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Gene silencing and gene drive in dengue vector control

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Vector-borne diseases are the most feared diseases throughout the world. Mosquitoes are the prime human disease vectors as they are responsible for nearly one million human deaths every year. So they are declared as the most dangerous insects to mankind. *Aedes aegypti* and *Ae. albopictus* are the most significant mosquito species, because of their role in transmitting dengue virus. These blood feeding ectoparasites of man and other vertebrates have developed excellent adaptations to survive and multiply in and around human habitations. Chemical-based mosquito control method does not give good results due to rapid development of pesticide resistance in mosquitoes. The past four decades have witnessed the development of several alternate mosquito control methods. Genetic control technologies have been recently developed as efficient and ecofriendly methods. Inundate release of genetically modified mosquitoes with lethal or pathogen-resistant genes for population reduction is a recent technology in mosquito control programme. Recent developments in molecular and genome editing technologies have made it easy to produce thousands of transgenic mosquitoes for field release. The present review highlights various scientific reports and research findings on gene silencing and gene drive techniques in dengue mosquito control.

Keywords: CRISPR/Cas9, Dengue, Genome Editing, miRNA, RNAi, siRNA.

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Introduction

Mosquitoes are the most harmful public health pests in the world as they are vectors of several dreadful diseases, viz. dengue, dengue haemorrhagic fever, malaria, West Nile fever, brain fever, and Yellow fever. In 2015, malaria alone caused 4,38,000 human deaths globally[1]. Dengue is also a fearful mosquito-borne disease, which causes over 20,000 deaths worldwide every year[2]. Some diseases like chikungunya, zika fever, and filariasis lead to physical damages and economic losses. Every year, incidences of dengue and other mosquito-borne disease are increasing due to climate change, globalization and viral evolution[2].

A total of 3,540 mosquito species grouped under two subfamilies and 112 genera have been documented throughout the world[3]. Indian mosquito fauna consists of 393 species, 49 genera, and 41 subgenera[4]. The world mosquito fauna includes 950 species of *Aedes*, out of which nearly 115 species of *Aedes* are reported from India[5]. *Aedes aegypti* and *Ae. albopictus* are the two major dengue vector mosquitoes widely distributed in India. Dengue fever virus (DENV) is spread by *Ae. aegypti* and *Ae. albopictus*. Every year, 390 million people are infected by DENV throughout the world[6]. Some species of the genus *Aedes*, including *Ae. aegypti* also transmit zika virus, the outbreak of which has been recently reported in Brazil. Dengue virus has spread into a wide geographical area in the world and its vector has spread into new areas. In 2012, the World Health Organization announced dengue as the "most important mosquito-borne viral disease in the world"[7-10]. In India, 18 states have been identified as endemic to dengue[11].

Till date, there is no antiviral drug or vaccine to combat DENV[12]. The two common ways of preventing mosquito-borne diseases are mosquito eradication and personal protection from mosquito bites. Insecticide application is the most effective way of mosquito eradication. Mosquitoes are developing resistance to many synthetic insecticides[13] more quickly than any other pests. So they are not easily controlled by insecticide application. Natural mosquito larvicides obtained from plant extracts and plant compounds[14-21], synthetic plant compounds[22], Actinomycetes[23-25], and marine sponge[26,27] have been reported as ecofriendly mosquito management products. Use of *Bacillus thuringiensis israelensis*

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(Bti) for dengue vector control has been reviewed[28] by Boyce *et al*.

Apart from natural pesticides, many other ecofriendly approaches have been researched for dengue mosquito control.

Wolbachia-based dengue vector control

Wolbachia, the gram negative bacteria are endosymbionts found in many insect species including some mosquito species. These bacteria pass down from one generation to the next through the eggs. This bacterium reduces the ability of *Aedes* mosquitoes to become infected with dengue virus. *Wolbachia* do not naturally infect dengue and malaria vector mosquitoes[29,30]. So *Wolbachia* are deliberately introduced into *Aedes* mosquitoes for the eradication of mosquito population. Many investigators have reported the use of *Wolbachia* bacteria in shortening the lifespan of vector mosquitoes. Dengue virus needs a definite period of development inside mosquitoes called extrinsic incubation period (EIP) before they are transmitted to a human host[31]. The EIP is long relative to the normal longevity of adult mosquito and so the pathogen transmission is more commonly done by older mosquitoes[32-35]. Hence, shortening of the vector mosquito lifespan will lead to the reduction of its vectorial capacity[36].

Xi *et al*. seeded an uninfected *Ae. aegypti* population with *Wolbachia* (wAlbB) through infected females and found that wAlbB caused high rates of cytoplasmic incompatibility in *Ae. aegypti*[29]. The laboratory studies by McMeniman *et al.*[33] also clearly showed that *Wolbachia* infection reduced adult life span in *Ae. aegypti* up to 50 %.

Genetic control methods

Ae. aegypti genome project has identified 15,419 gene models by using expressed sequence tags and gene structure information collected from other dipteran insects[37]. Genetic control methods aim either to destroy vector mosquitoes or to prevent the virus replication inside mosquitoes by inactivating the mosquito genes which support virus replication. Alphey *et al.* have reviewed different genetic control methods including sterile insect technique (SIT) and gene drive systems used in *Aedes* mosquito control[38]. SIT is a genetic control strategy, which is basically a population suppression method. It is a species specific and ecofriendly approach[39,40]. Sterilization of male mosquitoes by means of radiation or chemosterilants for field release is the basic requirement in SIT technique. The wild female mosquitoes that are mated with sterilized males will lay eggs, but the egg hatching or development of hatched larvae is arrested leading to population decline. In SIT technique, the separation of male mosquitoes is a difficult task. Moreover, the mating capability of sterilized males is affected because the radiation treatment damages the somatic cells of male insects[41], which necessitates release of more numbers of sterilized male mosquitoes[42].

