & Gould 2006). Due to the effects of CI, a *Wolbachia*-uninfected female will not breed with infected males successfully, reducing the frequency of uninfected individuals and increasing the frequency of *Wolbachia*-infected insects in a population (Sinkins & Gould 2006). Thus, other maternally inherited transformed symbionts would be spread within an insect population in association with *Wolbachia* (Aksoy et al. 2008), increasing the frequency of the transformed symbiont. This mechanism has been observed in *Ae. aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus* (Sinkins & Gould 2006), representing a potential manner to spread transformed symbionts, such as densovirus, in natural populations of mosquitoes.

A life-shortening strain of *Wolbachia* (*w*MelPop) identified in *D. melanogaster* was recently introduced into *Ae. aegypti* (McMeniman et al. 2009) and *An. gambiae* (Jin et al. 2009). Beyond promoting the spread of the transformed symbiont across the mosquito population (i.e., acting as a gene drive mechanism), this strain of *Wolbachia* was also thought to reduce the time frame available (i.e., mosquito life span) for pathogen development within the mosquito (known as the extrinsic incubation period or EIP) (McMeniman et al. 2009). Thus, the application of *w*MelPop to eliminate disease vectors may lead to a reduction of the pathogen developmental time (or EIP) within the vector (Read & Thomas 2009). Counter to this argument is the possibility that, *Wolbachia*, as well as densovirus and entomopathogenic fungi potentially target older mosquitoes over younger ones, and are considered evolution-proof mosquitocidal biocontrols agents (Read et al. 2009). For *Anopheles*, due to loss of fecundity per gonotrophic cycle in natural conditions (from 20% to 40% per gonotrophic cycle, (Killeen et al. 2000)), a selective pressure on pathogen developmental time already exists, especially in the case of *Plasmodium*-infected mosquitoes (Read et al. 2009). Thus, such selective pressure from the addition of *Wolbachia* is likely not strong enough to shorten the parasite life cycle within the vector.

*w*MelPop infection reduces the feeding ability of old mosquitoes [16] and activates the mosquito immune system (particularly antimicrobial peptides), leading to reduction of
filarial worms [17], dengue virus, and plasmodium parasite load (Moreira et al. 2009), including *P. falciparum* (Hughes et al. 2011).

As wMelPop and entomopathogenic fungi are capable of reducing vector competence (linear parameter) and vector survivorship (exponential parameter), these two effects combined may significantly reduce vectorial capacity and human malaria burden in endemic areas.

### 4. Transmission-blocking vaccines (TBVs)

Transmission-blocking vaccines (or TBVs) aim at interfering and/or blocking pathogen development within the vector, halting transmission to non-infected vertebrate host (depicted in Figure 1C). TBVs usually rely on immunization of vertebrate hosts (either infected or uninfected) with molecules derived from the pathogen or the vector in order to reduce pathogen transmission from infected to uninfected hosts. Such molecules (i.e., antigens) may be inoculated into the vertebrate host as purified proteins inducing the host immune system to produce specific antibodies (Singh & O'Hagan 1999). Alternatively, antibodies can be raised by inoculating the host with recombinant DNA plasmids containing the gene encoding such molecules (Lobo et al. 1999; Coban et al. 2004; Kongkasuriyachai et al. 2004; LeBlanc et al. 2008). The expression and secretion of the specific protein into host tissues induce the immune system to produce antibodies against such proteins (Abdulhaqq & Weiner 2008). To boost the immune response of the vertebrate, antigens are usually inoculated in conjunction with adjuvants. The mechanisms by which adjuvants improve the immune response are still poorly understood (Singh & O’Hagan 1999; Aguilar & Rodriguez 2007). The specific antibodies produced against pathogen and/or vector antigens will interfere with the development of the pathogen within the vector following a blood meal on a vaccinated and infected individual. Other insect-based vaccines, such as sialome-based vaccines (Valenzuela 2004; Oliveira et al. 2009) and insecticidal vaccines (Foy et al. 2003) that are, in some cases, dependent on cell-mediated immune-response in order to prevent vertebrate host infection and reduce insect lifespan, respectively, are not discussed in details this chapter. These can be found in (Willadsen 2004; Billingsley et al. 2006; Titus et al. 2006; Billingsley et al. 2008; Dinglasan & Jacobs-Lorena 2008; Oliveira et al. 2009). Insecticidal vaccines, due to their potential to reduce vectorial capacity exponentially (Billingsley et al. 2006), are briefly discussed.

