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Molecular analysis of pyrethroid resistance in *Cimex hemipterus* (Hemiptera: Cimicidae) collected from different parts of Iran

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Abstract

The present study was aimed to assess the bedbugs susceptibility to pyrethroid insecticides using molecular analysis. With the aid of pest control companies, adult bedbugs were collected from various places such as hotels, residential houses, and industrial buildings in seven cities highly crowded with domestic and foreign tourists in Iran from May 2016 to August 2017**.** Bedbugs were colonized in the laboratory to evaluate their resistance to pyrethroid using insecticide resistance bioassay. Genomic DNA was extracted from susceptible and resistant bedbugs. At first, specie specific primers targeting cytochrome oxidase subunit I (COI) gene was performed to confirm *Cimex hemipterus* species. Then, *kdr*-like gene was examined for point mutation using PCR and nucleotide sequencing. Bioassay showed that 11 out of 35 examined bedbugs were resistant to pyrethroids (31.43%; 95.00% confidence interval: 29.48-33.08%). The DNA sequencing showed that all examined bedbugs collected from Tehran province had homozygous V419L *kdr*-like gene mutations. The level of pyrethroid resistance found in the collected bugs from Tehran province indicated that this phenomenon has already been prevailed in the site and prompts the need to reevaluate the large use of pyrethroids to control the bedbugs.

Introduction

Two common and the most important species of *Cimicidae* family, members of cimicids are *Cimex lectularius* and *Cimex hemipterus*, known as bedbugs. The usual hosts for these blood-feeding ectoparasites are bats, birds, and particularly humans.[1] It has been well documented that bedbugs harbor at least 40 human pathogens, however, there is no tangible evidence regarding their ability to transmit these pathogens to human.[1-5] There are physical trauma, wheals (typically 5.00-20.00 mm), and itch from the bug's bites,[5] and the severity of the reaction depends on the individual's sensitivity.[6] Skin lesions in the bug infestations may include dermatitis, ecthyma, folliculitis, impetigo, and lymphangitis.[6] Some people suffer from allergic reactions due to the bedbug saliva, causing red inflammation and triggering anaphylaxis in certain cases.[6] There are also Reduviid bugs (Hemiptera:*Reduviidae*), including the bedbug distant cousin *Triatoma* infestans, also called the kissing bug, one of the species which spreads Chagas disease from the southern United States down through South America by passing the tropical parasite *Trypanosoma cruzi* to hosts as they sleep,[2] emphasizing that this family does not exist in Iran.

Cimex hemipterus, is a wingless, red-brown, blood-sucking insect with a maximum length of eight mm and a lifespan ranging from four months up to one year. All the daytime, bedbugs disappear and hide in inaccessible places such as cracks and crevices in beds, wooden furniture, floors, and walls and re-appear at night to feed on their preferred host, humans.[7]

A resurgence of bedbugs has been reported from the United States, Canada, Australia, Europe, and some Asian countries during the past 15 years.[8-14] The reasons for the explosion have not yet been clarified, however, several factors such as increased rate of international travel, reduced use of residual insecticides indoors, and insecticide resistance may play a role.[15]

Recent reports are claiming that several species of arthropod pests have developed considerable resistance to

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available insecticides, posing great danger to human life and needing immediate attention.[16-20] Mechanisms beyond the developed resistance in bedbugs against the pyrethroids, the most commonly used pesticide, including behavioral resistance,[21] cuticular resistance,[22] metabolic resistance,[23] and target-site insensitivity resistance are because of the substitute mutations at the DNA level.[24] The most prominent mechanism by which several species of insects develop resistance to insecticides is mutations occurred in the voltage-gated sodium channel (VGSC) reducing the affinity of the receptor.[20,25] Target site insensitivity of this type is known as 'knockdown' (or *kdr*-type) resistance, named firstly after a phenomenon observed in houseflies survived following being knocked down by DDT and subsequently demonstrating cross-resistance to pyrethrins.[26,27] The *kdr*-type target-site insensitivity resistance has been extensively studied in both *C. lectularius* and *C. hemipterus*, it is present everywhere within pyrethroid-resistant field-strains and plays a significant role in the expression of pyrethroid resistance. V419L and L925I mutations in a VGSC a-subunit gene of *C. lectularius*, have also been used as a molecular marker of resistance to pyrethroids in a few studies.[24,28,29] To date there is no study underlying the resistant mechanisms of bedbugs against pyrethroids in Iran. Therefore, the current study was aimed to assess the susceptibility of bedbugs collected from different parts of Iran to pyrethroid insecticides at the molecular level.

