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PYRETHROID RESISTANCE IN *CIMEX LECTULARIUS* (HEMIPTERA: CIMICIDAE) IN GERMANY

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Abstract The development of resistance against insecticides, which are primarily used for bed bug (*Cimex lectularius*) control, is considered to one of the main reasons for the worldwide expansion of this pest. This study provides evidence of the occurrence of resistance and the mechanisms associated with a decreased pyrethroid susceptibility of bed bugs collected in Berlin, Germany. In five of 20 bed bug field strains collected from infested apartments in Berlin, susceptibility to deltamethrin was determined in a filter contact bioassay. Maximum detected resistance ratio (Rr) was Rr>20 and considerably lower than reported from other countries. However, pyrethroid resistance was confirmed for the AS strain with Rr>20 in a *simulated use*-test. Pyrethroid resistance has been shown to be associated with the presence of the amino acid sequence exchanges V419L or L925I in voltage-gated sodium channel alpha-subunit gene as the target site of pyrethroids and an increased metabolic detoxification by cytochrome P450 enzymes. Pyrosequencing of genomic DNA fragments was performed in order to analyze the frequency of these polymorphisms in strains and field populations. The L925I exchange was present in 15 of 18 tested strains and populations with allele frequencies of up to 100%. Additionally, in one strain both males and females had the exchange V419L with allele frequencies up to 96%. Furthermore, each strain showed non-mutated, heterozygous and homozygous mutated bed bugs for the substitution L925I. Relative mRNA-expression levels of the four CYP-genes *cyp397a1*, *cyp398a1*, *cyp4cm1* and *cyp6dn1* were determined with reverse-transcription quantitative PCR (RT-qPCR) for seven bed bug strains. In four of seven strains a higher relative mRNA-expression level of *cyp397a1* was detected. One strain showed higher mRNA-expression levels of *cyp397a1* and *cyp398a1*.
Key words *kdr*-mutation, metabolic resistance, contact bioassay, *simulated use*-test

INTRODUCTION

Increased opportunities for dissemination and development of pyrethroid resistance are considered to be responsible for the ongoing worldwide expansion of the bed bug *Cimex lectularius* (Davies et al., 2012; Doggett et al., 2012). In recent years, German pest control companies have increasingly observed difficulties in controlling bed bugs using only pyrethroids (personal communications). For Germany, preliminary data about the occurrence of pyrethroid resistant bed bugs were presented at the ICUP 2014 (Vander Pan et al., 2014). Decreased susceptibility in bed bugs has been shown to be associated with the mutations V419L or L925I in the voltage-gated sodium channel α-subunit gene (Yoon et al., 2008; Zhu et al., 2010). An increased metabolic detoxification by cytochrome P450s (CYP) has been described as an additional mechanism causing pyrethroid resistance in bed bugs (Mamidala et al., 2011; Mamidala et al., 2012; Adelman et al., 2011; Bai et al., 2011; Zhu et al., 2013). The objective of the present study was to determine the presence, degree and mechanisms of pyrethroid resistance in bed bug field strains in Berlin.

MATERIALS AND METHODS

Bed Bug Strains

In the period 2009-2013, bed bugs were collected from 20 infested locations in Berlin. Of these 20, rearing of five bed bug field strains (SK, AS, OB, HO and LB) in the laboratory was successful. Sufficient numbers of bed bugs were obtained about one year after initial introduction into the laboratory. As a reference for the laboratory studies, the insecticide-susceptible bed bug strain of the German Environment Agency (UBA) was used, which has been kept since 1947, with several refreshments of the genetic pool. Rearing of all strains was performed as described in Vander Pan et al. (2014). Five field strains failed to breed under laboratory conditions and the remaining bed bug samples consisted of less than 20 individuals, which was insufficient for laboratory rearing in a reasonable time period. Thus, samples were frozen for further molecular analysis.

Filter Contact Bioassay

The 24-well filter contact bioassay was conducted as described by Vander Pan et al. (2014) and susceptibility to deltamethrin in five field strains (SK, AS, OB, HO and LB) was determined. EC_{50} values were calculated by using logit analysis and resistance ratios (Rr) were determined. Statistical differences between the EC_{50} values of the field strains and the susceptible UBA strain were calculated with a sum of square F-Test in Graphpad Prism 6.0 and p-values were adjusted using the Bonferroni-Holm correction.

