



## Original article

Mutations in *Rhipicephalus microplus* GABA gated chloride channel gene associated with fipronil resistanceE. Castro Janer<sup>a,\*</sup>, G.M. Klafke<sup>b,1</sup>, F. Fontes<sup>a</sup>, M.L. Capurro<sup>c</sup>, T.S.S. Schumaker<sup>c</sup><sup>a</sup> Department of Veterinary Parasitology, School of Veterinary, UDELAR. Av. Lasplacas 1620, CP 11600, Montevideo, Uruguay<sup>b</sup> Instituto de Pesquisas Veterinárias Desidério Finamor, Governo do Estado do Rio Grande do Sul, Estrada do Conde 6000, Eldorado do Sul, RS, Brazil<sup>c</sup> Department of Parasitology, Instituto de Ciências Biomédicas-USP, Av. Prof. Lineu Prestes 1374, Cidade Universitária, CEP. 05508-000, São Paulo, SP, Brazil

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## ABSTRACT

The tropical cattle tick, *Rhipicephalus microplus*, is one of the most damaging parasites that affects cattle in tropical and subtropical regions in the world. Tick resistance to acaricides is dispersed worldwide and a number of associated mutations in target site genes have been described. Phenylpyrazole (e.g. fipronil) and cyclodiene (e.g. lindane, dieldrin) insecticides both have the same mode of action blocking the GABA-gated chloride channel encoded by the *GABA-Cl* gene. A conserved mutation, *rdl* (resistance to dieldrin) is found across a number of arthropods resistant to cyclodienes and phenylpyrazoles. In ticks, the mutation T290 L, was identified in the second transmembrane (TM2) domain of the GABA-gated chloride channel of Australian cattle tick populations that are resistant to dieldrin. Recently, cross-resistance between fipronil and lindane was reported in *R. microplus* populations obtained from Uruguay and Brazil. The objective of the present study was to identify mutations in the *GABA-Cl* gene associated with fipronil resistance. Genomic DNA was obtained from engorged females from fipronil-susceptible and resistant populations sampled from Uruguay and Brazil (n = 166). Initially, it was searched the T290 L mutation described in Australia; however, this mutation was not detected in individuals from resistant populations from either country. The sequencing of a fragment of the *GABA-Cl* gene revealed nucleotide polymorphisms in fipronil- and lindane-resistant ticks in two populations from Uruguay and five from Brazil. Five amino acid substitutions were present in the resistant strains. Two different substitutions were found in an alanine residue (A286S and A286L) that is homolog to *rdl* mutations in fipronil-resistant individuals of other arthropod species. Four other amino acid substitutions (S281T, V317I, T328A and A329S) were present in some resistant strains, always with the mutation A286S. This is the first documentation of mutations in the *GABA-Cl* gene associated with fipronil-resistant in *R. microplus*.

## 1. Introduction

*Rhipicephalus microplus* (Canestrini, 1888) parasitism is one of the major limitations of cattle production in countries with subtropical and tropical climates. Resistance is a problem associated with the chemical control and it has been described against all chemical families of acaricides (e.g. organophosphates, synthetic pyrethroids, amidines, macrocyclic lactones, phenylpyrazoles and phenylureas). The existence of multiple acaricide-resistant tick strains has been frequently reported worldwide (Fernández-Salas et al., 2012; Muyobela et al., 2015; Rodríguez-Hidalgo et al., 2017; Fular et al., 2018) and recently, a tick strain resistant to all available classes of pesticides was reported in Brazil (Reck et al., 2014).

Early detection of acaricide resistance is fundamental to the

establishment of sustainable control strategies, mainly related to the choice of acaricide. Diagnostic methods based on molecular strategies are fast, highly sensitive and specific compared to bioassays (Pruett et al., 2002), as a single individual can be used to identify several mutant alleles. The diagnosis is based on the detection of resistance when there are low frequencies of alleles that confer resistance in a given population (Rosario-Cruz et al., 2005).

Fipronil is a phenylpyrazolic insecticide used to control *R. microplus* infestations in cattle. Its mode of action is attributed to the blocking of the gamma-Aminobutyric acid gated chloride ion-channel (GABA-Cl; Cole et al., 1993; Durham et al., 2001; Bloomquist, 2003). The resistance of *R. microplus* to fipronil was first described in Uruguay and developed relatively quickly (approximately 10 years after its initial marketing) compared to resistance to other acaricides (Castro-Janer

\* Corresponding author.