Materials and Methods

Regions and sample (bedbug) collection. There are four different geographical zones in Iran, known as region 1: Caspian Sea (temperature: 8.00 - 26.00 ˚C, annual rainfall: 400 - 1500 mm), region 2: Mountainous area (temperature: –5.00 - 29.00 ˚C, annual rainfall: 200-500 mm), region 3: Persian Gulf (temperature: 12.60 - 35.00 ˚C, annual rainfall: 200-300 mm), and region 4: The Central Desert (temperature: –4.00 - 44.00 ˚C, rainfall: less than 100 mm). The pattern of bedbug species distribution for the four different areas was determined according to the method of Skerman and Hillard.[30]

With the aid of pest control companies, adult bedbugs were collected from various places in each region such as hotels, residential houses, and industrial buildings before treating the infested areas with insecticide from May 2016 to August 2017. From each of the regions one or two cities were selected: Region 1: Sari (36.5659° N, 53.0586° E) and Rasht (37.2682° N, 49.5891° E), region 2: Tehran (35.6892° N, 51.3890° E) and Hamadan (34.7989° N, 48.5150° E), region 3: Ahvaz (31.3183° N, 48.6706° E) and Bandar Abbas (27.1832° N, 56.2666° E), and region 4: Semnan (35.2256° N, 54.4342° E).

Bedbug samples were transferred to the parasitology laboratory, Faculty of Veterinary Medicine, Urmia University for identification under a stereomicroscope (Olympus SZ61; Olympus Corporation, Tokyo, Japan), using morphological features and the taxonomic key of Usinger. Useful diagnostic features included rostrum length, mesothoracic coxae separation, antennal segment length, as well as pronotal anterior margin and hairs.[1]

Insecticide resistance bioassay. The resistance of the collected bedbugs from four regions were examined using the World Health Organization (WHO) insecticide-impregnated papers. Each paper (8.50 cm diameter) was impregnated with 500 µL of 10.00% cypermethrin (100 ppm; National Agrochemical Co., Tehran, Iran) according to the recommended dosage of the manufacturer. Each insecticide-impregnated paper was cut into 3.00 × 5.00 cm pieces, rolled, and placed inside a glass test tube (length × internal diameter: 12.50 × 1.50 cm). A piece of clean paper (3.00 × 5.00 cm) impregnated with isotonic sodium chloride solution was used as a control group similar to the impregnated paper was rolled and placed inside another glass test tube. Alive and motile adult bedbugs of either sex from each region (five samples per city) were carefully transferred into each tube and closed with fine-mesh gauze. The tubes were set upright in the holding rack and placed in a plastic box (20.00 × 20.00 × 15.00 cm) lined inside with damp cloths to obviate very low humidity. The box was closed for the appropriate exposure period. Bedbugs were maintained in an incubator at 24.00 ± 2.00 ˚C and 55.00 - 65.00% relative humidity. The concentrations of insecticides and exposure times used were recommended by WHO.[31] Mortality rates of the bedbugs exposed to cypermethrin were recorded after 24 hr of exposure to insecticide or isotonic chloride solution. Bedbugs were considered as dead if there were no vital signs or minor vital signs present, merely internal gut movements, movements of antennae, minimal leg movements with or without stimulation by a forceps. Each bioassay was replicated three times against each colony of bedbugs.

DNA extraction. Genomic DNA was extracted from individual tropical bedbug of each collection site using a DNA extraction kit (MBST, Tehran, Iran) following the manufacturer's instructions and as per the method described by Shayan and Rahbari.[32] Briefly, bug samples were ground by pestle and placed into 1.50 mL microcentrifuge tube and DNA extraction was performed according to the kit's protocol. Total DNA was eluted in 100 µL of elution buffer and its quality and concentration was determined using the NanoDrop (2000c; Thermo Fisher Scientific, Waltham, USA) and stored at – 20.00 ˚C for further procedures.

