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Molecular Mechanism for the Spread of Insecticide Resistance in *Ades Aegyti*

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ABSTRACT

Aedes aegypti, is a mosquito that spread, dengue virus, chikungunya, Zika fever, Mayaro and yellow fever viruses. While the mosquito originated from the African continent, but has spread in tropical, subtropical and temperate regions, causing havoc to 100s of millions of people. Different control mechanism(s) are employed to curb the mosquito population including a repertoire of insecticide but certain limitations and biological resistance are often associated. The survival of *A. aegypti* is dependent on number of environmental factors, including temperature, humidity, pH and salinity and the eggs of can, nonetheless, remain viable in a desiccated state for up to one year. Factors that contribute for the spread of insecticide resistance are very complex and are subject of concern for vector Biologists. Chemical nature and mode of action of different insecticides, mechanisms of resistance to insecticides, environmental pollution and toxicity to humans, and role of miRNAs in insecticide resistance are explored. Many miRNAs are differentially expressed between insecticide sensitive and resistant state. A new paradigm in the control of *A. aegypti* can be obtained with this study.

Keywords: *Aedes Aegypti*, Insecticides, Biological Resistance, Environmental Factors

INTRODUCTION

Massive urbanization and global warming have led to an explosion of vector-borne diseases in recent decades [1,2]. Several vector-borne diseases, such as Zika fever, dengue fever, yellow fever, and chikungunya, are transmitted by *Aedes aegypti* [3,4]. Most of these infections are not vaccine-preventable. Frequent genetic mutations in the antigenic regions of viral genomes and a wide diversity in viral serotypes pose elusive challenges in the development of vaccines [5-7]. Furthermore, a high cost of currently available vaccines also dissuades many third-world citizens from routine immunization. Therefore, pest management and control are presently the best options for the prevention of mosquito-borne diseases.

The lifespan of *A. aegypti* is brief, lasting from eight to ten days. However, it is dependent on a number of environmental factors, including temperature, humidity, pH and salinity [8,9]. The eggs of *A. aegypti* can, nonetheless, remain viable in a desiccated state for up to one year. This is an evolutionary advantage, the larvae emerge from the eggs as soon as extreme environmental conditions alleviate [10,11]. Only female *A. aegypti* mosquitoes are capable of biting since mouthparts of males cannot puncture the skin. Different chemical compounds present on human skin, for example, lactic acid, toluene, butanone, and octa decanoic acid, attract mosquitoes [12]. Several odorant receptors in mosquitoes, such as *Aaeg Or 4*, draw them to the skin [13].

Mosquito repellents usually contain twenty to thirty per cent N, N-diethyl metatoluamide (DEET) [14]. p-menthane-3, 8-diol, obtained from lemon eucalyptus (*Corymbia citriodora*), is also used in some insect repellents [15]. Commercial insecticides used in the Kingdom of Saudi Arabia contain either organ phosphorous or pyrethroids Cypermethrin, in addition to twenty to thirty per cent DEET. Mosquitoes often develop resistance to these lower concentrations of DEET and higher concentrations could not be used since they are toxic to human beings and mammals [16,17]. Therefore, the evolution of insecticide-resistant mosquitoes is a serious health concern in many regions of the world.

Consequently, unconventional approaches to control pests are being studied and tried in different parts of the world. One such approach is the use of the bacterium *Bacillus thuringiensis* for mass-killing of mosquitoes [18]. The spore-forming bacteria release crystal proteins (also known as Cry proteins or delta endotoxins) in the gut of mosquitoes. Cry proteins have broad-spectrum insecticidal activity; they can

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kill moths, butterflies, mosquitoes, flies, beetles, bees, wasps, and ants. Additionally, they are also effective against nematodes [19,20]. These toxins are encoded by the *cry* gene, which is usually carried on bacterial plasmids. The *cry* gene can, hence, be transferred from one strain to another by conjugation [21]. Using *B. thuringiensis*, several groups have targeted the *A aegypti* in both lab and field trials.

Wolbachia is another bacterium being studied in this context. It is an endo symbiont that confers upon mosquitos the resistance to several viruses, such as dengue and Zika [22-24]. In several countries of the world, different strains of the viruses have been targeted using this approach [25,26].

