

Review Article

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Target−site mediated insecticide resistance in major mosquito (Diptera: Culicidae) vectors: A systematic review

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ABSTRACT

The use of insecticides remain the most effective vector control approach for mosquito borne diseases like malaria, dengue fever, chikungunya, and Zika virus disease; however, their increasing resistance has complicated the management. Mutations in *kdr*, *ace−1*/*ace−2*, *rdl*, and nAChR, are involved in target-site mediated resistance and prevent the binding of pyrethroids and dichlorodiph enyltrichloroethane, organophosphorus pesticide, carbamates and cyclodienes, respectively. Here, we review the current knowledge on target-site mediated insecticidal resistance mechanisms in major mosquito vectors and the techniques used to identify these mutations. The results showed that *kdr* mutations are frequently reported in mosquito species, indicating the overuse of pyrethroid in mosquito control. Report on *ace* mutations is very limited, primarily detected in *Anopheles* and *Culex*, with extremely low detection rate in *Aedes*, despite the wide application of organophosphates in their control. Notably, reports of *rdl* mutations and changes in nAChR are rare, which provides an opportunity to exploit these target sites, particularly in the event of disease outbreaks. Among various detection methods such as RFLP, TaqMan, Multiplex PCR and FRET, allele-specific PCR is mostly employed, especially in detecting *kdr* mutation. However, allele-specific PCR cannot detect novel mutation. As a result, there is an urgent need to develop a scalable, cost-effective tool that can be widely applied especially as these mutations are genetic markers for early detection of insecticide resistance. Robust monitoring methods remain critical to manage insecticide resistance and effective control of mosquito-borne diseases.

KEYWORDS: Mosquito; *Culex*; *Anopheles*; *Aedes*; Insecticide resistance; *kdr*; Acetylcholinesterase; GABAA receptor

1. Introduction

Vector control is a pivotal component in managing mosquito-borne diseases, and the utilization of insecticides remains the foremost approach employed in this endeavor. For many years, different insecticides with a diverse mode of action were developed to control insects; some of them are employed worldwide to control mosquito vectors. Control of mosquito vectors is primarily based on the use of insecticides and larval habitat management. World Health Organization recommended the use of carbamates, neonicotinoids, organophosphates, and pyrethroids in this regard[1].

Though these control strategies are quite effective, extensive use and overutilization of these insecticides led to the development of insecticide resistance in mosquitoes, reported from different parts of the world[2−5]. Mosquitoes have various mechanisms to resist insecticides, ranging from physiological and morphological to behavioral. Physiological resistance consists of target-site modification and enzyme detoxification, whereas morphological resistance shows cuticular resistance (Figure 1)[6]. Mosquitoes evolved to use these mechanisms alone or in combination to show insecticide resistance[7].

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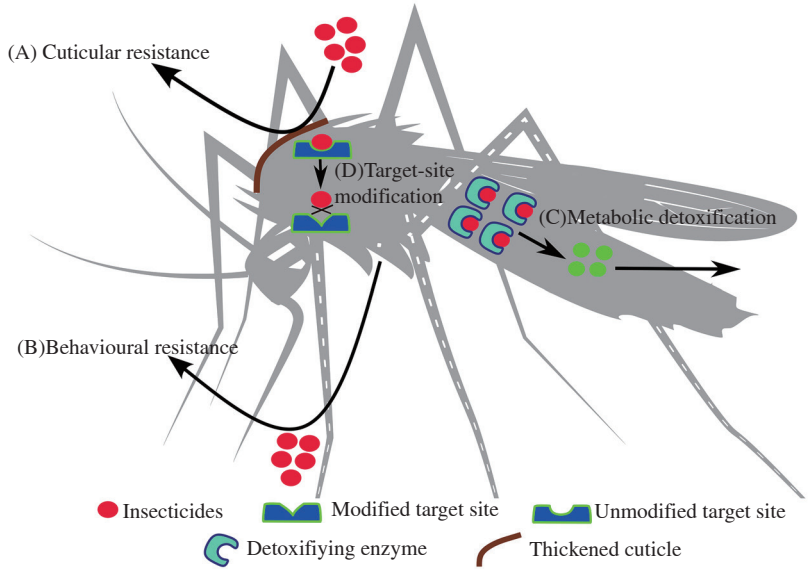

Figure 1. Schematic diagram of a mosquito outlining different modes of insecticides resistance. (A) Cuticular resistance: Thickened cuticle reduces penetration of insecticide. (B) Behavioral resistance: Mosquito avoids insecticide rich environment. (C) Metabolic detoxification: Insecticides metabolized by detoxifying enzymes into less toxic products and then excreted out. (D) Target-site modification: Modification in target-site prevent binding of insecticides (Image prepared in Inkscape 1.2.1).

