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Perspectives of antiviral RNA interference (RNAi) pathway of insects with special reference to mosquito in the context of dengue infection: a review

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Abstract
RNA interference is a post-transcriptional sequence selective gene control mechanism. Antiviral RNA interference (RNAi) pathway is one of the most momentous constituents of the insect innate immune system that can stymie versatile range of RNA virus like flavivirus. It has been demonstrated that RNA production by alphavirus replication is higher in proportion compared to flavivirus replication in mosquito cells. Studies demonstrated that infection by virus from *Togaviridae* and *Bunyaviridae* family of arbovirus to mosquito cells causes defect in RNAi response *in-vitro* but interestingly, it has also been stated that Dengue virus (DENV) could be actively inhibited by RNA interference (RNAi). This article is an endeavor to review the perspectives of the functional significance of antiviral RNA interference as a potent agent of controlling dengue infection in the vector.

Keywords: Antiviral RNA interference, siRNA, miRNA, piRNA, Flavivirus, Dengue, review.

1. Introduction
Mosquitoes play a significant role in the transmission of most human diseases than any other group of arthropods [1]. Mosquito-borne viral diseases are the most important emerging and re-emerging communicable diseases the world facing today at the beginning of the 21st century [2]. Mosquito borne diseases become a significant public health problem in the present situation it has been estimated that 2.5 billion people are inhabitants of dengue endemic areas with the daily risk of infection [3]. Southeast Asia harbors the greatest amount of dengue virus genetic diversity, suggesting it act as a viral 'source' population [4]. The dengue virus occurs as four antigenically distinct serotypes (DEN 1, 2, 3 and 4), that have emerged or re-emerged throughout the world since the 17th Century [5]. Current estimates of between 50 and 100 million cases of dengue fever per annum worldwide are reported. Of these cases 5, 00, 000 developed into severe forms of the disease such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [6]. Dengue virus is a single stranded virus with positive RNA belonging to the family *Flaviviridae*, genus *Flavivirus* [7]. The Dengue virus transmission has increased dramatically worldwide since 1970 along with the increase in the virulence and disease severity that has been attributed to the Southeast Asian Genotypes of dengue virus (DEN-2 and DEN-3) [8]. *Aedes* mosquito is susceptible to DEN virus [9]. Albeit *Aedes aegypti* and *Aedes albopictus* transmit DENV to human [10] but *Aedes aegypti* plays an important role in Dengue virus transmission [9]. Medically important arthropods like mosquitoes contain a range of versatile physiological mechanism to combat with viral infections and transmit the mosquito borne dengue virus to humans. RNA interference is a post-transcriptional sequence selective gene control mechanism [11, 12] and causes the elimination of virus infection [13]. In insects, RNA interference (RNAi) mechanism is considered as a major antiviral defense mechanism [14, 15, 16, 17, 18, 19]. In order to transmit into a suitable host, arbovirus like dengue must escape this anti-viral defense [20]. Interestingly, it has been reported that DEN virus could be actively inhibited by RNA interference (RNAi) [21, 22]. Additionally, researchers considered RNAi mechanism as a convincing method for treatment of flavivirus infection and to control the transmission of flavivirus by the vector [23, 24]. This article is an endeavor to review the perspectives of the functional significance of antiviral RNA interference as a potent agent of controlling dengue infection in the vector.

2. Diversity of antiviral RNAi mechanism

RNA interference is a post-transcriptional sequence selective gene control mechanism [11, 12]. The antiviral defense property of RNAi mechanism was first reported from plant [25] and from the nematode *Caenorhabditis elegans* [26, 27, 28]. But the RNA-dependent silencing of viral replication in insects was revealed by using sequences of recombinant SINV-expressing Dengue Virus 2 (DENV2). Interestingly, the infected mosquitoes with this recombinant SINV were found resistant to wild-type DEN2 virus infection through an RNA dependent mechanism [29, 30, 31]. The antiviral RNAi response has been shown by *Drosophila* [15, 17, 18, 19] and mosquitoes [14, 16,32, 33, 34]. Additionally, the significant RNAi mechanism also functions in various insects which might exhibit antiviral defense response (Table. 1).

RNAi or RNA silencing pathway comprises of a pool of ~ 21 to 30 nucleotides long and small RNAs, which are divided into three major classes: small interfering RNA (siRNA), microRNA (miRNA) and PIWI-interacting RNA (piRNA) [12].

Table 1: List of insects that exert RNAi response *in vivo* after administration of exogenous dsRNA by intra-*hemocoelic* injection at the larval or adult stages [49].