For a molecule to be an effective TBV candidate it has to induce high antibody titers in order to block completely pathogen development within the insect (Kubler-Kielb et al. 2007). Additionally, the antigen/adjuvant combination has to be safe enough to the vertebrate host so as to avoid side effects after immunization (Saul et al. 2007; Wu et al. 2008). Ideally, a TBV candidate antigen will display low levels of polymorphisms (in field isolates) so that a unique antigen may be used to produce a TBV capable of recognizing all the field variants of that specific antigen (Kocken et al. 1995; Drakeley et al. 1996; Duffy & Kaslow 1997; Sattabongkot et al. 2003). Alternatively, an effective TBV may need to combine different antigens because the combined action of the antibodies against such antigens may produce a more efficient transmission-blocking result (Duffy & Kaslow 1997; Gozar et al. 1998; Kongkasuriyachai et al. 2004).
4.1 Parasite antigen-based TBVs

Most of the studies on TBVs to date were conducted using antibodies targeting antigens expressed on the surface of sexual stage of malaria parasites (Figure 1C; Table 2). P. falciparum proteins Pfs25, Pfs28, Pfs48/45, and Pfs230, and their orthologs in Plasmodium vivax, have been tested in transmission-blocking assays (Quakyi et al. 1987; Kaslow et al. 1988; Duffy & Kaslow 1997; Hisaeda et al. 2000; Sattabongkot et al. 2003; Malkin et al. 2005; Outchkourov et al. 2008).

4.1.1 P. falciparum-derived TBV candidate – Pfs25

Pfs25 is a 25kDa protein expressed on the surface of zygote and ookinete stages of P. falciparum and consists of four tandem epidermal growth factor (EGF) domains (Kaslow et al. 1988). The TBV potential of Pfs25 was demonstrated using the Vaccinia virus as delivery systems of this antigen to mammalian hosts (Kaslow et al. 1991), or using recombinant Pfs25 expressed in yeast (Barr et al. 1991; Kaslow et al. 1991).

4.1.2 P. falciparum-derived TBV candidate – Pfs28

Pfs28 is a 28kDa P. falciparum conserved protein expressed on the surface of retorts, a transitional stage between zygote and ookinete. This antigen also was tested in transmission blocking activity assays. Antibodies produced by the injection of yeast-expressed Pfs28 (yPfs28), in the presence of alum, significantly reduced the infectivity of An. freeborni mosquitoes with P. falciparum. Lower infectivity was exhibited when vaccination was carried out with yPfs28 and yPfs25 antigens injected together (Duffy & Kaslow 1997).

Transmission blocking activity against P. falciparum was further improved when Pfs25 and Pfs28 were expressed as a unique chimeric protein in yeast, the 25-28c recombinant protein. Vaccination with the 25-28c recombinant protein led to complete arrest of oocyst development earlier, using a lower dose and for a greater amount of time, than vaccination with either Pfs25 or Pfs28 alone or a combination of both (Gozar et al. 1998).

4.1.3 P. falciparum-derived TBV candidate – Pfs48/45

Another TBV candidate to control spread of P. falciparum is Pfs48/45. The Pfs48/45 gene encodes a unique protein that migrates as a double band under non-reducing conditions (Milek et al. 2000). This protein is expressed on P. falciparum gametocyte and gamete surfaces and has a central role in male gamete fertility (van Dijk et al. 2001). Immunization of mice with this recombinant protein led to production of antibody titers that were capable of reducing P. falciparum oocyst intensity in An. stephensi by at least 88% in 11 out of 12 assays (Outchkourov et al. 2008).

