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Identification of Knockdown Resistance Mutations in the *Cimex hemipterus* (Hemiptera: Cimicidae) in Iran

Mehdi Tiotour,[1] Minoo Shaddel,[2] Mohammad Aminianfar,[3] Hadi Mirahmadi,[4] Gholamreza Barzegar,[5] Rahmat Solgi,[6]* and Mohammad Darvishi[3]*

[1]*Faculty of Medicine, AJA University of Medical Sciences, Tehran, Iran;* [2]*Department of Parasitology and Mycology, Faculty of Medicine, AJA University of Medical Sciences, Tehran, Iran;* [3]*Infectious Diseases and Tropical Medicine Research Center, Department of Aerospace and Subaquatic Medicine, AJA University of Medical Sciences, Tehran, Iran;* [4]*Infectious Diseases and Tropical Medicine Research Center, Research Institute of Cellular and Molecular Sciences in Infectious Diseases, Zahedan University of Medical Sciences, Zahedan, Iran;* [5]*Department of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran;* [6]*Cellular and Molecular Research Center, Birjand University of Medical Sciences, Birjand, Iran*

Abstract. The worldwide resurgence of tropical bed bug *Cimex hemipterus* beginning in the late 1990s has led to growing concern. Molecular data on pyrethroid resistance, which is essential for the control strategies, is unknown for *C. hemipterus* in Iran. The current study evaluated the deltamethrin resistance status of *C. hemipterus* by bioassay and molecular tests. Live bed bugs were collected from sleeping quarters (dormitories) in the city of Tehran and used for insecticide bioassay tests. For bioassay evaluation, mixed-sex pools of adult bugs were exposed to deltamethrin (0.025%)-treated paper. Polymerase chain reaction assay evaluated resistance-related mutations in the voltage-gated sodium channel gene (VGSC) gene of studied populations. On the basis of the bioassay test within the 48-h exposure to deltamethrin, *C. hemipterus* were determined to be resistant. Knockdown time ratios (KR_{50}) in the studied populations of *C. hemipterus* was 5.5-fold compared with those of the *C. lectularius* Teh strain. DNA sequencing of the VGSC gene revealed the presence of mutations at M918I and L1014 in *C. hemipterus.* According to the bioassay and molecular results of current study, *C. hemipterus* showed a high degree of pyrethroid resistance. The application of multiple approaches including physical, biological, and chemical tests should be regarded in future bed bug control strategies.

INTRODUCTION

Bed bugs (*Cimex lectularius* and *Cimex hemipterus*) are blood-feeding ectoparasites of significant public health concern around the world.[1] In recent years, bed bugs have reemerged in many countries. Bed bugs can cause serious indoor public health issues directly by nuisance biting and indirectly by hematophagic activity.[2] Their bites may cause allergic responses, including itching and erythematous nuisance biting, which can cause cutaneous and systemic reactions.[3] There is no evidence related to the transmission of pathogens by bed bugs to humans; however, many studies have shown that bed bugs may be a competent vector for *Bartonella quintana* and *Trypanosoma cruzi*.[4] The tropical bed bug *C. hemipterus* and common bed bug *C. lectularius* are mainly confined to tropical regions and temperate climates, respectively. Today, the tropical bed bug may be seen in subtropical or stable temperature regions due to globalization, climate change, tourism, and insecticide resistance.[5,6] Chemical agents in various forms, such as fumigants, powder dust, and liquids, as well as nonchemical techniques such as heat, steam, and vacuuming, have been used to manage this pest.[7,8] Pyrethroids are the most widely used chemical agent to control the bed bugs. These agents possess rapid knockdown activities and low mammalian toxicity, and they are relatively low cost.[8,9] However, their overuse and misuse have led to insecticide resistance in bed bugs.[9] Despite numerous studies performed on common bed bug resistance to pyrethroids,[6] few studies on the resistance status of the *C. hemipterus* have been performed. It was documented that various mutations in the voltage-gated sodium channel gene (VGSC) that cause a "knockdown resistance" (*kdr*) are responsible for insecticide resistance against pyrethroids.[10–13] One of the key factors to increase the bed bug infestation is poor bed bug management practices. Most residents of dormitories have no effective strategies to control bed bug infestation. This study aimed to evaluate the resistance to deltamethrin in collected tropical bed bugs by bioassay tests and molecular surveys from dormitories in Iran.

