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Inheritance of resistance to pyrethroids in *Triatoma infestans*, the main Chagas disease vector in South America

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ABSTRACT

An outbreak of pyrethroid resistance was recently detected in *Triatoma infestans* from northern Argentina. To analyze the inheritance of the resistant phenotype, we carried out experimental crosses between resistant (R) and susceptible (S) strains captured in Argentina during 2005. The R strain was collected from sprayed houses in the north of the province of Salta while the S strain was collected in the province of Chaco. Both strains were bred in the laboratory for reciprocal crosses (F1), intercrosses (F2) and backcrosses (BC). The descendents were tested by a standard insecticide resistance bioassay. Resistance ratios were 1 for S strain, 103.36 for R strain and 18.34 for F1. The regression lines of F1 generations (R × S and S × R) showed no significant differences and were closer to that of the R parents, indicating that inheritance of deltamethrin resistance in *T. infestans* is autosomal and incompletely dominant ($D = 0.20$). Chi-square analysis from responses of intercross and backcross progenies rejected the hypothesis of a single gene being responsible for resistance. The minimum number of independent segregation genes was three, as calculated with Lande's method. The genetic basis here described for the resistant phenotype indicate that, under pyrethroid selective pressure, the resistant genotypes could be easily spread to susceptible insects from resistant individuals, posing a major threat to vectorial control of Chagas disease.

1. Introduction

Triatoma infestans (Hemiptera, Reduviidae, Triatominae) is the principal vector of *Trypanosoma cruzi* – causative agent of Chagas disease – in Argentina, Brazil, Bolivia, Paraguay, Chile, Uruguay, and southern Peru. Pyrethroid insecticides have been widely used to control *T. infestans* (Zerba, 1999), but the appearance of resistance is not a common feature for Chagas disease vectors. Since 1997, this trait has been monitored in many areas of Argentina by measuring the resistance ratio (RR) to determine relative susceptibility of populations. Initially, in five studied provinces, low levels of resistance were sporadically detected (Vassena and Picollo, 2003). In 1999, RRs, averaging 1 in susceptible populations, ranged from 2 in one site of San Luis, to 7.9 in Salvador Mazza, a town in northern Salta on the Argentine-Bolivian border. In spite of these RR values, no operational control problems were reported by the National Control Service for Chagas disease until 2002, when this government service reported low efficiency of deltamethrin and other pyrethroids for the treatment of two rural sites close to Salvador Mazza. At that time, high resistance levels, with RRs ranging from 50.5 to 133.1, were recorded (Picollo et al., 2005). In addition, in some neighboring localities of Bolivia serious problems of vector control are currently being found in connection with high levels of resistance to deltamethrin. In order to understand the inheritance of insecticide resistance, we analyzed its transmission in insect populations collected at sites where the presence or absence of resistance was recorded, as well as in hybrid populations obtained by cross breeding experiments.

2. Materials and methods

2.1. Insects

Three separate populations of *T. infestans*, named "Control", "Susceptible" (S) and "Resistant" (R), were used. The Control colony derived from mixed parental populations collected in rural dwellings during the past 10 years in different localities of the province of Salta, where no insecticide resistance had been reported. Their level of susceptibility to deltamethrin represents

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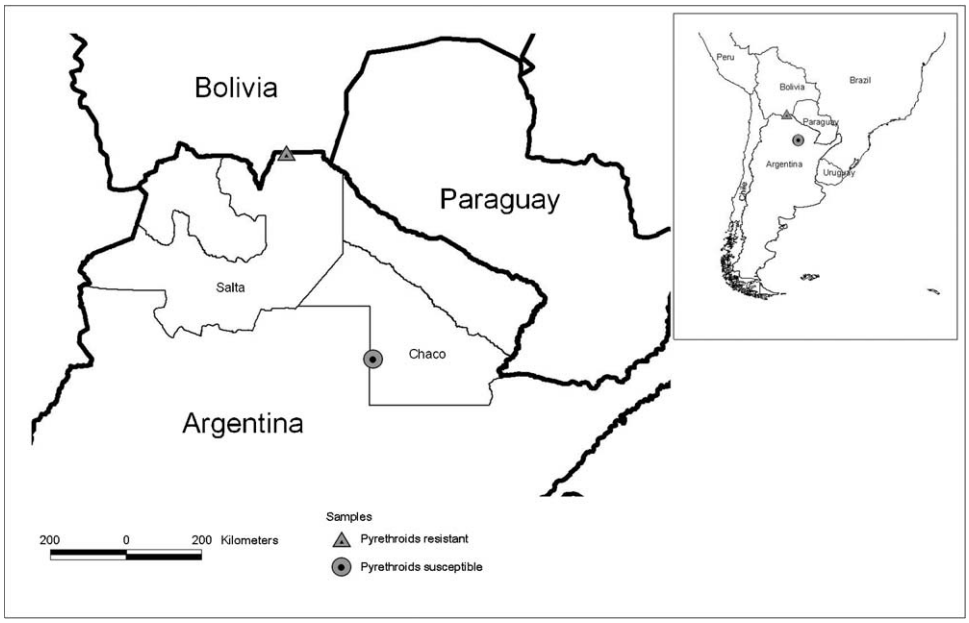

