7

Genetic approaches in *Aedes aegypti* for control of dengue: an overview

Ken E. Olson[1], *Luke Alphey*[2], *Jonathan O. Carlson*[3], *Anthony A. James*[4]

Abstract

The mosquito-borne dengue viruses (DV) cause an estimated 50 million human infections annually. The incidence of severe dengue disease in Southeast Asia and Latin America is increasing at an alarming rate. There are currently no vaccines or anti-viral therapies available to mitigate dengue disease. Current methodologies for controlling the principal vector, *Aedes aegypti*, are inadequate and ineffective. A potential solution to this growing human-health crisis is to develop new genetics-based vector control (GVC) approaches as part of an integrated control strategy. GVC includes both population reduction and population replacement strategies and represents a broad spectrum of genetic mechanisms at various stages in their development for field-testing. To realize the full potentials of these GVC strategies it is critical that we investigate, evaluate and, where appropriate, develop these strategies to the point where they can be deployed at field sites in one or more disease-endemic countries (DECs).
Keywords: dengue; *Aedes aegypti*; genetic-based vector control

Introduction

Dengue fever (DF) and its more serious form, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DHF/DSS) are caused by four closely related but antigenically distinct, single-strand RNA viruses transmitted by mosquitoes to humans. DVs cause more human morbidity and mortality than any other vector-borne viral disease with 2.5-3.0 billion people at risk of infection and 50-100 million DF and 250,000-500,000 DHF/DSS annual cases (Gubler 1996; 1998). All four DV serotypes cause disease and case-fatality rates for untreated DHF/DSS can be 30-40%. The risk of DHF/DSS is highest in areas where two or more DV serotypes are transmitted (Halstead 1988; Monath 1994; Rigau-Perez et al. 1998). At this time, there is no licensed vaccine and no clinical cure for the disease.

[1] Arthropod-borne and Infectious Diseases Laboratory, 3185 Rampart Road, Mail Delivery 1692, Foothills Research Campus, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523, USA. E-mail: kolson@colostate.edu
[2] Department of Zoology, Wellcome Trust Center, University of Oxford, Oxford OX1 3PS, UK. E-mail: luke.alphey@zoology.oxford.ac.uk
[3] Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO 80521-1692, USA. E-mail: jonathan.carlson@colostate.edu
[4] Department of Microbiology & Molecular Genetics, Department of Molecular Biology & Biochemistry, University of California, Irvine CA 92697-3900 USA. E-mail: aajames@uci.edu

Ae. aegypti is by far the most important and efficient vector of DV because of its affinity for humans (Gubler 1998). Dengue control currently depends on reduction or elimination of *Ae. aegypti*. In the 1940-1960s most tropical American countries used integrated programmes of environmental management and insecticides to eliminate mosquitoes (Gubler 1998), but many of these were abandoned in the early 1970s (Reiter and Gubler 1997). *Ae. aegypti* re-infested countries where it had been eliminated and dengue epidemics renewed. In 2004, *Ae. aegypti* is distributed more widely than it was before eradication began, and is now in large urban areas where a greater number of people than in the past are at risk (Gubler 2004). Remarkably, despite the successes of the past, current dengue vector control programmes are often nonexistent or ineffective (Reiter and Gubler 1997). Rather than maintaining integrated programmes that specifically target *Ae. aegypti*, ministries of health merged all mosquito control and relied on outdoor applications of aerosol insecticides to kill adult mosquitoes. This costly approach is ineffective in most cases because the majority of females rest indoors where they avoid insecticide contact (Reiter and Gubler 1997). Furthermore, many insecticides are useless due to the spread of resistance (Hemingway, Field and Vontas 2002).

Several GVC strategies for reducing DV transmission have been identified as potential dengue disease control methods and are designed either to reduce the overall population of DV-transmitting vectors or to replace existing vector populations with populations that cannot transmit the virus. Two vector population reduction approaches are currently being investigated and are in early laboratory cage trials. The first population reduction strategy is the development and use of natural or genetically engineered densoviruses that are pathogenic to *Ae. aegypti* (Carlson, Afanasiev and Suchman 2000). The second population reduction strategy is the development and use of insects carrying dominant lethal mutations (RIDL, see below, Thomas et al. 2000). This approach would require mating of genetically modified vectors (GMV)-RIDL males with local vector populations producing offspring that die prior to becoming adults. Both approaches are designed to reduce transmission of DVs by reducing the vector population. Approaches designed to replace populations of vectors are more long-term, but could have significant consequences for dengue disease control in the future (James 2000). In these approaches, an effector gene, such as an anti-DV gene, is appropriately expressed to block transmission by the vector. GVC approaches require identification of tissue-specific promoters, anti-pathogen effector genes, and genetic drive mechanisms such as synthetic transposable elements (TE) to introgress the effector gene into the population, eliminating vector competence. Successful GVC strategies will require knowledge of vector ecology in DECs and large cage trials in DECs prior to release of biocontrol agents or GMVs.