MATERIALS AND METHODS

Bed bug sampling. Bed bugs were collected from Tehran district, Iran, during 2021–2022. Live bed bugs were collected from seven sleeping quarters, and the maximum distance between these sites was 4 km. Bed bugs were collected by forceps from crevices and cracks in the furniture and walls. For morphological identification, collected bugs were transferred to the laboratory and identified under a stereomicroscope by identification keys.[15,16] Blood-fed samples were used for insecticide bioassays. Bed bugs were immediately frozen and kept at –20°C for molecular tests.

Bioassay test. The collected adult samples from dormitories were used for insecticide bioassay tests based on previous works.[12] In brief, at least three groups of 10 to 15 mixed-sex pools of adult bugs were exposed to deltamethrin-treated paper (0.025%) as a test, and an identical number of collected samples were exposed to paper impregnated with silicone oil as a control group. At continuous exposure, the knockdown and death rates were recorded. The dead, knockdown, and living samples were kept at –20°C for future analysis once the experiments were completed. Bioassay data for *C. lectularius* Teh strain (adult) were used as a pyrethroid-susceptible

*Address correspondence to Rahmat Solgi, PhD, Medical Parasitology and Mycology, assistant Professor, Medical Microbiology Groups, Birjand University of Medical Sciences, Ghafari Ave., Birjand, Iran Tel: +98 5632381517, Fax: +98 5632381517, E-mail: Rahmatsolgi@yahoo.com **or** Mohammad Darvishi, Associate Professor of Infectious Disease, School of Aerospace and Subaquatic Medicine Beasat Hospital AJA University of Medical Sciences, Tehran, Iran. Tel: 021 8802 8350, E-mail: darvishi1349@gmail.com

TABLE 1
Knockdown and mortality rates of bedbugs after exposure to discriminating dosages deltamethrin

Samples	Knockdown rate (%)		Mortality rate (%)	
	24 hours	48 hours	24 hours	48 hours
Cimex hemipterus	54	72	49	65
Cimex lectolarus	100	100	100	100

Exposure time was 24 hours. Data for the *C. lectularius* population are presented for comparison.

TABLE 2
Time (hours) taken to knockdown 50% (KT_{50}) and 95% (KT_{95}) of the *Cimex hemipterus* population when exposed to discriminating dosages of deltamethrin

Insecticide	*Cimex lectularius*		*Cimex hemipterus*		
	KT_{50}	KT_{95}	KT_{50}	KT_{95}	KR_{50}
Deltamethrin	4	19	22	75	5.5

KR_{50} = knockdown time ratios. Data from *C. lectularius* are presented for comparison.

reference. WHO classification was used to determine deltamethrin resistance as fallows; mortality rate $>$ 98% indicated susceptibility, and $<$ 98% proposed the presence of resistance.[15]

Detection of *kdr* mutations. The genomic DNA of isolated samples was extracted using a Qiagen extraction kit (Valencia, CA). In brief, the sample cutting into small pieces with a scalpel and then homogenized in 200 μL of a lysis buffer. Subsequently, 20 μg of reconstituted proteinase K was added and then mixed immediately and incubated until the tissue was digested completely. Further steps were undertaken based on manufacturer's instructions.

Genotyping of *kdr* gene. Amplification of two VGSC regions including 513-bp and 752-bp related to *kdr*-type resistance to pyrethroids was performed under following conditions: 1 μL of both forward and reverse primers (10 pM), 12.5 μL of Taq DNA Polymerase 2X Master Mix RED (Ampliqon, Denmark), and 2 μL of DNA template in a total volume of 25 μL (100–150 ng/reaction). Fragment amplification was carried out using the primers BBparaF1 and BBparaR1 for the first fragment and BBparaF3 and BBparaR3 for the second regions.[16] PCR for both reactions were performed under the following conditions: 95°C for 10 min for denaturation, followed by 40 cycles of 94°C for 20 seconds, 58°C for 20 seconds, and 72°C for 40 seconds, and the final extension of 70°C for 10 minutes. For detection of a point mutation at residues V410, V419, V421, and E435 at the first region and M918, L925, T929, L932, L936, and L1014 residue at the second region of VGSC gene, all the samples were sequenced (Daejeon, South Korea). The raw nucleotide sequences were aligned manually using ClustalW and analyzed by BioEdit software (version 7.0.9, Los Angeles, CA). The mutations were determined by comparing the translated sequences of samples compared with wild-type sequences (GenBank accession numbers GU123927 and GU123928) and *Musca demostica* (GenBank accession number AAB47604).