Insects with their orders		Silencing occurs In	Reference
Coleoptera	*Monochamus alternatus*	Epidermis	35
	Sitophilus spp.	Bacteriome tissue	36
	Tribolium castaneum	Progeny	37
Diptera	*Aedes spp.*	Fat body	38
	Anopheles gambiae	Fat body	39
	Armigeres subalbatus	Hemocytes	40
	Culex pipiens	Systemic	41
	Drosophila melanogaster	Central nervous system	42
Hymenoptera	*Apis mellifera*	Fat body	43
	Nasonia vitripennis	Progeny	44
Lepidoptera	*Bombyx mori*	Silk gland	45
	Helicoverpa armigera	Midgut	46
Orthoptera	*Locusta migratoria*	Progeny	47
	Schistocerca spp.	Eye	48

2.1 siRNA based RNAi

There are two subclasses of siRNA, which have been demonstrated on the basis of dsRNA origin, endo-siRNA and vsiRNA. The production of the endo-siRNA occurs from genome encoded inverted repeats of antisense transcripts from several loci where as vsiRNA or virus derived siRNA is produced by viral (RNA or DNA virus) genome [49]. However, vsiRNA has a significant role in the antiviral defense of insects [50]. The incorporation of dsRNA into the cytoplasm is the inaugural step of the RNAi mechanism as it stimulates to start the series of reactions of RNAi [16, 22, 29, 30, 31, 32, 34, 51, 52]. The dsRNA is then cut by dsRNA specific endonuclease called Dicer enzyme or DICER which is an RNAse III family protein and results in the generation of a pool of ~21 to 23 base pairs long and small interfering RNAs or siRNAs [53, 54, 55] which are accompanied by Argonaute protein (Ago 2 Protein) that stimulates RNA-Induced Silencing Complex (RISC) [56, 57, 58]. The RISC unwinds the siRNA and then the activated RISC uses one strand of siRNA as a RISC targeting co-factor. Thereafter, the RISC associated siRNA binds with a complementary target mRNA and produces a single-site cleavage on the target mRNA by its endonuclease activity [17, 59, 60, 61]. This results in the destabilization and degradation of mRNA [62].

2.2 miRNA based RNAi

miRNA causes the regulation of several cellular functions such as differentiation, development and metabolic homeostasis. Unlike the siRNA, the miRNA is present in both vertebrates and invertebrates [12]. RNAi mechanism involves the formation of ~22-23 nucleotide long microRNAs or miRNAs produced from the single arm of imperfect RNA hairpins which are ~80 nucleotides in length situated within polymerase II (pol II) -derived transcripts called primary miRNA or pre-miRNA[63,64] and results in repression of protein synthesis [21,65,66] . Pre-miRNA are stem loops which has 32 base pair imperfect stem and more than 10 nucleotides containing terminal loop that is cleaved by an RNase III enzyme Drosha in association with its cofactor DGCR8/Pasha [67, 68,69]. This results in the formation of a 2 nucleotide 3' overhang in pre-miRNA which is identified by Exportin 5 that helps in the transfer of pre-miRNA to the cytoplasm [70, 71]. Interestingly, in the cytoplasm, Dicer enzyme of RNA III family endonuclease also recognizes the 2 nucleotide 3' overhang of pre-miRNA in association with its cofactor. Then Dicer/TRBP complex bind to the base of pre-miRNA which is followed by the cleavage to release the terminal loop, forming a 20 base pair RNA duplex that is flanked by 2 nucleotide 3' overhangs [72]. After that the RNA strand which is quite loosely base paired with 5' end in association with dsRNA-binding protein R3D1 engage with the Argonaute-1 (Ago1) -containing RNA-induced silencing complex (RISC) [57, 73] and causes the formation of mature miRNA [74, 75]. Then the miRNA helps in guiding the RISC to a complementary target site of mRNAs and facilitate inhibition of protein synthesis of those mRNAs [76, 77]. A recent study revealed that Ars2 and the nuclear proteins CBP20 and CBP80 are components of the small RNA pathways establishing a link between siRNA and miRNA pathways [78]. (Figure: 1)