Current state of the art

Genetic approaches leading to vector population reduction

Mosquito densoviruses as tools for population reduction and transduction

The *Aedes densonucleosis virus* (AeDNV; family *Parvoviridae*) is mosquito-specific and does not infect vertebrates or non-target invertebrates. Larvae are infected in oviposition sites and die in a dose-dependent manner depending on viral titre and stage of infection. AeDNV is maintained through metamorphosis and is transmitted vertically to offspring (Barreau, Jousset and Bergoin 1997). Infected female mosquitoes deliver viruses to multiple breeding sites and viral concentrations

increase as larvae become infected and shed, thus increasing horizontal transmission to other larvae. Survival of infected adult females also decreases significantly in a dose-dependent manner (Kuznetsova and Butchasky 1988, Suchman and Carlson, unpublished). Shortening the female adult lifespan would reduce vectorial capacity since a significant proportion of females would not survive the extrinsic DV incubation period. Recently, a number of other densoviruses have been discovered that also may be adapted as biocontrol and transducing agents (Kittayapong, Baisley and O'Neill 1999).

AeDNV research has the most immediate potential to deliver products for an effective field trial once a field site is selected and more extensive cage experiments completed. Prototype population cage experiments testing the ability of AeDNV to persist, spread and reduce mosquito populations have already been performed and are encouraging: a relatively low inoculum of virus in a larval rearing site replicates to levels that reduce the mosquito population, and female mosquitoes originally from the site inoculate virus into new sites.

Critical laboratory needs and challenges for using densoviruses as biocontrol agents

Optimize densovirus preparations and use of AeDNV in cage experiments

Laboratory-based cage experiments need to be performed to determine 1) if other densoviruses persist and spread more efficiently than AeDNV; 2) if mosquitoes from DEC field sites are susceptible to AeDNV; 3) if different strains of mosquitoes vary in their susceptibility to other mosquito DNVs; 4) if large-scale production and use of the AeDNV bio-control agent is feasible; and 5) whether recombinant viruses expressing anti-vector effectors (such as RNAi interference targeting the expression of critical vector genes) can enhance lethality of the virus for *Aedes aegypti* larvae.

Large-scale cage trials to assess densovirus potential for persistence and spread

Large-scale cages can be used to replicate laboratory experiments with natural populations under field conditions. *Ae. aegypti* from long-term cage experiments need to be compared with the local populations outside the cage at regular intervals by genetic analyses to look for genetic effects of the virus on populations (Gorrochotegui-Escalante et al. 2002; Garcia-Franco et al. 2002; Root et al. 2003). These studies should yield valuable data on the ability of the virus to persist and spread in a wild mosquito population, and to control mosquito populations in the field. These studies will also help refine the experimental design for cage experiments for a number of GVC strategies.

Development and use of Release-of-Insects-carrying-a-Dominant-Lethal (RIDL), a GMV-based development of Sterile-Insect Technique (SIT).

Drosophila melanogaster has been engineered with the basic genetic properties of an RIDL strain (Thomas et al. 2000; Heinrich and Scott 2000), using a repressible gene expression system (‘tet-off’) based on the tetracycline-repressible transactivator tTA (Baron and Bujard 2000; Gossen and Bujard 1992). Mathematical modelling suggests that for insects with strong density-dependent regulation of population size, a RIDL system imposing lethality at a larval or pupal stage has major advantages over conventional SIT and will provide a simple and effective dengue control method. Preliminary studies in *Ae. aegypti* in which tTA is expressed under tetO/hsp control,

produced >95% lethality in larvae in the absence of tetracycline (Alphey et al., unpublished data). Two transgenic lines are currently being evaluated in cage trials. These lines are being introgressed into the genetic background of local transmitting strains of *Ae. aegypti* to study fitness issues, release parameters, and population-dynamics overtime.