Amplification of cytochrome oxidase subunit I (COI) gene. Amplification of a 685 bp fragment of the COI gene using species-specific PCR primers LEP-F (5′-ATTCA ACCAATCATAAAGATATNG G- 3′) and LEP-R (5′-TAWACT TCWGGRTGTCCRAARAAT CA-3′) described by Balvin *et al.* was performed to confirm C. *hemipterus* species.[33]

Genotyping of *kdr*-like gene. The PCR amplification of two 474-bp and 744-bp amplicons of the VGSC subunit gene (*kdr*-like gene) was performed using primers (forward KDR1: 5'AACCTGGATATACATGCCTTCAAGG3' and reverse KDR1: 5_TGATGGAGATTTTGCCACTGATG3 for fragment 1; forward KDR2: 5_GGAATTGAAGCTGCCAT GAAGTTG3 and reverse KDR2: 5_TGCCTATTCTGTCGAAA GCCTCAG3 for fragment 2) described previously.[34] PCR was carried out in 25.00 µL reaction volume consisting of 2.50 µL 10X PCR buffer, 2.00 µL $MgCl_2$ (50.00 mM), 0.50 µL dNTPs (10.00 mM), 3.00 µL DNA template, 10.00 pmol of each forward and reverse primer (0.50 µL for each) for either V419L and L925I, 0.50 µL Taq DNA polymerase (SinaClon, Tehran, Iran) and 15.50 µL ddH_2O. PCR cycling conditions were as follows: Initial denaturation at 94.00 ˚C for 10 min followed by 40 cycles of 94.00 ˚C for 40 sec, 52.00 ˚C (V419L) and 55.00 ˚C (L925I) for 40 sec, 72.00 ˚C for 40 sec and a final extension step of 72.00 ˚C for 10 min. PCR products were visualized on 1.50% agarose gel stained with safe DNA dye under UV-Transilluminator (20M; BTS, Tokyo, Japan).

The PCR products were sent to SinaClon Company (Tehran, Iran) for purification and nucleotide sequencing. The obtained sequences were compared to the wild-type sequences retrieved from GenBank (GU123927 and GU123928). The presence of single nucleotide polymorphisms (V419L and L925I) was confirmed by reading both forward and reverse strands.

Statistical analysis. The obtained data used for mortality bioassay was analyzed using the SPSS program (version 22.0; IBM Corp., Armonk, USA).

Results

Mortality bioassay. Only alive and motile adult bedbugs were selected for the test. In total, 40 bedbugs were subjected individually to the test. Five bedbugs were used as controls, of which none was dead at the end of the test. Among the 35 bedbugs exposed to insecticide, 11 (31.43%) were reported as resistant (95.00% confidence interval: 29.48-33.08%) and 24 (68.57%) were susceptible (95.00% confidence interval: 67.82-71.62%). The Most resistant bedbugs (5 out 11) were collected in two highly infested places located in Tehran province.

Genotyping of *kdr*-like gene. At first, amplification of the COI gene for bedbugs showed a consistent amplification with strong bands that specify all samples belong to *C. hemipterus*. Subsequently, the DNA of all 35 bedbugs exposed to insecticide during the mortality bioassay was extracted. Both parts of the *kdr*-like gene containing codons 419 and 925 were successfully amplified and sequenced for 20 bedbugs (Fig. 1), including nine susceptible and 11 resistant bedbugs according to the bioassays. In *C. hemipterus* field populations (Table 1), all sequenced bedbugs had homozygous wild-type L925I codon and homozygous mutated V419L codon related to Tehran province of *kdr*-like gene (Fig. 2). In other words, the existence of resistance alleles at the V419L mutation for all populations was 0.0 except for Tehran.

Fig. 1. Alignment of a part of the sequence of the *kdr*-like gene, encompassing codon 419 (in red), from a pyrethroid-susceptible bedbug [Genbank access number: GU123927] and adult bedbugs collected in the study.

Table 1. The *kdr*-type gene mutations found in *C. hemipterus* examined in the present study from May 2016 to August 2017.

Cities	V419L	L925I
Sari	-	-
Rasht	-	-
Hamadan	-	-
Ahvaz	-	-
Bandar Abbas	-	-
Semnan	-	-
Tehran	*	-

* Homozygous mutated V419L codon related to Tehran city of the *kdr*-like gene.

