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Original article

Investigation of knockdown resistance mutations in *Ctenocephalides felis* samples from the cat populations of Istanbul Province

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Abstract

Effective control of the cat flea (*Ctenocephalides felis*) infestation is essential for human and animal health. To date, several ectoparasitic drugs, such as carbamates, neonicotinoids, phenylpyrazoles, and pyrethroids have been used to control flea infestations; however their widespread use has led to resistance, hampering treatment success. In this study, the resistance potential of *C. felis* collected from cats to fipronil (FIP), imidacloprid (IMI), flumethrin (FLU), and propoxur (PRO), which are the commonly used compounds for flea control, was investigated by molecular analyses. Bioassays encompassed exposure of adult cat fleas to FIP (2%), IMI (6%), FLU (0.1%), and PRO (0.1%)-impregnated papers using an insecticide susceptibility test kit according to the WHO protocol. Afterward, PCR was performed to identify mutations indicating resistance to FIP and FLU. Based on molecular analyses, mutations in the *para* gene of *C. felis* were identified as L1014F and T929V, indicating FLU resistance. Frequencies of T929V and L1014F mutations in fleas were 2.5% and 10%, respectively. On the other hand, two fleas that survived after eight hours of exposure to FIP lacked the A302S mutation, which was partially associated with FIP resistance. Even though our results revealed no resistance to FIP, IMI, and PRO in fleas collected from the study's cat population, the occurence of FLU resistance due to mutations in the *para* gene of the fleas was demonstrated at the molecular level.

Keywords: bioassay, ectoparasiticide, flea, mutation, resistance

Introduction

Ectoparasites are responsible for several common diseases in companion animals and humans worldwide, compromising animal welfare and immunity (Beugnet and Franc 2012). Fleas, the most clinically significant ectoparasites infesting domestic animals, may potentially infest the species that are taxonomically or physiologically related and inhabit the same environment due to the non-specificity in host selection (Erkunt Alak et al. 2020).

Ctenocephalides felis, commonly known as the cat flea, is a widely distributed species with an extensive host diversity, including humans, dogs, sheep, goats, roosters, and chickens as well as cats (Erkunt Alak et al. 2020). Cat flea infestations, previously defined to be varying between 18.9% to 71.6% may trigger disorders ranging from simple irritation to flea allergic dermatitis, which is a crucial immunological response characterized by hypersensitivity reactions caused by the transmission of the fleas' salivary gland antigens to the host (Slapeta et al. 2011, Thomas et al. 2016, Akküçük et al. 2019, Genchi et al. 2021). Apart from their direct pathogenicity, fleas are recognized as a significant health concern in cats since they are the well-known vectors of miscellaneous diseases, such as feline bartonellosis, feline hemoplasmosis and feline rickettsiosis (Lappin et al. 2020, Rajamannar et al 2022).

Although numerous drugs acting through different mechanisms have been used to control cat fleas for many years, the pitfalls in treating and preventing flea infestations remain a significant concern for pet owners and veterinarians (Beugnet and Franc 2012). The most critical conflict is the potential drug resistance, which hampers the continuous and effective use of these drugs (Beugnet and Franc 2012). To date, numerous studies have been carried out to monitor flea populations for the early detection of insecticide resistance, which allows alternative treatment options and extends the longevity of the current host-targeted therapies (Bass et al. 2004, Rust et al. 2015, Ghavami et al. 2018, Erkunt Alak et al. 2020). According to the documented data, structural changes in the target protein of insecticides result in an increased frequency of less sensitive individuals (Coles and Dryden 2014). The most frequently used compounds in the control of flea infestations are reported as fipronil (FIP), imidacloprid (IMI), flumethrin (FLU), and propoxur (PRO) (Rust 2016). These drugs have become the most widely used class of insecticides worldwide, with large-scale applications and offer a broad-spectrum of efficacy against the main parasites of cats, including ectoparasites (Rust 2016). Despite the widespread use of FIP, IMI, FLU and PRO to control cat flea infestation, no scientific report is available documenting whether or not the fleas in Turkey's cat population have developed resistance to these drugs. Accordingly, we designed this research to assess the susceptibility of cat fleas in Istanbul province, Turkey, to FIP, IMI, FLU, and PRO using biological assays and PCR-based diagnostic tests. Even though the study was conducted within a respectively narrow geographical area, considering the significance of global drug resistance and substantial animal movement in Istanbul province, which constitutes a strategic location between Europe and Asia, we anticipate that the outputs of our study will serve as preliminary data in the assessment of the resistance status in different regions (Leul et al. 2024).

