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First evidence of the A302S *Rdl* insecticide resistance mutation in populations of the bed bug, *Cimex lectularius* (Hemiptera: Cimicidae) in North America

Camille J. Block¹, Lindsay S. Miles^{1,✉}, Cari D. Lewis², Coby Schal^{3,✉}, Edward L. Vargo⁴, Warren Booth^{1,*,✉}

¹Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

²Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM, USA

³Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, NC, USA

⁴Department of Entomology, Texas A&M University, College Station, TX, USA

*Corresponding author. Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA (Email: warrenbooth@vt.edu).

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The common bed bug, *Cimex lectularius* (L.) (Hemiptera: Cimicidae), is a pervasive indoor pest with prominent medical, veterinary, and economic impacts. Bed bug infestations are controlled by a wide range of insecticides, including pyrethroids, neonicotinoids, pyrroles, and phenylpyrazoles; however, bed bugs have evolved resistance mechanisms to most of these insecticides. Mutations in the *Rdl* (resistance to dieldrin) gene, located in a subunit of the γ -amino butyric acid (GABA)-gated chloride channel, have been identified in several pest insects, including the German cockroach. These have been found to confer resistance to fipronil, a phenylpyrazole insecticide commonly used in urban environments, in addition to cyclodienes (eg dieldrin), a class of insecticides banned in most countries since the 1990s. While resistance to dieldrin and fipronil has been reported in bed bugs, both *C. lectularius* and the tropical bed bug, *C. hemipterus*, the occurrence of mutations in the *Rdl* gene has yet to be thoroughly investigated. In this study, we sequence a fragment of the *Rdl* gene commonly found to harbor cyclodiene and phenylpyrazole conferring mutations from 134 unique populations collected across the United States and Canada spanning a 14-yr period. Homozygous genotypes for the A302S mutation were found in 2 geographically distinct populations. This finding represents the first record of a non-synonymous *Rdl* mutation in bed bugs and identifies another mechanism by which insecticide resistance may be conferred in this species.

Keywords: target-site mutation, GABA-receptor, fipronil, phenylpyrazole, dieldrin, cyclodiene

Introduction

Globally, insecticide resistance is one of the most significant challenges associated with the control of insect species with organic insecticides (Whalon et al. 2008). This is particularly problematic for pest insects (Zhu et al. 2016), including the common bed bug, *Cimex lectularius* (Dang et al. 2017, Romero 2018, Booth 2024). Bed bugs are a hematophagous insect with a long history of association with humans (Panagiotakopulu and Buckland 1999, Balvín et al. 2012). They have the potential to disperse both actively and passively (Wang et al. 2010, Cooper et al. 2015) and due to their

proclivity to resist the detrimental effects of inbreeding (Booth et al. 2012, Saenz et al. 2012, Fountain et al. 2015), infestations establish rapidly; each then acting as a new propagule pool from which further infestations may be founded (Booth 2024). In recent decades, a near-global resurgence of bed bugs has occurred (Doggett et al. 2018a), resulting in significant medical, veterinary, and economic impacts (Doggett 2018, Doggett et al. 2018b, Hwang et al. 2018, Perron et al. 2018, Szalanski 2018).

Bed bug infestations have been controlled using a variety of insecticides (Doggett and Lee 2023), most notably the organochlorine

DDT (dichloro-diphenyl trichloroethane); a powerful chemical that almost succeeded in eradicating bed bug infestations in the early 1950s (Potter 2011). However, within only a few years of its introduction to control bed bugs, populations displaying resistance to this chemical began to appear (Busvine 1958), thus eliciting a change in the insecticides used to control outbreaks (Potter 2011). Insecticide resistance continues to be a significant issue in the control of bed bugs, with infestations exhibiting resistance to an array of commercially available insecticide classes, including pyrethroids, neonicotinoids, pyrroles, cyclodienes, and phenylpyrazoles (Romero and Anderson 2016, Dang et al. 2017, González-Morales et al. 2021).