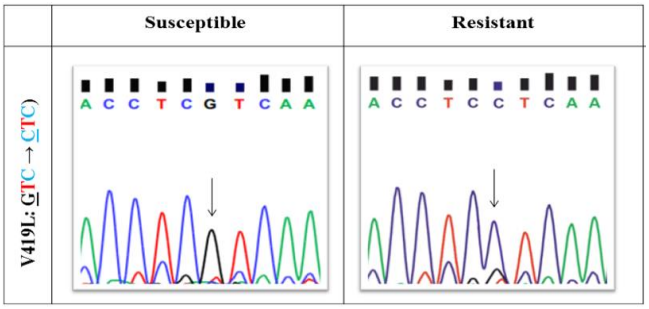

Fig. 2. Chromatograms showing differences between *kdr*-type gene sequences of pyrethroid susceptible and resistant bedbugs. On the left-hand side, the VGSC amino acid substitution that results in a kdr mutation is indicated, and the corresponding base pair change is bolded and underlined. The same base pairs are indicated by an arrow in the chromatogram sequence, and its identity is boxed on the chromatogram base-pair readout.

Discussion

Bedbugs were a common pest up to the mid-20th century, however, with the advent of synthetic insecticides, their population considerably was declined particularly in the developed countries.[35] On the other hand, during the past two decades a resurgence of bedbugs infestations have been reported simultaneously

all around the world,[5] including Europe,[36,37] Africa,[38] South America,[39] Asia,[40,41] Australia,[42] and North America.[43] It is estimated that the resurgence of bedbugs has incurred severe economic losses which cost billions of dollars,[11] including 200 million AUD in Australia since the resurgence started in 2011.[44] The incurred losses were due to eradication charges, lost revenue, and discarded infested items.[45] It has been claimed that a blend of various factors such as insecticide resistance, increased international travel, and poor control practices probably have led to the resurgence of bedbugs.[25]

Bioassays determined that pyrethroid-resistant bedbugs were present in several cities in Iran. PCO (pest control operators) usually uses pyrethroid insecticides in different regions of Iran. They use mostly 10.00% cypermethrin). People frequently use these products, which are easily available in local stores. As a result, pyrethroid insecticides are often improperly used in terms of concentrations and the number of applications, which may induce unnecessary insecticide pressure on bedbugs and the other pests.

Some bedbugs examined in the present study showed a point mutation in V419L location leading to the resistance to pyrethroids. Resistance to pyrethroids was not always associated with the V419L mutation in bedbugs in the USA and this mutation was not always associated with the L925I mutation to form a resistant haplotype.[24,28] The present data is the only results of genotyping of *C. hemipterus* based on a *kdr*-like gene in Iran. The presence of *kdr*-type mutations was detected for the first time in *C. lectularius* collected in New York, USA, using two target sites in the *kdr*-like gene for point mutations (V419L and L925I).[24] Then a high rate of the mutation, almost 88.00% was detected in 110 field-strains of *C. lectularius* collected across the USA.[23] Furthermore, researches have subsequently disclosed the presence of mutations in *kdr*-type genes in parathyroid-resistant bedbugs around the world. For example, in France, all specimens of *C. lectularius* collected from a single multi-occupancy high-rise apartment complex were reported to homogeneously have only haplotype B (L925I) *kdr*-type mutations.[33] Besides, time changing mutations of both V419L and L925I were revealed in *C. lectularius* in Korea[28]. The *kdr*-type gene analysis of *C. hemipterus* strains determined that the V419L, L925I, and I936F mutations were absent from the specimens investigated, although two other mutations, M918I and L1014F, were putative *kdr*-type mutations as they appear in other pyrethroid-resistant insects.[46]

Our results showed that pyrethroids resistant bedbugs were common in many places of Tehran, since PCOs mostly use pyrethroid insecticides in this city and its suburbs. Also, due to heavily populated and passenger traffic, people often use pyrethroid insecticides which are easily available in the local stores. Although studies are limited, to date all investigations examining *kdr*-type genes have revealed a high proportion of the mutations in this gene suggesting that these mutations are probably widespread in bedbugs across the world. The continuous use of pyrethroid insecticides, despite not being very efficacious, might be contributed to the maintenance of high frequency of *kdr*-type gene mutations.

The evolutionary mechanisms beyond the respective *kdr*-type gene mutations among bedbug species have not been completely understood yet and this must be clarified in future studies. Furthermore, it is important that pest control managers tightly adhere to "best practice" as defined in the various bedbug management industry standards to reduce the risk of treatment failure. In conclusion, the current study was the first effort to investigate the distribution and extent of *kdr*-type gene mutations in bedbug populations across Iran. Also, the results obtained from this study suggested that the bedbugs in Tehran province have developed resistance to various groups of pyrethroid insecticides which are the most common insecticides used for pest control.

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Conflict of interest

The authors declare no potential conflict of interest.

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