Furthermore, with regards to the application of Pfs48/45 as a potential TBV against malaria, the variability of Pfs48/45 from culture and field isolates from many countries was analyzed (Kocken et al. 1995; Drakeley et al. 1996). The results obtained indicated low levels of polymorphism in the overall gene among either in vitro cultures or field isolates (Kocken et al. 1995; Drakeley et al. 1996).
4.1.4 *P. falciparum*-derived TBV candidate – Pfs230

Another *P. falciparum* protein tested in TBV assays was Pfs230, a 230kDa protein expressed on the surface of gametocytes. Although antibodies against Pfs230 blocked the development of *P. falciparum* in the midguts of *An. freeborni*, the transmission blocking activity of anti-Pfs230 monoclonal antibodies was completely lost when complement was inactivated. Thus, the blocking activity of anti-Pfs-230 antibodies was detected only when complement proteins were present (Quakyi et al. 1987).

4.1.5 *P. vivax*-derived TBV candidates – Pvs25 and Pvs28

*P. vivax* sexual stage surface proteins, orthologs of *P. falciparum* TBV candidates, also have been isolated and tested in transmission blocking experiments. Pvs25, a Pfs25 ortholog, is expressed on the surfaces of the insect-stages, zygotes and mature ookinetes, whereas Pvs28, a Pfs28 ortholog, is mainly expressed on retorts and mature ookinetes (Hisaeda et al. 2000).

Transmission blocking experiments using antibodies against either Pvs25 or Pvs28 were tested (Hisaeda et al. 2000). Four species of mosquitoes were artificially fed on a mixture of *P. vivax*-infected chimpanzee blood in the presence of antibodies (raised in mice co-injected with alum). *P. vivax* ookinete development was completely blocked by the anti-serum against Pvs25 (Hisaeda et al. 2000). Vaccination against Pvs25 and Pvs28 also presented efficient transmission blocking activity against *P. vivax* isolated from human patients, despite polymorphism in these proteins (Sattabongkot et al. 2003).

Transmission blocking activity of Pvs25 has been evaluated in phase 1 human trials. The results from the study revealed significant interference in *P. vivax* development within mosquito midgut caused by the human anti-Pvs25 sera. Additionally, long lasting antibody titers were elevated, and no reactogenicity (side effects) was observed (Malkin et al. 2005). Nevertheless, higher antibody titers are necessary for successful control of *P. falciparum* transmission by mosquitoes in endemic areas (Malkin et al. 2005). Unfortunately, a second phase 1 trial, using Pvs25 as a potential TBV using Montanide ISA 51 as an adjuvant was halted due to induced local and systemic reactions in the vaccinees (Wu et al. 2008).

4.2 Other pathogen molecule-based TBV candidates

In regard to proteins expressed on the surface of parasites (other than *Plasmodium*) transmitted to humans by insect vectors (Table 2), only a limited number has been tested as potential TBVs (Tonui et al. 2001a; Saraiva et al. 2006).

In *Leishmania major*, the two most abundant surface antigens, LPG and gp63, were tested as transmission blocking vaccines. *Phlebotomus duboscqi* sand flies were partially fed on mice immunized with purified native LPG, recombinant gp63 (rgp63) expressed in bacteria, crude *L. major* lysate (WPA), or a cocktail of LPG and rgp63. The sand flies were subsequently fed on *L. major*-infected mice. The results indicated that serum against WPA and the two protein-cocktail exhibited greater *L. major* blocking activity than sera against either LPG or gp63 (Tonui et al. 2001a). However, blocking of *L. major* development was due...
to damage of the midgut epithelial layer, probably caused by immune-active substances present in the blood of the pre-vaccinated mice (Tonui et al. 2001b).

Interestingly, a commercially available treatment for canine visceral leishmaniasis (Leishmune®) was recently shown to function as a TBV in sand flies (Saraiva et al. 2006). Leishmune® (FML-vaccine) is a protective vaccine made of L. donovani fucose-manose ligand and the adjuvant saponin, which was successfully tested in a phase III vaccine trial (da Silva et al. 2000). Although the surface molecule (FML) was isolated from L. donovani, Leishmune® exhibited transmission blocking activity in the New World sand fly Lutzomyia longipalpis when infected with Leishmania infantum chagasi (Saraiva et al. 2006). Antibodies produced in dogs following Leishmune® injection reduced Lu. longipalpis infectivity by 79.3% and parasite load by 74.3% even after 12 months of immunization (Saraiva et al. 2006).