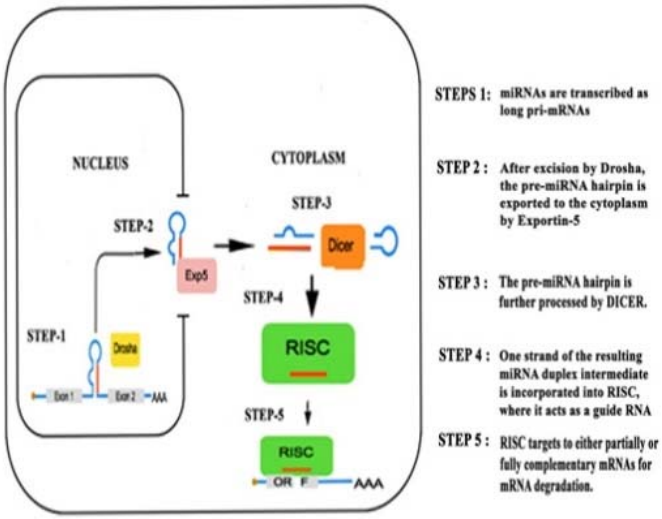

FIGURE 1 : miRNA MEDIATED PATHWAY OF RNAi [50]

2.3 piRNA based RNAi

Although the PIWI pathway of RNAi is less understood [20], it has been reported that it also has a significant role in the antiviral defense of mosquitoes [79]. Additionally, the presence of viral piRNAs in mosquitoes has been suggested by several researchers [20, 80, 81]. In PIWI pathway ~24-30 nucleotides length piRNA are produced by a mechanism which is independent of the involvement of Dicer [82].It has been thought that the piRNAs are processed from single stranded primary transcripts [20, 83]. The characteristic feature which makes this piRNA unique involves strand biasness i.e., many of the reads match against one viral genome strand [84] and has strong nucleotide biasness [85].It has been reported that upon viral infection in the C6/36 cell line of *Aedes albopictus*, primary and secondary piRNA were produced [81].

3. Principal components of siRNAi: The key regulator of antiviral defense mechanism in insects

The siRNA pathway of RNAi is considered as the key regulator of antiviral defense in insects. Dcr-2 and Ago2 containing RISC are the two major components of the siRNA pathway which play significant role in antiviral defense [49]. (Figure: 2)

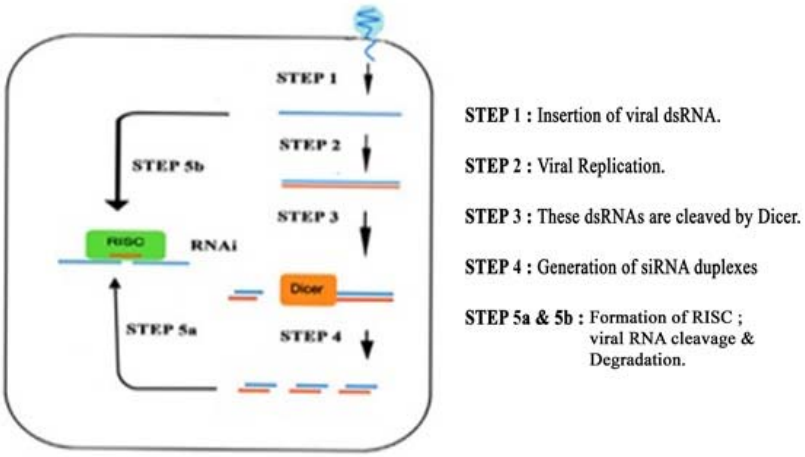

FIGURE 2 : ANTIVIRAL RNA INTERFERENCE PATHWAY [50]

3.1 Dicer-2

The dsRNA after viral infection originates from viral genome or as a replication intermediate [49]. The Dicer-2 with the help of Loquacious, act to process the dsRNA to produce siRNA [86]. The structural characteristics of Dcr-2 from N-terminus to C-terminus end involves: a domain of the DExH-box; two RNase III domains which exhibit dicing of dsRNA and a binding domain with dsRNA [87]. The various functional aspects of Dcr-2 in antiviral defense mechanism include the dicing up or breakdown of viral dsRNA of its RNase III activity [88], production of siRNA by the cleavage of viral dsRNA which is necessary for the

development of active RISC complex as a component of RNAi and finally involvement in the regulation of some antiviral genes like *vago* [54].

3.2 RNA-induced silencing complex or RISC

RISC is considered as a "ribonucleoproteic complex" [49] which contains various functional elements like siRNA, Ago2 [89], dFXR (*Drosophila* ortholog of fragile X mental retardation protein, [90]), *vig* (vasa intronic gene, [90]). Additionally, there are some constituents which promote siRISC assembly or activation [49] involving aubergine [91], C3PO [92] or Hsp90 [93]. After cleavage of viral dsRNA by Dicer-2 the produced siRNAs are shifted to siRISC in an asymmetric way by the Dicer-2 [75, 94] in association with R2D2 [56, 95]. After loading of siRNA into siRISC, the passenger strand of double stranded siRNA is then cleaved by Ago2 [96, 97]. Thereafter the resulting active siRISC complex searches for target viral RNAs and degrades them in a sequence specific manner [98].

