Proceedings of the Eighth International Conference on Urban Pests
Gabi Müller, Reiner Pospischil and William H Robinson (editors) 2014
Printed by OOK-Press Kft., H-8200 Veszprém, Papái ut 37/a, Hungary

STUDIES ON PYRETHROID RESISTANCE IN CIMEX LECTULARIUS (HEMIPTERA: CIMICIDAE), IN BERLIN, GERMANY

[1]ARLETTE VANDER PAN, [1]CAROLA KUHN, [1]ERIK SCHMOLZ, [1]JUTTA KLASEN, [2]JÜRGEN KRÜCKEN, AND [2]GEORG VON SAMSON-HIMMELSTJERNA

[1]Federal Environment Agency, Bötticher Str. 2, Haus 23, 14195 Berlin, Germany
[2]Institute for Parasitology and Tropical Veterinary Medicine, Free University Berlin, Robert-von-Ostertag-Str. 7-13, 14163 Berlin, Germany

Abstract Pyrethroid-resistance in bed bugs, *Cimex lectularius,* has been described in many countries, but up to now, for Germany no data are available. However, pest control companies increasingly report difficulties in controlling bed bugs with pyrethroids. In the present study four bed bug strains were collected from infested apartments in Berlin and reared in the laboratory without insecticide selection pressure. In this work bed bug colonies are referred to as strains, which denotes their origin but individuals within a strain and not genetically uniform. A filter contact bioassay was developed and susceptibility of the collected bed bug strains against deltamethrin was determined in comparison to a pyrethroid susceptible laboratory strain. Resistance ratios, calculated from LD_{50}-values were between R_r 3.8 and R_r 5.1. Molecular studies regarding two mutations V419L and L925I in the voltage gated sodium channel α-subunit gene, which have been reported to be involved in knockdown resistance (*kdr*) in bed bugs collected from the USA, were also performed. Pyrosequencing of genomic DNA fragments showed the presence of mutation L925I in each of the four studied field populations with allele frequencies between 30% and 59%, while it was not detectable in the laboratory strain. Furthermore, none of the tested strains had the substitution V419L. The results demonstrate that decreased pyrethroid susceptibility of bed bugs is present in Germany but resistance levels are considerably lower than reported from the USA and Australia.
Key words Pest control, contact bioassay, Pyrosequencing, *kdr*-mutation.

INTRODUCTION

Over the last ten years there has been an upturn in reports on bed bug infestations in hotels, public buildings and private houses worldwide. International travel and migration, the trade of second-hand articles, regulatory restriction of chlorinated hydrocarbon-, organophosphate- and carbamate- insecticides and the evolution of insecticide resistance against all of them are considered to be responsible for the expansion of the bed bug *Cimex lectularius* (Davies et al., 2012; Doggett et al., 2012). Due to their low mammalian toxicity and a rapid effectiveness (knock down effect), pyrethroids are mainly used for bed bug control. However, the presence of pyrethroid-resistant bed bugs has frequently been reported in recent years (Moore and Miller, 2006; Romero et al., 2007; Boase, 2008; Yoon et al., 2008; Lilly et al., 2009; Zhu et al., 2010; Suwannayod et al., 2010; Tawatsin et al., 2011). German pest control companies have also observed increasing difficulties in controlling bed bugs with pyrethroids, but for Germany no published data are available yet. Knockdown resistance (*kdr*) to pyrethroids has been shown to be associated with the presence of two point mutations (V419L or L925I) in the voltage gated sodium

channel α-subunit gene (Yoon et al., 2008; Zhu et al., 2010; reviewed by Davies et al., 2012). In the present study, susceptibility to deltamethrin in four field strains of *C. lectularius* was determined in a filter contact bioassay. Furthermore, pyrosequencing of genomic DNA fragments of single and pooled bed bug samples was performed in order to analyze the presence of the two polymorphisms (V419L, L925I) and to evaluate the genotypes by which they appear.