Lately, a genetically engineered strain of *A. aegypti*, OX513A, was developed by a British biotech company. In these mosquitoes, a self-limiting gene is turned on, which diminishes survival in offspring. Male OX513A mosquitoes were released in the fields in Brazil and Panama since they are incapable of biting humans and animals. Ninety per cent reduction in the population of wild-type mosquitoes was observed. To further propagate OX513A in labs, the self-limiting gene is turned off using the tetracycline antidote. The mosquitoes are then released in fields. In the absence of the antidote in the environment, the self-limiting gene gets turned on again [27-29].

The most advanced technology is targeting microRNAs (miRNAs). Different miRNAs modulate different growth process in mosquitoes. For instance; miRNA-7, miR-8, miR9a, and miR124 regulate development; miR-124, miR-310–313 clusters, Bantam, Let-7, and miR1 regulate neurogenesis; miR278 and miR309 muscle and exoskeleton formation; and bantam and let7 wing development [30]. The molecular mechanisms underlying these developmental processes are not clearly understood. However, miRNAs are known to have a strong association with the evolution of insecticide resistance [31,32].

Insects can potentially counter both the approaches, i.e., insecticide-based as well as bio control, by developing resistant strains. Therefore, it is imperative to look at the molecular and cellular mechanisms underlying the development of resistance.

CHEMICAL NATURE AND MODE OF ACTION OF DIFFERENT INSECTICIDES

Insecticides can be organic or inorganic and natural or synthetic. Organ chlorides, organophosphates, carbamates, pyrethroids, neonicotinoids, and ryanoids are the main classes of insecticides [33]. Virtually all insecticides target the nervous system of insects, however, their mode of action slightly differ [34].

Some of the oldest and most widely used insecticides are organ chlorides. Organ chlorides, as the name suggests, are chlorinated hydrocarbons. They have very low water solubility and resist degradation in the environment. Organ chlorides include dichlorodiphenyltrichloroethane (DDT), aldrin, dieldrin, and lindane among others. Organ chlorides are divided into two main subgroups: DDT-like compounds and chlorinated alicyclic [35]. DDT-like compounds target the peripheral nervous system (PNS) of insects. In axons, they hamper the closure of sodium channels and, hence, membrane depolarization. Sodium ions keep leaking through the neurons and form a destabilizing negative after-potential, causing hyper excitability of the neurons [36]. Chlorinated alicyclic, on the other hand, bind to gamma-Amino butyric acid (GABA) A receptor ($GABA_A$ receptor), inhibiting chloride ions to flow into the neurons. After 2-8 h of exposure, the activity of insects' central nervous system is depressed. This follows hyper excitability, tremors, and seizures [35,37].

Organophosphates are esters of phosphoric acid. As much as fifty per cent of synthetic insecticides are organophosphates. Some of the commonly used organophosphates include parathion, malathion, chlorpyrifos, phosmet, and diazinon [38]. Organophosphates disrupt the neuromuscular enzyme, acetyl cholinesterase, by binding to it via covalent irreversible inhibition [39]. When the enzyme is disrupted, nerve impulses are inhibited. Cholinergic neurons use acetylcholine as a transmitter substance. Acetyl cholinesterase catalyses' the hydrolysis of acetylcholine at the synaptic gap, thus controlling the transmission of nerve impulses. When acetyl cholinesterase is inhibited by organophosphates, acetylcholine accumulates. Consequently, the receptors get saturated with acetylcholine, making nerve impulses inoperative. Many vital systems are affected simultaneously. However, the respiratory system shuts down first, causing the insect to die of respiratory failure [40-42].

Carbamates are derivatives of carbamic acid. Carbamate's insecticides have the functional group carbamate ester. Some of the most familiar carbamate insecticides are aldicarb, carbofuran, ethienocarb, and fenobucarb [43]. The mode of action of carbamates and organophosphates is essentially the same. Carbamates also disrupt acetyl cholinesterase. The only difference is that organophosphates cause phosphorylation of acetyl cholinesterase while carbamates cause carbamylation [39,44].

Synthetic pyrethroids are structural derivatives of pyrethrins, which are naturally produced by the pyrethrum flowers (*Chrysanthemum cinerariifolium* and *Chrysanthemum coccineum*). Some of the commonly used pyrethroids include allethrin, imiprothrin, permethrin, and cypermethrin [45]. Household insecticides and insect repellants are pyrethroids in nature. Pyrethroids are basically axonic excitotoxins. They target the voltage-gated sodium channels of axons, keeping them open and hence preventing the repolarization of neurons. Insects exposed to pyrethroids paralyze consequently [46,35].

