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Research

Knockdown Resistance-Associated Mutations Dominate Populations of the Common Bed Bug (Hemiptera: Cimicidae) Across the South Central United States

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Abstract

Despite awareness of the mutations conferring insecticide resistance in the bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), within the United States few studies address the distribution and frequency of these. Within the United States, studies have focused on collections made along the East Coast and Midwest, documenting the occurrence of two mutations (V419L and L925I) within the voltage-gated sodium channel α-subunit gene shown to be associated with knockdown resistance (*kdr*) to pyrethroids. Here, the distribution and frequency of the V419L and L925I site variants is reported from infestations sampled within Oklahoma and its immediately adjacent states. Additionally, the presence of a mutation previously undocumented in the United States (I935F) is noted. While novel in the United States, this mutation has previously been reported in Australian and Old World populations. No infestations were found to harbor wild-type individuals, and hence susceptible, at each of the three sites. Instead, ~21% were found to possess the resistant mutation at the L925I site (haplotype B), ~77% had mutations at both the V419L and L925I sites (haplotype C), and 2% possessed the mutation at the L936F site (haplotype A^b). The high frequency of haplotype C corresponds to previous studies in the United States, and contrasts dramatically with those of the Old World and Australia. The data presented here provide insight into the contemporary occurrence of *kdr*-associated insecticide resistance in the South Central United States, a region for which data have previously been absent. These data suggest that New World and Old World/Australian infestations are likely to have originated from different origins.

Key words: *Cimex lectularius*, voltage-gated sodium channel, *kdr*, pyrethroid resistance, insecticide resistance

Despite its Old World origin, the common bed bug, *Cimex lectularius* L. has a long history of association with the United States; likely introduced unintentionally by early European settlers (Usinger 1966). Following its introduction, it spread from east to west, infesting human-associated dwellings (Miller 2018); however, a rapid decline occurred in the late 1950s. In recent years, a dramatic resurgence has occurred within temperate regions on a near-global scale (Davies et al. 2012) and some South American countries (e.g., Brazil, Nascimento et al. 2011). This resurgence has been mirrored by the reappearance or introduction of the tropical bed bug, *Cimex hemipterus* F. (Hemiptera: Cimicidae) in tropical and subtropical regions (Davies et al. 2012, Doggett et al. 2012, Campbell et al. 2016). In the United States, this resurgence began in the late 1990s and by 2006 infestations had been reported in all 50 states (Miller 2018). This has largely been attributed to an increase in both national and international travel, the frequent exchange of secondhand furniture, ineffective pest control, and poor knowledge of what bed bugs actually look like, hence resulting in low early detection rates (Boase 2001, Doggett et al. 2004, Reinhardt et al. 2008). However, the evolution of mechanisms conferring resistance to a variety of insecticides (Busvine 1958, Romero et al. 2007, Yoon et al. 2008, Koganemaru et al. 2013, Zhu et al. 2013, Lilly et al. 2016, Romero and Anderson 2016, Dang et al. 2017) has also likely contributed to the rapid and widespread resurgence observed.

Early instances of resistance can be traced back to failed attempts to control *C. lectularius* using dichloro-diphenyl trichloroethane (DDT) (Johnson and Hill 1948, Busvine 1958). Introduced in the mid 1940s, DDT initially proved to be an effective means of controlling infestations; the widespread application resulting in the near-complete eradication of the species in the United States within just 5 yr of its introduction as an insecticide (Potter 2011). However, as the overall population numbers began to decline reports of failure to control infestations using DDT began to surface, indicating that mechanisms had likely evolved conferring resistance to the

insecticide (Potter 2011). Through sequencing of the coding gene of the voltage-gated sodium channel (VGSC), Yoon et al. (2008) identified two nonsynonymous mutations (V419L [valine to leucine] and I925L [isoleucine to leucine]) within a pyrethroid-resistant population that were absent in a pyrethroid-susceptible population. These mutations reduce target-site sensitivity to pyrethrin, pyrethroids, and organochlorides, conferring knockdown resistance (*kdr*) (Davies and Williamson 2009). A third mutation (I936F [isoleucine to phenylalanine]) has recently been identified (Dang et al. 2015); the role it may play in conferring insecticide resistance requires further assessment.