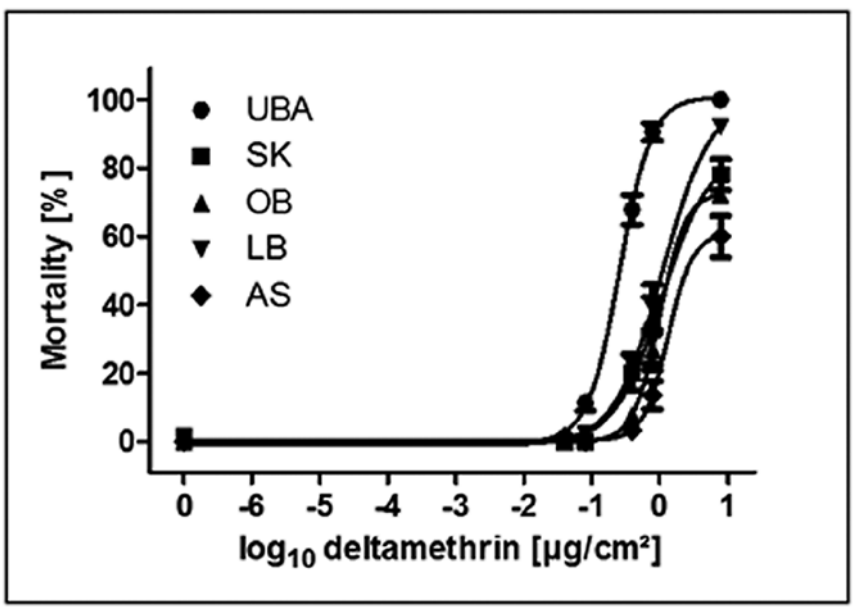

Figure 1. Dose response curves for effects of deltamethrin on mortality (%) of five Cimex lectularis strains in a filter contact bioassay. Male bed bugs were exposed to the active substance for 24 h. UBA is the susceptible reference strain, strains SK, OB, LB and AS were recently collected and isolated in Berlin and successfully adapted to laboratory maintenance.

MATERIALS AND METHODS

Bed bug populations. In this work bed bug colonies are referred to as strains, which denotes their origin but individuals within a strain and not genetically uniform. Bed bug field strains were collected from 20 infested apartments in Berlin. Of these 20, twelve collected samples consisted of less than 30 bed bugs, which was insufficient to establish a laboratory strain. From the eight remaining strains, laboratory rearing of four field strains was successful up to now (SK, OB, LS and AS strain). Three strains failed to breed, and one strain has not been included in our study due to technical reasons. Rearing was performed without insecticide selection pressure. Sufficient numbers of bed bugs were obtained about one year after initial introduction to the laboratory. The insecticide-susceptible laboratory *C. lectularius* strain of the Federal Environment Agency (UBA) has been kept since 1947, with several refreshments of the genetic pool. All strains were held in petri dishes with filter paper (Whatman® round filter, 70 mm Ø, 87 g/m², thickness 180 µm) kept in an incubator (24 h darkness; 25 ± 3°C and 45 ± 10% humidity) and were fed weekly on rabbits.

Filter contact bioassay. Male bed bugs of both field and laboratory strains were exposed for 24 h to deltamethrin on filter papers (Whatman® filter, 87 g/m², thickness 180 µm) in 24-well cell culture plates (adapted from Romero et al., 2007). The bed bugs had a total of seven feedings and were tested eight days after their last blood meal. Concentrations of technical grade deltamethrin (99% active ingredient,

Bayer Environmental Science) were generated by dilution in acetone (for analysis, 99.8%, Merck KGaA) and ranged from 0.0005 µg/µl, 0.001 µg/µl, 0.005 µg/µl, 0.01 µg/µl to 0.1 µg/µl. An amount of 50µl was pipetted on each filter paper disc (0.64 cm^2). Controls consisted of acetone treated filter papers only. All filter papers were allowed to dry for 30 minutes, before being placed in the bottoms of 24-well cell culture plates with forceps. Each concentration was tested on a plate with 18 individual bed bugs which were exposed to deltamethrin and six bed bugs as a control. In order to prevent aggregation, only one bed bug was placed into each well. Plates were closed and stored in darkness at room temperature. Testing of each concentration was repeated five times (total number of bed bugs: n = 90 insecticide-exposed bed bugs and n = 30 as controls for each concentration). The UBA strain has always been tested in parallel to the field strains. Mortality was determined after 24 h exposure by gently touching each male bed bug with a forceps. Individuals were categorized as vital by showing a normal movement behavior or as dead when no or only uncoordinated movement was observed and bed bugs in dorsal position were not able to turn back into ventral position (bed bugs did not recover). LD_{50} values were calculated by using logit analysis and resistance ratios (R_r =LD_{50} resistant strain/ LD_{50} susceptible strain) were determined. Statistical differences between the LD_{50} values for each field strain and the susceptible UBA strain were calculated with a sum of square F-Test in Graphpad Prism 5.0 and p-values were adjusted using the Bonferroni correction.