4.3 Vector-based TBVs

Proteins expressed within insect vector tissues and that may interact with pathogens also have been tested as TBV candidates (Table 3). Vector-based TBV candidates include (structural) proteins that are expressed by the insect midgut (Lal et al. 2001), midgut enzymes that play a role in blood digestion (Lavazec et al. 2007), and parasite receptors expressed by the epithelial cells lining the midgut (Kamhawi et al. 2004; Dinglasan et al. 2007).

In mosquitoes, polyclonal antibodies against An. gambiae midgut proteins nearly completely reduced the intensity of P. falciparum oocysts (98%) and sporozoites (96%) within An. stephensi tissues. Also, An. gambiae-derived anti-midgut monoclonal antibodies inhibited development of P. falciparum and P. vivax in different Anopheles species (Lal et al. 2001). Additionally, these antibodies also can be used to reduce insect vector densities (vector-blocking vaccines) because they reduce vector survivorship and fecundity (Lal et al. 2001). Antibodies against carboxypeptidase cpbAg1 from An. gambiae reduced P. falciparum infectivity by more than 92% seven days after an infectious artificial blood feeding (Lavazec et al. 2007). In addition to the effect on the number of oocysts per infected mosquito, anti-cpbAg1 strongly reduced mosquito progeny (Lavazec et al. 2007). Antibodies to a midgut aminopeptidase (AgAPN1), which is one of the P. falciparum receptors in the An. gambiae midgut, were used to reduce P. falciparum oocyst intensity in An. gambiae and An. stephensi by 73% and 67%, respectively (Dinglasan et al. 2007).

Another molecule expressed on the surface of midgut cells that may serve as receptor for parasite attachment has also been assessed as a TBV candidate (Kamhawi et al. 2004). The galectin-like PpGalec characterized from the midgut of the sand fly P. papatasi is a receptor for L. major lipophosphoglycan (LPG). P. papatasi artificially fed on blood mixed sera from PpGalec-immunized mice displayed a reduction of 86% of L. major midgut infection. Moreover, no infectious metacyclic forms were detected from the flies fed on anti-PpGalec sera (Kamhawi et al. 2004).

4.4 Future of TBVs

In addition to identifying TBV candidates that are effective and may span different insect vector species, studies on TBV development must include antigenic variability present in
field isolates (Kocken et al. 1995; Drakeley et al. 1996; Duffy & Kaslow 1997; Sattabongkot et al. 2003), immunogenicity of such antigens (Kubler-Kielb et al. 2007), reactogenicity caused by adjuvants (Saul et al. 2007; Wu et al. 2008), non-specific responses (Quakyi et al. 1987; Tonui et al. 2001a), and improper folding of antigens (Kaslow et al. 1994; Milek et al. 1998a; Milek et al. 1998b; Milek et al. 2000). Natural antigenic boosting is another important issue that must be dealt with (Arevalo-Herrera et al. 2005).

Antigens expressed on the surface of insect-stage parasites have been postulated as TBV candidates because they seem not to be under the selective pressure mediated by the vertebrate immune system.

Another interesting aspect of TBVs is the possibility of natural boosting of the immune response of animals infected with a pathogen (i.e., pre-immunized) (Milek et al. 1998a; Arevalo-Herrera et al. 2005). Hence, candidate TBV proteins expressed on the surface of both insect-stage and blood-stage pathogens may induce activation of the immune response in infected hosts vaccinated with the same antigens (Arevalo-Herrera et al. 2005). However, this approach may not be suitable to every TBV, such as Pvs25 which displays low expression in blood-stage *P. vivax* (Arevalo-Herrera et al. 2005), and has yet to be demonstrated for the *Plasmodium* TBV-antigen candidates that are expressed during gametocytogenesis, for example, Pfs230 (Quakyi et al. 1987) and Pfs48/45 (Milek et al. 1998a). Proper folding of the TBV candidate protein following expression via recombinant techniques also may affect the efficacy of the vaccinating antigen. Thus, the system of choice for recombinant expression can significantly affect the outcome of the TBV candidate.