Recent developments in molecular engineering have made it possible to eradicate mosquitoes or to restrict the pathogen carrying capacity of vector mosquitoes by genetic control methods. RNA interference and gene drive systems are postulated as important genetic methods applicable to dengue mosquito control.

RNA interference method of dengue mosquito control

RNA interference (RNAi) is a novel genetic control method, which can be utilized to eradicate dengue mosquitoes by knockdown of essential mosquito genes. RNAi–mediated gene silencing is achieved by the introduction of double-stranded RNA (dsRNA). The introduced dsRNA will silence the translation of target mRNA in a sequence-specific manner. Vector competence in *Ae. aegypti* to DENV is a natural phenomenon and marked differences in VC is found among *Ae. aegypti* populations. RNAi is a natural process and a major antiviral innate immune pathway in mosquitoes for antagonizing the arboviruses[43-46]. In RNAi-mediated gene silencing, either the target mRNA is degraded or the translation is inhibited. Whyard *et al*. observed that 37 genes were expressed in the testes of *Ae. aegypti,* of which 6 genes were found expressed in both testes and ovaries[47]. They silenced the testis genes and female sex determination gene by delivering dsRNA through oral feeding of larvae, which resulted in the arrest of sperm production and male-biased population.

siRNA-based interference

Small interfering RNAs (siRNA) are important components in RNAi process. They are short dsRNAs with 20-25 bp resulting from the cleavage of dsRNAs. Essential mosquito genes responsible for normal growth, development, reproduction, and other physiological processes of mosquitoes are the important targets of siRNA. Some earlier reports on RNAi-based *Aedes* control are presented in Table 1. Delivery of dsRNA into mosquito larvae is a challenging task; it is achieved by different methods,

viz. oral treatment, topical application, and microinjection. Microinjection mode of dsRNA delivery needs technical skills and is time consuming[48]. Oral and topical applications of dsRNA are feasible. Microalgae and other microorganisms in water are the major food sources for the mosquito larvae[49] and such unicellular organisms can be used as vectors for the transfer of dsRNA into mosquito larvae.

Kumar *et al*. selected the micro algae *Chlamydomonas reinhardtii* as a delivery system for dsRNA into mosquito larvae through ingestion to silence the 3-hydroxykynurenine transaminase (3-*HKT*), an essential gene of mosquitoes[50]. In the study, they developed dsRNA from 328 bp region of *Anopheles gambiae 3-HKT* gene and integrated with the chloroplast genome of *C. reinhardtii*. Maximum larval mortality of 53 % was reported in this study when transgenic microalgae were eaten by *An. stephensi* larvae.

Zhang *et al*. used chitosan nanoparticle as a delivery system for siRNA. They targeted two chitin synthase genes namely *AgCHS1* and *AgCHS2* in *An. gambiae*[51]. The results clearly showed that *AgCHS1* transcript level and chitin content in the larvae were reduced by 62.8 and 33.8 %, respectively. Kumar *et al*.[52] investigated the efficacy of oral administration of dsRNA through chitosan nanoparticle to suppress the expression of wing development gene in *Ae. aegypti*. The oral treatment with dsRNA caused abnormal wing growth and down regulated the transcription of wing development gene.

In a recent study, the dsRNA delivery efficiency of three types of nanoparticles namely chitosan, carbon quantum dot and silica nanoparticles into *Ae. aegypti* was tested to knockdown *SNF7* and *SRC* genes. The results clearly showed that carbon quantum dot nanoparticles were the most efficient carriers for dsRNA to cause silencing of target genes and larval mortality[53]. Topical application is also an efficient method of delivering dsRNA to inactivate essential mosquito genes in *Aedes* mosquitoes[54]. Silencing of the *Ae. aegypti* inhibitor of apoptosis protein 1 gene (*AaeIAP1*) by topical application of dsRNAs caused up to 48 % mortality[54].

Though siRNA-based gene knock-down is a rapid method, it is a temporary one and not inherited to next generation.

miRNA-based interference

Micro RNAs (miRNAs) are short single-stranded RNAs with 20-22 nucleotides formed from the processing of noncoding RNAs. They have been shown to regulate apoptosis, viral infection, and other critical biological events in animals and plants[55-58]. In mosquitoes, a large number of miRNAs have been reported to exhibit altered profiles during infection and regulate the host immune responses[59-63]. Mosquito miRNAs can either positively or negatively control the host response to pathogen infection.

