Role of Enhanced Detoxication in a Deltamethrin-resistant Population of Triatoma infestans (Hemiptera, Reduviidae) from Argentina

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Deltamethrin and other pyrethroids have been extensively used in Argentina since 1980, for the chemical control of Triatoma infestans Klug (Hemiptera: Reduviidae). Recently, resistance to deltamethrin was detected in field populations by the survival of bugs exposed by topical application to the diagnostic dose estimated on the CIPEIN susceptible strain. Results of the current study showed low resistant ratios (RRs) to deltamethrin for the resistant populations (RR ranged from 2.0 for San Luis colony to 7.9 for Salta colony). Biochemical studies were made on the most resistant colony (Salta) and the susceptible strain (CIPEIN), in order to establish the importance of degradative mechanisms as a cause of the detected resistance.

Esterase activity was measured on 3 days old first instars through phenyl-thioacetate and α-naphtyl acetate activities. The results showed a significant difference in no cholinesterase esterase activity from susceptible (7.6 ± 0.7 µM S./i.min.) and Salta resistant colony (9.5 ± 0.8 µM S./i.min.).

Cytochrome P450 mono-oxygenase (P450) activity was measured on individual insects through ethoxyccomarine deethylase (ECOD) activity using a fluorescence micro plate reader. The dependence of ECOD activity on age and body region of the nymphs, and pH and time of incubation were studied in order to optimize the measurement. As a result, comparative studies were performed on abdomens of 2 days old first instars at pH 7.2 and 4 h incubation time. ECOD activity of first nymphs was significantly lower in the susceptible colony (61.3 ± 9.08 pg ECOD/ insect) than in the resistant one (108.1 ± 5.7 pg ECOD/ insect).

These results suggest that degradative esterases (no-cholinesterase) and mono-oxygenases cytochrome P450, play an important role in the resistance to deltamethrin in Salta colony from Argentina.

Key words: deltamethrin resistance - Triatoma infestans - cytochrome P450 mono-oxygenase - Argentina

Despite prolonged and intensive control campaigns against Chagas disease vectors, few studies have been reported on the possible development of insecticide resistance in triatomines (Nelson 1994). The first well documented evidence of field resistance was the resistance to dieldrin detected in Venezuelan Rhodnius prolixus from Trujillo, and later reported for the states of Yaracuy, Tachira, Cojedes, and Portuguesa from Venezuela (González Valdizieso et al. 1971, Cockburn 1972, Nocerino 1976). More recently, resistance to pyrethroid insecticides was reported in Brazilian Triatoma infestans from Rio Grande do Sul and a Venezuelan R. prolixus from Carabobo (Vassena et al. 2000). Resistance Ratios (RR) to the pyrethroids in the Brazilian colony could be associated to the intensive use of deltamethrin (RR 7), and cypermethrin (RR 3.3), and to the lower application of lambda-cyhalothrin (RR 1.7). Otherwise the Venezuelan R. prolixus showed high resistance levels to all pyrethroids evaluated (RR from 11.4 for deltamethrin to 4.5 for lambda-cyhalothrin), and low resistance to dieldrin (RR 3), in spite of the use of cyclodienes for the control of R. prolixus in that state. Toxicological studies with the monoxygenase inhibitor piperonyl butoxide, demonstrated that the resistance to pyrethroids observed in resistant T. infestans and R. prolixus, was caused by an increase in the activity of the biodegradation enzymes.

In Argentina, a monitoring study for the detection of changes in the insecticide susceptibility of T. infestans field populations, was developed since 1997. Monitoring of deltamethrin resistance in collected insects from 13 provinces (170 samples), by the topical application of diagnostic dose (DL99) for the susceptible CIPEIN strain, showed incipient resistance in 4 localities of Argentina: Mendoza, San Luis, Catamarca, and Salta (Picollo 2001). The deltamethrin resistance can be correlated with the National Campaigns for Vector Control made with this insecticide and other pyrethroids in those provinces. The deltamethrin resistance detected for these field samples was not yet correlated with failures in the field chemical control, but demonstrated the genetic potential for resistance in this species.