Critical laboratory needs and challenges for using RIDL to reduce vector populations

Increase the penetrance of RIDL-induced lethality

An ideal RIDL system would kill 100% of the individuals supposed to be affected. This is not essential for population suppression or to prevent the spread of the transgene within the target population, and indeed is not provided by current radiation-based SIT programmes for other insects. However, the system can in principle be refined in this regard by using alternative RIDL effectors, such as pro-apoptotic genes (Heinrich and Scott 2000), generating and testing more strains with the current constructs, or combining more than one insertion or construct to give a more highly penetrant and redundant system.

Construct a female-specific RIDL system

Most known female-specific promoters from *Aedes* are induced after the uptake of the blood meal. A RIDL system could potentially be developed around such a promoter to cause females to die soon after biting. Such females would be unable to transmit DV, which have a 10-14 day extrinsic incubation period. Alternatively, it should be possible to identify the sex-specific elements of *Aedes Actin-4*, a gene that expresses an actin in the female pupal developing flight muscles (Muñoz et al. 2004), to drive pre-adult lethality and thereby prevent biting-female development. Such a system would also avoid the need for physical sexing of the release generation and potentially allow the release of any of a wide range of developmental stages.

Determine key parameters for eventual use of RIDL technology in the field

These parameters include the economic and fitness costs of mass rearing of GMV-RIDL strains, the effect of the release ratio (GMV-RIDL / wild-type) of release into cage populations for optimal (most cost-effective) population reduction, and the ability of GMV-RIDL to compete with local mosquitoes for mating and resources. These parameters will feed into a suitable combined epidemiological and entomological model of dengue transmission, the development of which is another key requirement. This will provide a realistic estimate of the cost-effectiveness of RIDL, and a rational method for comparing this to other approaches, applied singly or in combination, in different transmission regimes.

Genetic approaches leading to vector population replacement

Much work has focused on developing GMVs that are refractory for DV transmission by developing germ-line-transformed *Ae. aegypti* that appropriately express an anti-pathogen effector gene. By targeting the pathogen, rather than the vector, expression of the effector gene should have minimal impact on the reproductive fitness of the GMV. The long-term goal is to replace existing transmission-competent vector populations with GMV populations that are no longer permissive for DV transmission. Replacement of *Ae. aegypti* populations to block DV transmission may be a real alternative to current vector control strategies. *Ae. aegypti*

is responsible for most of the severe dengue epidemics, it is relatively easy to manipulate genetically and maintain in the laboratory, and the vectors continuously exchange genes locally and appear to have few gene flow barriers within 150 km (Gorrochotegui-Escalante et al. 2002). At least three genetic-transformation systems have been described and used successfully in *Ae. aegypti* to generate GMVs. These transformation systems are based on the Class II TEs *Mos1* (Mariner), *Hermes* and *piggyBac* (Jasinskiene et al. 1998; Coates et al. 1998; Kokoza et al. 2000). *Mos1* and *piggyBac* are the most commonly used TEs for generating GMVs.

Anti-dengue virus effector genes – RNAi

During the last three years, considerable progress has been made toward identifying effector genes that can profoundly reduce *Ae. aegypti* competence for DV transmission (Adelman et al. 2001; 2002; Olson et al. 2002; Tavanty et al. 2004). The major thrust of research has been to design and express double stranded RNAs (dsRNAs) that make DV-susceptible cells non-permissive for virus replication. This strategy is based on RNA interference (RNAi), an ancient potent, innate immune response in insects and a related response termed post-transcriptional gene silencing in plants (Tijsterman, Ketting and Plasterk 2002).

We now know that *Drosophila melanogaster*, *Caenorhabditis elegans*, humans and plants have the RNAi pathway, which is triggered by the presence of intracellular double-stranded RNA (dsRNA). The presence of dsRNA in cells is an early warning signal of RNA-virus invasion that directs an innate response resulting in destruction of any mRNA having sequence identity with the dsRNA. Many RNA viruses generate dsRNA in infected cells as a byproduct of replication and these replicative intermediates serve as potent recognition patterns for inducing the RNAi intracellular response. If RNA viruses trigger RNAi, why are mosquitoes such efficient vectors of arboviruses? We do not know for sure, but DV may escape the antiviral effects of RNAi in competent mosquitoes either by failing to present the threshold concentration of dsRNA molecules required for triggering the response or by encoding a viral protein that suppresses the RNAi response. Currently, there is no evidence for a DV RNAi suppressor protein. However, Uchil and Satchidanandam (2003) have recently shown that the dsRNA replicative form (RF) of DVs is sequestered in double-membrane structures in the cytoplasm of infected cells which may limit RF exposure to the RNAi pathway.