According to the Insecticide Resistance Action Committee, target-site resistance is a modification of target-site proteins to either prevent the binding of insecticides at its site of action or inhibit their interaction with the target site, thereby reducing or eliminating the pesticidal effect of the insecticide (Figure 2)[8]. In mosquitoes, the most widely used insecticides target four major sites and recurrent application of this insecticide led to the development of resistance in those target sites. Two of these are well known: altered acetylcholinesterase (AChE) and knockdown resistance (*kdr*). However, the other two types, which involve mutations in the gamma-aminobutyric acid (GABA) gated chloride channel and mutations in the nicotinic acetylcholine receptor (nAChR) gene, are not as frequently documented. Knockdown resistance (*kdr*) refers to all the sodium channel mutations primarily associated with resistance against pyrethroids and organochlorines. On the other hand, AChE is connected to resistance against carbamates and organophosphates. Additionally, resistance to dieldrin (*rdl*) involves a mutation in the GABA-gated chloride channel. nAChR mutation is associated with resistance to cyclodiene and neonicotinoids[9,10]. This review aimed to investigate the prevalence of target-site mutations (such as *kdr*, *ace-1*, *ace-2*, *rdl*, mutation in nAChR) associated with insecticide resistance in *Anopheles*, *Aedes*, and *Culex* mosquito species. This article also further concentrates on diverse methodologies implemented to detect these resistance mechanisms in natural populations.

2. Materials and methods

Two online databases, 'ResearchGate' and 'Google Scholar' were used to gather most of the articles whereas other articles were downloaded from the reference list of these existing manuscripts[11,12]. The search was conducted using keywords such as "*kdr*," "*ace-1*," "*ace-2*," "*rdl*," and "nicotinic acetylcholine receptor" with mosquito genus (*Culex*/*Anopheles*/*Aedes*).

The inclusion criteria for this review article are:

i. Reports of *kdr* mutations in *Anopheles* and *Aedes* from 2012-early 2022 and for *Culex* up to early 2022.

ii. Other target-site mutations (*ace-1*/*ace-2*/*rdl*/ mutation in nicotinic acetylcholine receptor) up to early 2022.

iii. Articles detailing molecular method like RFLP, TaqMan, Multiplex PCR, FRET, allele-specific PCR, *etc*. used to identify the above-mentioned target-site mutations.

iv. Excluded articles were also reviewed to ensure that any relevant article was not ignored.

Studies were excluded based on the following criteria:

i. If the study did not report any mutations or did not use molecular techniques to detect mutations.

ii. If the same mutation was reported from the same location multiple times, only recent ones are included and older reports were excluded.

iii. Any duplicates were removed.