In regards to vector-based TBV candidate molecules, the number of TBV antigens available is limited and must to be increased to target other vector species. In addition to assessing a TBV candidate molecule that prevents pathogen development within insect vector tissues, an effect on the vector survivorship is also one of the main objectives.

Reduction of vector survival is thought to interfere exponentially with vectorial capacity (Black IV & Moore 2004; Billingsley et al. 2006; Billingsley et al. 2008), as the time available for pathogen development within the vector is significantly shortened. Despite several studies showing that insect feeding on blood of animals immunized with insect tissue homogenates exhibit reduced survivorship, most of these studies suffered from high experimental variability (Billingsley et al. 2006). However, one study has shown that immunization with a unique insect molecule (mucin) can induce an immune response capable of killing insect vectors via a cell-mediated response (Foy et al. 2003). Thus, an ideal TBV antigen should reduce parasite development, reducing vector competence (a linear parameter in the vectorial capacity equation), as well as vector survivorship (the exponential parameter). These two effects associated can lead to thorough reduction of vectorial capacity and disease burden in endemic areas.

TBV could also be able to reduce survivorship of different species of insect vectors, via immunization with conserved antigens, as proposed by (Canales et al. 2009), providing protection to pathogens transmitted by different vectors. However, significant cross-species effects have yet to be demonstrated.
<table>
<thead>
<tr>
<th>TBV antigen (antigen origin)</th>
<th>Antigen name</th>
<th>Vaccinated animal (adjuvant)</th>
<th>Sera dilution (concentration)</th>
<th>Targeted pathogen</th>
<th>Insect vector</th>
<th>Mean oocyst number</th>
<th>Infectivity %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfs25 (P. falciparum) Virus (Vaccinia virus)</td>
<td>rPfs25*</td>
<td>Mouse (Ribi)</td>
<td>1:2 (200μg/ml)</td>
<td>P. falciparum</td>
<td>An. freeborni</td>
<td>0.1</td>
<td>1%</td>
<td>(Kaslow et al. 1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:4 (100μg/ml)</td>
<td></td>
<td></td>
<td>1</td>
<td>11%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:16 (50μg/ml)</td>
<td></td>
<td></td>
<td>1.4</td>
<td>16%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.5</td>
<td>39%</td>
<td></td>
</tr>
<tr>
<td>Yeast (S. cerevisiae) Pfs25-B</td>
<td>Mouse (FCA)</td>
<td>P. falciparum</td>
<td>1:2</td>
<td>An. freeborni</td>
<td>0</td>
<td>&lt;1%</td>
<td>(Barr et al. 1991)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(MTP*)</td>
<td></td>
<td>1:2</td>
<td></td>
<td>0</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monkey*</td>
<td></td>
<td>1:2</td>
<td></td>
<td>0-2.6*</td>
<td>0-10%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast (S. cerevisiae) Pfs25-B</td>
<td>Mouse (Alum)</td>
<td>P. falciparum</td>
<td>Neat</td>
<td>An. freeborni</td>
<td>0</td>
<td>0%</td>
<td>(Kaslow et al. 1994)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monkey* (Alum)</td>
<td></td>
<td>Neat</td>
<td></td>
<td>0-3.9*</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.02-125*</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19-28.25*</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA vaccination VR1020/25</td>
<td>Mouse</td>
<td>P. falciparum</td>
<td>1:5</td>
<td>An. stephensi</td>
<td>0.17-0.26</td>
<td>3.4-3.8%</td>
<td>(Lobo et al. 1999)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:10</td>
<td></td>
<td>0.19-0.39</td>
<td>2.6-4.3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA vaccination/ (S. cerevisiae) VR1020/25 and Yeast Pfs25</td>
<td>Monkey*</td>
<td>P. falciparum</td>
<td>1:2</td>
<td>An. stephensi</td>
<td>0.9-3.1</td>
<td>5-17%</td>
<td>(Coban et al. 2004)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Montanide ISA 720)</td>
<td></td>
<td>1:4</td>
<td>An. gambiae</td>
<td>2.4-6.4</td>
<td>14-38%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:8</td>
<td></td>
<td>3.2-10.4</td>
<td>19-62%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast (P. pastoris) Pfs25</td>
<td>Mouse (cholera toxin)</td>
<td>P. falciparum</td>
<td>1:2</td>
<td>An. dirus</td>
<td>0</td>
<td>0%</td>
<td>(Arakawa et al. 2005)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:8</td>
<td></td>
<td>0</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:32</td>
<td></td>
<td>10</td>
<td>21.3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast (P. pastoris) Pfs25</td>