Table 1—Different genome editing tools and target genes of vector mosquitoes reported by previous workers

Targeted gene	Method used	Target mosquito	Reference
Testis genes and female sex determination gene	RNAi	*Aedes aegypti*	47
3-hydroxykynurenine transaminase	Gene silencing	*Anopheles stephensi*	50
Chitin synthase genes (*AgCHS1*&*AgCHS2*)	RNAi	*Anopheles gambiae*	51
Wing development vestigial gene	RNAi	*Aedes aegypti*	52
SNF7 and SRC	RNAi	*Aedes aegypti*	53
Inhibitor of apoptosis protein 1 gene (*AaeIAP1*)	RNAi	*Aedes aegypti*	54
miR-1174	miRNA	*Aedes aegypti, Anopheles gambiae*	67
miR-281	RNA silencing	*Aedes albopictus*	68
Orco (odorant receptor coreceptor)	ZFN	*Aedes aegypti*	75
miR-275	miRNA	*Aedes aegypti*	75
AaegGr3 (*Ae. aegypti* gustatory receptors)	ZFN	*Aedes aegypti*	77
Aaeg-wtrw	CRISPR/Cas9	*Aedes aegypti*	79
Male determining (*nix*) genes	CRISPR/Cas9	*Aedes aegypti*	80
ECFP (Enhanced Cyan Fluorescent Protein gene)	CRISPR/Cas9	*Aedes aegypti*	81
kmo, *loqs*, *r2d2*, *ku70*, *lig4* and *nix*	CRISPR/Cas9	*Aedes aegypti*	82
Kmo (kynurenine 3-monoxygenase) gene	TALEN	*Aedes aegypti*	85
v-ATPase, subunit-A gene	RNAi	*Aedes aegypti*	92
axon guidance gene *semaphorin-1a* (*sema1a*)	RNAi	*Aedes aegypti*	93
ectoderm-derived *AgCHS1* transcripts as well as midgut-specific *AgCHS2*	RNAi	*Anopheles gambiae*	93
AeCPA-1 (Carboxy Peptidase A-1)	RNAi	*Aedes aegypti*	94

Wolbachia uses *aae-miR-2940* to regulate a methyltransferase gene for blocking DENV replication[30,64]. The miRNAs, *aga-miR-2304*, *aga-miR-2390*, and *aae-miR-375* are involved in the modulation of host immune response in mosquitoes and *aae-miR-375* enhances DENV-2 infection in an *Ae. aegypti* cell line[65,66]. The mosquito- and gut-specific miRNA, *miR-1174* is essential for proper sugar absorption, fluid excretion, blood intake, egg maturation, and survival in female mosquitoes. The *miR-1174* is very much expressed and localized in the posterior midgut, where blood digestion takes place in mosquito. The depletion of *miR-1174* resulted in severe defects in sugar absorption and blood intake. Serine hydroxymethyltransferase (*SHMT*) is a direct *miR-1174* target and *miR-1174* is essential for fine-tuning the *SHMT* transcript to levels necessary for normal mosquito gut functions[67].

In *Ae. albopictus*, *miR-281* expression was found in the midgut and was up-regulated by dengue virus infection in the mosquito after 4 days. But the knock-down or silencing of *miR-281* by antagomiRs injection into infected mosquitoes down-regulated the virus replication[68]. In most of the blood feeding mosquito species, the eggs mature only after blood meal intake by the females[69]. Blood feeding activates the release of neuropeptides from neurosecretory cells, which in turn stimulates the ecdysteroid hormone production in the ovaries[70-73]. Studies showed that the expression of *miR8/miR*-200 family of miRNAs was very high in the fat bodies of female mosquitoes after blood meal and *miR8* was found to be directly regulating a molecule known as 'swim' (secreted wingless-interacting molecule) in the fat body of female mosquitoes; high levels of 'swim' is considered detrimental to egg development. So the knockdown of *miR8* leads to abnormal ovarian development[74]. The blood meal digestion is also regulated by miRNAs. The knockdown of *miR275* in female mosquitoes by injecting specific antagomiR of *miR275* after blood meal caused defects in blood digestion, fluid excretion and egg development in *Ae. aegypti*[75].

Gene drive

The gene drive systems are designed to enhance the inheritance of specific alleles in the vector mosquito populations[76]. The developments in genome editing technologies help the scientists to make changes in the genome of desired organisms. Mass release of transgenic *Ae. aegypti* mosquitoes that carry antipathogenic constructs can replace wild-type mosquito populations and thus prevent dengue epidemics[43]. Previously, some investigators tried to generate transgenic mosquitoes using transposon-based systems and their efforts resulted in some rare successes[77-79]; after 10 years of their investigations efficient methods for gene transformation were developed in mosquitoes[80,81]. In genome editing, the genetic code can be modified and gene knockout is possible. A double-strand break (DSB) in the chromosomes is possible by any one of the three common tools namely zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), or clustered regularly interspaced palindromic repeat associated (CRISPR/Cas) proteins.

ZFN binds on target site of DNA and cleaves at desired positions of the genome. It consists of a DNA binding domain and nuclease domain. The nuclease domain consists of *FokI* restriction enzyme which cleaves the target site of DNA. DeGennaro *et al*. targeted *Ae. aegypti* odorant receptor coreceptor (*orco*) gene to investigate the role of *orco* gene in the odorant receptor pathway during host identification[82]. The study also focused on finding the sensitivity to N,N-diethyl-meta-toluamide (DEET). For this experiment, initially they injected the designed ZFN into *Ae. aegypti* embryos. The resulted *orco* mutants showed reduced odour-evoked activity than normal wild type *Ae. aegypti*. Liesch *et al*. used ZFN to produce neuropeptide Y-like receptors 1 (*npylr1*) null mutants[83]. In the study, 200 ng/µL designed ZFN and 850 ng/µL homologous recombination vector was injected into *Ae. aegypti* embryos and it was noticed that the host-seeking behavior of *npylr1* mutants was not inhibited. In another experiment, McMeniman *et al*. used ZFN to mutate *Ae. aegypti* gustatory receptors (*AaegGr3*) gene by injecting the designed ZFNs into *Ae. aegypti* embryos[84]. *AaegGr3* is a subunit of the heteromeric CO_2 receptor. The *AaegGr3* mutants produced were found to have reduced electrophysiological and behavioral responses to CO_2.