Both cyclodienes and fipronil, a phenylpyrazole insecticide, have been shown to have the potential to effectively control bed bugs in laboratory studies (Lofgren et al. 1957, Sierras and Schal 2017). They share a mode of action that works on a subunit of the γ -amino butyric acid (GABA)-gated chloride channel. While cyclodiene insecticides have been banned since the late 1990s, fipronil is commonly used to control pest insects in the urban environment (Kaakeh et al. 1997, Hooper-Bui and Rust 2000, Wiltz et al. 2010, Vargo and Parman 2012), including fleas on domestic cats and dogs (Dryden et al. 2013). That said, resistance to both cyclodiene (eg dieldrin) and phenylpyrazole (eg fipronil) insecticides have been reported in the common bed bug, *C. lectularius*, and the tropical bed bug, *C. hemipterus* (Fabricius) (WHO 1976, González-Morales et al. 2021).

Two mechanisms have been identified that confer resistance to cyclodiene and phenylpyrazole insecticides: (i) target-site mutations that result in non-synonymous amino acid substitutions in ion channels associated with the nervous system (eg A302S [alanine to serine]) and (ii) the upregulation of detoxification enzymes (eg cytochrome P450 monooxygenases, carboxylesterases, and glutathione *S*-transferases). In the German cockroach, *Blattella germanica* (L.), the brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), and *Drosophila* spp. (Diptera: Drosophilidae), resistance has been partially explained by *Rdl* (resistance to dieldrin) point mutations, which alter GABA receptor function (Le Goff et al. 2005, Ang et al. 2013, Garrood et al. 2017). However, prior to this study,

similar point mutations have not been detected in fipronil-resistant bed bugs (González-Morales et al. 2021). The aim of this study was to determine if *Rdl* mutations are present in bed bug populations collected within North America over a 14-yr period.

Materials and Methods

Samples were collected by pest management companies (Supplementary Table S1) within the United States and Canada between 27 June 2008 and 3 August 2022 (Fig. 1, Supplementary Table S2). These collections represent 134 unique populations that span 22 U.S. states ($n = 117$) and 4 Canadian Provinces ($n = 17$) (Supplementary Table S2). After collection, specimens were preserved in 100% ethanol and stored at -20°C until DNA extraction. A previous study confirmed that all samples were *C. lectularius* based on cytochrome oxidase I haplotypes (Lewis 2024). DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (QIAGEN) following the manufacturer's instructions, with DNA elution in 3 volumes of 25 μl of warm AE buffer. Extracted DNA was stored at -20°C . A fragment of the *Rdl* gene known to include the A302S/G mutation site was PCR-amplified using primers BG-*Rdl*-F (5'-GTGCGGTCCATGGGATACTA-3') and BG-*Rdl*-R (5' AACGACGCGAAGACCATAAC-3') (Hansen et al. 2005). Amplification of the genomic fragments was performed using the following conditions: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min, with a final extension period of 72°C for 7 min. Negative controls were incorporated into each PCR. Fragment amplification was confirmed through electrophoresis on 2% agarose gels. Polymerase chain reaction products were purified using Exo-SAP-IT (ThermoFisher Scientific) and sequenced using the BG-*Rdl*-F primer with the Big Dye Terminator v3.1 cycle sequencing kit (ThermoFisher Scientific). Sequencing products were purified using the Big Dye Xterminator kit (ThermoFisher Scientific) and sequencing was performed on a SeqStudio Genetic Analyzer (ThermoFisher Scientific). Sequences were visualized and aligned in Geneious (Geneious Prime 2024.0; <https://www.geneious.com>).