Within the scope of the study, initially undertaken biological assays, facilitated a less time-consuming *in vitro* system for simultaneous screening of several samples, providing preliminary data, in which susceptibility was established according to the mortality rate and they were measured as percentage mortalities and evaluated to determine lethal doses of drugs which were determined by the probit analysis (Bossard 1997). In the second stage of this study, PCR-based analyzes that allowed the individual screening of fleas were carried out to identify the mutations that potentially indicate resistance to these compounds. The present study mainly aimed to investigate the potential inefficacy of antiparasitic drugs in treating flea infestations in cats, respond to veterinary professionals' complaints concerning their treatments' failure, and provide scientific data to evaluate the significance of drug resistance. We anticipate that a better understanding of the dynamics of resistance will contribute to ensuring the efficacy of a broader selection of veterinary products and establishing of more effective therapeutic approaches for flea control in cats.

Materials and Methods

Ethical Statement

This study was approved by the Unit Ethics Committee of Istanbul University-Cerrahpasa (Approval no: 2022-63).

Flea collection and morphological identification of flea samples

Flea samples (n = 235) were collected by a flea comb equipped with metal teeth (13 teeth per cm) to a glass jar from cats between 2022 and 2023 in two different animal shelters located in Istanbul Province, Turkey (41°0′44″ North, 28°58′34″ East). Thirty-five out of 82 inspected cats were infested with fleas with an overall prevalence rate of 42.68%. The fleas were

collected using an air vacuum pump (Heidolph, Germany) attached to glass jars and a pipette through a hose. The mouths of the jars were covered with fine-mesh gauze to facilitate continued airflow. Subsequently, these jars were placed in a light-proof box and transferred to the laboratory, where bioassays were performed. A single flea from each infested cat was placed in a separate glass jar containing 70% alcohol for taxonomic identification. Identification of flea samples was achieved by light microscopy based on the parasite's morphological features involving the head shape, presence of genal and pronatal combs, and length of the first and second spines of the genal combs.

Bioassays

The bioassays encompassed the exposure of the adult cat fleas to FIP (2%), IMI (6%), FLU (0.1%), and PRO (0.1%)-impregnated papers using an insecticide susceptibility test kit (WHO/VBC/81.815). The test kit was supplied by the University of Sains Malaysia (USM), and the protocol was conducted according to instructions of the World Health Organization (WHO 1970). Initially, the test paper (6 x 1.5 cm) was impregnated with the tested insecticide and inserted into the test tubes. For each tested insecticide, four groups of fleas, each including 10 fleas, were put into 17 x 1.6 cm clean glass test tubes covered by the fine-mesh gauze and left in contact with impregnated papers. The tubes were stationed vertically in the rack so that the fleas were placed at the bottom half the kit box remaining in total darkness during the whole exposure period of eight hours. Dead or paralyzed fleas were recorded at 15-min intervals. After the exposure period ended, the fleas were transferred to clean holding tubes containing pieces of clean filter paper (6 x 1.5 cm). The assessment of resistance status was conducted following the classification system outlined by WHO, where susceptibility was inferred by mortality rates ranging from 98% to 100%, confirmation of resistance was suggested by mortality rates falling between 90% and 98%, and resistance was indicated by mortality rates below 90%. Knockdown time 50 (KD_{50}, the time by which 50% of fleas were dead or paralyzed) and KD_{90} (the time by which 90% of fleas were dead or paralyzed) were estimated from counts during the exposure period. Susceptibility to substance was assessed after one, 8 and 24-hour observations. Consequently, collected fleas (alive and dead) were processed for further PCR based experiments. Abbott's formula was applied when the negative control mortalities ranged between 5% to 20%. The time and knockdown regression parameters were estimated using PeriProbit version 1.63 software (Sakuma 1998).

DNA Extraction

PureLink® Genomic DNA Mini Kit (Thermo Fisher, USA) was used for DNA extraction to obtain genomic DNA from flea samples, and the procedures were performed using the manufacturer's protocol. The purified DNAs were then stored at -20ºC until use.