The results of the study selection process, data extraction, and data

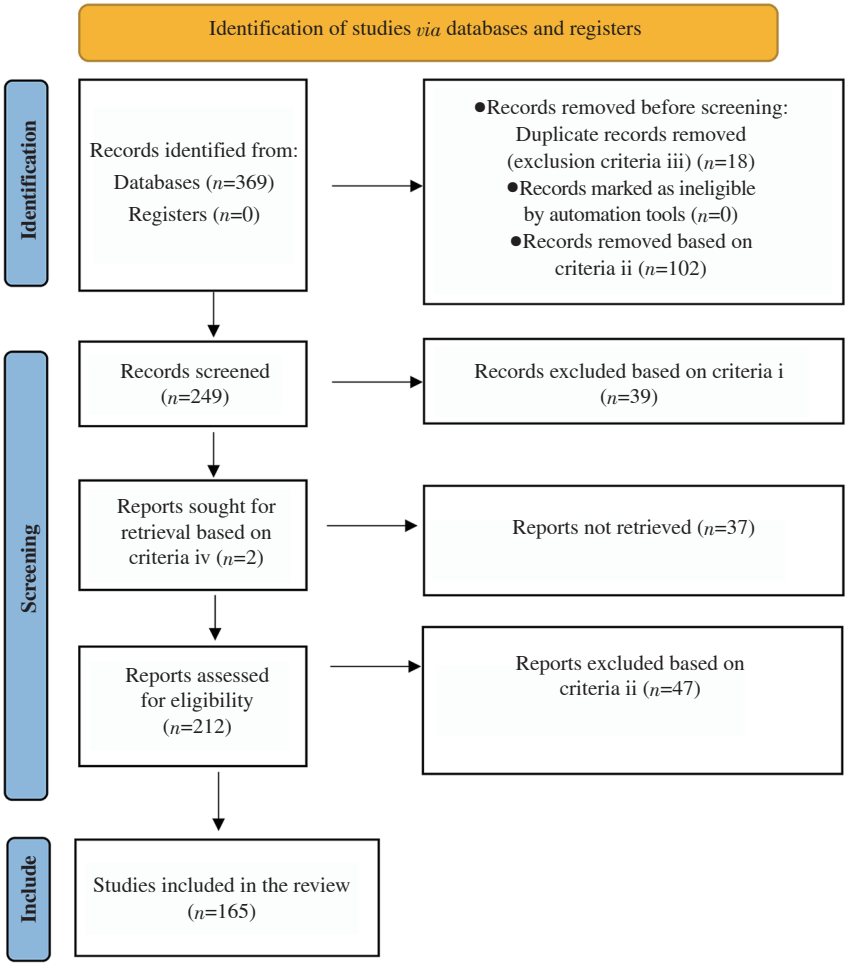

Figure 2. PRISMA 2020 flow diagram for systematic review.

synthesis were reported according to the Preferred Reporting Items for Systematic Reviews statement. A flow diagram was used to summarize the search and selection process (Figure 3)[13].

3. Target-site mediated resistance mechanisms: Mutations of genes encoding insecticide target proteins

A single alteration in the amino acid sequence can cause substantial changes to the protein's structure and function. This is particularly important in insecticide binding sites. Minor changes in the amino acid sequence can lead to variations in the size and shape of the site, which can potentially interfere with the protein's capacity to bind to insecticides. For instance, research has demonstrated that changes in the DNA sequence of the voltage-gated sodium channel can modify the pyrethroid and DDT binding sites, impacting their ability to attach to the channel. This, in turn, can influence the vulnerability of insects to these insecticides. Similarly, with acetylcholinesterase, a solitary alteration in the enzyme's active site can change the size of the insecticide binding pocket, thereby affecting the interaction with organophosphates and carbamates[14].

3.1. Voltage-gated sodium channel (VGSC)

Pyrethroid and dichlorodiphenyltrichloroethane (DDT) bind with VGSC to exert their insecticidal effects. VGSC is a transmembrane protein composed of four homologous domains and each domain consists of six transmembrane segments connected by loops (Figure 4). There are two proposed sites of pyrethroid binding in VGSC. O'Reilly *et al*., suggested that helices of segment five of the second domain (IIS5) and segment six of the third domain (IIIS6), additionally with segment four (IS4) and five (IS5) of the first domain serve as the binding site for DDT and pyrethroid[15]. Du *et al*. suggested another binding site in which, the pyrethroid binds to a pocket formed by linker of segment four (IS4)-five (IS5) of the first domain and the helices between segment five of the first