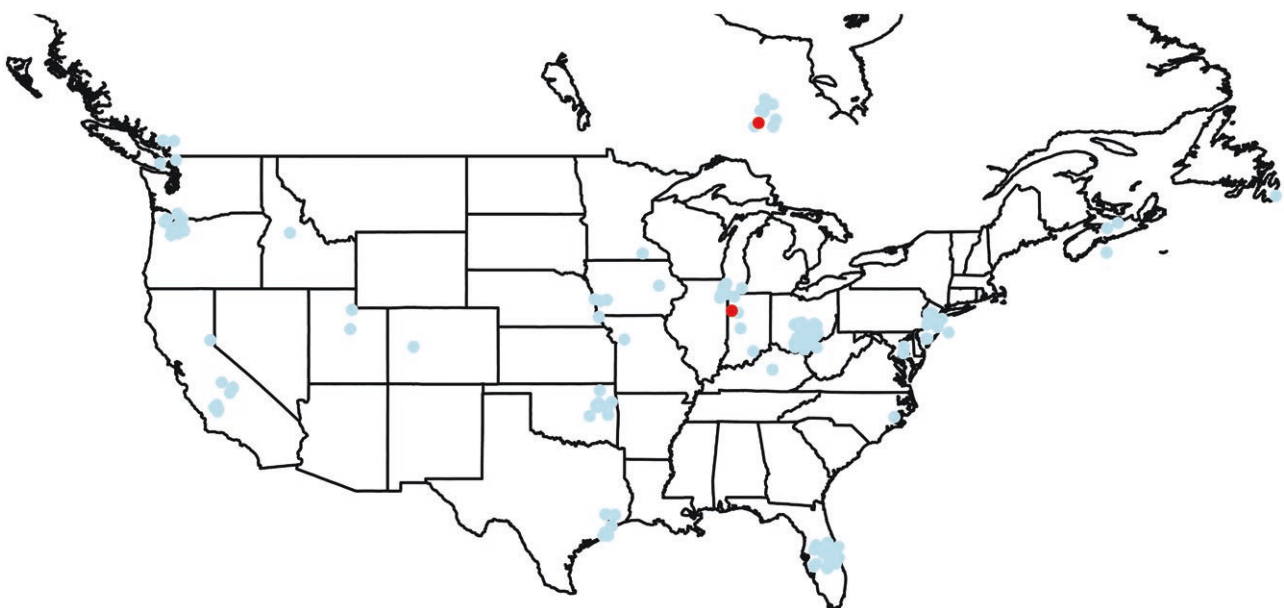


Fig. 1. Map of North America depicting sample locations (circles) of *Cimex lectularius* sequenced here for the A302S *Rdl* mutation. Homozygous wild type (A302) infestations are depicted in blue (light circles), and homozygous resistant mutations (302S) in red (dark circles).

Table 1. Collections from which multiple bed bug (*Cimex lectularius*) specimens were genotyped at the *Rdl* locus. In parenthesis—Population genotype frequency.

City	State/Province	Country	Sample size	Genotype
Glendale Heights	Illinois	United States	6	Mutant (100%)
Columbus	Ohio	United States	10	Wild type (100%)
Portland	Oregon	United States	3	Wild type (100%)
Windsor	Ontario	Canada	10	Wild type (100%)
Niagra Falls	Ontario	Canada	4	Wild type (100%)

Results

An unambiguous 245-bp genomic fragment of the GABA-receptor gene that includes the A302S/G mutation site was amplified and sequenced for all specimens. Due to the genetic uniformity within bed bug infestations resulting from extensive inbreeding (Booth et al. 2018), one specimen was screened per collection site. Sequence alignment identified a GCC (Adenine) to TCC (Serine) transition at amino acid position 302 in 2 samples; one collected in Ontario, Canada, on 2 October 2009, and one collected in Glendale Heights, Illinois, on 10 November 2009 (Supplementary Table 2). Both individuals exhibited the mutation in the homozygous state. From 5 populations additional individuals were genotyped to confirm within population allele frequency. The number of samples sequenced per population ranged from 3 to 10, depending on pest controller sample returns (Table 1). These included one *Rdl* mutant (Glendale Heights, Illinois) and 4 putatively wild-type populations. Results confirmed the findings of sequencing one individual per population. All individuals in the *Rdl* mutant population were homozygous for the mutation, whereas all other individuals were homozygous wild-type (Table 1).