Polymerase Chain Reaction (PCR)

Flumethrin

PCR was performed following the instructions of a previous study (Bass et al. 2004) to identify mutations indicating resistance to FLU using primers CF1-F and CF2-R to amplify a 982 bp fragment of the *para* gene encompassing the T929 and L1014F mutations using Wee 32 Thermal Cycler (HiMedia, India) (Table 1). The 982 bp PCR product was also used as a DNA template. New fragments were amplified using alternative Group 1 or Group 2 primers to reveal homozygous susceptible, heterozygous resistant, and homozygous resistant individuals (Table 1). New fragments were amplified to identify the T929V mutation using Group 1 primers. New fragments were amplified using Group 2 primers to identify the L1014F mutation. Group 1 amplification resulted in fragments of 303 bp, 261 bp, and 80 bp; concurrently, Group 2 amplification resulted in fragments of 220 bp, 160 bp, and 100 bp in size. The internal controls of these fragments were 303 bp in size for T929V and 220 bp in size for L1014F. PCR amplification was utilized to detect the presence or absence of fragments, indicating the presence or absence of mutations in individuals (Table 2).

Fipronil

PCR to identify mutations indicating resistance to FIP was performed according to the previous study (Gonzales-Morales et al. 2022). A fragment of 245 bp in size of the GABA receptor gene, which contains the A302S mutation site, was amplified using a primer pair previously used in a study (Table 1).

Results

Flea collection and morphological identification of flea samples

Fleas were found in 35 out of 82 cats (%42.68). Two hundred thirty-five fleas were collected from 35 infested cats. A single flea from each animal was subjected to morphological analysis (35 fleas in total). Species identification showed that All fleas collected

Table 1. Primers used for detection of flumethrin (FLU) and fipronil resistance.

Flumethrin Oligo Name	Sequences	100 µM stock-µl TE
CF1-F	5-TGGCCAACGCTGAATTTGC-3	440
CF2-R	5-TGTTTCATTATCCGCTGTTGG-3	919
CF1b-F	5-CAACGCTGAATTTGCTT-3	455
CF8-R	5-CTCTCCACAAAGCACAGG-3	398
SkdrRF-F	5-GTGCCTTGGGTAATCTAGTG-3	898
SkdrSR-R	5-TAGATAATACACAACACAAACGT-3	602
CF7-F	5-TAGAGTCTATGTGGGATTGCA-3	470
CF11-R	5-TTAATTAGCACGATAGTTC-3	354
PkdrR-F	5-CTACTGTTGTCATTGGTAATT-3	568
PkdrS-R	5-CCCACTAATTTAGTCACAAG-3	666

Fipronil Oligo Name	Sequences	100 µM stock-µl TE
Md_Rdl_F1	CCCTCTGGACTGATCGTTGT	892
Md_Rdl_R1	GCATTTGTCGATGACATCAAAG	486
MdRdlF2	TCTTACAGGAAATTATTCACATC	529
MdRdlR2	ATGGCAAAGACCATCACGAAACAC	592

Table 2. Expected sizes of PCR products for mutation profiles.

	Expected PCR product size		Susceptible/Resistant(S/R)	
T929V	303	261	80	
	+	-	+	Homozygous susceptible
	+	+	+	Heterozygous resistant
	+	+	-	Homozygous resistant
L1014F	220	160	100	
	+	-	+	Homozygous susceptible
	+	+	+	Heterozygous resistant
	+	+	-	Homozygous resistant

were identified as *C. felis* based on morphological features characterized by the presence of eyes, an elongated head, genal/pronotal combs, horizontal genal combs with five or more pointed spines, and genal combs with one and two spines of the same length.

Bioassays

Mortality rates after one hour exposure to FIP, IMI, FLU, and PRO were recorded as 42.5%, 72.5%, 35% and 47.5%, respectively; after eight hours exposure as 95%, 100%, 72.5% and 100%, respectively; and after 24 hours of exposure as 100%, 100%, 87.5%, and 100%, respectively. Variation in reactions to FIP, IMI, FLU, and PRO were noted in KD values, with KD_{50} ranging from 60 to 105 min, from 45 to 55 min, from 75 to 210 min, and from 45 to 75 min, respectively, and with KD_{90} ranging from 165 to 360 min, from 105 to 150 min, from 510 to 840 min, and from 135 to 240 min, respectively.