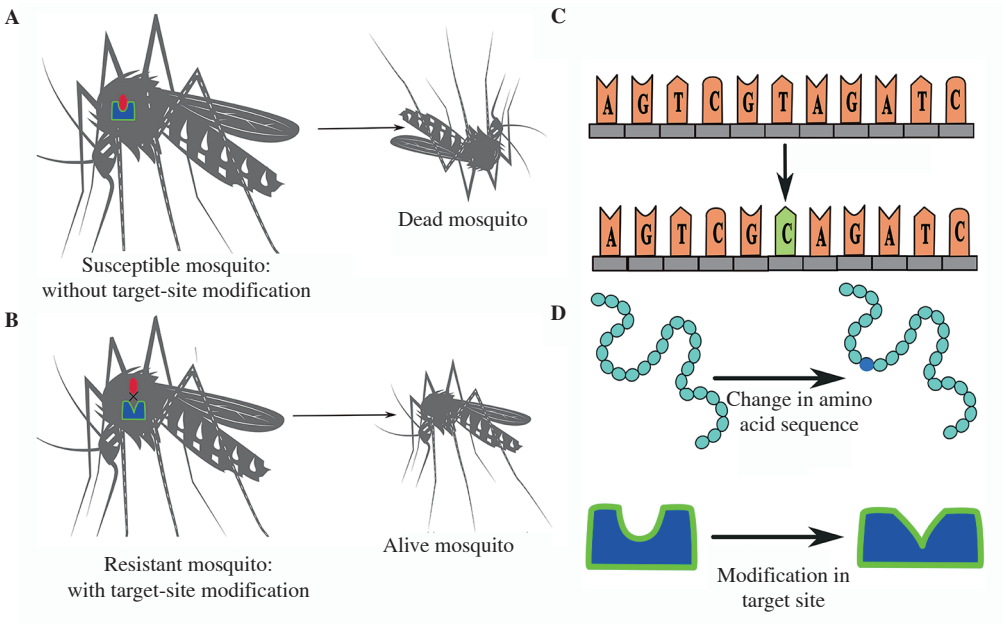

Figure 3. Mechanism of target-site mediated insecticide resistance in mosquitoes. (A) A susceptible mosquito becomes dead when the insecticide binds to its target site. (B) In a resistant mosquito (C) a single mutation in the gene encoding the target protein changes the amino acid sequence in the binding region, preventing the insecticide from binding to the target site and leading to the survival of the mosquito (Image prepared in Inkscape 1.2.1).

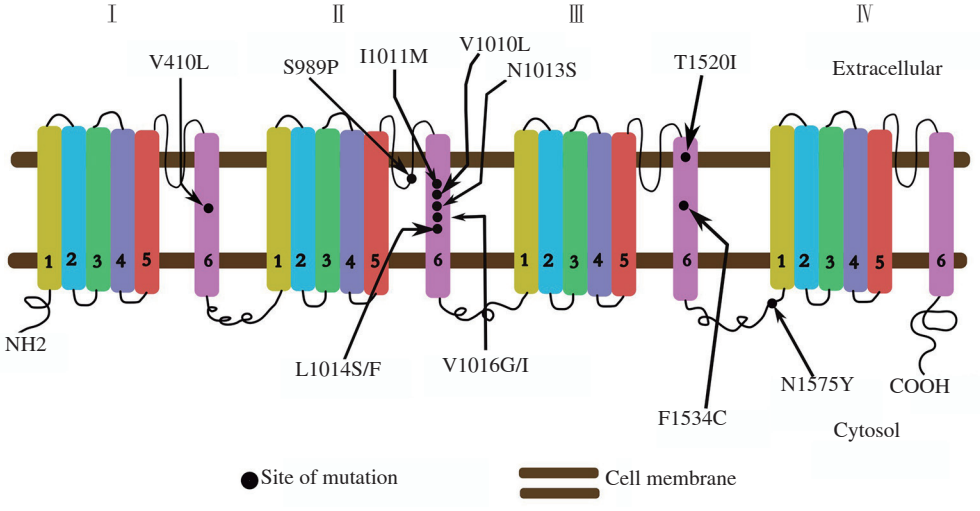

Figure 4. Voltage-gated sodium channel with potential *kdr* mutations. Roman numerical (Ⅰ-Ⅳ) denotes each domain of VGSC, Arabic number (1-6) refers to segment of respective domains (Image prepared in Inkscape 1.2.1).

domain (IS5) and segment six of the second domain (IIS6)[16]. The binding of these insecticides result in loss of function that cause paralysis of mosquitoes, also referred as 'knockdown'[2]. A point mutation in the voltage-gated sodium channel gene altered these binding sites leading to alterations in the sodium channel's gating properties[7]. *Kdr* mutations were first observed in Musca domestica and are considered to be associated with resistance[15]. Substitution of lysine with phenylalanine (L1014F) was reported in segment six of the second domain (IIS6) in different *Anopheles* species. Other substitutions in the same position (L1014C/S/W) were also reported worldwide. Mutations L1014F and L1014S were first reported from west and east Africa, which is why these mutations are known as *kdr*-east (*kdr*-e) and *kdr*-west (*kdr*-w), respectively[2]. Functional characterization of these pyrethroid resistance-associated mutations in *Xenopus oocytes* revealed that most *kdr* mutations alone (F1534C, V1016I/G, T1520I, S989P, V410L, I1011V, I1011M) or in combination (V1016I+F1534C, T1520I+F1534C, V1016G+S989P, V1016G+S989P+F1534C, V410L+F1534C) are involved in

pyrethroid resistance[18].