Neonicotinoids are related to nicotine. Neonicotinoids have a nitro-methylene, nitro-imine or cyanoimine group. The use of these insecticides is getting increasingly common. Most common neonicotinoids are Imidacloprid, nithiazine, acetamiprid, and clothianidin. These are agonists at nicotinic acetylcholine receptors (nAChRs), interacting with both alpha and beta unit of the receptor [47,48]. In contrast to vertebrates, nAChRs exclusively occur in the central nervous system (CNS) of insects. In fact, the nervous system of insects is very rich with nAChRs. The neurotransmitter acetylcholine activates these receptors [49,50]. Low and moderate concentrations of acetylcholine trigger nerve impulses. However, high concentrations of acetylcholine or neonicotinoids block nAChRs. The organism dies of paralysis. Furthermore, while acetyl cholinesterase breaks down acetylcholine, it is incapable of degrading Nicotinoids [47]. Some neonicotinoids, such as Imidacloprid, depolarizes the motor neurons [51].

Ryanoids are synthetic analogues of ryanodine, which is a diterpenoid found in *Ryania speciose* [52]. Chlorantraniliprole is the most commonly used ryanoids [53]. The mode of action of ryanoids and ryanodine is the same. Both the compounds bind with open ryanodine receptors with very strong affinity. Ryanodine receptors are a class of calcium receptors that occur in muscle cells. Ryanoids either partially or completely close the ryanodine receptors, depending upon their concentrations. Consequently, calcium ions are released from calcium stores in the muscle cells. First, massive muscle contractions occur and then paralysis ensues [54,52].

MECHANISMS OF RESISTANCE TO INSECTICIDES

In recent years, resistance to almost all insecticides has been observed. Resistance to an insecticide usually develops when expression of a xenobiotic-degrading enzyme gets elevated or the enzyme develops a better affinity for a xenobiotic compound. Alternatively, the receptor that an insecticide target may also undergo structural and compositional changes so that it no longer binds to that insecticide.

Esterase, Glutathione S-Transferases, and Monooxygenases are three main groups of enzymes associated with resistance to Organochlorines, organophosphates, carbamates, and pyrethroids. Esterase's bound and turns over insecticides. They do not degrade insecticides instead, they sequester them [55,56]. The genes involved in esterase-based resistance are *estα* and *estβ*. In mosquitoes, most commonly elevated phenotypes are estα2[1] and estβ2[1] [57]. Mutations in the regulatory elements of esterase's, causing their up regulation, have also been reported [58]. Furthermore, in resistant strains of mosquitoes, esterase's that bind to organophosphates with very high affinity have been documented [59]. Resistant strains are also frequently reported to have high levels of glutathione S-transferases [60,61]. This enzyme detoxifies a large number of xenobiotic compounds [62]. They bring about the nucleophilic attack of reduced glutathione on electrophilic centers of lipophiles [56]. Two glutathione S-transferases with elevated levels have been identified in DTT-resistant *A. aegypti* [63]. Monooxygenases occur in numerous animal species, including insects [64-66]. These enzymes are involved in the degradation of xenobiotic and endogenous metabolism. One of the best-known monooxygenases is P450, which metabolizes virtually all insecticides. P450 first captures molecular oxygen and then it receives electrons from NADPH, finally incorporating an oxygen molecule into its substrate. Elevated levels of this enzyme are linked with resistance to pyrethroids. Certain monooxygenases also convert organophosphates into their respective Oxon analogues before organophosphates can inhibit acetyl cholinesterase [67,68].

Acetyl cholinesterase, GABA receptors, and sodium channels are known to develop resistance to insecticides by undergoing changes in their amino acid composition. Receptors and enzymes with altered amino acid composition either do not bind to insecticides at all or bind them with reduced affinity [56]. For example, sodium channels in mosquitoes acquire 'kdr'-like resistance to both DDT and pyrethroids. An example of kdr mutation is the substitution of phenylalanine instead of leucine in the sixth trans membrane segment of sodium channels [69,70]. Often single amino acid substitutions are involved. E3 esterase, for instance, develops resistance to malathion when tryptophan at the 251st position gets replaced with leucine [71]. Likewise, in E3 esterase, glycine at the 137th position may also get replaced with aspartate conferring resistant to several organophosphates [72]. In dieldrin-resistant *A. aegypti*, an alanine to serine substitution occurs in channel lining domain of GABA receptors [73].