Statistical analysis. The times of KT_{50} and KT_{95} in which 50% and 95% of bed bugs were paralyzed, respectively, were calculated by PriProbit version 1.63 software. Abbott's formula was used if the control knockdown mortality was $<$ 20%. Populations were statistically compared using chi-square test. The resistance ratio at KT_{50} (KR_{50}) was calculated by comparing the rates of KT_{50} values of the field strains of tropical bed bugs with the corresponding knockdown time for *C. lectularius* Teh strain.

RESULTS

One thousand three hundred bed bugs were collected from three out of seven (42%) infested sites. On the basis of morphological identification keys, 35% and 65% of them were related to *C. hemipterus* and *C. lectularius*, respectively. From the bed bugs samples, 200 individuals of *C. hemipterus* were isolated randomly and used to bioassay test. On the basis of the bioassay test within the 48-h exposure to the deltamethrin, *C. hemipterus* were determined to be resistant. The difference between knockdown and mortality rates of *C. hemipterus* and *C. lectularius* at 24 and 48 hours were significant ($P < 0.05$). Knockdown and mortality rates of collected bed bugs to deltamethrin are shown

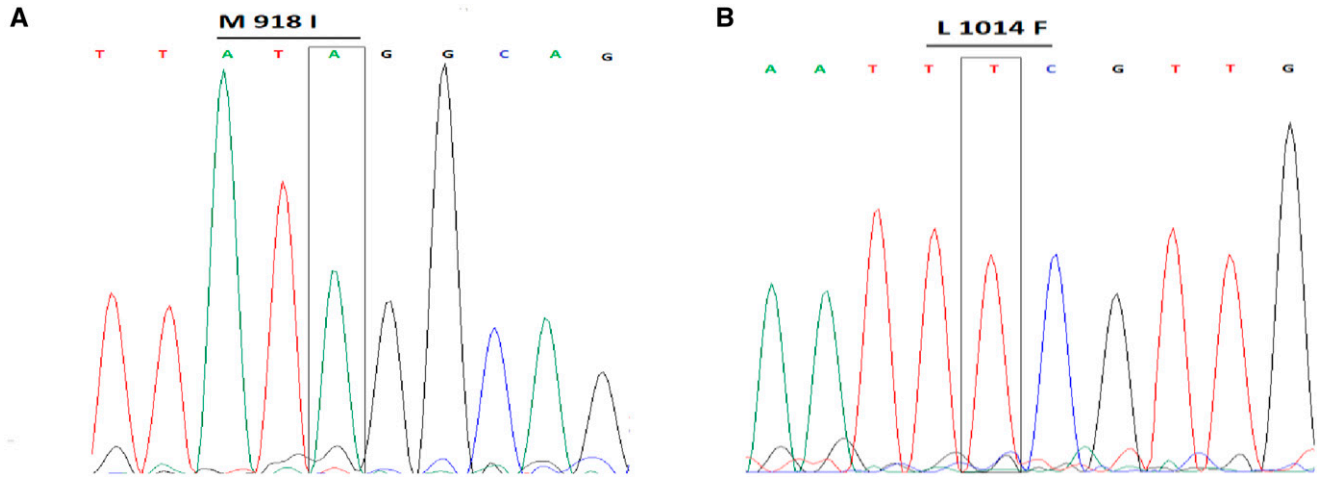

FIGURE 1. Chromatograms showing kdr genotypes of *Cimex hemipterus.* A:M 918 I, transitions with change in amino acid at sites I918 (isoleucine/I: ATA) compare with sites M918 (methionine/M: ATG); B: L1014F, transitions with change in amino acid at sites F1014 (phenylalanine/F: TTC) compare with L1014 sites (leucine/L: CTG). This figure appears in color at www.ajtmh.org.