Site-specific nucleases namely TALENs and CRISPR/Cas9 are considered as important nucleases used in genome editing. Gene drive systems based on TALENs and CRISPR/Cas9 can spread desirable mosquito phenotypes in wild populations. TALENs are two component site specific nuclease systems. They have been used in genomic editing of many different genomes including *Ae. aegypti*[85]. TALEN contains DNA binding domain that consists of 33-34

amino acids. There are two variable sites in the domain at 12th and 13th amino acids, which recognize specific nucleotide in the target genome. Successful knockout of kynurenine 3-monoxygenase (*kmo*) gene, which is essential for eye pigmentation in insects using TALEN has been reported[85]. Knockout of *kmo* gene resulted in 20-40 % survival in *Ae. aegypti* and nearly 20 % of the survived mosquitoes had white eyes.

CRISPR/Cas9 is a flexible tool for making breaks in the genomes of wide variety of organisms including vector mosquitoes. CRISPR simplifies the process of deleting, adding, or modifying genes. CRISPR/Cas9 system has been reported as very effective genome editing tool to make site-specific mutations in *Ae. aegypti*[86]. The function of the Cas9 nuclease is to make double stranded break near to proto spacer adjacent motif sequence. As of 2014, CRISPR/Cas9 had successfully been tested in cells of 20 species; the edits modified their germline, which were found to be inherited. In a study, the male determining (*Nix*) gene was targeted by CRISPR/Cas9-mediated gene editing method via embryonic microinjection[87]. The resulted mutants were feminized genetic males. Dong *et al*. used the CRISPR/Cas9-based gene editing to alter Enhanced Cyan Fluorescent Protein (*ECFP*) gene in *Ae. aegypti*[88]. In this experiment, they used Cas9 enzyme and two single guided RNAs (sgRNAs) and targeted different regions of *ECFP* gene. The first generation mutants showed 5.5 % knockout efficiency. Successful integration of 200-bp single-stranded DNA oligodeoxynucleotide (ssODN) donor as a template for homology-directed repair was done in *Aaeg-wtrw* locus and mutations were generated using CRISPR/Cas9 in *Ae. aegypti* mosquitoes[86]. Basu *et al*. used CRISPR/Cas9 tool to modify 6 different genes namely *kmo*, *loqs*, *r2d2*, *ku70*, *lig4*, and *nix*in in *Ae. aegypti*[89]. In this study, the 40 different sgRNAs designed were assessed for their editing potential in transient embryo and as a result they achieved somatic and germ line mutations in *Ae. aegypti* mosquitoes.

Since genome editing technique by using CRISPR/Cas9 system is done at DNA level, it does not interfere with the endogenous machinery of cell[90]. Off-target effect is a major problem in CRISPR/Cas9 technique and this problem can be overcome by the introduction of mutations in sgRNAs and delivery of multiple sgRNAs for a single target[91].

Limitations of gene drive

Gene drive system has some limitations. In many cases, gene drive is not reversible and so much care is required when organisms with a gene drive are released into the environment. Gene drive requires many generations to spread through wild mosquito populations and may be affected by several factors including the survival rate of drive carrying mosquitoes, their fitness for mating with wild population and gene flow in the natural population. Drive-resistant alleles may arise for gene drives. Cross-breeding or gene flow may allow a gene drive to move outside the target population. Release of gene drive mosquitoes in the environment may have harmful side effects on other organisms. Further studies are needed to overcome these challenges.

Conclusion

Climate change, global warming, and urbanization are supporting the dengue outbreaks in new areas. Every year, the number of dengue cases and human deaths due to dengue fever is increasing. Hence, prevention of dengue virus spread and eradication of *Aedes* mosquitoes are urgent needs throughout the world. In addition, non-chemical strategies are need of the hour to avoid pesticide resistance development in *Aedes* mosquitoes. RNAi and gene drive systems are emerging as promising alternate strategies of *Aedes* mosquito eradication. In India, research in these new areas of mosquito control research should be intensified.

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References

1 World Health Organization, World Health Statistics, 2015.
2 Murray N E A, Quam M B and Smith A W, Epidemiology of dengue: Past, present and future prospects, *Clin Epidemiol*, 2013, **5**, 299–309.
3 Harbach R E, Mosquito Taxonomic Inventory, 2014, http://mosquito-taxonomic-inventory.info (Accessed on 17 October, 2014).
4 Bhattacharyya D R, Rajavel A R, Natarajan R, Mohapatra P K, Jambulingam P, Mahanta J, *et al*., Faunal richness and the checklist of Indian mosquitoes (Diptera: Culicidae), *Check List,* 2014, **10**(6), 1342–1358.
5 Laxmikant S, Distribution of *Aedes aegypti* and *Aedes albopictus* from Jalna District (MS) India, *Biosci Disc*, **5**(1), 11–14.
6 Bhatt S, Gething P W, Brady O J, Messina J P, Farlow A W, Moyes C L, *et al*., The global distribution and burden of dengue, *Nature*, 2013, **496**(7446), 504–507.

7 Gubler D J, Dengue, urbanization and globalization: The unholy trinity of the 21st century, *Trop Med Health*, 2011, **39**, 3–11.