Fig. 1. Geographical locations of *Triatoma infestans* capture sites.

the baseline for relative comparison in this work. The Susceptible (S) population derived from insects collected in houses of the Chacabuco department, province of Chaco (26°55′11″S, 61°37′42″W), an area where no problems in insecticide control have been reported. The "Resistant" (R) population consisted of insects collected in sprayed houses at the neighborhood of the town of Salvador Mazza, province of Salta (22°01′22.9″S, 63°41′24.5″W). The distance between the latter two capture sites is approximately 600 km (Fig. 1). Previous measurements performed on these populations showed complete susceptibility in the former two ($RR \approx 1$) and high levels of resistance in the third ($RR > 100$). All insects were kept in the insect facility of our institute, under controlled temperature, humidity and photoperiod conditions (27 ±2 °C, 60 ± 5% relative humidity, 12:12 h light:-darkness). Insects were fed on mouse blood once a week.

2.2. Experimental matings

Male and female fifth-instar nymphs were maintained individually, according to Brewer et al. (1981), until the imaginal moult. Virgin adult bugs were then introduced into plastic cages (8 cm diameter, 10 cm height) with a cardboard stand inside. Five cages containing one pair of adults were prepared for each of the following groups: R × R, S × S and reciprocal crosses (R females × S males, and S females × R males) to produce F1 hybrids. Because there was no evidence for sex linkage (see below), the F1 progeny from both reciprocal crosses was pooled and reared to adult stage for backcross to parental strains: BC1 (pooled F1 males × R females, and R males × pooled F1 females combined) and BC2 (pooled F1 males × S females, and S males × pooled F1 females combined). Members of the F1 (F1 males × F1 females) progeny were intercrossed to produce the F2 progeny. Both backcross and F2 progeny insects were used to test the hypothesis of monogenic inheritance.

2.3. Bioassays

The levels of resistance to deltamethrin were analyzed in first instar nymphs, descendant from each of the experimental crosses, following the recommended protocol (Picollo et al., 2005; WHO, 1994). Serial dilutions of technical grade deltamethrin (Bayer, Argentina) were used, at concentrations chosen to obtain a convenient mortality range M ($0\% < M < 100\%$). Each dilution was applied to at least 10 nymphs, and each test was done in triplicate. Insecticide concentrations in bioassays ranged from 6×10^{-2} to 200 ng/insect. Nymphs were treated by topical application of 0.2 μL of each dilution on the dorsal abdomen and mortality was checked 24 h later. The control insects received similar treatment with acetone.

2.4. Data analysis

Mortality data from bioassays were corrected for natural control mortality using Abbot's formula (Abbott, 1987). Bioassay data from each *T. infestans* group were pooled for probit analysis (Lichfield and Wilcoxon, 1949) to obtain the LD_{50} (50% lethal dose) and its 95% confidence limit (CL) using a POLO software (LeOra Software Inc., Cary, NC).