Despite interest in understanding the frequency and distribution of VGSC-associated mutations in *C. lectularius* (Yoon et al. 2008; Seong et al. 2010; Zhu et al. 2010; Durand et al. 2012; Booth et al. 2015, 2018; Dang et al. 2015; Pelenchar et al. 2015; Balvin and Booth 2018), studies within the United States are scarce and focus on populations primarily distributed within eastern and mid-western states (Zhu et al. 2010, Vargo et al. 2011). These studies revealed that populations exhibiting pyrethroid-susceptible VGSC profiles were relatively rare. For example, Zhu et al (2010) reported that only 12% of 110 populations sampled lacked both V419L and L925I VGSC mutations. Similarly, in a study of 38 populations, sampled across a comparable geographic range as Zhu et al (2010), Vargo et al. (2011) found 15.8% lacked both V419L and L925I. Neither study reported on the frequency of the I936F mutation. An understanding of the distribution and frequency of these mutations within populations infesting the South Central states is currently lacking. Furthermore, the I936F mutation, recently reported in populations of *C. lectularius* collected in Australia, Israel, and Europe (Dang et al. 2015, Palenchar et al. 2015, Balvin and Booth 2018), has yet to be documented among samples collected in the United States.

The aim of this study was to generate data that further our understanding of VGSC-associated mutation frequencies and distribution, specifically within the South Central United States. Focus is directed toward Oklahoma and its immediately adjacent states (Kansas, Missouri, Arkansas, Texas, New Mexico, and Colorado). The three previously identified mutation sites were assessed (V419L and I925L [Yoon et al. 2008], I936F [Dang et al. 2015]) through Sanger sequencing. Given the lack of information regarding VGSC-associated mutations in this geographic region, these findings provide novel information that may be informative to pest management professionals in the development of control strategies aimed at current and future infestations. Additionally, these findings may enhance our understanding of local and global variation in VGSC-associated mutation frequency, and thus provide insight into both continental and transcontinental patterns of *C. lectularius* spread.

Materials and Methods

Sample Collection and DNA Extraction

Samples of *C. lectularius* (n = 343) were collected from a total of 97 infested structures within 34 cities across seven South Central states (Table 1; Supp Table 1 [online only]); specifically Oklahoma (n = 49), Kansas (n = 9), Missouri (n = 11), Arkansas (n = 3), Texas (n = 13), New Mexico (n = 9), and Colorado (n = 3) (Fig. 1), between 7 March 2014 and 23 July 2015. Samples were confirmed as *C. lectularius* using barcoding primers LEPF (5′-ATT CAA CCA ATC ATA ATA AAG ATA TNG G-3′) and LEPR (5′-TAW ACT TCW GGR TGT CCR AAR AAT CA-3′). In association with local pest control companies (see Acknowledgments), samples (mainly adults of mixed sex) were collected from single rooms, immediately preserved in 95% ethanol, and subsequently stored at –20°C prior to DNA extraction. Genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Germantown, MD). DNA concentration was standardized to ~50 ng/µl and stored at –20°C until use.

Detection of VGSC Mutations

For each specimen, two genomic fragments previously shown to exhibit VGSC-associated mutations (Yoon et al. 2008, Dang et al. 2015) were PCR amplified. Primer combinations BBParaF1/BBParaR1 (V419L) and BBParaF3/BBParaR3 (L925I, I936F) were used, as previously described by Zhu et al. (2010). PCR products were subsequently purified using Exo-SAP-IT (Affymetrix Inc., Santa Clara, CA) and sequencing reactions performed with primers BBparaF1 for the V419L mutation site and BBparaR3 for the L925I and I936F mutation sites, using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Samples were sequenced on an ABI 3130xl Genetic Analyzer (Applied Biosystems), the resulting chromatograms visualized using CLC Genomic Workbench (https://www.qiagenbioinformatics.com), and the presence/absence of mutations scored.