Discussion

Through Sanger sequencing of 134 *C. lectularius*, each representing a unique population spanning 22 U.S. states and 4 Canadian provinces, we provide the first record of the *Rdl* A302S mutation in bed bugs. Of the 134 bed bugs sequenced, two were found with the A302S mutation, and both were in the homozygous state. From one resistant population for which multiple specimens were available (Glendale Heights, Illinois), all individuals ($n = 6$) were found to exhibit the mutant *Rdl* A302S genotype in the homozygous state. From 4 additional populations (2 U.S. and 2 Canadian) from which multiple individuals were sequenced ($n = 3-10$), all specimens were homozygous wild-type genotype. While it would be ideal to sequence multiple individuals for each infestation, this is rarely possible due to the low number of specimens per infestation that are returned by pest management professionals. That said, previous studies have provided justification that a single individual per population is representative of the population genotypic frequency (Booth et al. 2015, Balvin and Booth 2018, Holleman et al. 2019).

The detection of the *Rdl* mutation in only 2 populations is interesting, given that both cyclodiene and fipronil resistance have previously been documented in bed bugs, yet the A302S *Rdl* mutation has not been reported (WHO 1976, González-Morales et al. 2021). This raises questions regarding target-site mutations in the GABA-gated chloride channel and fipronil resistance in bed bugs. First, it is possible that exposure to dieldrin decades ago was selected for the *Rdl* mutation in broadly distributed bed bug populations; however, due to negative fitness effects, this mutation was selected against after dieldrin use was discontinued. Fitness costs associated with *Rdl* point mutations have been reported in mosquitoes and

planthoppers (Platt et al. 2015, Zhang et al. 2016, Rigby et al. 2020, Gomard et al. 2022), and these may be additive in the presence of *kdr*-associated mutations (Platt et al. 2015). With 2 *kdr*-associated mutations common in bed bugs in the U.S. (Lewis et al. 2023), this may have created a selection pressure to eliminate *Rdl* mutations from populations. Sequencing specimens collected prior to 2008 may help address this. Second, while the A302S mutation has been linked to resistance in other species, it is possible that additional resistance-conferring mutations within the GABA-receptor gene but outside of the *Rdl* fragment exist but have yet to be identified. Clearly, these findings suggest that further studies are warranted that investigate target-site mutations in the GABA-gated chloride channel.

Finding the A302S mutation in bed bugs suggests that insecticide exposure has occurred and may be continuing today. Cyclodienes were common insecticides used in the indoor environment prior to being discontinued in the late 1990s (Whitehead 1962, Kristensen et al. 2005), and fipronil is still commonly used in anti-flea medication in companion animals. As co-sleeping with dogs or cats is common in the United States (estimates range from 48% to 56% of households with pets co-sleep [Krahn et al. 2015, Chin et al. 2024]), there is potential for fipronil transfer to bedding (Dyk et al. 2012, Cochran et al. 2015).

Overall, these findings expand our understanding of the mechanisms that confer insecticide resistance in bed bugs and document the presence of a target-site mutation previously linked to cyclodiene and phenylpyrazole resistance in other insect pests. Further research aimed at determining the extent to which the A302S mutation confers resistance in bed bugs in addition to a more comprehensive global assessment of mutation frequency and distribution is warranted.

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Author contributions

Camille Block (Conceptualization [equal], Data curation [lead], Formal analysis [lead], Investigation [lead], Methodology [equal], Writing—original draft [equal], Writing—review & editing [equal]), Lindsay Miles (Data curation [supporting], Formal analysis [supporting], Investigation [supporting], Methodology [supporting], Writing—review & editing [supporting]), Cari Lewis (Data curation [supporting], Writing—review & editing [supporting]), Edward Vargo (Data curation [supporting], Funding acquisition [equal], Investigation [supporting], Methodology [supporting], Writing—review & editing [supporting]), Coby Schal (Data curation [supporting], Funding acquisition [equal], Investigation [supporting], Methodology [supporting], Writing—review & editing [supporting]).

[supporting]), and Warren Booth (Conceptualization [lead], Data curation [equal], Formal analysis [supporting], Funding acquisition [equal], Investigation [supporting], Methodology [supporting], Project administration [lead], Resources [lead], Software [lead], Supervision [lead], Validation [lead], Visualization [supporting], Writing—original draft [supporting], Writing—review & editing [equal])

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Conflicts of interest. The authors declare no conflicts of interest.

Supplementary material

Supplementary material is available at *Journal of Medical Entomology* online.

Data Availability

Data supporting the results can be found in the [Online Supplemental Material](#).

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