To compare lethal doses and to estimate whether the LD_{50} of any group was significantly different from the control group, the ratio of LD_{50} and the 95% CI for the ratio were calculated. If the 95% confidence interval included 1, then the LD_{50} values of the two groups were considered not significantly different (Robertson and Preisler, 1992). The degree of dominance (D) was determined from mortality data according to the method of Stone (1968) using the pooled data of the reciprocal F1 crosses:

$$D = \frac{2X3 - X2 - X1}{X2 - X1} \quad (1)$$

where $X1$: Log_{10} LD_{50} in S, $X2$: Log_{10} LD_{50} in R, and $X3$: Log_{10} LD_{50} in the reciprocal progeny (F1). This equation was proposed for dominance estimation in monogenic characters, but is also used widely for dominance estimation in polygenic characters (Raymond et al., 1987; Preisler et al., 1990; Bouvier et al., 2001). The variance of dominance was calculated using the equation from Preisler et al. (1990):

$$\mathrm{Var}D = \frac{4}{K[\sigma_3^2 + (X3 - X1)^2 \sigma_2^2/K + (X3 - X2)^2 \sigma_1^2/K}$$

where $K = (X2 - X1)$, σ_1^2: character variance in S, σ_2^2: character variance in R, and σ_3^2: character variance in the reciprocal progeny (F1). Dominance level of survival at a given insecticide dose (in our

case, 25 mg deltamethrin active ingredient/m^2 applied under field conditions), referred to as effective dominance (D_{ML}), and was calculated according to the formula cited in Bourguet et al. (2000):

$$D_{ML} = \frac{MF1 - MS}{MR - MS} \quad (2)$$

where MS, MR and MF1 represent the mortality percentages for the susceptible, the resistant and heterozygous F1 progenies respectively. D_{ML} ranges between 0 (survival is recessive) and 1 (survival is complete dominant). The hypothesis of monogenic resistance was tested from mortality data of backcrosses and F2 progeny, as compared to theoretical expectations using the chi-square test (Tabashnik et al., 1992).

The minimum number of independently segregating genes (η) contributing to resistance was estimated according to Lande (1981):

$$\eta = \frac{(X2 - X1)^2}{8 \times \sigma_s^2} \quad (3)$$

where $\sigma_s^2 = \sigma_{BC1}^2 + \sigma_{BC2}^2 - [\sigma_3^2 + 0.5\sigma_1^2 + 0.5\sigma_2^2]$, σ_{BC1}^2 and σ_{BC2}^2 were the character variance in the backcrosses.

3. Results

The LD_{50} of the R population was 103.36 times higher than the LD_{50} of the control colony, while there was no difference between the control colony and S group. The R × R matings maintained the resistant phenotype in the four inbred generations tested (date not shown). Fig. 2 shows that the dose–response lines of both parental populations and F1 were straight, suggesting that either susceptible or resistant strains were homogeneous. The LD_{50} values (see Table 1) and probit regression lines of reciprocal (R × S and S × R) crosses between susceptible and resistant insects were not significantly different ($\chi^2 = 5.1$; d.f. = 2; $p = 0.08$), indicating that resistance to deltamethrin is inherited as an autosomal trait with no maternal effect or sex linkage. For this reason, F1 bioassay data were pooled for further calculations.

The degree of dominance of the R strain was 0.2 ± 0.02, indicating that the resistant character is incompletely dominant.

Determinations of mortality in response to the dose of 25 mg/m^2, used in field operations for vector control [Formula (2)] (MS = 93.3; MR = 0%; MF1 = 36.7), produced a DML = 0.61, indicating a predominance of resistant insects in the F1 heterozygous progeny.

Evidence for monogenic inheritance, resulting from the Chi-square goodness of fit test, indicated that observed mortality differs significantly from expected mortality in the backcross (BC1 and BC2) ($\chi^2_{BC1} = 25.12$ and $\chi^2_{BC2} = 56.94$). We obtained similar results when the test of a monogenic model was performed on the F2 progeny ($\chi^2_{F2} = 194.78$, $p < 0.0001$), suggesting that there is more than one gene controlling the resistance in the R strain (Fig. 3). In addition, the results of the estimation used the Lande method (1981) and showed the resistance was controlled by at least three factors ($\eta E = 3.04$, calculated using data in Table 1).