Molecular Assays

Flumethrin

The T929V and L1014F mutations were present in all *C. felis* (n = 5) that survived 24 h of exposure to 0.1% FLU impregnated paper in 982-bp-sized fragment of the *para* gene (Fig. 1). A 303-bp-sized fragment for the T929V mutation was found in all flea samples (n = 5). Another fragment of 80 bp, indicating susceptibility, was detected in five flea samples. A third fragment of 261 bp in size, corresponding to resistance, was found in only one flea sample (Fig. 1). According to these results, four of the five flea samples were homozygous for the susceptible codon (Threonine), one flea sample had susceptible/resistant codons (Threonine/ /Valine), and no flea possessed homozygous resistant codon (Valine) (Table 3). The 220 bp fragment for the L1014F mutation was found in all flea samples (n = 5). Another fragment of 100 bp, indicating susceptibility,

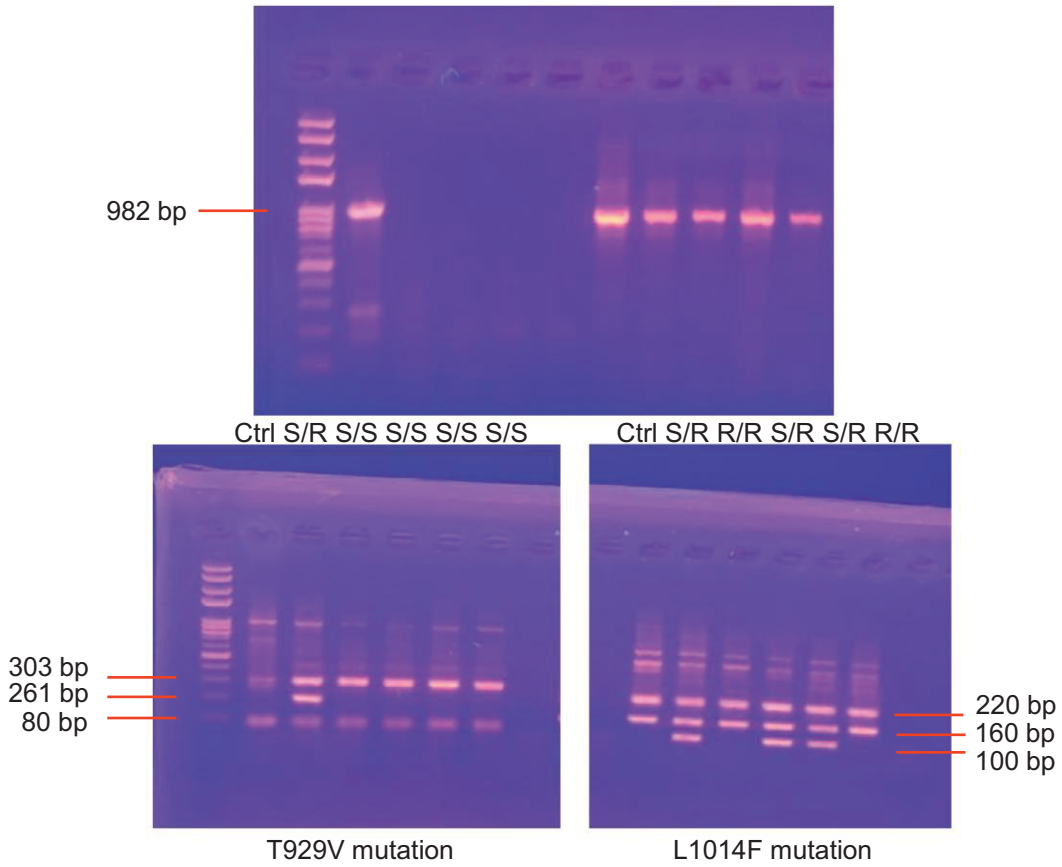

Fig 1. Data regarding gel electrophoresis for FLU.
Top; PCR products exhibiting the para-gene with the L1014F and T929V mutations indicating FLU resistance. Left; PCR products were analyzed to genotype individual cat fleas for FLU resistance. A 303 bp "control" fragment was consistently amplified in all reactions. The presence of T929 (S) and V929 (R) alleles was indicated by the selective amplification of 80 bp and 261 bp fragments. Right; PCR products were analyzed to genotype individual cat fleas for FLU resistance. A 220 bp "control" fragment was consistently amplified in all reactions. The presence of L1014 (S) and F1014 (R) alleles was indicated by the selective amplification of 100 bp and 160 bp fragments.