<td>Human (Montanide ISA 51)</td>
<td>P. falciparum</td>
<td></td>
<td>An. stephensi</td>
<td>-</td>
<td>&lt;10%</td>
<td>(Wu et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>DNA vaccination (in vivo electroporation) Pfs25</td>
<td>Mouse</td>
<td>P. falciparum</td>
<td>1:2*</td>
<td>An. gambiae</td>
<td>1.0</td>
<td>2.5%</td>
<td>(LeBlanc et al. 2008)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:4*</td>
<td></td>
<td>3.4</td>
<td>9%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:8*</td>
<td></td>
<td>9.5</td>
<td>24%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:16*</td>
<td></td>
<td>51.9</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBV antigen (antigen origin)</td>
<td>Antigen production</td>
<td>Antigen name</td>
<td>Vaccinated animal (adjuvant)</td>
<td>Sera dilution (concentration)</td>
<td>Targeted pathogen</td>
<td>Insect vector</td>
<td>Mean oocyst number</td>
<td>Infectivity %</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-------------------</td>
<td>--------------</td>
<td>------------------------------</td>
<td>-----------------------------</td>
<td>-------------------</td>
<td>--------------</td>
<td>------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Pfs28 (P. falciparum)</td>
<td>Yeast</td>
<td>yPfs28</td>
<td>(FCA+Ribi) (Alum)</td>
<td>Neat²</td>
<td>P. falciparum</td>
<td>An. freeborni</td>
<td>0.33*</td>
<td>0.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>yPfs25+yPfs28</td>
<td></td>
<td>1:40</td>
<td></td>
<td></td>
<td>0.047-0.16</td>
<td>7.6-9%</td>
</tr>
<tr>
<td>Pfs25-Pfs28 (P. falciparum)</td>
<td>Yeast</td>
<td>25-28C</td>
<td>Mouse (Alum)</td>
<td>Neat²</td>
<td>P. falciparum</td>
<td>An. freeborni</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Pf45/48 (P. falciparum)</td>
<td>Bacteria (refolded in vitro)</td>
<td>Pf45/48-10C</td>
<td>Mouse (FCA)</td>
<td>1.2</td>
<td>P. falciparum</td>
<td>An. stevensi</td>
<td>0.45</td>
<td>0.06%</td>
</tr>
<tr>
<td></td>
<td>Bacteria (w/chaperonins)</td>
<td>Pf45/48-10C</td>
<td>Mouse (FCA)</td>
<td>1.2</td>
<td>P. falciparum</td>
<td>An. stevensi</td>
<td>0.5-1</td>
<td>0-12%</td>
</tr>
<tr>
<td></td>
<td>Purification</td>
<td>Pf230</td>
<td>Rabbit (FCA)</td>
<td>-</td>
<td>P. falciparum</td>
<td>An. freeborni</td>
<td>0.2-4</td>
<td>0.3-5.8%</td>
</tr>
<tr>
<td>Pvs25 (P. vivax)</td>
<td>Yeast</td>
<td>Pvs25</td>
<td>Mouse (Alum)</td>
<td>1.2</td>
<td>P. vivax</td>
<td>An. stevensi</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mouse (Alum)</td>
<td>1.2</td>
<td>P. vivax</td>
<td>An. dirus</td>
<td>0.18</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rabbit (Alum)</td>
<td>2.2</td>
<td></td>
<td></td>
<td>4.25</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rabbit (Alum)</td>
<td>1.8</td>
<td></td>
<td></td>
<td>4.06</td>
<td>-</td>
</tr>
<tr>
<td>DNA vaccination</td>
<td>DV25</td>
<td>Mouse</td>
<td>P. vivax</td>
<td>1.8</td>
<td>An. freeborni</td>
<td></td>
<td>3.17</td>
<td>86%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P. vivax</td>
<td>1:10</td>
<td>An. freeborni</td>
<td></td>
<td>0.4</td>
<td>87%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P. vivax</td>
<td>1:10</td>
<td>An. gambiae</td>
<td>An. freeborni</td>
<td>1.5</td>
<td>86%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Padishvili (Alhydrogel)</td>
<td>1:1</td>
<td>An. gambiae</td>
<td>An. gambiae</td>
<td>0.8</td>
<td>93%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Monkey (Montanide ISA 720)</td>
<td>1:4</td>
<td>P. vivax</td>
<td>An. dirus</td>
<td>0.0-0.04</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Human (Alhydrogel)</td>
<td>1:1</td>
<td>P. vivax</td>
<td>An. albimanus</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Monoclonal antibodies were used in transmission blocking assays; (†) Freund’s complete adjuvant; (¶) Muramyl tripeptide; (¶) Aotus trivirgatus; (¶) Oocysts present in midguts of mosquitoes fed on sera from monkeys immunized 22 weeks before challenge. No oocysts were present in mosquitoes that fed on sera from animals immunized 12 weeks before challenge; (-) Undetermined; (‡) Seven days after 3rd immunization; (§) Sixty one days after 3rd immunization; (¶¶) Eighty nine days after 3rd immunization; (¶¶¶) Macaca mulatta; (¶¶¶) Similar results using An. stephensi; (¶¶¶¶) Immunization with 20µg of plasmid; (¶¶¶¶) Sera diluted 1:40; (¶¶¶¶¶) TBV assayed 204 days after immunization; (¶¶¶¶¶¶) Aotus lemurinus griseimembr; (¶¶¶¶¶¶¶) Sera were not previously diluted prior to mixing with equal or greater amount of blood for insect feeding.