Anopheles (*An.*) *sinensis* and *An. gambiae* showed the highest prevalence of *kdr* variants including L1014F, L1014C, L1014S, and L1014W. Additional mutations like N1013S and V1010L have also been documented. The presence of the V1010L mutation occurred solely in *An. culicifacies* specimens from India. A novel N1575Y mutation in the same domain was also observed in *An. coluzzii* and *An. gambiae* (Supplementary Table 1).

In *Aedes* populations, 11 substitutions have been reported alone or in combination. Among them, S989P, I1011M/V, V1016G/Ⅰ in domain Ⅱ and F1534C in domain Ⅲ are mostly reported. The F1534C and V1016G/I mutations confer resistance against pyrethroids when present alone, but their efficacy is further enhanced in the presence of S989P and/or T1520I mutations[19]. T1520I was previously described in domain Ⅲ of *Aedes* (*Ae.*) *aegypti* but was recently found in *Ae. albopictus* from India and the Lao People's Democratic Republic (Supplementary Table 1)[20,21].

Until 2020, both the L1014S and L1014F haplotypes were thought to be solely related to pyrethroid resistance in *Culex*, but recently, novel mutations (L932F+I936V) were discovered from Brazil, which are presumed to be connected with pyrethroid resistance, along with L1014F/S in *Culex* (*Cx.*) *quinquefasciatus* (Supplementary Table 1) (Figure 5A)[20].

3.2. Acetylcholinesterase (AChE)

Mosquitoes show resistance against organophosphates and carbamates by substitutions in acetylcholinesterase coding genes, *ace-1* and *ace-2*. A single amino acid substitution from a glycine to serine position 119 (G119S) (*ace-1*) results in steric hindrance, leading to reduced accessibility of insecticide to bind to 119 position, which is in proximity to catalytic serine (S200)[23].

Another mutation in the *ace-1* gene, resulting in the substitution of phenylalanine for valine (F290V), reduces insecticide interaction efficiency with catalytic serine (S200), probably due to the orientation of the valine side chain. As these residues are important characteristics of cholinesterase, particularly acetylcholinesterase, which has been selected for its high catalytic activity, these AChE mutations may impair normal cholinergic function, resulting in a selective disadvantage[23]. Another mutation changing phenylalanine at 331 positions (*ace-2*) to tryptophan (F331W), which is present in the choline-binding site, reduces the accessibility of insecticides due to the bigger size of tryptophan[24]. The phenylalanine at 455 position (*ace-2*), which changes to tryptophan (F455W) (homologous to the Phe331 of AChE in *Torpedo californica*), is also thought to be responsible for AChE's insecticide insensitivity[25].

The G119S substitution has been detected in *An. coluzzii*, *An. arabiensis* and *An. gambiae*, mainly from different African nations like Sudan, Mali, Togo, South Cameroon, Congo *etc*. (Supplementary Table 2).

G119S mutation was also found in *Ae. aegypti* populations from India and Indonesia (Supplementary Table 2)[26,27]. However, no mutations in *Ae. albopictus* has been recorded yet.

The three substitutions (G119S, F455W, F431W) reported in *Culex* were thought to be associated with alterations of acetylcholinesterase. Among them, G119S was reported frequently, but F455W was solely reported in *Cx. tritaeniorhynchus* from Japan and another F431W mutation was also reported in *Cx. tritaeniorhynchus* from India and China (Supplementary Table 2).

Duplication of the AChE gene was also reported in the West African population of *An. gambiae* and *An. coluzzii* by which they increase their resistance ability. *An. gambiae* shows far more complexity in gene duplication. Among *Culex* species, the highest copy number was reported from *Cx. pipiens* (Figure 5B)[28].