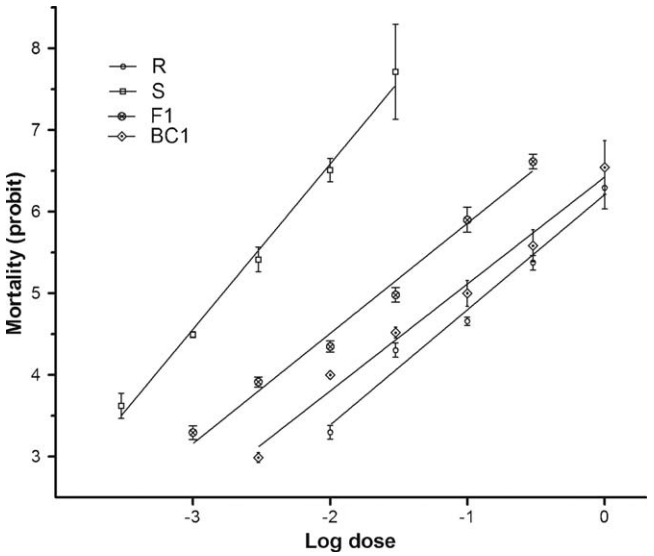

Fig. 2. Dose–response curves of *T. infestans* mortality after deltamethrin treatment. Mortality (in probit units) of susceptible (S), and resistant (R) parentals, heterozygous (F1) and backcross (BC_1) is presented. Data points are means from biological replicates ± SEM.

4. Discussion

In spite of widespread use of pyrethroids for the control of Chagas disease vectors, no insecticide resistance was confirmed for *T. infestans* during the 20th century (Picollo, 2004). However, in 2002, high levels of resistance were detected during spraying operations in two focal sites in the neighborhood of Salvador Mazza, in northern Salta, at the Argentine-Bolivian border. These insects were shown to be resistant not only to deltamethrin but also to the entire set of pyrethroid insecticides used in their control (Picollo et al., 2005). The study presented here was done with insects whose progenitors were captured in those sites. According to Santo Orihuela et al. (2008), RR values of up to 21 may not lead to

Table 1
Bioassay statistics and resistant ratios for deltamethrin in first instars *Triatoma infestans*.

Strain or cross	*N*	LD_{50} ng/insect (95% CL)	Slope ± SE	RR (95% CL)[a]
Control strain	180	0.24 (0.084–0.386)	1.75 ± 0.23	
S (susceptible strain)	658	0.36 (0.29–0.42)	1.90 ± 0.12	1.23 (0.76–2.00)
R (resistance strain)	890	29.44 (24.66–35.47)	1.36 ± 0.08	103.36 (62.71–170.34)*
F1				
$R_{females} \times S_{males}$	860	4.58 (3.80–5.41)	1.36 ± 0.08	15.89 (11.00–22.96)*
$S_{females} \times R_{males}$	900	6.00 (4.84–7.49)	1.36 ± 0.08	21.07 (12.95–34.27)*
F1 (pooled)[b]	1760	5.22 (4.54–6.02)	1.36 ± 0.05	18.34 (10.67–31.52)*
F2[c]	6111	3.81 (3.48–4.17)	1.19 ± 0.03	13.36 (8.39–21.28)*
BC1[d]	1430	16.71 (12.06–23.46)	1.23 ± 0.06	58.73 (36.25–95.15)*
BC2[e]	1256	1.24 (0.95–1.62)	1.49 ± 0.07	3.16 (1.92–5.21)*

[a] Resistance ratios (RR) and 95% confidence limits (CL) relative to control strain, calculated according to Robertson and Preisler (1992).
[b] Combined data from the two reciprocal crosses.
[c] F1 ($F1_{male} \times F1_{female}$) pooled intercrossed.
[d] (Pooled $F1_{males} \times R_{females}$) and ($R_{males} \times$ pooled $F1_{females}$) combined.
[e] (Pooled $F1_{males} \times S_{females}$) and ($S_{males} \times$ pooled $F1_{females}$) combined.
* Significant differences from control by this method.