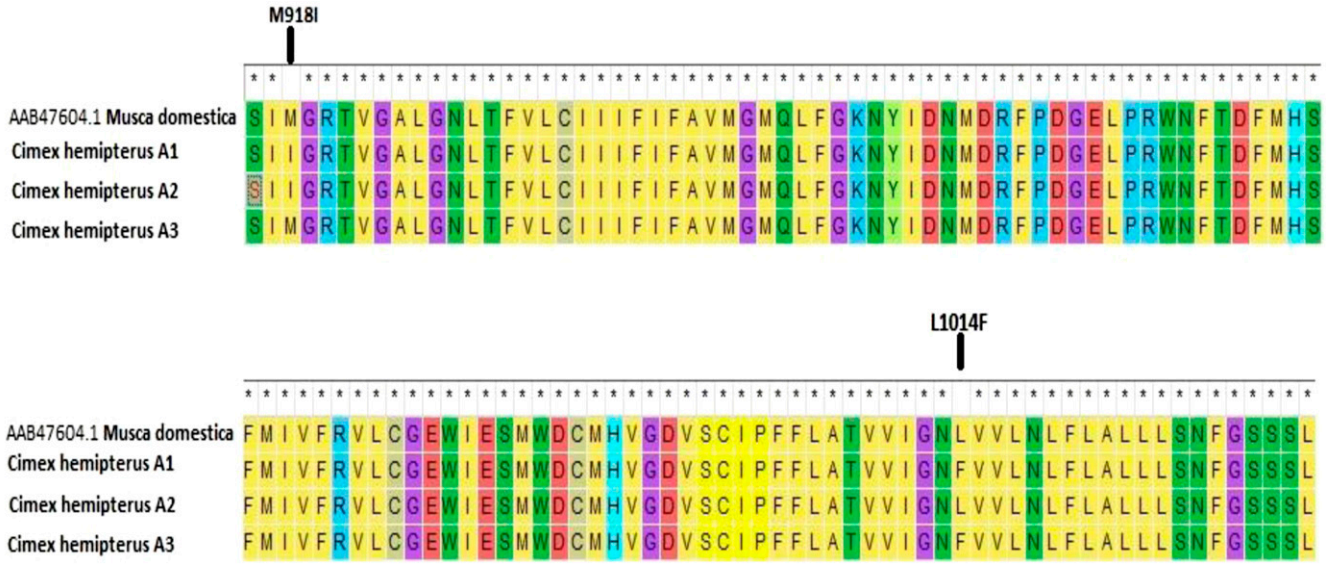

FIGURE 2. Gene sequences of the voltage-gated sodium channel of *Cimex hemipterus* aligned with that of *Musca domestica* (GenBank accession number: AAB47604) showing the mutations M918I and L1014F. Three haplotypes (A1, A2, and A3) were reported. This figure appears in color at www.ajtmh.org.

in Table 1. No mortality was noted in the control bioassay experiments. KR_{50} indicates that resistance to deltamethrin of *C. hemopterus* has increased 5.5-fold compared with the common bed bug (Table 2). DNA sequencing of the domain IS6 and part of the domain I–II linker region as a first region and the domain IIS4–IIS6 as a second region of the VGSC gene revealed the presence of mutations at M918I and L1014 in *C. hemipterus* (Figure 1).

Twenty-five amplicons were randomly selected for sequencing. *Kdr* mutations of only M918I were observed in 12 of 25 (40.9%), M918I and L1014F in six of 25 (24%), and only L1014F in seven of 25 (28%) (Figure 2). All of these *kdr* mutation sites of VGSC gene seem to be associated with *kdr*-type resistance to pyrethroids.