Table 2. Transmission blocking vaccines based on pathogens molecules (Modified from Coutinho-Abreu & Ramalho-Orthigao 2010).
5. Conclusion

The technology for generating transgenic and paratransgenic insects to fight vector borne diseases is well established in several laboratories, and the use of such strategies is upon us. However, many studies still need to be performed in order to improve both the design and the efficiency of transgenic insects in preventing disease transmission. In addition, many aspects related to potential environmental impacts of the release of transgenic insects in nature need to be clarified. On the other hand, TBVs may also emerge as a feasible approach against several vector borne diseases, including leishmaniasis and malaria (Malkin et al. 2005; Saraiva et al. 2006). This assumption is supported by at least two recent cases. The first, a fucose-mannose ligand-based TBV was previously tested during a phase III trial (da Silva et al. 2000; Saraiva et al. 2006) and became a commercialized drug in Brazil (Palatnik-de-Sousa et al. 2008) against canine visceral leishmaniasis. Another is based on the P. vivax Pvs25 antigen use as a TBV, based on which was approved during a phase I trial (Malkin et al. 2005; Saraiva et al. 2006).

However, regardless of the approach to be developed, it is clear to many investigators that new technologies to be combined with existing approaches against vector-borne disease are necessary to reduce the burden of such diseases.

6. References


Pvs25 and Pvs28-evaluation of homologous and heterologous antigen-delivery prime-boost strategy."  


Proc Natl Acad Sci U S A 98(9): 5228-33.


Li, C., M. T. Marrelli, et al. (2008).  "Fitness of transgenic Anopheles stephensi mosquitoes expressing the SM1 peptide under the control of a vitellogenin promoter."  
J Hered 99(3): 275-82.


Vaccine 23(24): 3131-8.

Marrelli, M. T., C. Li, et al. (2007).  "Transgenic malaria-resistant mosquitoes have a fitness advantage when feeding on Plasmodium-infected blood."  


Science 323(5910): 141-4.


www.intechopen.com
Transgenesis, Paratransgenesis and Transmission Blocking Vaccines to Prevent Insect-Borne Diseases


Integrated Pest Management is an effective and environmentally sensitive approach that relies on a combination of common-sense practices. Its programs use current and comprehensive information on the life cycles of pests and their interactions with the environment. This information, in combination with available pest control methods, is used to manage pest damage by the most economical means and with the least possible hazard to people, property, and the environment.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:
