

Metabolic resistance to deltamethrin is mediated by P450 and esterases in common bed bugs *Cimex lectularius* L. (Heteroptera: Cimicidae)

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RESEARCH ARTICLE

Abstract

The infestations of *Cimex lectularius* L. (Heteroptera: Cimicidae) registered in the last decades have been influenced by several human activities, including international tourism and commerce. Moreover, the development of insecticide resistance and careless pest control strategies contributed to the dispersal of bed bugs. Given the complexity of the topic, distinguishing physiological and molecular mechanisms involved in resistance can help design proper control tools and limit the resistance spread. Here we determined the susceptibility to deltamethrin and imidacloprid in bed bugs collected in Italy. Also, we assessed the role of esterases and P450 monooxygenases by direct enzymatic activity measurement and inhibition by synergism bioassays. Our results showed that the field-collected colony exhibited high resistance ratios to imidacloprid and deltamethrin (757 and >60,000 times, respectively) compared to the susceptible colony. Moreover, resistant bed bugs showed increased activity of esterases and P450 monooxygenases. The synergistic effect of piperonyl butoxide (PBO) suggests the significant contribution of both enzymatic groups as detoxification pathways implicated in pyrethroid-resistant bed bugs. Further investigations are needed to unravel the biochemical and molecular basis involved in the resistant phenotype for developing novel strategies for pest control.

Keywords: insecticide resistance, enzymatic activity, deltamethrin, imidacloprid

1. Introduction

During the past twenty years, the common bed bug *Cimex lectularius* L. (Heteroptera: Cimicidae) has become a notorious urban pest in several countries, and there are many factors involved, such as furniture recycling, international travel, and commercial exchange (Dang *et al.* 2017). Moreover, there is a high contribution of insecticide resistance, accompanied by inefficient monitoring strategies and decreasing use of broad-spectrum insecticides in human dwellings (Alizadeh *et al.* 2020).

Pyrethroids are the most common insecticides used in public health pest management (Wang *et al.* 2015). Recently, the application of neonicotinoids in urban environments

has increased, particularly in formulations combined with pyrethroids (Davies *et al.* 2007). However, many urban and medical insect pests developed resistance to pyrethroids (Zhu *et al.* 2016), and increasing evidence supports the evolution of resistance to neonicotinoids in target species (Bass *et al.* 2015). In pyrethroid-resistant bed bugs, the major contributor is the knockdown resistance mutations (*kdr*) that result in target-site insensitivity (Dang *et al.* 2014, Yoon *et al.* 2008). Also, different detoxification mechanisms are implicated in insecticide resistance, as was evidenced by gene overexpression of glutathione S-transferases, cytochrome P450 monooxygenases, and carboxylesterases (Adelman *et al.* 2011, Lilly *et al.* 2016a, Mamidala *et al.* 2012).

Since insecticide resistance due to multiple mechanisms seems to be the rule rather than the exception, accurately identifying the mechanisms involved in resistant bed bugs could be valuable for designing control practices for these insects. Here, we determined the resistance to two active ingredients with different modes of action, deltamethrin (sodium channel modulator – 3A) and imidacloprid (nicotinic acetylcholine receptor competitive modulator – 4A), in *C. lectularius* adults collected in Italy. Also, P450 monooxygenase and esterase activities and enzymatic inhibition by piperonyl butoxide (PBO) were evaluated to identify the mechanism involved in metabolic resistance.

2. Materials and methods

Insects

We used two different bed bug colonies for toxicity and biochemical assays. The ‘Harlan’ colony (hereafter HH-S) is the susceptible strain that has been maintained in laboratory conditions without insecticide exposure for more than four decades (Feldlaufer *et al.* 2014). The origin of the field-collected colony (IT-C) was specimens sampled in a hotel infestation in Venice (Italy) in 2016. Insects were kept in plastic vials with corduroy fabric inside as refuge and oviposition surfaces and kept at 25±1.5 °C, 40±15% relative humidity (RH), and 12:12 (L:D) h. The feeding procedure consisted of a pigeon-blood source offered weekly and supervised by the CIPEIN Institutional Animal Care and Use Committee (Cáceres *et al.* 2019). Stage V nymphs (n=600) were collected from both colonies and fed *ad libitum* to guarantee their molting. Afterward (7-10 d), freshly molted males and females (1:1) were used for each bioassay.

Chemicals

Deltamethrin (99%) and imidacloprid (98.4%) were provided by Chemotecnica S.A. (Buenos Aires, Argentina) and used for toxicity bioassays. Insecticides were diluted in analytical-grade acetone (Sintorgan SACIF, Buenos Aires, Argentina) and tested in the following concentrations: 0.00005-20 mg/ml and 0.0001-5 mg/ml for deltamethrin and imidacloprid, respectively. PBO (99%) (Chemotecnica S.A.) was diluted in acetone to obtain an effective dose of 3.2 mg/ml that produced a synergistic effect without mortality (Cáceres *et al.* 2019). For biochemical assays, the 7-Ethoxycoumarin (7-EC), 7-Hydroxycoumarin (7-OHC), the Bradford reagent, and standard bovine protein solution (BSA) were purchased at Sigma-Aldrich (Saint Louis, MO, USA). The 7-coumaryl permethrate (7-CP) was previously synthesised in our laboratory (Santo Orihuela *et al.* 2006).

Toxicity assays

Insecticides were topically applied to determine the resistance profile of field-collected bed bugs (Cáceres *et al.* 2019). An aliquot of 0.2 µl of insecticide dilution was applied using a micro-applicator with a 10 µl syringe (Hamilton, Reno, NV, USA) on the abdominal tergum. The final doses tested were 0.02, 0.2, 2, 20, 200, 2,000 ng/insect for imidacloprid and 0.02, 0.2, 2, 20, 200, 2,000, 4,000, and 6,000 ng/insect for deltamethrin. Control insects received acetone only. Then, groups of 10 treated insects were placed in a clean vial and maintained under rearing conditions. Mortality was evaluated after 24 h, by direct observation of insects that died or did not walk. At least three independent replicates for each insecticide dose were performed.

Detoxifying enzyme assays

We analysed two foremost enzymatic families associated with metabolic resistance: pyrethroid-esterase (EST) and cytochrome P450 monooxygenases (P450). Males and females were evaluated (n=100 per strain) in both enzymatic assays. Fluorescence measurements with 390-nm excitation and 440-nm emission filters were determined using a microplate fluorometer (FluoroSkan, Thermo Scientific, Waltham, MA, USA).

The pyrethroid-esterase activity evaluation uses the fluorescent 7-OHC product obtained in the hydrolysis of 7-CP (adapted from Santo Orihuela *et al.* 2006). The procedure consists of bed bugs individually homogenised in 220 µl of 50 mM phosphate buffer (pH 7.2). The reaction started by adding 7-CP (10 µl) to 190 µl of each homogenate placed in a 96-well microplate. Fluorescence measurement was made for 33 min at 3-min intervals. Specific activity was calculated using units of EST activity relative to protein concentration per sample (µmoles/min/mg protein). Also, the protein concentration of bed bug samples was determined using the Bradford method, which considers the protein content proportional to the absorption. In a 96-well microplate, each well consisted of 250 µl of Bradford reagent with an aliquot (10 µl) of the bed bug sample previously used in esterase assays. Also, a calibration curve was performed with four BSA dilutions (0.25, 0.5, 1.0, 1.4 mg/ml) loaded in 5 µl aliquots into the microplate wells along with the reagent. The absorption measurements were made at 595 nm in a BIO-RAD 680 XR microplate reader (Hercules, CA, USA).

The procedure for cytochrome P450 monooxygenase activity consisted of the O-demethylation of 7-EC and the fluorescence of the reaction product (7-OHC) (Santo Orihuela *et al.* 2008). The insect abdomen was cut and placed individually into a 96-well microplate containing 500 µl of 7-EC (4 mM) solved in a phosphate buffer (50 mM) pH 7.2. The reaction stopped by adding 500 µl of ethanol-

glycine buffer (pH 10.4) following incubation at 30 °C for 4h. Microplates were centrifuged (3,500 rpm) before and after the incubation period. Blank wells received the same amount of ethanol-glycine buffer before incubation.

In both enzymatic assessments, the relative fluorescence units (RFU) were adjusted for background hydrolysis and nonspecific fluorescence of the substrate. A conversion of RFU data to 7-OHC picomoles per minute (activity units) was made using a calibration curve with the following dilutions of 7-OHC: 73.13, 365.66, 731.33, 1,462.65 (for EST), and 60.19, 117.50, 172.50, 224.33 (for P450) total picomoles per well.

Synergistic assay

To study the implication of enzymatic mechanisms in the pyrethroid resistance, insects from both colonies were exposed to glass surfaces coated with PBO. Previously, 0.05 mg/m² of PBO was determined as the effective dose that produces a synergistic effect without detrimental survivorship in the susceptible colony (Cáceres *et al.* 2019). The treatment with PBO consisted of glass vials (95 cm²) covered with the PBO solution and dried for 60 min. After that, 14 adults (sex ratio: 1:1) were introduced to the vial and kept for 1 h. Then, deltamethrin was topically applied, and mortality was assessed after 24 h, as was previously described in toxicity bioassays. The final doses of deltamethrin tested were 2, 20, 200, and 2,000 ng per insect. In control treatments, groups of insects were exposed only to PBO and pure acetone. The experiments were replicated four times for each colony.

Statistical analysis

We estimated the lethal dose 50 (LD₅₀) and 95 (LD₉₅) for both colonies and insecticides. The resistance ratio (RR) for the field-collected insects was calculated by the ratio LD₅₀ IT-C / LD₅₀ HH-S. If the 95% confidence interval includes 1, then the LD was not significantly different. Also, to evaluate the PBO synergistic effect, we estimated the synergist ratio by the quotient LD₅₀ without and with PBO

pretreatment. To determine statistical differences produced by PBO the Test of equality was used. The hypothesis of equality shows statistical differences between treatments by rejecting the equality of slopes and intercepts in the dose-mortality regressions for each treatment (Robertson *et al.* 2007). All of these parameters were obtained by Probit analysis using PoloPlus 2.0 software (LeOra Software LLC, Berkeley, CA, USA).

To find differences in enzymatic activities of P450 and EST, we performed a generalised linear model (GLM), setting the function 'Varident' to model the variance for each enzymatic group with colonies as a factor. Significant differences (*P*-value<0.05) were confirmed with a posteriori Fisher's test for least significant differences (LSD). The statistical analyses of enzymatic activities were made with the software Infostat (Di Rienzo *et al.* 2014).

3. Results and discussions

The acute toxicity of imidacloprid and deltamethrin was evaluated in susceptible and field-collected colonies. Table 1 summarises LD₅₀, LD₉₅, and RR data. For the IT-C strain, our results showed that LD₅₀ values for both insecticides were significantly high. In particular, we confirmed deltamethrin resistance in bed bug adults of IT-C, with RR that was 40,000 times higher than that of HH-S. Also, the IT-C colony demonstrated a high resistance level to imidacloprid, which was 757 times more resistant than HH-S (Table 1).

The field-collected colony IT-C was highly resistant to imidacloprid and deltamethrin. Numerous investigations evidenced that pyrethroid resistance is common and widespread in *C. lectularius* and *C. hemipterus* infestations (Dang *et al.* 2017). Otherwise, neonicotinoid-resistant bed bugs were scarcely documented (Cáceres *et al.* 2019, Gordon *et al.* 2014, Romero and Anderson 2016). Romero and Anderson (2016) evaluated the susceptibility to various neonicotinoids active ingredients in bed bugs sampled in the USA. Their findings revealed resistance ratios to imidacloprid ranging from 160 to 440 times in two different

Table 1. Deltamethrin and imidacloprid toxicity on susceptible (HH-S) and field-collected (IT-C) adult *Cimex lectularius*.

Insecticides	Colonies	n ¹	Slope ± SE	LD ₅₀ (CL 95%) ²	LD ₉₅ (CL 95%) ²	χ ² (df)	RR (CL 95%) ³
Deltamethrin	HH-S	150	2.92±0.36	0.15 (0.13-0.18)	0.96 (0.66-1.72)	1.01 (4)	-
	IT-C	120	NA	>6,000	NA	NA	>40,000
Imidacloprid	HH-S	120	1.01±0.15	0.24 (0.11-0.52)	15.4 (4.8-124.08)	5.70 (6)	-
	IT-C	110	0.95±0.15	181.73 (45.7-1,044.5)*	266,658 (19,128-25,640)*	4.06 (7)	757.21 (115.9-1,714)

¹ Number of insects tested. No mortality or less than 10% was observed in the control groups.

² LD₅₀ and LD₉₅ values in ng per insect. Asterisks (*) indicate significant differences for each insecticide LD₅₀ and LD₉₅ values between colonies.

³ RRs (95% CL) for each combination of colony and insecticide were calculated according to Robertson *et al.* (2007).

bed bug colonies compared to the Harland susceptible strain. In addition, our previous research on the insecticide resistance in bed bugs collected in Argentina showed moderate resistance levels to imidacloprid, with RR ranging from 24 to 196 (Cáceres *et al.* 2019). Therefore, the presence of neonicotinoid-resistant bed bugs collected in Italy and those described in the New World suggests resistance to these insecticides spread worldwide. Also, these findings suggest the potential effect of cross-resistance between both active ingredients, which are often included in commercial products and recommended for bed bug control (Gordon *et al.* 2014, Wang *et al.* 2015).

Using PBO as an enzymatic inhibitor is an accurate tool for synergising pyrethroids. Here we found that the application of deltamethrin in the resistant colony pretreated with PBO produced a significant reduction in the LD₅₀ (Hypothesis of equality: $\chi^2=45.84$, d.f.=2, $P<0.05$), which was a reduction of 15.6-times compared to the maximum dose of deltamethrin applied alone (Figure 1). Mutations or overexpression in detoxification enzyme genes is one kind of insecticide resistance mechanism, and diverse metabolic pathways have been previously reported (Bass *et al.* 2015).

Herein, we performed biochemical assays to quantify the activity of two main enzymatic groups (cytochrome P450 monooxygenases and esterases) in susceptible and resistant bed bugs. Our results show that resistant colony IT-C has significantly increased enzyme activities compared to susceptible HH-S. Figure 2 shows the results of enzymatic activity related to detoxification in bed bugs. We found that P450 and EST activities in adult bed bugs of IT-C were significantly increased and ranged from 2.9- and 1.3-fold,

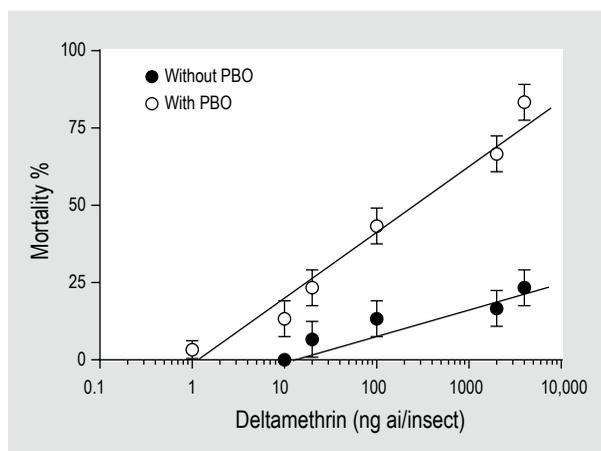


Figure 1. The effect of enzymatic inhibition by piperonyl butoxide (PBO) in *Cimex lectularius* field-collected colony (IT-C). Doses of deltamethrin are represented on a logarithmic scale (ng of deltamethrin – active ingredient – per insect). Probit regression lines indicate the PBO pre-treatment (○) and without PBO pre-treatment (●). Maximum-likelihood ratio test equality of slopes and intercepts of the regression lines, $P>0.05$.

respectively. P450 activity (Figure 2A) recorded for IT-C and HH-S was 161.66 ± 8.92 and 55.88 ± 6.15 picomoles/min./insect (mean \pm SEM), respectively ($T=9.77$, $P\text{-value}<0.0001$). also, EST activity (Figure 2B) was significantly different between bed bug colonies, with 3.75 ± 0.17 and 2.96 ± 0.15 picomoles/min./ μg protein (mean \pm SEM) in IT-C and HH-S, respectively ($T=3.48$, $P\text{-value}=0.0007$).

Similarly, Adelman *et al.* (2011) assessed cytochrome P450 and esterases in pyrethroid-resistant bed bug strain and found that those enzymatic groups increased 30- to

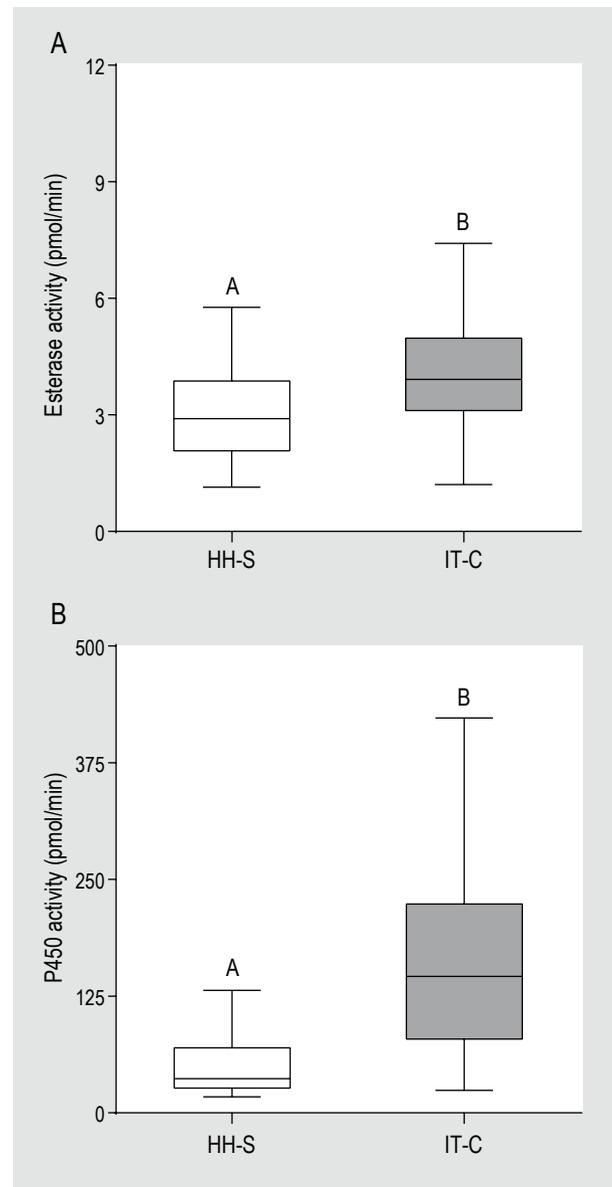


Figure 2. Enzymatic detoxification activity in *Cimex lectularius* field-collected (IT-C) and susceptible colonies (HH-S). (A) Esterase activity. (B) P450 monooxygenase activity. Each bar represents the mean activity \pm SEM in pmol/min. Different letters indicate significant differences ($P>0.05$) among colonies, analysed by generalised linear model and Fisher's LSD test.

40-fold compared to HH-S. Also, Romero and Anderson (2016) studied the role of P450, esterases, and glutathione S-transferases in neonicotinoid-resistant bed bugs. Their results showed that multiple detoxification pathways could be involved in the resistance of *C. lectularius* colonies collected through the USA. Otherwise, Yoon *et al.* (2008) reported the absence of statistical differences in enzymatic activities of susceptible and resistant bed bug strains.

Even when insecticide resistance could be due to enzymatic detoxification, multiple mechanisms could display together in the resistant insects. *Kdr*-mutations were reported in *C. lectularius* from the USA, Australia, and Germany (Dang *et al.* 2014, Vander Pan *et al.* 2014, Yoon *et al.* 2008, Zhu *et al.* 2010). Also, the bed bug cuticle has a significant role in resistance by cuticle thickening (Lilly *et al.* 2016b) and the expression of CPR-cuticle proteins in resistant strains (Benoit *et al.* 2016). Using biochemical and molecular techniques could improve our knowledge of the metabolic pathways involved in the resistance phenomenon. Adelman *et al.* (2011) demonstrated that resistance to pyrethroids in bed bugs was associated with esterase and P450 gene overexpression. Additionally, Zhu *et al.* (2012) concluded that P450s were involved in resistant bed bugs by C1CPR protein gene expression interference. Recently, the genomic study in more than 20-bed bug colonies from the USA performed by Benoit *et al.* (2016) revealed the overexpression of several genes associated with insecticide resistance in the *Cimex* genome.

4. Conclusions

Our results demonstrated resistance to two insecticides (pyrethroid and neonicotinoid) in field-collected bed bugs from Italy. It is noteworthy that IT-C bed bugs were sampled and established in rearing laboratory conditions for three years without any insecticide exposure. Thus, the high resistance ratios displayed here could be associated with the strong selection pressure produced by the former insecticide treatments delivered in the field. Moreover, the resistant colony show increased esterases and P450 monooxygenases activity compared to the susceptible strain. The high levels of enzymatic activities could be related to their involvement in insecticide resistance. However, more bed bug samples must be collected to provide a complete picture of the resistance status and to monitor the resistance in bed bugs from Italy. Also, the molecular target for these insecticides has to be identified to better understand the high resistance levels observed. Further research is needed to achieve new alternatives for bed bug infestation management.

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Conflict of interest

The authors declare no conflict of interest.

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