Individuals were identified as susceptible or resistant as follows: V419L – GTC = valine, CTC = leucine; L925I – CTT = leucine, ATT = isoleucine (Yoon et al. 2008); I936F – ATT = isoleucine, TTT = phenylalanine (Dang et al. 2015). For each, the former amino acid represents the wild-type (susceptible) state and the latter the mutant (resistant) state. The presence of heterozygotes would be identified as overlapping peaks at the respective nucleotide position. To maintain consistency with previous studies of VGSC mutation frequency and distribution, we use the haplotype designations assigned by Zhu et al. (2010): haplotype A = susceptible at both 419 and 925; B = 419 susceptible, 925 resistant; C = resistant at both 419 and 925; and D = 419 resistant, 925 susceptible. However, haplotype designation A through D provides no information regarding the presence or absence of the mutation at site I936F. As such, we follow the additional notation of Dang et al. (2015), in which a superscript 'b' is added to the haplotype designation of Zhu et al. (2010). For example, haplotype A^b represents susceptible alleles at both 419 and 925, and the resistant allele at 936.

Results

All samples yielded unambiguous sequences for both PCR fragments. Sanger sequencing of these amplified fragments revealed the presence of three haplotypes among the 343 samples examined (Table 1; Fig. 1; Supp Table 1 [online only]). Per population, on average 3.5 specimens were screened (range 1–11) (Supp Table 1 [online only]). No individuals were found exhibiting either haplotypes A or D. Haplotype B was found in 20 (20.6%) populations, spanning all sampled states except Kansas and Arkansas. Haplotype C, which exhibits resistant alleles at both 419 and the 925, was found in 75 (77.3%) populations, spanning all sampled states. Interestingly, haplotype A^b (susceptible at both 419 and 925, but resistant at site 936) was found in two populations in Albuquerque, NM (Table 2; Supp Table 1 [online only]). Heterozygotes for both susceptible and resistant alleles at site 419 were found in only a single population (Supp Table 1 [online only]). Additionally, a sample reported to be from a single population collected in an apartment in Tulsa, OK, consisting of nine individuals, exhibited two haplotypes. Two individuals exhibited haplotype B, while the remaining seven exhibited haplotype C (Supp Table 1 [online only]). Given that we cannot confirm that these were indeed collected from a single

Table 1. *kdr* haplotype information per city and state

Fig. 1 ref.	Location	State	No. of unique infestations	Total no. of infestations examined in state	Haplotypes present in state
1	Greeley	CO	1	3	C
2	Denver	CO	2		B, C
3	Albuquerque	NM	9	9	A^b, B, C
4	Wilson	KS	1	9	C
5	Hoisington	KS	1		C
6	Great Bend	KS	4		C
7	Galva	KS	1		C
8	Kansas City	KS	2		C
9	Denton	TX	3	13	B, C
10	Temple	TX	1		C
11	Austin	TX	6		B, C
12	Houston	TX	3		B, C
13	Craig	MO	1	11	C
14	O'Fallon	MO	2		C
15	Ballwin	MO	1		C
16	St. Louis	MO	7		B, C
17	Booneville	AR	1	3	C
18	Little Rock	AR	1		C
19	Benton	AR	1		C
20	Guthrie	OK	1	49	C
21	Edmond	OK	1		C
22	Bethany	OK	2		C
23	Oklahoma City	OK	6		C
24	Chickasha	OK	1		C
25	Blanchard	OK	2		C
26	Norman	OK	4		C
27	Shawnee	OK	1		B
28	Skiatook	OK	1		B
29	Collinsville	OK	1		C
30	Sand Springs	OK	2		B
31	Bristow	OK	3		B, C
32	Tulsa	OK	17		B, C
33	Broken Arrow	OK	3		C
34	Muskogee	OK	4		B, C

apartment, it is possible that these were collected in separate apartments within this complex. Adjacent apartments harboring genetically divergent (and thus potentially exhibiting different *kdr* haplotypes) have been noted in apartment buildings previously (see Booth et al. 2012).