8 World Health Organization, Global Strategy for Dengue Prevention and Control, 2012-2020. Geneva, WHO Press, 2012.

9 WHO TDR Global Alert and Response Dengue/Dengue HaemorrhagicFever, WHO, Geneva, 2013, http://www.who.int/csr/disease/dengue/en/index.html (Accessed on 02 March, 2014).

10 Gibbons R V and Vaughn D W, Dengue: An escalating problem, *BMJ*, 2002, **324,** 1563–1566.

11 Dawn A, A spatio-temporal analysis of dengue fever in West Bengal with special reference to Kolkata municipal corporation area, *IOSRJ Human Soc Sci*, 2014, **19**(1), 46–55.

12 Roche R R and Gould E A, Understanding the dengue viruses and progress towards their control, *BioMed Res Int*, 2013, http://dx.doi.org/10.1155/2013/690835.

13 Brown A W, Insecticide resistance in mosquitoes: A pragmatic review, *J Am Mosq Cont Assoc*, 1986, **2**, 123–40.

14 Shaalan E A S, Canyon D, Younes M W F, Abdel-Wahab H and Mansour A H, A review of botanical phytochemicals with mosquitocidal potential, *Environ Int*, 2005, **3**, 1149–1166.

15 Ghosh A, Chowdhury N and Chandra G, Plant extracts as potential mosquito larvicides, *Indian J Med Res*, 2012, **135**, 581–598.

16 Sivaraman G, Gabriel Paulraj M, Rajiv Gandhi M, Reegan A D and Ignacimuthu S, Larvicidal potential of *Hydnocarpus pentandra* (Buch.-Ham.) Oken seed extracts against *Aedes aegypti* Linn. and *Culex quinquefasciatus* (Say) (Diptera: Culicidae), *Int J Pure Appl Zoo*, 2014, **2**(2), 109–112.

17 Reegan A D, Gabriel Paulraj M and Ignacimuthu S, Larvicidal, ovicidal, repellent and histopathological effects of orange peel (*Citrus sinensis* Osbeck) extracts on *Anopheles stephensi* Liston mosquitoes (Diptera: Culicidae), *Int J Appl Biol*, 2013, Special issue No. 1, 24–29.

18 Reegan A D, Rajiv Gandhi M, Gabriel Paulraj M and Ignacimuthu S, Larvicidal activity of medicinal plant extracts against *Culex quinquefasciatus* Say and *Aedes aegypti* L. mosquitoes (Diptera: Culicidae), *Int J Pure Appl Zoo*, 2014a, **2**(2), 205–210.

19 Reegan A D, Rajiv Gandhi M, Gabriel Paulraj M and Ignacimuthu S, Ovicidal and oviposition deterrent activities of medicinal plant extracts against *Aedes aegypti* L. and *Culex quinquefasciatus* Say mosquitoes (Diptera: Culicidae), *Osong Pub Health Res Perspect*, 2014b, http://dx.doi.org/10.1016/j.phrp.2014.08.009.

20 Rajiv Gandhi M, Reegan A D, Sivaraman G, Sivasankaran K, Gabriel Paulraj M and Ignacimuthu S, Larvicidal and repellent activities of *Tylophora indica* (Burm. F.) Merr. (Asclepiadaceae) against *Culex quinquefasciatus* Say and *Aedes aegypti* L. (Diptera: Culicidae), *Int J Pure Appl Zoo*, 2014, **2**(2), 113–117.

21 Rajiv Gandhi M, Reegan A D, Ganesan P, Sivasankaran K, Gabriel Paulraj M, Balakrishna K, Ignacimuthu S and Al-Dhabi N A, Larvicidal and pupicidal activities of Alizarin, isolated from roots of *Rubia cordifolia* against *Culex quinquefasciatus* Say and *Aedes aegypti* (L.) (Diptera: Culicidae), *Neo Ent*, 2016, DOI 10.1007/s13744-016-0386-x.

22 Gabriel Paulraj M, Reegan A D and Ignacimuthu S, Toxicity of benzaldehyde and propionic acid against immature and adult stages of *Aedes aegypti* (Linn.) and *Culex quinquefasciatus* (Say) (Diptera: Culicidae), *J Entomol,* 2011, **8**(6), 539–547.

23 Anwar S, Ali B, Qamar F and Sajid I, Insecticidal activity of Actinomycetes isolated from Salt Range, Pakistan against mosquitoes and Red Flour Beetle, *Pakistan J Zool*, 2014, **46**(1), 83–92.

24 Gabriel Paulraj M, Saravana Kumar P, Ignacimuthu S and Sukumaran D, Natural insecticides from Actinomycetes and other microbes for vector mosquito control*, In*: Herbal insecticides, repellents and biomedicines: Effectiveness and commercialization, Vijay Veer and Reji Gopalakrishnan, Eds, Springer Publications, 2015, 85–99.

25 Vijayakumar R, Murugesan S, Cholarajan A and Sakthi V, Larvicidal potentiality of marine Actinomyctes isolated from Muthupet mangrove, Tamil Nadu, India, *Int J Microbiol Res*, 2010, **1**, 179–183.

26 Reegan A D, Kinsalin V A, Gabriel Paulraj M and Ignacimuthu S, Larvicidal, ovicidal, and repellent activities of marine sponge *Cliona celata* (Grant) extracts against *Culex quinquefasciatus* Say and *Aedes aegypti* L. (Diptera: Culicidae), *ISRN Entomology*, 2013, doi.org/10.1155/2013/315389.