ENVIRONMENTAL POLLUTION AND TOXICITY TO HUMANS

Each year 4.6 million tons of pesticides are introduced into the environment globally. Interestingly, 99 per cent of these pesticides come in contact with non-target soil, water bodies, and atmosphere [74]. Consequently, these strayed chemicals are absorbed by organisms. Annually, three million cases of acute pesticide poisoning are documented, resulting in two hundred and fifty thousand deaths [75,76]. Even in developed countries like the United States, pesticides have been isolated from a majority of wells in rural areas. Ocean currents and atmospheric circulation have even conducted pesticides, such as DDT, to sheets of ice in Greenland and bodies of Antarctic penguins [74]. Needless to say, residual pesticides are today detected in all soil types, including vegetable fields and forest lands and deep groundwater [77,78].

The adverse effects of pesticides on domesticated animals and wildlife are conspicuous. Organochlorines, such as DDT, cause thinning of eggshells in birds [79] and acute

mortality in small animals due to inhibition of acetyl cholinesterase [80]. Furthermore, DDT is also a well-known carcinogen and endocrine disruptor [81]. Organophosphates cause immunotoxicity in chordates by inhibiting serine hydrolases and esterase's [82]. Parathion, for example, causes susceptibility to fungal infections. Organochlorines, such as Chlordane, also have adverse effects on the immune system of vertebrates [83]. Nicotinoids affect all the major organ systems of mammals, for instance, cardiovascular, immune, and nervous system [84]. Imidacloprid and pyrethroids severely affect foraging and growth rate in insect colonies [85]. Thiamethoxam, another Imidacloprid, causes homing failure and thus mortality in worker bees [86].

Since there is an enormous similarity between the metabolism and organ systems of humans and insects, pesticides are frequently reported to have detrimental effects on human health. For example, organ chlorides have been implicated in several human cancers, including pancreatic cancer, non-Hodgkin's lymphoma, and breast cancer. They are also associated with impaired lactation and reduced fertility in men [87]. Organophosphates are also known to have adverse effects on the reproductive health of humans [88]. Furthermore, they also affect fetal and infant development and cognitive development in children [88,89]. Pyrethroids are infamous for their neurotoxic reactions [90]. Neonicotinoids is a relatively newer class of insecticides. Initially, they were thought to be safe. However, their neurotoxic and genotoxic effects in humans have also been documented recently [91].

ROLE OF miRNAS IN INSECTICIDE RESISTANCE

miRNAs are small noncoding RNA molecules that are involved in post-transcriptional regulation and RNA interference [92,93]. To do so, they base-pair with the complementary sequences on the target mRNA molecule. Consequently, the target mRNA is cleaved and/or its translation is impaired [94,95]. However, many recent studies have found that miRNAs also up regulate the translation of their target mRNAs [96-99]. Lately, miRNAs have been implicated in promoting and inhibiting the translation of various genes that resist insecticides.

The common fruit fly (*Drosophila melanogaster*) is a model organism for studying insecticide resistance. It is the DDT resistance that is usually studied in the fruit fly. Previously, detoxification genes, such as cytochrome P450, glutathione S-transferases, ATP binding cassette transporters, and esterase's were implicated in the DDT resistance in the fly. Recently, however, a few studies have focused on miRNAs. Pittendrigh [100] found that ten miRNAs were differentially expressed between the DDT resistant and DDT sensitive strain of *D. melanogaster* [100]. These miRNAs targeted transcripts encoding different detoxification genes. For example, miR-311-3p, miR-312-3p, and miR-313-3p which target cytochrome P450 monooxygenases were down regulated in resistant flies. Cytochrome P450 monooxygenases detoxify different insecticides, including DDT. MiR-986-5p, miR-995-3p, miR-312-3p, and miR-2a-3p, on the other hand, were found to interact as well as affect the transcriptional level of multidrug resistance-associated protein B7. MiR-986-5p was found to be the most highly expressed among all the differentially expressed miRNAs in the resistant strain. The function of miR-986-5p is not clearly understood, however, the study found that it interacts with the transcripts of multiple detoxification genes.

Cotton aphids (*Aphis gossypii*) parasite on dozens of edible plants, such as watermelons, squash, cantaloupes, and asparagus. Spirotetramat is a keto-enol insecticide that inhibits the lipid biosynthesis in sucking insects (including aphids) by inhibiting the Acetyl-CoA carboxylase enzyme [101]. It was recently found that miR-276 and miR-3016 up regulate the *Acetyl-CoA carboxylase* gene post-transcriptionally, hence rendering the insects resistant to the insecticide [102].