was detected in three flea samples. A third fragment of 160 bp, indicating resistance, was found in all five flea samples (Fig. 1). According to these results, none of the five flea samples were homozygous for the susceptible codon (Leucine), three flea samples had susceptible or resistant codons (Leucine and Phenylalanine). The remaining two flea samples were homozygous for the resistant codon (Phenylalanine) (Table 3).

Fipronil

No cat fleas treated with 2% FIP-impregnated paper survived after 24-h of exposure. Nevertheless, a molecular assay was conducted on two fleas that survived after an eight hours of treatment. No discernible band suggestive of a mutation was noted in the PCR results analyzed by electrophoresis.

Discussion

The occurrence of resistance to insecticides registered as veterinary medicinal products is a complex process depending on many variables, such as genetic, physiological, and ecological aspects, as well as the insecticides' concentrations and application frequency (Karunaratne et al. 2018). Continuous monitoring of drug resistance, a global health concern posing a constant threat to the performance of these compounds, is a primary crucial approach to flea control. In this context, this study investigated the potential resistance of fleas collected from cats in Istanbul province to FLU, PRO, FIP, and IMI, subsequently elucidating the underlying molecular mechanisms.

The first step of the present study involved bioassays using various substrates and configurations to investigate the potential insecticide resistance of the cat flea. In this context, biological analyses were performed according to the WHO protocols (1970) using insecticide-impregnated test papers, in which fleas were

Table 3. PCR product size of T929V and L1014F.

	PCR product size - T929V			Susceptible/Resistant(S/R)
	303	261	80	
Sample 1	+	+	+	Heterozygous resistant
Sample 2	+	-	+	Homozygous susceptible
Sample 3	+	-	+	Homozygous susceptible
Sample 4	+	-	+	Homozygous susceptible
Sample 5	+	-	+	Homozygous susceptible

	PCR product size - L1014F			Susceptible/Resistant(S/R)
	220	160	100	
Sample 1	+	+	+	Heterozygous resistant
Sample 2	+	+	-	Homozygous resistant
Sample 3	+	+	+	Heterozygous resistant
Sample 4	+	+	+	Heterozygous resistant
Sample 5	+	+	-	Homozygous resistant

trapped for a short period. Although using bioassays to monitor flea susceptibility is not a novel approach, this monitoring method's high coordination and longevity renders it unique, particularly in veterinary parasitology practise (Kopp et al. 2013). In the present study, all the fleas were identified as *C. felis*, a worldwide prevalent species (Xhaxhiu et al. 2009, Slapeta et al. 2011, Gracia et al. 2012, Chandra et al. 2017, Van der Mescht et al. 2021, Zhang et al. 2021). The susceptibility status was established according to the WHO (2016) guidelines for insecticide susceptibility tests. Considering the WHO guidelines, the mortality rate reaching 100% after eighth and 24-h exposure to PRO and IMI indicated that the tested isolates were not resistant to these insecticides (WHO 2016). Previous reports concerning PRO resistance have highlighted a rapid emergence of resistance to the relevant compound (El- Gazzar et al. 1988). In this regard, we assumed that the susceptibility of the tested isolates to PRO was associated with the nonuse of PRO in this region for years (Costa et al. 2014). On the other hand, since the mortality rate below 90% is defined as resistance and 90-97% indicates suspiciousness (WHO 2016), the PCR method was applied to investigate the mutations associated with FLU and FIP resistance in the suspected specimens to verify potential resistance.