In this work, deltamethrin resistant levels were assessed in the Argentinean resistant colonies, and the es-
terase and cytochrome P 450 monooxygenase (P450) activity was studied, as a probable cause of resistance in the highest resistant field population (Salta).

**MATERIALS AND METHODS**

*Insects* - Resistant and susceptible *T. infestans* used in this study were obtained from our laboratory culture at 28 ± 1°C, 50% RH and 12:12 h (L:D) photoperiod (WHO 1994). CIPEIN is an susceptible strain maintained in our laboratory since 1975 without exposure to insecticides (Picollo et al. 1976). Deltamethrin resistant colonies were originated from field populations collected by specialized personnel of the National Campaigns for the Control of Chagas Vectors, and maintained in our laboratory. Mendoza colony was collected in Guaymallén, Mendoza in June 1997, San Luis colony in Belgrano, San Luis in July 1997, Catamarca colony in San Martín, Salta in September 1999. Deltamethrin resistance in these field populations was assessed at the time of collection by the appearance of alive nymphs treated with diagnostic dose of the insecticide for the susceptible strain (WHO 1994, Picollo 2001).

From all colonies, 3 days old, first instars, starved since eclosion (mean weight 1.2 ± 0.2 mg) were selected for toxicity test, according the WHO protocol (1994). First and second instars, 3 days old and starved since eclosion, from CIPEIN and Salta colonies, were selected for esterase and P450 activity measurement. First instars from CIPEIN strain were used for studying the dependence of de-ethylase activity with pH, incubation time, age, stadium, and body region.

*Chemicals* - Technical grade deltamethrin (97% [AI], Aventis, Buenos Aires, Argentina). The 5,5′-dithiobis-2-nitrobenzoic (DTNB), 7-ethoxyxoumarin (7-EC), 7-hydroxyxoumarin (umbelliferone), eserine and Fast Blue B were from Sigma (St. Louis, MO), Phenylthioacetate (PTA) and lauryl sodium sulphate from Aldrich (Milwaukee, WI). All solvents were analytical grade and purchased from Merck (Buenos Aires, Argentina).

*Bioassays* - Serial dilutions of deltamethrin in acetone were prepared and applied topically with a 10 µl Hamilton micro syringe provided with a repeating dispenser. Each nymph was treated with 0.2 µl of the solution on the ventral abdomen. Final dose ranged from 0.02 to 6 nanogram/insect.

Each concentration was replicated three times using 10 insects per replicate. Treated insects were placed onto filter paper discs and housed in an environmental chamber (Lab-Line Instruments, Melrose Park, IL), at 28 ± 1°C, 55 ± 5% RH and 12:12 h photoperiod. Mortality was recorded at 24 h after treatment. The criterion for mortality was the inability of the nymphs to walk from the center to the border of a 7 cm filter paper disc (Vassena et al. 2000). Mortality data were corrected using Abbott formula (Abbott 1925).

*Enzyme activity* - Esterases were determined on first instars from susceptible and resistant populations according to Ellman’s colorimetric method (Ellman 1961) adapted to *T. infestans* (Casabé & Zerba 1981).

Phenylthioacetate (PTA) - The filtrated homogenate (100 µl), and 300 µl of phosphate buffer (pH 7.2) containing DTNB (10 mg/100 ml), were added in a semi micro cell (500 µl capacity). Reaction was started by adding 50 µl of 20 mM PTA. Changes in absorbance were measured for 5 min at 412 nm on a Shimadzu UV-160. Measurements were repeated after pre-incubation with 50 µl of 10⁻⁴ M eserine for 15 min at 25°C.

Alpha-naphyl acetate (α-NA) - The color reagent was prepared just before the assay, by mixing 1 part of fast blue solution in phosphate buffer 0.2 M, and 5 parts of sodium dodecyl sulphate (SDS) 5%. Filtered homogenate (100 µl), 1.4 ml of phosphate buffer 0.2 M pH 7.2, and 100 µl of 4 mg/ml α-NA, were added in the reaction tube. Samples were incubated at 30°C for 3 min. At initial time and after 3 min of incubation 400 µl of the mixture homogenate/substrate was added to 80 µl of color reagent. After the 10 min necessary for the stabilization of the color, the absorbance was measured at 600 nm.