4. Defensive strategies of Virus against antiviral RNAi

Most RNA virus produce dsRNA as a byproduct of replication .This dsRNA after the invasion of virus acts as a signal that triggers the RNAi [62]. It is thought to happen in between the viral replication and viral RNA uncoating [99]. However, it has been stated that insect viruses by the production of specific proteins called Suppressors of RNA Silencing (SRS) or Viral Suppressors of RNAi (VSR) could also repress the antiviral RNAi response of insects [49,100, 101, 102]. The SRS or VSR protein of virus generally binds to the dsDNA and prevents the Dicer mediated processing of dsDNA after invasion [103]. But in contrast, none of the mature dengue viral proteins could repress the mechanism of RNAi [101]. Albeit VSR is found in insect virus like Cricket Paralysis virus (dsDNA), Flock House Virus (FHV) but VSR that works during the infection of mosquito for any arbovirus has not been found yet [101, 104, 105].

5. Conclusion

Antiviral RNA interference (RNAi) pathway is one of the most momentous constituents of insects innate immune system that can stymie versatile range of RNA virus like flavivirus [34, 106] whereas Toll and JAK-STAT [107] pathways have also a significant contribution in flavivirus infection control within the mosquito body [108]. It has been reported that antimicrobial pathways like Toll and Imd play significant antiviral role in *Aedes aegypti* [7]. Moreover, the study on silencing the RNAs of *dcr2, ago2* and *r2d2* genes confirmed the role RNAi in facilitating virus resistance in mosquito vectors [106]. It has been demonstrated that restraining of infectious virus production and dissemination in vector mosquitoes require functional Dcr2, R2D2 and Ago2. [14, 16, 34,109]. In this context, it has been reported that the RNAi mechanism plays a significant antiviral function in mosquitoes [14,16,32,33,34]. Arboviral diseases emerged as a serious public health related problem in the present days [62]. Dengue seems to be the most prevalent disease, causing significant morbidity and mortality among humans worldwide, particularly in countries situated in the tropics. Dengue incidence has increased in recent decades with about two-fifths of the world population estimated to be at risk. The expeditious growth of dengue fever as a global pandemic exacerbated the manifestations of the disease accompanied by Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) [110]. Interestingly, it has been demonstrated that RNA production by alphavirus replication is higher in proportion when compared to flavivirus replication in mosquito cells [106]. This is probably caused by either difference in RNAi machinery accessibility to dsRNA of the virus during replication or availability of abundant Dcr2 substrate as a consequence of rapid viral replication to higher titers of mosquito cells infected by alphavirus [22,111] or because of the differences in RNAi evasion capability of alphavirus and flavivirus [106]. Studies demonstrated that infection by virus from *Togaviridae* and *Bunyaviridae* family of arboviruses to mosquito cells causes defect in RNAi response *in-vitro* [106] but studies also suggested that DEN virus could be actively inhibited by RNA interference (RNAi) [21, 22]. It has been revealed that genes of both siRNA and miRNA pathway undergo diversifying selection which might cause possible concurrence of small RNA pathways [106]. Therefore RNAi may act as an efficient mechanism to restrict the Dengue. Additionally, the abundance of piRNA in gonads of insect vectors suggests the plausible role of piRNA pathway of RNAi in the restriction of the vertical transmission of arboviruses [84]. Thus, this could probably provide a satisfactory explanation in favor of the severity of Dengue infection. The significant development of several virulent strains due to high rate of mutation in the RNA genome of the RNA virus like Dengue [112, 113] may be an adaptive strategy of this virus that enhances its fitness to survive, exist, and propagate in the environment by avoiding the RNAi mechanism of mosquitoes. However, researchers reiterated that RNAi response resulted in DENV2 resistance in mosquitoes [114]. It has been demonstrated that constitutive expression of plasmid containing 500 base pairs inverted repeat sequence derived from DENV2 genome cause complete and heritable resistance to infection of DENV2 [115]. Albeit the Dengue virus could maintain its existence by developing virulent strains to survive and sustain, the above notion might support the plausible role of RNAi in the interruption of DENV infection and transmission by helping the mosquito vector to acquire a genetic resistance from Dengue infection. Further studies are required to understand clearly the different aspects of the molecular nature of various components like DICER and RISC of the RNAi pathway in order to know their potential role against the arboviral infection such as dengue.

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