Molecular analyses for FLU revealed that L1014F (%10, 5/5) mutation was more frequent than T929V (2.5%, 1/5) (Table 3). Likewise, resistance to pyrethroids in *P. irritans* fleas was previously investigated by bioassays and molecular methods in Zanjan province of Iran, and two mutations, L1014F and T929V, were observed in the *para* gene. Due to the low mortality rates and presence of two mutation sites in the gene sequences, the researchers concluded that insecticide resistance was caused by two mutations in the target sites of pyrethroids (Ghavami et al. 2018). Another study conducted in the west and northwest of Iran aimed to identify the kdr mutation in the VGSC gene in *P. irritans* and *C. canis*. The VGSC sequence analysis showed two mutation sites in resistant fleas and one in susceptible fleas. The susceptibility was homozygous in 5.26% of *C. canis* and heterozygous in 94.73%. The occurrence of flea resistance is associated with the high-frequency L1014F mutation, hampering the effectiveness of the treatments (Seidy et al. 2022). According to Rust et al. (2015), the frequencies of L1014F and T929V mutations in fleas were 72% and 77.4%, respectively. In another study, the frequency of the L1014F mutation was 74.1%, while the T929V mutation was 50% in flea samples collected from England (Bass et al. 2004). In a study carried out with *C. felis* collected from seven goat farms in Turkey, two pyrethroid resistance-associated mutations, L1014F and T929V, were identified in the *para* gene of cat fleas with the frequencies of 95.7% and 92.6%, respectively, which was linked with extensive cypermethrin, a synthetic pyrethrin (Erkunt Alak et al. 2020). These findings suggest that the extensive use of pyrethroids leads to the occurrence of both mutations. In our study, regarding the T929V mutation, one of the flea samples was heterozygous for resistance, while the remaining samples were homozygous for susceptibility. Regarding the L1014F mutation, three samples were heterozygous resistant, while the remaining two flea samples exhibited homozygous resistance. A single flea sample was heterozygous for both L1014F and T929V mutations. Since the coexistence of mutations increases the resistance of fleas to pyrethroids, the presence of a single resistance allele associated with any of these mutations

is sufficient for the occurrence of pyrethroid resistance, and fleas can survive after contact with pyrethroids.

The *Rdl* gene encodes a subunit of the GABA receptor, which is involved in insect resistance to dieldrine (Ffrench-Constant et al. 1993). The encoded *Rdl* subunit combines with the GABA receptor subunit, creating a specific binding site for two classes such as cyclodiene and phenylpyrazole. Resistance to the target location is caused by specific changes in the *Rdl* gene, where the amino acid alanine 302 is replaced with either serine or glycine. This mutation, which is highly prevalent in different insect groups and was initially associated with cyclodiene resistance, confers cross-resistance to FIP, a phenylprazole class insecticide (Hosie et al. 1995, Ffrench-Constant et al. 2000). In our study, no FIP resistance was detected since the mortality rate reached 100% within 24 hours of exposure to the compound. Molecular analysis on two fleas that survived after eight hours exposure revealed a slight prolongation of the knockdown period; however, no evidence of any genetic alteration was noted at the molecular level. The observed outcome was compatible with the anticipated result, as the mortality rate following a 24-hour exposure was 100%. If potential resistance to FIP-containing products is suspected due to decreased drug efficacy, product failures, such as improper storage, incorrect dosage, application errors, or atypical climatic or environmental conditions, should initially be ruled out. The primary factors contributing to pet owners' perception of treatment inefficacy are attributed to inconsistent application of the product or inadequate control of fleas in the surrounding environment. Additionally, in our study, the fleas' mortality rate was 100% after 8-hour exposure to PRO and IMI, excluding potential resistance. Based on bioassays regarding the mortality rates at one h exposure, the use of IMI and FIP can be considered an effective strategy for flea control.

In conclusion, our study demonstrated that *C. felis* may be the primary cause of flea infestations in cats in Istanbul province, Turkey. Out of the four ectoparasiticides studied, FLU was discovered to possess the highest level of resistance despite being the most recent ectoparasiticide administered to cats compared to the others. We assumed that the widespread use of pyrethroids such as permethrin, deltamethrin, cypermethrin, and FLU for flea control since the mid-1970s due to their affordable price might have contributed to this outcome. The data collected from studies on the dynamics of resistance will offer substantial insight for developing efficient treatment strategies, which will contribute to the combat against cat fleas. We consider even though this study has been a local investigation, considering the geographical location of Istanbul province with massive animal movements, the outputs of our study serve as a significant representative example of resistance status in the larger scale regions, offering valuable data for veterinary practice in improving therapeutic approach against cat fleas. However, further studies are needed to clarify potential correlations between environmental factors (such as insecticide usage patterns) and the prevalence of kdr mutations in the sampled populations.

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