Similarly, non cholinesterase esterase activity was measured after previous inhibition with eserine. For this, 1.25 ml of the mixture homogenate and substrate, was incubated with 150 µl eserine for 15 min. After this period color reagent was added as described before.

Cytochrome P450 monooxygenase - Activity was measured using 7-ethoxyxoumarin O-deethylation (ECOD) on intact tissue on micro plate reader (Bouvier et al. 1998). Fluorescence was determined using a micro plate fluorescence reader Packard Fluorocount, with 400 nm excitation and 440 nm emission filters.

First instar abdomens were placed individually into the wells of a 96-well micro plate containing 100 µl of 0.05 M phosphate buffer, and 7-EC 0.04 mM. The reaction was stopped after incubation time by adding 100 µl of glycine buffer (10⁻⁴ M), pH 10.4. In order to bring down the abdomens in the wells, micro plates were centrifuged at 2000 g for 30 s in a refrigerated centrifuge for micro plates ALC 4237R prior to and after the incubation of the enzymatic reaction at 30°C. For each population, similar wells receiving glycine buffer previous to incubation were used for blanks.

Statistical analysis - Dose-mortality data from each *T. infestans* colony were subjected to probit analysis (Litchfield & Wilcoxon 1949). Lethal dose 50% (LD50) values obtained in probit were expressed as nanograms of deltamethrin per insect. After probit analysis, resistance ratios with 95% CL (confidence limit) were calculated for each colony, by comparing results from resistant insects to corresponding results from the susceptible strain as described by Robertson and Preisler (1992).

Biochemical data were analyzed by analysis of variance (ANOVA) to assess significant differences in enzyme activity between susceptible and resistant population (Sokal & Rohlf 1980).

**RESULTS**

The resistance ratio (RR) to deltamethrin for each resistant population relative to the CIPEIN susceptible strain, were established (Table I). All samples showed low resistance levels ranging from 2.0 to 7.9. The highest resistant Salta colony was selected for biochemical studies.

The measured activity of esterases from susceptible (CIPEIN) and resistant (Salta) *T. infestans* colonies, was
summarized in Table II. There was not difference between the total esterase activity from both colonies, using PTA or α-NA as substrates. No significant differences were found between no-cholinesterase esterases (after eserine inhibition) for both colonies (p > 0.05) using α-NA as substrate. Otherwise significant difference was found between esterase no-cholinesterase from susceptible and resistant populations using PTA as substrate (p = 0.0006). Measured activity in resistant nymphs was higher than susceptible ones.

The optimal methodological and biological variables for measuring P450 activity were determined on T. infestans susceptible strain. Methodological variables studied were the pH of the incubation buffer and the reaction time. The results showed that the maximal reading of ECOD activity was obtained using pH 7.2 (Figure) although the differences were no significant (p > 0.05). Concerning incubation times, fluorescence increased with time increase and showed a plateau at an incubation time of 3-5 h (Figure). The biological variable studied was the age of T. infestans first instar. The ECOD activity measured on 1, 2, 3, and 4 days old starved nymphs are shown in Figure. In spite of the lack of statistical significance, the results suggested a trend to maximal activity towards the second day after hatching. Moreover lesser dispersion in ECOD activity records was observed for 1 and 2 days old nymphs.

As a result of the described standardization according to the methodological and biological variables, we used abdomens of 2 days old first instars T. infestans, buffer pH 7.2, and 4 h incubation time. Under these conditions, deethylation of 7-ethoxycoumarin appeared significantly higher in the resistant colony than in the susceptible one (Table III). The enzyme activity ratio (R/S) between R and S colonies was 1.9. In spite of the significant difference of the higher ECOD activity of the Salta strain (p = 0.03) we attempted to confirm in another instars the difference in ECOD activity of T. infestans resistant and susceptible first instar. For that purpose we applied the fluorometric technique to measure ECOD activity, to 2 days old second instar of the resistant and susceptible populations. Results are shown in Table III. The higher ECOD activity obtained in the resistant colony was 2.000 (1.275 – 3.138) and 7.891 (4.999 – 12.455) for susceptible and resistant populations, respectively.