Pyrosequencing of genomic DNA. Genomic DNA of 100 pooled male *C. lectularius* per strain was isolated with the NucleoSpin® 8 Tissue Kit (Macherey-Nagel) and examined for the presence of the two *kdr*-polymorphisms (V419L and L925I) and their allele frequencies. For the study on single bed bugs, DNA from ten males per population was isolated individually with the same method. DNA fragments with the potential point mutations V419L and L925I were amplified by PCR (Phusion Hot Start II High-Fidelity DNA Polymerase, Thermo Scientific or Biozym) using sequence specific primer pairs (V419Lup: 5´- GTGGCACATGTTGTTCTTCATAGT-3´, V419Llo(biotin) 5´-CGCCTTCTTTTGCAGTTCA-3´; L925Ilo: 5´- CCCATCACAGCAAAGATGAAAAT-´3, L925Iup(biotin): 5´-ATTATGGGCAGAACAGTGGGT-3´). For the subsequent quantitative analysis of both genetic DNA modifications, pyrosequencing (PyroMark® Q24 System and Software, Qiagen) was performed as described in the PyroMark® Q24 User Manual (sequencing primer for V419L: 5´-CCTGGGATCATTCTACC-3´and L925I: 5´-ACACAAAAGTTAAATTACCA-3´). For each of the four field strains five technical repeats were analyzed. Differences between the susceptible UBA strain and the field strains were tested for statistical significance with a one way ANOVA and p-values were adjusted with Tukey's multiple comparison post test (Graphpad Prism 5.0).

RESULTS AND DISCUSSION

Susceptibility to Deltamethrin

The differences in deltamethrin LD_{50} values between the UBA strain and the four field strains were found to be statistically highly significant ($p<0.0053$ for all strains; Fig. 1; Table 1). As expected, the LD_{50} value of the insecticide susceptible laboratory strain was low (0.258 µg/cm^2), confirming high susceptibility against deltamethrin. LD_{50} values of the field strain bed bugs were 1.072 µg/cm^2 for the SK strain, 0.989 µg/cm^2 for the OB strain, 1.095 µg/cm^2 for the LB strain and 1.319 µg/cm^2 for the AS strain. However, there was no significant difference in the LD_{50} values between the four field strains. Resistance ratios of the field populations relative to the UBA control strain ranged between 3.8 and 5.1 (Table 1).

Table 1. Comparison of deltamethrin LD50 values (n=90 males) between the susceptible reference strain (UBA) and the four recently to the laboratory adapted strains collected in Berlin (The differences in LD50 values were found to be statistically highly significant with $p<0.0053$ for all strains). Resistance ratios (Rr =LD50 resistant strain/ LD50 susceptible strain) and coefficients of determination (R2) for all dose response curves are given.

Strain	LD_{50} (µg/cm²)	R^2	Rr
UBA	0.258	0.95	-
SK	1.072	0.8844	4.2
OB	0.989	0.9632	3.8
LB	1.095	0.0637	4.2
AS	1.319	0.9192	5.1