Simulated Use-Test

Actual presence of pyrethroid resistance in the AS strain, which showed the highest Rr in the filter contact bioassay, was tested under conditions of practice (choice trial system), using a pyrethroid insecticide primarily used for bed bug control. The susceptible UBA strain was used as reference strain. In the *simulated use*-test, a total of 100 bed bugs (equal sex ratio) in a harborage were attracted by CO_2 and heat in order to mimic a host. Between the CO_2 and heat source, an insecticide treated wallpaper was placed (freshly sprayed, aged for one and two weeks) over which the bed bugs were lured. The time period bed bugs spent on the treated surfaces in the test system did thus vary and corresponded to their natural seeking behavior. As control, 100 bed bugs of the AS and UBA strain in an equal sex ratio were placed in petri dishes in direct proximity to the trial system. Only the bed bugs which crossed the treated surface were included in effectiveness evaluation, animals that stayed in the harborage were neglected. Numbers of lethally affected bed bugs were determined after 24 h and afterwards daily inspected by forceps stimulation of the bed bugs for a period of seven days. Statistical differences in lethal effect between the susceptible UBA strain and the AS field strain were calculated using mid P exact tests (α=0.05) with the free online statistic software OpenEpi (version 3.03). The resulting mid P values were adjusted using Bonferroni-Holm correction.

Pyrosequencing Of Genomic DNA

Pyrosequencing of genomic DNA fragments of individual (10 field strains and the UBA strain; 10 male and female bed bugs each) and pooled (18 field strains and the UBA strain; 100 male and female bed bugs each) bed bug samples was performed in order to analyze the presence of the two polymorphisms (V419L, L925I) and to evaluate the genotypes. Pyrosequencing, corresponding assays and statistics were conducted as described by Vander Pan et al. (2014).

Reverse-Transcription Quantitative PCR

Relative mRNA-expression levels of the CYP genes *cyp397a1*, *cyp398a1*, *cyp4cm1* and *cyp6dn1* were determined using RTqPCR for seven bed bug field strains and the susceptible UBA strain (10 individual male bed bugs each, two technical repeats) using *rps16*, *rpl8* und *rpl11* as reference genes (Zhu et al., 2012). Sequence specific primer pairs used for RT-qPCR were designed as described by Zhu et al. (2012) for the three reference genes and by Adelman et al. (2011) for the four CYP genes. At the end of every extension phase, the amount of double-stranded PCR product was measured by the increase in fluorescence and the quantification cycle (Cq) was determined. Relative quantitative analysis of amplification data was done using CFX Manager Software. Statistical differences (α=0.05) between the resulting mRNA expression levels of the four CYP genes from the seven strains and those from the susceptible UBA strain were calculated with a Kruskal-Wallis test and Dunn´s post hoc test.

RESULTS AND DISCUSSION

Differences in EC_{50} values between the UBA strain and the five field strains in the filter contact bioassay were found to be statistically highly significant ($p<0.0005$ for all strains). Highest Rr was Rr>20. Furthermore, pyrethroid resistance in the AS strain (Rr>20) was confirmed in the *simulated use*-test, as up to 50% of the bed bugs crossing the sprayed surface survived the pyrethroid insecticide treatment. Differences in the number of lethally affected UBA and AS strain bed bugs were found to be highly statistically significant ($p=0.0000021$), regardless of the ageing stage of the insecticide.

Pyrosequencing of pooled bed bugs showed the presence of mutation L925I in 15 of 18 examined field strains with allele frequencies up to 100%. In one of these strains, the mutation V419L was additionally detected in both males and females with allele frequencies up to 96%. Pyrosequencing of single bed bugs showed non-mutated, heterozygous as well as homozygous genotypes for the polymorphism L925I (V419L was not detected in these tests).

In comparison to the UBA strain, an up to 56fold higher relative mRNA-expression level of *cyp397a1* was detected in five field strains. Four of these five strains additionally showed the mutation L925I and the fifth a 4.9fold higher transcript level of *cyp398a1*.