The diamondback both (*Plutella xylostella*) is an infamous pest of cruciferous vegetables. The resistance of this insect to synthetic insecticides and *B. thuringiensis* derived toxins is legendary [103]. Chlorantraniliprole, a ryanoids insecticide, alters the expression of over one hundred miRNAs in the diamondback moth. A 2017 study by Zhu *et al* identified dozens of miRNAs differentially expressed between Chlorantraniliprole sensitive and Chlorantraniliprole resistant strains of the diamondback moth [32]. The targets of these differentially expressed miRNAs were identified by miRanda and RNA hybrid. A majority of the miRNAs targeted genes encoding cytochrome P450, glutamate-gated chloride channel, glutathione S-transferases, ATP-binding cassette transporters, and cuticle proteins. For instance; pxy-miR-8533-3p targeted larval cuticle protein 30 [104]; pxy-miR-100-5p, glutamate-gated chloride channel [105]; pxy-miR-275-5p, multidrug resistance-associated protein 4 [106]; and pxy-miR-1175-5p, esterase FE4 [107]. Etebari [108] found that enriching the diet of deltamethrin resistant *P. xylostella* larvae with miR-2b-3p increases mortality of the pest when it is exposed to deltamethrin. MiR-2b-3p down regulates the transcript levels of *CYP9F2*. *CYP9F2* is a cytochrome P450 gene and it may have a role in metabolic resistance to insecticides [108].

The northern house mosquito (*Culex pipiens*), transmits a large number of infectious diseases, for example, West Nile fever, Japanese encephalitis, St Louis encephalitis, Sindbis fever, and lymphatic filariasis. Pyrethroids, such as deltamethrin, are used to eradicate the pest. However, the mosquito has developed a strong resistance to pyrethroids. Many recent studies implicated miRNAs in pyrethroids resistance in *C. pipiens*. For instance, in a 2016 study by *Liu et al*, the expression levels of miRNA-938 were found 1.8 times higher in deltamethrin resistant strains of *C. pipiens pallens*. Contrarily, the expression levels of *CpCPR5*, the

target of miRNA-938, were 2.8 times lower in the resistant strains [109]. The CpCPR5 protein plays an important role in the formation of the cuticle. It is postulated that the protein may render the cuticle permeable to deltamethrin [110]. Likewise, miR-92a that targets *CpCPR4*, another cuticle protein, was found unregulated in deltamethrin resistant *C. pipiens* [111]. Many other miRNAs are differentially expressed between deltamethrin sensitive and resistant *C. pipiens* strains. For instance, a 2014 study found that cpp-miR-71 is considerably under-expressed in the deltamethrin resistant strains. cpp-miR-7 targets *CYP325BG3*, a cytochrome P450 gene involved in detoxification [112]. Lately, miR-278-3p [113] and miR-285 [114] were implicated in pyrethroids resistance in *C. pipiens* via post-transcriptional regulation of *CYP6AG11* and *CYP6N23*, respectively. Both these genes are also members of the cytochrome P450 family.

The Asian corn borer (*Ostrinia furnacalis*) is a pest of corn in South and South-East Asia. The pest has caused devastating loses in corn fields, such as eighty per cent in the Philippines, ninety-five per cent in Taiwan, and a hundred per cent in the Marianas [115]. Asian corn borer feeds on almost all parts of the plant, especially kernels. Mostly, bio control methods, such as *B. thuringiensis*-based Cry toxins, are used to eradicate the pest. However, in recent decades, the pest developed strong resistance to them [116]. Globally, Cry1Ab is the most widely commercialized Cry toxin for *B. thuringiensis* corn and Asian corn borer has developed a strong resistance to it. In a recent study, Xu *et al* found 22 miRNAs (21 known and 1 novel) that were differentially expressed in the resistant and sensitive pest strains. Half of the miRNAs were overexpressed in the resistant and half in the sensitive strains. The transcriptome profiling revealed that most of these miRNAs targeted *B. thuringiensis* toxin receptor genes, for example, amino peptidase N and cadherin-like protein. The other miRNAs targeted amino peptidases, chymotrypsin-like enzyme, alkaline phosphatases, ATP-binding cassette transporters, and trypsin-like enzyme. In the resistant strains, expression of amino peptidases 1 to 4 was unregulated and miRNAs targeting them, Ofu-miR-3851c–5p, Ofu-miR-963–3p, Ofu-miR-927–3p, and Ofu-miR-2731, was down regulated. Amino peptidases metabolize Cry toxins. Likewise, expression of trypsin-like serine protease and chymotrypsin-like protease was high in the resistant strains and Ofu-miR-6038 and Ofu-miR-3897–3p, which target them respectively, was low [117].

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