It has to be noted that the resistance ratios observed herein are considerably lower than those reported from the USA, amounting to e.g. 5,200 (Adelman et al., 2011) and 12,800 (Romero et al., 2007) or resistant ratios higher than 432,000 in Australia (Lilly et al., 2009). A possible explanation for low R_rs might be the rearing of the field strains without insecticide selection pressure over a period of more than one year, causing loss of mutations causing resistance (Zhu et al., 2013). The latter explanation is supported by the observation that resistance ratios in the first collected field strain SK considerably decreased between initial filter contact bioassays in 2009 (unpublished data) and the re-examining in 2012 as presented in this paper. Another reason could be that in our studies only R_rs of those field populations were determined, which we were able to reproduce in the laboratory. Rearing of three field strains could not be accomplished, possibly due to fitness disadvantages caused by the presence of insecticide-resistance, which has been observed in other insects (Roush and McKenzie, 1987; Brito et al., 2013). This would implicate, that in our studies sample selection of the field populations was not random and R_rs were selectively determined in populations with lower insecticide susceptibility. Up to now, nothing is known about the genetic structure of German bed bug populations. It is also possible that the field strains in our study descended from local reservoirs where the prevalence of pyrethroid-resistance causing alleles is low. However, the differences in R_rs as seen in our study compared to those reported from other countries may also reflect the actual occurrence of R_rs and may not be influenced by methodological biases. If this would be the case, a possible explanation for lower R_rs in Germany would be a result of differing bed bug control methods. Up to now, no comparative data on practical control routines in different countries are available.

Allele Frequencies of the Two Point Mutations V419L and L925I

Pyrosequencing revealed the absence of the two mutations in the susceptible laboratory strain. Only the L925I (CTT→ATT) substitution was identified in the four collected field strains. Pyrosequencing indicated allele frequencies of 30% mutated fragments in the LB strain. The OB strain showed the highest frequency of the mutated allele with 59%. The allele frequencies of the SK and AS strain were intermediate with 44% and 51%. None of the tested strains had the V419L substitution (GTC→CTC), which generally rarely occurs in bed bug populations (Table 2). These results are

supported by several studies from the United States (Zhu et al., 2010; Zhu et al., 2013). Statistically significant differences in allele frequencies were found between the susceptible UBA strain and each of the four field strains ($p<0.0005$ for all strains). In addition, significant differences between SK and OB strain ($P<0.0005$), SK and LB strain ($p<0.005$), OB and LB strain ($p<0.0005$) and LB and AS strain ($p<0.0005$) were observed. These results demonstrate that the point mutation L925I is present in German bed bug populations. However, the finding of Zhu et al. (2010) that even the single mutation L925I could confer significant deltamethrin resistance and thus high resistance ratios could not be confirmed. In contrast, it was found that although the allele frequencies of the four field strains differed significantly between the strains there were no significant differences in their in vitro susceptibility to deltamethrin. None of the published studies provides information about the impact of homo- and heterozygosity of both point mutations on the resistance status of bed bug populations. Results from ongoing tests on single bed bugs showed in each field strain non-mutated as well as heterozygous bed bugs for the allele L925I were present. Additionally, *kdr* homozygous bed bugs were found in the OB, LB and AS strains (Table 2). Therefore, a possible explanation for the low resistance ratios observed in our study could be that although the mutation L925I was detected in each tested field strain, only bed bugs which are homozygous regarding the mutated allele show high tolerance against pyrethroids. This hypothesis is supported by results regarding other *kdr* mutations in insects which indicate that the *kdr* allele is fully recessive resulting in a close positive correlation between high LD_{50} values for pyrethroids and the frequency of *kdr* homozygotes in flies or mosquitoes (Huang et al., 2004; Saavedra-Rodriguez et al., 2007). This may also hold true for the *kdr* alleles in bed bugs and could explain the considerably lower susceptibility to pyrethroids which we found in our study.

Table 2. Frequencies (among n=100 males) of both SNPs (V419L and L925I) and the different genotypes (homozygous susceptible (HS), heterozygous (H) and homozygous mutated (HM)) of the L925I mutation in 10 single male bed bugs of the four field strains and the susceptible UBA strain, respectively. Background signals of approximately 10% for both alleles were often detected in the absence of the mutation.