27 Reegan A D, Kinsalin V A, Gabriel Paulraj M and Ignacimuthu S, Larvicidal, ovicidal and repellent activities of marine sponge *Cliona celata* (Grant) extracts against *Anopheles stephensi* Liston (Diptera: Culicidae), *Asian Pac J Trop Med*, 2015, **8**(1), 29–34.

28 Boyce R, Lenhart A, Kroeger A, Velayudhan R, Roberts B and Horstick O, *Bacillus thuringiensis israelensis* (*Bti*) for the control of dengue vectors: Systematic literature review, *Trop Med Int Health*, 2013, **18**(5), 564–577.

29 Xi Z, Khoo C C and Dobson S L, *Wolbachia* establishment and invasion in an *Aedes aegypti* laboratory population. *Science*, 2005, **310**, 326–328.

30 Zhang G, Hussain M, O'Neill S L and Asgari S, *Wolbachia* uses a host microRNA to regulate transcripts of a methyltransferase, contributing to dengue virus inhibition in *Aedes aegypti, Proc Natl Acad Sci*, 2013, **110**(25), 10276–10281.

31 Watts D M, Burke D S, Harrison B A, Whitmire R E and Nisalak A, Effect of temperature on the vector efficiency of *Aedes aegypti* for dengue 2 virus, *Am J Trop Med Hyg*, 1987, **36**, 143–152.

32 Popovici J, Moreira L A, Poinsignon A, Ormaetxe I, McNaughton D and O'Neill S L, Assessing key safety concerns of a *Wolbachia*-based strategy to control dengue transmission by *Aedes* mosquitoes, *Mem Inst Oswaldo Cruz*, 2010, **105**(8), 957–964.

33 McMeniman C J, Lane R V, Cass B N, Fong A W C, Sidhu M, Wang Y, *et al*., Stable introduction of a life-shortening *Wolbachia* infection in to the mosquito *Aedes aegypti*, *Science*, 2009, **323**, 141–144.

34 Brownstein J S, Hett E and O'Neill S L, The potential of virulent *Wolbachia* to modulate disease transmission by insects, *J Invert Pathol,* 2003, **84**, 24–29.

35 Cook P E, McMeniman C J and O'Neill S L, Modifying insect population age structure to control vector-borne disease, *Adv Exp Med Biol,* 2008, **627**, 126–140.

36 Moreira L A, Iturbe-Ormaetxe I, Jeffery J A, Lu G, Pyke A T, Hedges L M, *et al*., A Wolbachia symbiont in *Aedes aegypti*

limits infection with dengue, Chikungunya, and *Plasmodium*. *Cell*, 2009, **139,** 1268–1278.

37 Nene V, Wortman J R, Lawson D, Haas B, Kodira C, Tu Z J, *et al.*, Genome sequence of *Aedes aegypti*, a major arbovirus vector, *Science*, 2007, **316**, 1718–1723.

38 Alphey L, McKemey A, Nimmo D, Oveido M N, Lacroix R, Matzen K, *et al*., Genetic control of *Aedes* mosquitoes, *Pathog Glob Health*, 2013, **107**, 170–179.

39 Knipling E, Possibilities of insect control or eradication through use of sexually sterile males, *J Econ Entomol,* 1955, **48**, 459-62.

40 Phuc H K, Andreasen M H, Burton R S, Vass C, Epton M J, Pape G, *et al.,* Late-actingdominant lethal genetic systems and mosquito control, *BMC Biol,* 2007, **5,** 11.

41 Proverbs M D, Induced sterilization and control of insects, *Annu Rev Entomol*, 1969, **14**, 81–102.

42 Helinski M E H and Knols B G J, Mating competitiveness of male *Anopheles arabiensis* mosquitoes irradiated with a semi- or fully-sterilizing dose in small and large laboratory cages, *J Med Entomol*, 2008, **45**, 698–705.

43 Franz A W E, Sanchez-Vargas I, Adelman Z N, Blair C D, Beaty B J, James A A, *et al*., Engineering RNA interference-based resistance to dengue virus type2 in genetically modified *Aedes aegypti*, *Proc Natl Acad Sci*, 2006, **103**, 4198–203.

44 Campbell C L, Keene K M, Brackney D E, Olson K E, Blair C D, Wilusz J, *et al*., *Aedes aegypti* uses RNA interference in defense against Sindbis virus infection, *BMC Microbiol,* 2008, **8,** 47.

45 Sanchez-Vargas I, Scott J C, Poole-Smith B K, Franz A W E, Barbosa-Solomieu V, Wilusz J, *et al*., Dengue virus type 2 infections of *Aedes aegypti* are modulated by the mosquito's RNA interference pathway, *PLOS Pathog,* 2009, **5,** e1000299.

46 Khoo C C H, Piper J, Sanchez-Vargas I, Olson K E and Franz A W E, The RNA interference pathway affects midgut infection and escape barriers for Sindbis virus in *Aedes aegypti*, *BMC Microbiol,* 2010, **10,** 130.

47 Whyard S, Erdelyan C N G, Partridge A L, Singh A D, Beebe N W and Capina R, Silencing the buzz: A new approach to populationsuppression of mosquitoes by feeding larvae double-stranded RNAs, *Paras Vect*, 2015, **8**, 96, DOI 10.1186/s13071-015-0716-6.

48 Gu J, Liu M, Deng Y, Peng H and Chen X, Development of an efficient recombinant mosquito densovirus-mediated RNA interference system and its preliminary application in mosquito control, *PLoS ONE*, 2011, **6**(6), e21329, doi:10.1371/journal.pone.0021329.