RNAi is activated by dsRNA and results in a reduced steady-state level of specific RNA molecules with sequence similarity to the dsRNA (Cogoni and Macino 1997; Vaucheret et al. 1998). The mechanism of RNAi has been studied in some detail in *Drosophila melanogaster*. In the fruitfly, the RNase III enzyme Dicer is responsible for digesting dsRNA into 21-23 bp small interfering RNAs (siRNAs). The siRNAs are then unwound into single-stranded siRNAs in an ATP-dependent step and incorporated into an enzyme complex termed the RNA-induced silencing complex (RISC). The single-stranded siRNAs guide RISC to the target mRNA and the complex cleaves the message or inhibits its translation (Schwarz et al. 2002). This strategy has been used in transgenic plants to develop resistance to a number of RNA-virus pathogens. Several groups now have evidence that mosquito species such as *Ae. aegypti*, *Anopheles stephensi* and *An. gambiae* develop an RNAi response very similar to that found in *D. melanogaster*. These vectors are capable of silencing endogenous gene expression or virus replication after introduction of dsRNA targeted to a specific gene (Adelman et al. 2002; Travanty et al. 2004; Brown et al. 2003). Replication of several arboviruses appears to trigger the RNAi response in mosquito cells and we

now have evidence for the genes involved in the *An. gambiae* antiviral response to the arbovirus *O'nyong-nyong alphavirus* (*Togaviridae*) (Sanchez-Vargas et al. 2004; Keene et al. 2004).

RNAi maybe an Achilles heel for replication of RNA viruses and we should be able to induce a robust RNAi response to DVs in the midgut or other relevant tissues of a transgenic mosquito by expressing DV-specific dsRNA. This strategy would sensitize the cells to the presence of the RNA virus leading to the destruction of the virus genome either as the virus uncoats or following virus transcription in the cell. The midgut is a likely target for mounting this line of defence because it is the first tissue the virus encounters in the vector and is the major determinant of vector competence in the mosquito. In addition, oral infection of midguts with high concentrations of virus begin with relatively few foci of infection of epithelial cells that spread throughout the gut over a 5-7 day period prior to dissemination. A virus-specific dsRNA should be able to suppress DV replication during that time frame. Both *Ae. aegypti* midgut and salivary-gland promoters are available to test whether RNAi can be used to promote resistance to DVs in the vector (James et al. 1991; Moreira et al. 2000).

The RNAi approach of developing resistance in *Ae. aegypti* has the following advantages: 1) RNAi does not require expression of a potentially antigenic protein; 2) the strategy utilizes the machinery of a natural innate immune response that is present in the mosquito (Sanchez-Vargas et al. 2004); 3) a number of anti-DV dsRNA effector sequences have already been identified that cause profound resistance in mosquito cell culture and in adult mosquitoes (Adelman et al. 2001); 4) the anti-DV dsRNA effector sequence (500-600 bp) should be less prone to the effects of single-point virus mutations and selection since the active units of RNAi activity are 21-23 bp siRNA blocks formed from the dsRNA trigger (Travanty and Olson, unpublished data, Blair, Adelman and Olson 2000); 5) transgenic lines that express dsRNAs from several non-*Ae. aegypti* promoters have now been generated (Travanty et al. 2004); 6) DV-2 pathogenesis studies of virus in *Ae. aegypti* have been performed to determine the temporal and spatial infection patterns of the virus after oral infection (Sanchez-Vargas and Olson, unpublished data); 7) DV challenge protocols for assessing resistance in transgenic mosquitoes are available (Sanchez-Vargas and Olson, unpublished data).

Critical laboratory short-term needs and challenges for using RNAi-based disease control strategies and other effector gene strategies

Identify Ae. aegypti midgut and salivary gland promoters that can be utilized to deliver anti-DV at the correct time and place in the mosquito tissue.

We are currently evaluating the *Ae. aegypti ferritin heavy chain, carboxypeptidase, GFAT* and *glutamine synthetase* midgut promoters and the *D7* and *apyrase* salivary-gland promoters for gene-expression potential. To test both RNAi and promoter activity we are developing transgenics that express GAL4 and transgenics with anti-DV dsRNA expression under UAS control (Brand and Perrimon 1993). The two lines can be crossed and offspring evaluated for RNAi efficacy. Identifying suitable promoters is a key to this strategy. It is apparent that the siRNA 23-nucleotide signal is not amplified in insects as it is in plants and *C. elegans* therefore RNAi probably does not spread from cell to cell in mosquitoes (Hoa et al. 2003). This makes it critical that the antiDV dsRNA is expressed in the same vector cells that are critical for DV infection and replication.