Discussion

Through Sanger sequencing of 97 *C. lectularius* infestations, spanning seven South Central U.S. states, we provide the first record of the distribution and frequency of knockdown resistance-associated VGSC mutations in this region. We found that haplotype C (characterized by both V419L and L925I mutations) predominates, being present in ~77% of infestations surveyed. Haplotype B (L925I mutation only) was found in ~21% of infestations. Of interest, haplotype A^b (I936F mutation only), was recorded for the first time in U.S. populations, being found in two infestations sampled in Albuquerque, NM. It is currently unknown what level of resistance haplotype A^b imparts, if any; however, haplotypes B and C have been shown to elevate pyrethroid resistance up to or above several hundred-fold (Romero 2011).

While data are lacking for *C. lectularius* infestations previously sampled across South Central states, data are available for infestations in the eastern and mid-western regions (Zhu et al. 2010, Vargo et al. 2011, Booth et al. 2018). Despite being both temporally and geographically differentiated, these findings contrast with those presented in this study. Specifically, while haplotypes B and C were found in high frequency in the previous U.S. studies, a shift toward haplotype C was observed in the South Central samples screened here (see Table 2). Furthermore, haplotypes A and D were not recorded in this study. Interestingly, while recorded here, haplotype A^b has not been reported previously in the United States. It is impossible to determine whether this was absent within the eastern and mid-western samples previously screened by Zhu et al. (2010) and Vargo et al. (2011), or simply evaded detection. While Vargo et al. (2011) employed Sanger sequencing and would likely have reported this mutation if present among the 38 infestations investigated, Zhu et al. (2010) employed the use of allele-specific PCR. This method precludes the identification of additional mutations outside of the priming sites. As such, they report only on the frequency and distribution of the V419L and L925I mutations.

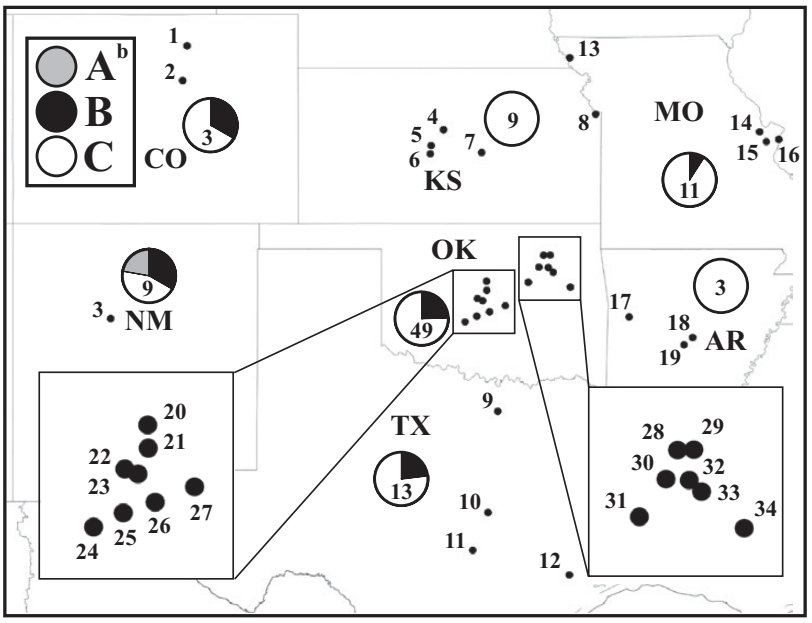

Fig. 1. Map of the South Central United States identifying sampling locations of *C. lectularius* (black dots, numbers beside dots relate to city names indicated in Table 1). Pie charts indicate VGSC mutation frequency (numbers within pies represent sample size within each state). Sampling locations within Oklahoma subdivided and represent Tulsa and its surrounding locations, and Oklahoma City and its surrounding sampling locations.