49 Merritt R W, Feeding behavior, natural food, and nutritional relationships of larval mosquitoes, *Ann Rev Entomol*, 1992, **37,** 349–376.

50 Kumar A, Wang S, Ou R, Samrakandi M, Beerntsen B T and Sayre R T, Development of an RNAi based microalgallarvicide to control mosquitoes, *Malaria World J* (GCE Special Issue), 2013, **4**(6), 1–7.

51 Zhang X, Zhang J and Zhu K Y, Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito *Anopheles gambiae, Ins Mol Biol*, 2010, **19**(5), 683–693.

52 Ramesh Kumar D, Saravana Kumar P, Rajiv Gandhi M, Al-Dhabi N A, Gabriel Paulraj M and Ignacimuthu S, Delivery of chitosan/dsRNA nanoparticles for silencing of wing development vestigial (vg) gene in *Aedes aegypti* mosquitoes*, Int J Biol Macromol*, **86**, 89–95.

53 Das S, Debnath N, Cui Y, Unrine J and Palli S R, Chitosan, carbon quantum dot, and silica nanoparticle mediated dsRNA delivery for gene silencing in *Aedes aegypti*: A comparative analysis, *ACS Appl Mater Interfaces*, 2015, **7**(35), 19530–19535.

54 Pridgeon J W, Zhao L, Becnel J J, Strickman D A, Clark G G and Linthicum K J, Topically applied AaIAP1 double-stranded RNA kills female adults of *Aedes aegypti*, *J Med Entomol*, 2008, **45,** 414-420.

55 Chen T, Huang Z, Wang L, Wang Y, Wu F, Meng S, *et al*., MicroRNA-125a-5p partly regulates the inflammatory response, lipid uptake, and ORP9 expression in oxLDL-stimulated monocyte/macrophages, *Cardiovasc Res*, 2009, **83**(1), 131–139.

56 Kincaid R P and Sullivan C S, Virus-Encoded microRNAs: An overview and a look to the future, *PLoS Pathog*, 2012, **8**(12), e1003018.

57 Bushati N and Cohen S M, microRNA functions, *Annu Rev Cell Dev Biol,* 2007*,* **23***,* 175–205.

58 Carrington J C and Ambros V, Role of microRNAs in plant and animal development, *Sci*, 2003, **301**, 336–338.

59 Wang X, Zhang J, Li F, Gu J, He T, Zhang X, *et al*., MicroRNA identification based on sequence and structure alignment, *Bioinform*, 2005, **21**, 3610–3614.

60 Mead E and Tu Z, Cloning, characterization, and expression of microRNAs from the Asian malaria mosquito, *Anopheles stephensi,* BMC Genomics, 2008, **9**, 244, Doi: 10.1186/1471-2164-9-244.

61 Li S, Mead E A, Liang S and Tu Z, Direct sequencing and expression analysis of a large number of miRNAs in *Aedes aegypti* and a multi-species survey of novel mosquito miRNAs, *BMC Genomics*, 2009, **10**, 581, Doi: 10.1186/1471-2164-10-581.

62 Winter F, Edaye S, Huttenhofer A and Brunel C, *Anopheles gambiae* miRNAs as actors of defence reaction against *Plasmodium* invasion, *Nucleic Acids Res,* 2007, **35**, 6953–6962.

63 Skalsky R L, Vanlandingham D L, Scholle F, Higgs S and Cullen B R, Identification of microRNAs expressed in two mosquito vectors, *Aedes albopictus* and *Culex quinquefasciatus, BMC Genomics*, 2010, **11**, 119, Doi: 10.1186/1471-2164-11-119.

64 Hussain M, Frentiu F D, Moreira L A, O'Neill S L and Asgari S, *Wolbachia* uses host microRNAs to manipulate host gene expression and facilitate colonization of the dengue vector *Aedes aegypti, Proc Natl Acad Sci,* 2011, **108**, 9250–9255.

65 Hussain M, Walker T, O'Neill S L and Asgari S, Blood meal induced microRNA regulates development and immune associated genes in the dengue mosquito vector, *Aedes aegypti, Insect Biochem Mol Biol,* 2013, **43**, 146–152.

66 Thirugnanasambantham K, Hairul-Islam V I, Saravanan S, Subasri S and Subastri A, Computational approach for identification of *Anopheles gambiae* miRNA involved in modulation of host immune response, *Appl Biochem Biotechnol,* 2013, **170**, 281–291.

67 Liu S, Lucas K J, Roy S, Ha J and Raikhel A S, Mosquito-specific microRNA-1174 targets serine hydroxymethyl

transferase to control key functions in the gut, *Proc Natl Acad Sci,* 2014, **111**(40), 14460–14465.

68 Zhou Y, Liu Y, Yan H, Li Y, Zhang H and Xu J, Puthiyakunnon S and Chen X, miR-281, an abundant midgut-specific miRNA of the vector mosquito *Aedes albopictus* enhances dengue virus replication, *Parasit Vectors*, 2014, **7**, 488.

69 Roubaud E, Cycle autogene d'attente generations hivernales suractives inaparentes chez le moustique commun *Culex pipiens, C R Acad Sci Paris,* 1929,**180**, 735–738.

70 Attardo G M, Hansen I A and Raikhel A S, Nutritional regulation of vitellogenesis in mosquitoes: Implications for anautogeny, *Insect Biochem Mol Biol*, 2005, **35**, 661–675.