3.3. Gamma-aminobutyric acid gated chloride channel

Cyclodiene is another class of insecticide that targets the GABA-gated chloride channel (GABAA) in insects. GABAA has 5 subunits, each consisting of a single extracellular cysteine loop and four transmembrane domains (M1-M5). Mosquitoes show resistance against cyclodienes by resistance against dieldrin (*Rdl*) mutation, an alanine to serine/glycine substitution (A302S/A302G) [9]. Previous research done by Thompson *et al.* in *Drosophila* (*D.*) *melanogaster* demonstrated that A302S mutation is associated with resistance because alanine 302 is a conserved residue present in the M2 transmembrane domain, which lines the chloride ion channel[29]. Another research on the GABAA receptor of *Drosophila* revealed that this conserved alanine residue serves two functions: as an insecticide binding site and to allosterically stabilize the GABAA receptor in a sensitive state so that once it binds to its ligand, the gate opens to chloride for a longer duration[9].

Dieldrin resistance is linked to A302S (named after *rdl* mutation in *D. melanogaster*) substitution in *Ae. aegypti*, *An. stephensi* and *An. arabiensis*[9,30]. An A302G substitution (or *Anopheles* cytotype A296G/S) has been observed in *An. gambiae* and *An. funestus* while the A302S substitution was reported in *An. arabiensis* (Supplementary Table 3)[9,31]. The occurrence of *rdl* in several *Anopheles* populations in Indonesia, even though dieldrin has been banned for 15 years, maybe due to the use of cyclodienes in agriculture[32].

A302S was reported in both *Ae. aegypti* and *Ae. albopictus*. The persistent presence of A302S may be due to the long-term existence of organochlorines in the environment or to a low fitness cost[28].