### Table I

<table>
<thead>
<tr>
<th>Population</th>
<th>n</th>
<th>Slope ± SE</th>
<th>LD₅₀ (ng/i) (95% CL)</th>
<th>RR (95% CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIPEIN</td>
<td>145</td>
<td>1.76 ± 0.25</td>
<td>0.099 (0.072 – 0.137)</td>
<td>—</td>
</tr>
<tr>
<td>Catamarca</td>
<td>150</td>
<td>1.42 ± 0.23</td>
<td>0.266 (0.181 – 0.385)</td>
<td>2.679 (1.660 – 4.324)</td>
</tr>
<tr>
<td>Mendoza</td>
<td>160</td>
<td>1.97 ± 0.25</td>
<td>0.375 (0.180 – 0.773)</td>
<td>3.771 (2.462 – 5.779)</td>
</tr>
<tr>
<td>San Luis</td>
<td>145</td>
<td>1.95 ± 0.28</td>
<td>0.199 (0.141 – 0.269)</td>
<td>2.000 (1.275 – 3.138)</td>
</tr>
<tr>
<td>Salta</td>
<td>149</td>
<td>1.69 ± 0.25</td>
<td>0.784 (0.553 – 1.077)</td>
<td>7.891 (4.999 – 12.455)</td>
</tr>
</tbody>
</table>

Topical application of 0.2 µl acetone solution to dorsal abdomen of first instar (aged 3 days). LD₅₀ value ng ai/nymph; RR ± 95% CI calculated by method of Robertson and Preisler (1992); CIPEIN: susceptible laboratory strain

### Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (µM S/insect. min)</th>
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<tbody>
<tr>
<td>Phenylthioacetate (PTA)</td>
<td>CIPEIN</td>
</tr>
<tr>
<td>PTA + eserine</td>
<td></td>
</tr>
<tr>
<td>α-naphthylacetate (α-NA)</td>
<td></td>
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<tr>
<td>α-NA + eserine</td>
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</tbody>
</table>

Substrate + eserine: esterase (no-cholinesterase) activity. Values followed by the same letter were not statistically different according to ANOVA.
activity was also observed in second instar of the Salta resistant population (p = 0.006).

**DISCUSSION**

Since 1960’s, the chemical control of Chagas disease vectors was based on insecticides (Zerba 1999). The continuous use of insecticides produced the development of resistance in *R. prolixus* field populations from Venezuela (Nocerino 1976, Vassena et al. 2000) and resistance to pyrethroids in *T. infestans* from Brazil (Vassena et al. 2000). An extensive monitoring of resistance was made in Venezuela (Molina & Soto Vivas 2001), Colombia (Sandoval 2001), and Argentina (Picollo 2001). Resistance to deltamethrin was assessed in field populations from 4 localities of Argentina, by the survival of bugs exposed to the diagnostic dose (LD99) of the susceptible strain.

The Sa colony observed in first and second instars could be a contributive cause of the resistance phenomena.

For biochemical studies we found that miniaturized and automated assays of P450 activity, offer some major advantages over conventional ones. It is simpler and more rapid because no substrate-product organic separation step is necessary before measuring. It is also much more performing since on microsomal preparations the ECOD assay sensitivity is usually many times lower (de Sousa et al. 1995). This great sensitivity allows the quantification of ECOD activities on whole individual insect abdomens directly placed in a micro titer plate well, without any prior preparation of microsomal membranes.

**ACKNOWLEDGEMENTS**

To Sonia Blanco and Mario Zaidemberg from the Ministry of Health of Argentina for the coordination of the insect sampling.

**REFERENCES**