71 Lea A O, The medial neurosecretory cells and egg maturation in mosquitoes, *J Insect Physiol*, 1967, **13**, 419–429.

72 Matsumoto S, Brown M R, Suzuki A and Lea A O, Isolation and characterization of ovarian ecdysteroidogenic hormones from the mosquito, *Aedes aegypti, Insect Biochem*, 1989, **19**, 651–656.

73 Brown M R, Graf R, Swiderek K M, Fendley D, Stracker T H, Champagne D E, *et al*., Identification of a steroidogenic neurohormone in female mosquitoes, *J Biol Chem*, 1998, **273**, 3967–3971.

74 Lucas K J, Roy S, Ha J, Gervaise A L, Kokoza V A and Raikhel A S, MicroRNA-8 targets the Wingless signaling pathway in the female mosquito fat body to regulate reproductive processes, *Proc Natl Acad Sci USA*, 2015, **112**(5), 1440–1445.

75 Bryant B, Macdonald W and Raikhel A S, microRNA miR-275 is indispensable for blood digestion and egg development in the mosquito *Aedes aegypti, Proc Natl Acad Sci USA,* 2010, **107**(52), 22391–22398.

76 Alphey L, Genetic control of mosquitoes, *Ann Rev Ent,* 2014, **59**, 205–224.

77 Miller L H, Sakai R K, Romans P, Gwadz R W, Kantoff P and Coon H G, Stable integration and expression of a bacterial gene in the mosquito *Anopheles gambiae*, *Sci*, 1987, **237**(4816), 779–781.

78 McGrane V, Carlson J O, Miller B R and Beaty B J, Microinjection of DNA into *Aedes triseriatus* ova and detection of integration, *Am J Trop Med Hyg*, 1988, **39**(5), 502–510.

79 Morris A C, Eggleston P and Crampton J M, Genetic transformation of the mosquito *Aedes aegypti* by micro-injection of DNA, *Med Vet Entomol,* 1989, **3**(1), 1–7.

80 Coates C J, Jasinskiene N, Miyashiro L and James A A, *Mariner* transposition and transformation of the yellow fever mosquito, *Aedes aegypti, Proc Natl Acad Sci USA*, 1998, **95**(7), 3748–3751.

81 Jasinskiene N, Coates C J, Benedict M Q, Cornel A J, Rafferty C S, James A A, *et al*., Stable transformation of the yellow fever mosquito, *Aedes aegypti,* with the Hermes element from the housefly, *Proc Natl Acad Sci U S A***,** 1998**, 95**(7), 3743–3747.

82 Dennis E J, Goldman C, *et al*., Orco mutant mosquitoes lose strong preference for humans and are not repelled by volatile DEET, *Nat*, 2013, **498**, 487–491.

83 Liesch J, Bellani L L and Vosshall L B, Functional and genetic characterization of neuropeptide Y-like receptors in *Aedes aegypti, PLoS Negl Trop Dis*, 2013, **7**, e2486.

84 McMeniman C J, Corfas R A, Matthews B J, Ritchie S A and Vosshall L B, Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans, *Cell,* 2014, **156**, 1060–1071.

85 Aryan A, Anderson M A, Myles K M and Adelman Z N, TALEN-based gene disruption In the dengue vector *Aedes aegypti*, *PLoS One*, 2013, **8**(3), e60082.

86 Kistler K E, Vosshall L B and Matthews B J, Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti, Cell Reports*, 2015, **11**, 51–60.

87 Hall A B, Basu S, Jiang X, Qi Y, Timoshevskiy V A, Biedler J K, *et al*., A male-determining factor in the mosquito *Aedes aegypti*, *Sci*, 2015, **348**, 1268–1270.

88 Dong S, Lin J, Held N L, Clem R J, Passarelli A L and Franz A W, Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, *Aedes aegypti, PLoS One*, 2015, **10**, e0122353.

89 Basu S, Aryan A, Overcash J M, Samuel G H, Anderson M A E, Dahlem T J, *et al*., Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in *Aedes aegypti,* 2015, *Proc Natl Acad Sci USA,* **112**, 4038–4043.

90 Arunkumar K, Parallels between CRISPR-Cas9 and RNAi, Insect genetic technologies Research Coordination net work, igtrcn.org/parallels-between-crispr-cas9-and-rnai.

91 Shen B, Zhang W, Zhang J, Zhou J, Wang J, Chen L, et al, Efficient genome modification by CRISPR–Cas9 nickase with minimal off-target effects, *Nat Methods,* 2014, **11,** 399–402.

92 Coy M R, Sanscrainte N D, Chalaire K C, Inberg A, Maayan I, Glick E, *et al*., Gene silencing in adult *Aedes aegypti* mosquitoes through oral delivery of double-stranded RNA, *J Appl Ent,* 2012, **136**(10), 741–748.

93 Zhang X, Mysore K, Flannery E, Michel K, Severson D W, Zhu K Y, *et al*., Chitosan/interfering RNA nanoparticle mediated gene silencing in disease vector mosquito larvae, *J Vis Exp*, 2015, **97**, 10.3791/52523.

94 Khoo C C H, Doty J B, Heersink M S, Olson K E and Franz A W E, Transgene-mediated suppression of the RNA interference pathway in *Aedes aegypti* interferes with gene silencing and enhances Sindbis virus and dengue virus type 2 replication, *Insect Mol Biol*, 2013, **22**(1), 104–114.