A302S was also reported in *Cx. quinquefasciatus* and *Cx. pipiens*

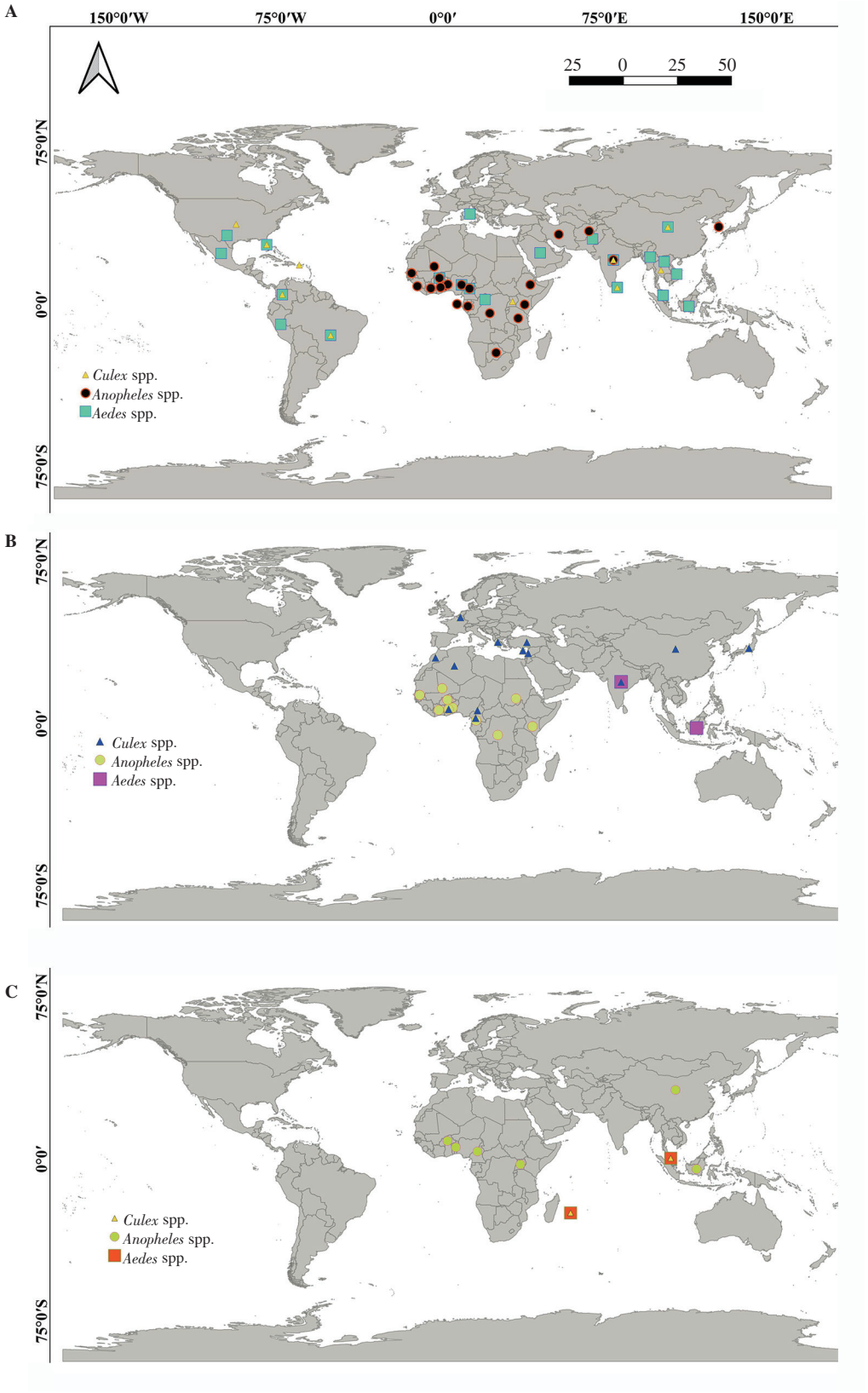

Figure 5. A: Distribution of *kdr* mutations reported in different mosquito species (*Aedes* from 2017 to early 2022; *Anopheles* from early 2014 to early 2022; *Culex* up to early 2022). B: Distribution of *ace–1*, *ace–2* mutations reported in different mosquito species (up to early 2022). C: Distribution of *rdl* mutations reported in different mosquito species (up to early 2022). The maps were created using QGIS software version 3.22.

from Malaysia and La Réunion Island (France), respectively (Supplementary Table 3). Detection of A302S/G is very low among different mosquito species (Figure 5C).

3.4. Resistance by nicotinic acetylcholine receptor

Neonicotinoids show their effects by binding with nAChR. nAChR belongs to the cystine-loop superfamily of ligand-gated ion channels and is composed of five subunits, which form an ion pore, but the number of subunits varies in different species. Each subunit is made of four transmembrane domains (TM1-TM4) and an *N*-terminal extracellular domain, which includes an acetylcholine binding site[12]. Neonicotinoids mimic acetylcholine and act as antagonists to activate nAChR. Mutation in nAChR has been reported in different agricultural pest like *Tuta absoluta* [substitution of glycine to glutamic acid (G275E) in α6 and in *Nilaparvata lugens* (mutation Y151S is associated with resistance to imidacloprid][10,33]. Study in *D. melanogaster* revealed that these mutations are associated with insecticide resistance shown by nAChR[34].

Studies in *Drosophila* revealed that mutation in the nAChR gene has been associated with neonicotinoid resistance[35]. nAChR targeting insecticides are widely applied in insect pest control and some of the pest species reported to have a mutation in the nAChR gene, such as *Plutella xylostella*, *Bactrocera dorsalis*, *Ceratitis capitata*, *Tuta absoluta etc*[36]. Though the spinosad-resistant *Ae. albopictus* population has been reported from Pakistan, to date, no mutation in nAChR has been reported in any mosquito species[37].

4. Molecular tools to detect target–site mutations

The resistance phenomenon can be studied at many different levels, ranging from insecticidal bioassays to determining insecticide susceptibility/resistance status at the molecular level. In the beginning, any target-site mutation was detected by cloning and sequencing the cDNA of the target site. This technique is very accurate for the detection of novel mutations as well as previously known ones. However, some easily replicable and cost-effective methods are needed to evaluate any large population. As these target sites are genetic markers to detect insecticidal resistance, many DNA-based techniques have been developed, which are discussed below.

One such method is polymerase chain reaction (PCR), commonly used to amplify specific target genes, followed by DNA sequencing or restriction fragment length polymorphism analysis[38]. PCR-based methods offer several advantages, including high sensitivity, specificity, and cost-effectiveness. However, the presence of inhibitors or low-quality DNA can compromise the accuracy of PCR-based methods. Restriction fragment length polymorphism analysis can be time-consuming and mainly depends on the presence of a restriction site in target mutation, which limits its uses.