Identify the most efficient construct format for delivering the dsRNA

Currently we are designing effector RNAs that comprise 300 bases of DV target sequence in a sense orientation followed by *Ae. aegypti* intron sequence and an exact antisense complement of the sense RNA (Adelman et al. 2002; Travanty et al. 2004). There may be a need to develop new constructs for expression in mosquitoes that form larger dsRNAs in the 500-600 bp range. Does the intron size matter, since it is ultimately cleaved? What untranslated sequences are needed to stabilize expression of the effector gene in target tissues?

Identify the specificity of an effector dsRNA based on DV2 sequence

Will it protect the mosquito from infection with other DV2 genotypes or other DV serotypes? There is indication that it is possible to target multiple serotypes by carefully choosing DV-specific target sequences (Sanchez-Vargas et al. 2004). Will this approach drive selection of DV with altered infection characteristics?

Develop a recombinant/reporter virus to rapidly assess RNAi in transgenic mosquitoes

Researchers have considerable experience developing infectious cDNA clones of flaviviruses and alphaviruses and have developed alphaviruses that express eGFP as a marker of infection (Foy et al. 2004; Keene et al. 2004). The development of a DV-expressing GFP as a marker would greatly facilitate identification and characterization of transgenic lines for virus resistance.

Development of protein-based effector genes

A number of effector-gene strategies will most likely need to be developed to engineer resistance effectively into vector populations. Ito et al. (2002) showed that peptides recognizing mosquito-tissue surface proteins block entry of a malaria sporozoite into the salivary glands of a transgenic mosquito. The challenge here is to identify effector proteins that block DV transmission yet can be effective against a rapidly evolving RNA virus. These peptide-based effectors could take the form of single-chain antibodies (Cappuro et al. 2000??) that bind to and neutralize DV or mimic the envelope glycoprotein domain-III region of DVs (Hung et al. 2004).

Long-term research challenges for GVC-replacement technology

Development of an efficient anti-DV effector gene is only the first step towards the long-term goal of using genetically manipulated insects to control DV. We also need to demonstrate that transposon-mediated systems or other genetic drive systems will successfully invade field populations. The first step in this process is to evaluate transposon-mediated drive of genes through mosquito cage populations. In *D. melanogaster*, studies with autonomous (self-mobilizing constructs that carry a copy of their transposase within the transposon) and non-autonomous (stable constructs mobilized only by externally supplied transposase) TEs carrying marker genes have shown that elements will increase the frequency of the marker gene when introduced into cage population of flies (Carareto et al. 1997). This mobility was characterized by a tight linkage of the transposon with an active marker gene for as many as 40 generations. However, stability of the marker gene varied inversely with the size of the final, ‘loaded’, autonomous element. Researchers need to conduct cage experiments to evaluate the mobility and stability of loaded autonomous TEs as they spread through cage populations of mosquitoes; maintenance of the integrity of the ‘loaded’ TE during population replacement and beyond is one of several major

challenges to the development of usable gene drive systems. Obviously serious discussions must take place to identify potential field sites for evaluation of control, especially those strategies involving vector replacement strategies.

Future directions for research and capacity/partnership building

Discussion of other laboratory and field research that will need to be performed to realize GVC approaches fully is found elsewhere in this book. Critical research needs include the development and the characterization of genetic drive mechanisms, the development of a much more complete understanding of the ecology of dengue disease transmission in DECs, and the formation of full and meaningful partnerships with DECs to evaluate GVC approaches. To realize the full potentials of GVC strategies it is critical that we investigate, evaluate and, where appropriate, develop GVC strategies to the point where they can be deployed at field sites in one or more DECs. A number of gaps in knowledge have slowed or prevented the development of genetic control methods. These gaps exist between the state-of-the-art laboratory development of novel anti-DV tools and knowledge of field properties of mosquitoes that will affect their use, and between scientists in the developed world and the DEC scientists who would be responsible for implementing the technology. Further gaps exist among scientists and the agencies that would be responsible for the deployment of any genetic control strategy, and in policies and procedures for evaluating how genetic control methods fit into the overall strategy of existing or planned control programmes; these problems have become acute as the tools have now been developed to allow implementation of some methods. Finally, gaps exist between the enthusiasm of scientists for these genetic methods and the level of awareness of potential end-users of the risks and benefits of using them for controlling dengue transmission.

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