Strain	Allele frequencies (%)				Genotypes of L925I		
	V419L	SD	L925I	SD	HS	H	HM
UBA	4	2.96	11	2.07	+	-	-
SK	4	2.34	44	4.82	+	+	-
OB	4	2.97	59	6.11	+	+	+
LB	4	3.03	30	4.28	+	+	+
AS	5	3.63	51	6.87	+	+	+

It has been shown that the *kdr* mutations are not the only mechanisms which can cause pyrethroid resistance in bed bugs. An increased expression of genes coding for cuticular proteins and an up-regulation of ABC transporters which are responsible for the translocation of many substrates and xenobiotics causing lower concentration of insecticides at their target site should also be considered to be responsible for pyrethroid resistance in *C. lectularius*. An increased metabolic detoxification

by esterases, glutathione S-transferases, and especially by cytochrome P450s have been described as additional mechanisms causing pyrethroid resistance in bed bugs (Bai et al., 2010; Adelman et al., 2011; Mamidala et al., 2011; Mamidala et al., 2012; Zhu et al., 2013). To determine whether multiple resistance mechanisms are interacting in the bed bug strains collected in Berlin, further analyses of enzyme expression patterns are currently in progress.

REFERENCES CITED

Adelman, Z.N., K.A. Kilcullen, R. Koganemaru, M.A.E. Anderson, T.D. Anderson, and D.M. Miller. 2011. Deep Sequencing of Pyrethroid-Resistant Bed Bugs Reveals Multiple Mechanisms of Resistance within a Single Population. PLoS One 6(10): e26228. doi:10.1371/journal.pone.0026228.

Bai X., P. Mamidala, S.P. Rajarapu, S.C. Jones, and O. Mittapalli. 2011. Transcriptomics of the Bed Bug (*Cimex lectularius*). PLoS One 6(1): e16336. doi: 10.1371/ journal.pone.0016336.

Boase, C. 2008. Bed Bugs (Hemiptera: Cimicidae): An Evidence-Based Analysis of the Current Situation, pp- 7-14. *In*: Proceedings of the Sixth International Conference on Urban Pests, Robinson W.H. and D. Bajomi, Budapest, Hungary.

Brito, L.P., J.G.B. Linss, T.N. Lima-Camara, T.A. Belinato, A.A. Peixoto, J.B.P. Lima, D. Valle, and A.J. Martins. 2013. Assessing the Effects of *Aedes aegypti kdr* Mutations on Pyrethroid Resistance and Its Fitness Cost. PloS One 8(4): e60878. doi: 10.1371/journal.pone.0060878.

Davies, T.G.E., L.M. Field, and M.S. Williamson. 2012. The re-emergence of the bed bug as a nuisance pest: implications of resistance to the pyrethroid insecticides. Medical and Veterinary Entomology 26(3): 241-254.

Doggett, S.L., D.E. Dwyer, P.F. Peñas and R.C. Russel. 2012. Bed bugs: clinical relevance and control options. Clinical Microbiology Reviews 25(1): 164-192.

Huang, J., M. Kristensen, C.L. Qiao and J.B. Jespersen. 2004. Frequency of kdr gene in house fly field populations: correlation of pyrethroid resistance and kdr frequency. Journal of Economic Entomology 97(3): 1036-1041.

Lilly, G.D., S.L. Doggett, M.P. Zalucki, C.J. Orton, and R.C. Russell. 2009. Bed bugs that bite back, confirmation of insecticide resistance in the common bed bug, *Cimex lectularius*. Professional Pest Manager 13: 22–24.

Mamidala, P., S.P. Rajarapu, S.C. Jones, and O. Mittapalli. 2011. Identification and validation of reference genes for quantitative real-time polymerase chain reaction in *Cimex lectularius*. Journal of Medical Entomology 48(4): 947-951.

Mamidala, P., **A.J. Wijeratne**, **S. Wijeratne**, **K. Kornacker**, **B. Sudhamalla**, **L.J. Rivera-Vega**, **A. Hoelmer**, **T. Meulia**, **S. C. Jones, and O. Mittapalli. 2012.** RNA-*Seq* and molecular docking reveal multi-level pesticide resistance in the bed bug. BMC Genomics 13(6): 1-16.

Moore, D.J. and D.M. Miller. 2006. Laboratory Evaluations of Insecticide Product Efficacy for Control of *Cimex lectularius*. Journal of Economic Entomology 99(6): 2080-2086.

Romero, A.M.F., D. Potter, and K.F. Haynes. 2007. Insecticide resistance in the bed bug: a factor in the pest´s sudden resurgence? Journal of Medical Entomology 44(2): 175-178.

Roush, R.T. and J.A. McKenzie. 1987. Ecological genetics of insecticide and acaricide resistance. Annual Review of Entomology 32: 361-380.