The results of the *simulated use*-test demonstrate that pyrethroid resistance in bed bugs is present in Germany. Moreover, different potential resistance mechanisms like mutations in the voltage-gated sodium channel and an increased metabolic detoxification by cytochrome P450s (Adelman et al., 2011, Zhu et al., 2013) have been proven in bed bugs occurring in Germany. Other xenobiotic metabolising enzymes (Zhu et al., 2013; Fountain et al., 2016) or mechanisms such as cuticle thickening (Lilly et al., 2016) have not been examined in the present study but may also have an influence on the susceptibility of bed bug strains in Germany and should be investigated in the future.

CONCLUSIONS

Although the frequency of resistance-associated alleles is high and can occur in combination with an increased metabolic detoxification, Rr´s in all tested bed bug field strains were considerably lower than reported from other countries (e.g. USA Rr=5,200 (Adelman et al., 2011) and Australia Rr=432,000 (Lilly et al., 2009)). This might be due to the fact that bed bugs were reared without selection pressure in the laboratory. However, the results of the *simulated use*-test indicated that even bed bug populations with comparatively low Rr´s can survive a treatment with pyrethroids. This is an obvious explanation for recent reports of German pest control companies about increasing problems or failures in bed bug control using pyrethroids. Development and spread of resistant bed bugs can only be prevented with integrated control approaches including insecticide-free methods (e.g. heat treatment).

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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.

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. Med. Vet. Entomol. 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. Clin. Microbiol. Rev. 25(1): 164-192.

Fountain, T., M. Ravinet, R. Naylor, K. Reinhardt, and R.K. Butlin. 2016. A Linkage Map and QTL Analysis for Pyrethroid Resistance in the Bed Bug *Cimex lectularius*. G3: Genes|Genomes|Genetics 6(12):4059-4066. doi:10.1534/g3.116.033092.

Lilly, 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.

Lilly, D.G., S.L. Latham, C.E. Webb, and S.L. Doggett. 2016. Cuticle Thickening in a Pyrethroid-Resistant Strain of the Common Bed Bug, *Cimex lectularius* L. (Hemiptera: Cimicidae). Vontas J, ed. PLoS ONE. 11(4):e0153302. doi:10.1371/journal.pone.0153302.

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*. J. Med. Entomol. 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.

Vander Pan, A., C. Kuhn, E. Schmolz, J. Klasen, J. Krücken, and G. von Samson-Himmelstjerna. 2014. Studies on Pyrethroid Resistance in Cimex lectularius in Berlin, Germany. Paper presented at the *Sixth International Conference on Urban Pests*, H-8200 Veszprém, Pápai út 37/a, Hungary.

Yoon, K.S., D.H. Kwon, J.P. Strycharz, C.S. Hollingsworth, S.H. Lee, and J.M. Clark. 2008. Biochemical and molecular analysis of deltamethrin resistance in the common bed bug (Hempitera: Cimicidae). J. Med. Entomol. 45(6): 1092-1101.

Zhu, F., J. Wigginton, A. Romero, A. Moore, K. Ferguson, R. Palli, M.F. Potter, K.F. Haynes, and S.R. Palli. 2010. Widespread distribution of knockdown resistance mutations in the bed bug, *Cimex lectularius* (Hemiptera: Cimicidae), populations in the United States. Arch. Insect Biochem. Physiol. 73(4): 245-257.

Zhu, F., S. Sams, T. Moural, K.F. Haynes, M.F. Potter, and S.R. Palli. 2012. RNA Interference of NADPH-Cytochrome P450 Reductase Results in Reduced Insecticide Resistance in the Bed Bug, *Cimex lectularius*. Smagghe G, ed. PLoS ONE. 7(2):e31037. doi:10.1371/journal.pone.0031037.

Zhu, F., H. Gujar, J.R. Gordon, K.F. Haynes, M.F. Potter, and S.R. Palli. 2013. Bed bugs evolved unique adaptive strategy to resist pyrethroid insecticides. Sci. Rep., 3, 1456. doi:10.1038/srep01456.