Another commonly used molecular tool is allele-specific PCR, which is used for the detection of known *kdr* mutation and is helpful for large-scale sample detection. However, to perform this technique, prior knowledge of the mutation site is required, and it may not be suitable for detecting novel mutations. Another drawback of allele-specific PCR is the requirement of separate reactions to detect multiple *kdr* mutations in a single mosquito vector. To overcome this limitation, a multiplex PCR technique has been developed, which helps to detect several co-existing *kdr* mutations within a single mosquito population. For instance, Saingamsook *et al.* designed a multiplex PCR method to detect both V1016G and F1534C mutations in *Ae. aegypti* simultaneously[39]. Another multiplex based method developed by Villanueva-Segura *et al.*, which can detect V410L, V1016I, and F1534C in *Ae. aegypti* in single PCR reaction[40].

Alternatively, a PCR Dot Blot method called PCR-based SSOP (sequence-specific oligonucleotide probes) offers convenience in detecting *kdr* mutations without electrophoresis. Kolaczinski *et al.* introduced this technique, which utilizes allele-specific probes to identify *kdr* mutations using a dot blot format[41].

In the case of *An. gambiae*, Lynd *et al*. developed a high-throughput method known as Hot Ligation Oligonucleotide Assay to detect east and west *kdr* mutations[40]. This method provides enhanced reliability compared to conventional PCR, as the presence of mutations can be visually scored, and it can be efficiently conducted using essential laboratory equipment. A similar method known as SSOP-ELISA utilizes SSOP to visualize PCR products in an ELISA format, as developed by Kulkarni *et al*.[43].

A more reliable and sensitive method is Fluorescence Resonance Energy Transfer/Melt Curve analysis which uses two different fluorophores to detect east and west *kdr* through melting temperature analysis[44]. This method relies on a number of nucleotide mismatches to produce differences in the melting temperature of PCR products.

Taqman-based SNP genotyping is a comprehensive method for the detection of different 270 target-site mutations (*kdr*, *rdl*, *ace*), which uses probes labelled with different fluorophores for wild type and mutant-alleles[45–47]. This method is more sensitive and specific as the detection can be done during PCR, and no subsequent processing of the PCR product is needed.

Despite all these effective methods, whole-genome sequencing (WGS) is the ultimate tool for detecting new mutations that may be involved in resistance development. WGS provides a comprehensive view of the mosquito genome, enabling the identification of all

potential resistance genes, and novel mutations. However, WGS is more expensive and time-consuming than PCR-based methods. The methods mostly employed in detecting *kdr*, *ace-1*, *ace-2*, and *rdl* mutations in mosquitoes are listed in Supplementary Table 4.

5. Conclusions

This review is an initiative to summarize all the information to understand target-site mutation and their worldwide report and with required techniques to detect them. Target-site resistance, has been extensively studied, mainly focusing on *kdr* mutations in the *vgsc* genes of pyrethroid-resistant mosquitoes, potentially overshadowing other crucial target-site mutations. Therefore, this review can be very helpful to get a comprehensive idea of any target-site mutation in mosquitoes in a single manuscript. The prevalence of *kdr* mutation raises concerns about synthetic pyrethroid use, emphasizing the need to explore alternative methods of mosquito control. The significance of *Ae. albopictus*, with its role in arbovirus maintenance, calls for more research, particularly in screening for ace mutations. This is especially relevant due to the increasing use of OPs in *Aedes* control worldwide. But insecticide resistance is a complex phenomenon involving different resistance mechanisms. Enzymatic detoxification, cuticular thickening and behavioral resistance also play significant roles, but they are beyond this study's scope, potentially limiting its comprehensiveness. All the available techniques for molecular marker detection are also consolidated. But there was unavailability of data on practical application of several methods in field conditions or in low-resource environments. Further research needs to understand field application of those techniques. This review highlights genetic markers specific to resistance mutations which can also help to understand not only global trend of resistance but also the population specific resistance pattern. Moreover, understanding which target sites are currently under heavy selection pressure and where mutations are less common, new insecticides can be designed targeting less mutated sites.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

D.S. conceptualized the study. Literature search and first draft prepared by S.D., A.S. and P.D. Review and editing were done by S.D., A.S., P.D. and D.S. Figures were prepared by S.D. All authors read and approved the submitted version.

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