DISCUSSION

Many arthropods, such as mosquitoes, ticks, scabies, and fleas are major pests and threats to human health.[17] So far, several insecticides have been used to control these pest species in Iran. Repeated use of these agents to control pests has led to the development of resistant.[18] For many years, bed bugs were controlled in Iran through various methods, such as boiling, using minerals, biological control with fungi, and cleaning of equipment. Then, the insecticidal properties of DDT were discovered and introduced to control the pests. DDT was widely used for control of malaria vector in Iran. With the emergence of insect resistant to DDT, other insecticidal agents were introduced.[19] Pyrethroids were introduced in 1994 and became the most important group of synthetic insecticides against medical pests. In the past decade, the widespread use of these compounds against malarial vectors in Iran has led to resistance in other insects, including bed bugs.[20] The current research is the first attempt to investigate pyrethroid resistance in *C. hemipterus* isolated from several dormitory locations in Iran. Knockdown time ratios (KR_{50}) in the studied populations of *C. hemipterus* was 5.5-fold compared with those of the *C. lectularius* Teh strain. The results showed that the resistance to deltamethrin in the studied populations has increased. The bioassay results in this study were similar to previous studies in different parts of the world.[6,21,22] Thus, reconsideration of new strategy to control *C. hemipterus* is important in the studied areas. The previous study by Punchihewa et al. to evaluate the insecticide resistant of *C. hemipterus* to deltamethrin in Sri Lanka showed a high percentage of resistance among 2016 isolated strain compared with susceptible strains of 2002, which were the same as our results.[24]

The *C. lectularius* Teh strain was used as an insecticide-susceptible control sample because no susceptible strain of *C. hemipterus* could be sourced. Similar use of this strain as a reference has been made in other investigations.[12,18]

The main point mutations related to insecticidal resistant in a range of important agricultural pests and disease vector to pyrethroids found in domain IS6 and part of the domain I–II linker region as a first region (V410,V419,V421, and E435) and the domain IIS4–IIS6 as a second region of the VGSC gene (M918, L925, T929, L932, and L1014).[23,24] Another recent study has introduced novel mutations of *C. hemipterus*, including L899V and D953G.[9] The first characterization of the VGSC gene of both resistant and susceptible strains of C. *lectularius* was established in United States,[25] and then many similar studies were accomplished in different parts of the world.[10,11,20,24] On the basis of the current study, three haplotypes, including haplotype A (only M918I; 40.9% [12/25]), B (M918I and L1014F; 24% [6/25]), and C (L1014F; 28% [7/25]) were observed. L1014F mutation was observed for the first time from housefly *Musca domestica*, and then it was determined as super-*kdr* mutation from other insect pest including bed bugs.[16] The appearance of super-*kdr* mutation including M918I and L1014F from this study emphasizes the possibility of expansion of pyrethroid resistance isolate in Iran. Similar to our study, M918I and L1014F mutations were observed in another public health pest.[9,16,26,27]

CONCLUSION

The application of effective insecticides, as well as consideration of biological and socioeconomic risk factors and the side effects of current public pest control measures, need to be considered in the establishment of revised control strategies. On the basis of the bioassay and molecular results of the current study, *C. hemipterus* has shown a high degree of resistance to pyrethroid. The application of multiple approaches, including physical, biological, and chemical tests, should be regarded in future bed bug control.

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Authors' addresses: Mehdi Tiotour, Minoo Shaddel, Mohammad Aminianfar, and Mohammad Darvishi, Infectious Diseases and Tropical Medicine Research Center, Department of Aerospace and Subaquatic Medicine, AJA University of Medical Sciences, Tehran, Iran, E-mails: i.parasite@yahoo.com, navidinparast@yahoo.com, microbiology3961@yahoo.com, and darvishi1349@gmail.com. Hadi Mirahmadi, Infectious Diseases and Tropical Medicine Research Center, Resistant Tuberculosis Institute, Zahedan University of Medical Sciences, Zahedan, Iran, and Department of Parasitology and Mycology, Faculty of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran, E-mail: hmirahmadi59@gmail.com. Gholamreza Barzegar, Department of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran, E-mail: rezabarzegar51@gmail.com. Rahmat Solgi, Infectious Disease Research Center, Birjand University of Medical Sciences, Birjand, Iran, E-mail: rahmatsolgi@yahoo.com.

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