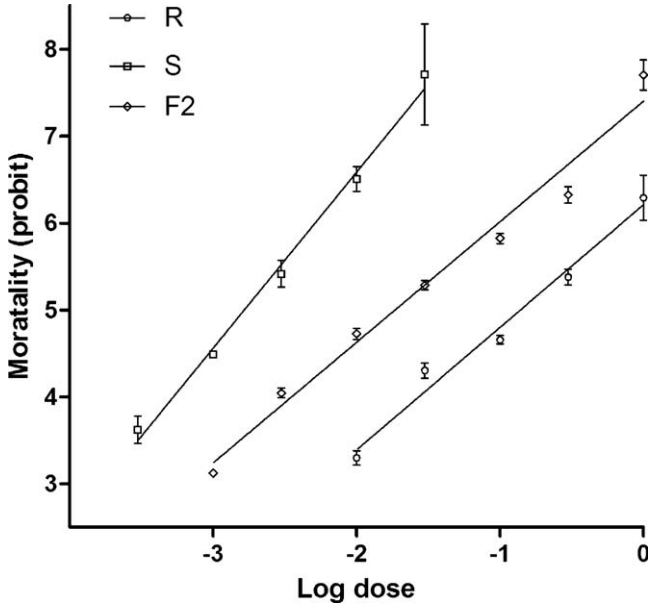

Fig. 3. Dose–response curves of *T. infestans* mortality after deltamethrin treatment. Mortality (in probit units) of resistant (R), susceptible (S) and F2 crosses (F2 obs). Data points are means from biological replicates ± SEM.

noticeable problems of field control, but values of 50 or higher – as shown for these insects – may hamper control campaigns.

The transmission of a pyrethroid-resistant phenotype for two generations derived from R × R crossings, sustains the idea of a genetic basis for this resistance, as opposed to temporary physiological adaptation. Moreover, adaptive changes can be ruled out, since the resistant insects maintained in the laboratory for over a year in the absence of pyrethroids remained resistant. In contrast, S × S crosses kept under similar conditions in the laboratory remained highly susceptible. No significant difference was observed between R × S and S × R progeny in this work, indicating that transmission of the R character is not associated to sex and maternal effects. In a recent paper Germano et al. (2010) also suggests that the resistance to deltamethrin in *T. infestans* is controlled by autosomal and semi-dominant factors.

Resistance to insecticides has often been attributed to insensitivity of target-site proteins or functional boosting of detoxification systems at the genomic level (Li et al., 2007).

The pattern of inheritance of resistance in the F2 and backcross progeny found in our study is consistent with a multigenic system as described for the regulation of detoxification pathways in other insects (Li et al., 2007). On the other hand, a large decrease in slope (increased *i* variance) from F1 to F2 generation is expected if a major locus is involved (Lande, 1981). In this work the slope did not decrease (in R to F1), decreased slightly (R to F2) or increased (R to BC2). The Lande's method (Formula (3)) indicated that resistance to deltamethrin in the R strain appeared to be controlled by at least three factors.

Several studies (González Audino et al., 2004; Picollo et al., 2005; Santo Orihuela et al., 2008) have examined the activity of some detoxifying enzymes in R and S *T. infestans* strains. Whereas esterase functions did not display clear differences, P450 activity, as measured by the 7-ethoxycoumarin-O-deethylation (ECOD) assay, revealed a significant increase in the R strain. However, the modest magnitude of this increase led the authors to postulate that involvement of monooxygenase interacts with other resistance mechanisms, such as insensitivity of nervous membranes (related to the presence of the *kdr* gene), or cuticular permeability. In a recent study (Pedrini et al., 2009), a standardized procedure was developed to measure cuticle thickness at the second tergite of *T. infestans* by scanning electron microscopy. Using the same S and R strains as in this work, this measurement indicated that the tegument of R insects was thicker than that of S insects (32.1 ± 5.9 versus 17.8 ± 5.4, $p < 0.0001$). Moreover, different parameters of weight-adjusted hydrocarbon content of the cuticle showed significant increases in the R strain, suggesting that a selective hydrocarbon transport to the surface might be related to the R phenotype (Pedrini et al., 2009). Interestingly, Cockburn (1976) also found that the organochlorine dieldrin resistance in a strain of *Rhodnius prolixus* was primarily due to changes in cuticular penetration.

Our results show that the regression line of the F1 generation was closer to the R parents, indicating that inheritance of resistance is more dominant than recessive. The results based on the dominance degree (Formula (1)), indicate that the character was incompletely dominant in the R strain ($D = 0.20$). However, the effective dominance (Formula (2)) $D_{ML} = 0.61$, shows a higher prevalence of resistant individuals in F1 at the dose applied in the field. Similar D_{ML} (0.66) have been found for pyrethroid insecticides by Germano et al. (2010) supporting the incomplete dominance of the trait. Assuming that the determinism of resistance to pyrethroids in field populations and in our laboratory R strain is similar the resistant phenotype might rapidly be selected in pyrethroid-sprayed areas.

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