It should be noted that the results of these earlier studies relate to samples collected between 5 and 9 yr prior to those screened in the present study. As such, temporal change in the frequency of these mutations may be expected given the continued application of pyrethroid-containing insecticides (Potter et al. 2012, Gordon et al. 2014), and the potential for intracontinental/intercontinental dispersal events (Boase 2001, Davies et al. 2012). Such a temporal change has previously reported for the North American head louse, *Pediculus humanus capitis* De Geer (Anoplura: Pediculidae), a species frequently treated with pyrethroids. For head lice samples collected between 1999 and 2008 from populations spread across 12 U.S. states, the knockdown resistance allele frequency was 84.4%, whereas for those collected after 2007 it was 99.6% (Yoon et al. 2014). Similarly, finding that knockdown resistance mutations were present in all, or nearly all populations sampled here (depending on whether the I936F mutations convey resistance), may be unsurprising, given that a similar pattern was recently reported across all 138 populations of the North American head louse sampled from 48 U.S. states (Gellatly et al. 2016).

The detection of the I936F mutation here therefore raises the question of whether this mutation was previously absent in the United States, was simply undetected in these previous studies, or may be regionally confined. Where reported, this mutation has been found in between 9% and 16% of infestations screened. The former representing infestations sampled in Australia (Dang et al. 2015), and later in Europe (Balvin and Booth 2018). Of the 32 Australian samples investigated by Dang et al. (2015), the A^b haplotype was found only in samples collected in the field during 1994 and 1998. Balvin and Booth (2018) reported the A^b haplotype in nine infestations of 105 screened, corresponding to collections made in five countries during 2011 and 2012. While we lack contemporary data for the east, west, and mid-western United States, its rarity within this current study spanning the South Central United States, with representation in only two populations in one city, might suggest an introduction through intercontinental movement. A molecular investigation of intercontinental population genetic structure may shed more light into this potential scenario; however, the noticeable variation that exists in the frequencies of the *kdr* haplotypes between the New World and both the Old World and Australia suggests such movements, contrary to current opinion, may be relatively infrequent (Boase 2001, Davies et al. 2012) (see Table 2).

By comparing *kdr* profiles of *C. lectularius* sampled across Europe (Durand et al. 2012, Booth et al. 2015, Balvin and Booth 2018), the Middle East (Palenchar et al. 2015), Asia (Seong et al. 2010, Tomita et al. 2012), Australia (Dang et al. 2015), and the United States (Zhu et al. 2010, Vargo et al. 2011, Booth et al. 2018), we largely see a clear distinction between the New World and both Old World and Australian populations (Table 2). Across the Old World and Australia, haplotype B has been found in ~90% of populations. Furthermore, remarkably similar frequency distributions are reported for haplotypes A, C, and D (Tomita et al. 2012, Booth et al. 2015, Dang et al. 2015, Balvin and Booth 2018). Durand et al. (2012) and Palenchar et al. (2015) failed to detect haplotypes A, and A and C, respectively; however, the remaining haplotypes closely followed the frequencies of other Old World and Australian populations (i.e., haplotype B predominates). In contrast, in the United States, the frequency of haplotype C is elevated and found in ~58% of populations (range 38.7% and 77.3%), while haplotype B was found in ~32.5% of populations (range 20.6% and 45.2%). The elevated frequency of haplotype C in the United States, and the suppressed frequency of haplotype B clearly diverge from the Old World and Australian populations where haplotype B dominates; suggesting intercontinental gene flow may in fact be rare. Comparable to the U.S. profiles, Seong et al. (2010) reported haplotype C in two of four samples collected in Korea in 2007/2008. It should be noted, however, that all four samples reported within this study were collected from the U.S. Army Garrison, Yongsan, Seoul, between 1993 and 2008 and samples collected in 1993. As such, it is possible that these samples originated from the United States and were introduced to the army garrison by posted U.S. soldiers; hence, they may not be representative of Korean bed bug infestations.

Table 2. Review of frequencies of *kdr*-associated haplotypes reported in previous studies of *Cimex lectularius* (adapted from Balvin and Booth 2018)

Study	Region	No. of populations screened	No. of heterozygous populations	Haplotype						
				A	B	C	D	A[b]	B[b]	C[b]
				V419 L925	V419 925I	419L 925I	419L L925	V419 L925 936F	V419 925I 936F	419L 925I 936F
This study	United States	97	1	0	20 (20.6)	75 (77.3)	0	2 (2.1)	0	0
Zhu et al. (2010)[a]	United States	93	0	12 (12.9)	42 (45.2)	36 (38.7)	3 (3.2)	0	0	0
Vargo et al. (2011)[a]	United States	38	4	4 (10.5)	12 (31.6)	22 (57.9)	0	0	0	0
Dang et al. (2015)	Australia	32	4	3 (9.4)	25 (78.1)	2 (6.3)	0	5 (15.6)	1 (3.1)	0
Seong et al. (2010)[a]	Korea	4	1	1 (25)	1 (25)	2 (50)	0	0	0	0
Tomita et al. (2012)[a]	Japan	60	NA	6 (10)	50 (83.3)	4 (6.7)	0	0	0	0
Palenchar et al. (2015)	Israel	12	12	0	11 (91.6)	1 (8.3)	0	0	11*c* (91.6)	1 (8.3)
Durand et al. (2012)[a]	France	198*b*	0	0	198 (100)	0	0	0	0	0
Booth et al. (2015)[a]	Europe	49	2	4 (8.1)	46 (93.9)	1 (2.0)	0	0	0	0
Balvin and Booth (2018)	Europe	105	9	4 (4.2)	98 (93.3)	2 (1.9)	0	9 (8.6)	0	0

Numbers for each haplotype include heterozygous locations. Representation by percentage is in parentheses (note that due to the presence of heterozygous populations, these values may exceed 100% for a given study).

[a]Studies not reporting the I936F site.

[b]Two high-rise multi-apartment buildings sampled, totaling 198 apartments (102 and 96, respectively).

[c]I936F mutation reported to occur at only low levels.

Reviewing previous studies of *kdr* frequency and distribution in *C. lectularius*, an interesting finding is the paucity of individuals exhibiting heterozygosity at VGSC genes. Given that these genes are located within the nuclear genome, heterozygosity is possible, identified by overlapping peaks in the sequence chromatogram, or amplified PCR products for both allele-specific primers. That said, of 684 infestations for which *kdr* haplotypes are reported, only 32 (4.7%) proved heterozygous for one or more mutations (Table 2). This finding may reflect the process by which new infestations are founded from lone gravid females or small, highly inbred propagules already homozygous at a given gene (Booth et al. 2012, 2015, 2018; Saenz et al. 2012). Furthermore, given that gene flow among infestations appears uncommon (Saenz et al. 2012, Booth et al. 2018), the opportunity for heterozygotes to be formed appears rare. Alternatively, if heterozygotes exhibit a reduced level of resistance when exposed to insecticides relative to those homozygous for the mutations (i.e., mortality of heterozygotes is elevated relative to homozygous resistant mutants due to incomplete dominance at the gene), those individuals may be selected against during treatments, resulting in the rapid shift to homozygosity within an infestation. Indeed, it has been shown in the southern cattle tick (*Boophilus microplus* Canestrini, Ixodida: Ixodidae) and the sub-Saharan mosquito *Anopheles gambiae* Giles. (Diptera: Culicidae), that individuals heterozygous at genes conferring pyrethroid resistance exhibit levels of mortality lower than those homozygous susceptible, but greater than those homozygous resistant (Corbel et al. 2004, Aguilar-Tipacamú et al. 2008, Li et al. 2008). These findings suggest an incomplete-dominant mode of inheritance of these genes in these species; the authors are not aware of similar data for *C. lectularius*. Ultimately, the paucity of heterozygotes likely results from a combination of factors, all of which translate into knockdown resistant infestations.

Clearly, these current findings present an alarming picture of the present status of VGSC-associated mutations in the South Central United States. Furthermore, the discordant patterns seen in the distribution and frequency of these mutations when comparing populations sampled from the United States, Europe, Israel, Asia, and Australia, suggests movement between the U.S. and the Old World and Australian populations may be limited. In light of this, future research using both mtDNA and polymorphic nuclear markers is necessary to clarify the genetic relationships among the New World, Old World, and Australian populations, and thus infer dispersal patterns. Furthermore, data relating to VGSC-associated mutations in *C. lectularius* populations in the Western United States are lacking, and warrant investigation.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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