E-mail address: [elinorcastro59@gmail.com](mailto:elinorcastro59@gmail.com) (E. Castro Janer).<sup>1</sup> These authors contributed equally.

et al., 2009), such as organophosphates and pyrethroids (12–16 years; Cardozo and Franchi, 1994). This rapid development of resistance could be explained by cross-resistance determined by metabolic detoxification, as described in the cattle tick resistance against organophosphates (Miller et al., 1999), or by target site insensitivity due to mutations selected by others chemical groups with the same mode of action, as described in the cross-resistance between pyrethroids and DDT (Nolan et al., 1977; Schnitzerling et al., 1983). The cross-resistance between organochlorine cyclodiene derivatives and phenylpyrazoles insecticides has been demonstrated in several studies with insects (Colliot et al., 1992; Cole et al., 1993, 1995; Bloomquist, 1994; Scott and Wen, 1997; Brooke et al., 2000) and recently, in *R. microplus* field strains from Brazil and Uruguay (Castro-Janer et al., 2015).

The genetic basis of resistance to dieldrin has been described for several insect species as a mutation in the transmembrane domain II of the *GABA-Cl* or *rdl* (resistance to dieldrin) gene (ffrench-Constant et al., 2004; Bass et al., 2004; Du et al., 2005). A mutation in the *GABA-Cl* gene was found at nucleotide positions 868 and 869 (AC to CT) in dieldrin-resistant *Rhipicephalus australis* Fuller, 1899 [former *R. microplus*, see Estrada-Peña et al. (2012)]. The mutation codes for an amino acid change from threonine (T) to leucine (L) at position 290, located four amino acids from the *rdl* loci, 302 of the *Drosophila melanogaster* ortholog (Hope et al., 2010). It can be hypothesized that if there were cross-resistance between fipronil and cyclodienes, the same mutation would confer resistance to both products as demonstrated in some insects. In this study, we describe the existence of mutations in the *GABA-Cl* gene of fipronil- and lindane-resistant strains and field populations of *R. microplus* from Uruguay and Brazil.

## 2. Materials and methods

### 2.1. Ticks

#### 2.1.1. Tick colonies

The Mozo strain was used as a susceptible tick reference strain. It is maintained as a laboratory colony without acaricide treatment in Uruguay since 1973 (Cardozo et al., 1984). RFSan (Castro-Janer et al., 2009), Juarez, and Jaguar (Reck et al., 2014) were used as fipronil-resistant strains. RFSan ticks were obtained from a previous study (Castro-Janer et al., 2009) and were conserved in 70% ethanol at -20 °C since then. The Juarez and Jaguar strains were maintained in cattle at the Instituto de Pesquisas Veterinárias Desidério Finamor, in Eldorado do Sul, Brazil, as described previously (Reck et al., 2009). All reference strains were evaluated by larval immersion tests (LIT) and adult immersion tests (AIT), as described in Castro-Janer et al. (2009). To obtain the DNA, between 10 and 25 whole engorged females were used from the susceptible (Mozo) and fipronil-resistant (also lindane-resistant) references (RFSan, Juarez and Jaguar).

#### 2.1.2. Field populations

Ticks from 14 different populations from Uruguay (identified with the prefix “U”) and Brazil were tested by *in vitro* bioassays (Castro-Janer et al., 2010a, 2010b, 2015). These ticks were collected between 2007 and 2013 and conserved in 70% ethanol at -20 °C. To obtain DNA, between two to 23 engorged females from each population were used according to availability. We used genomic DNA from whole ticks that survived fipronil lethal concentrations of 50% (0.75 ppm), 90% (1.5 ppm) or at the discriminating concentration (5 ppm) to the AIT, determined previously with the Mozo strain by Castro-Janer et al. (2009). Surviving ticks were conserved in 70% ethanol at -20 °C. Two populations resistant to fipronil and lindane (U-DUR, LP2081), three populations resistant to fipronil but susceptible to lindane (U-Am10, SGF1 and DES) and one population susceptible to both fipronil and lindane (U-FRIG) were used. Eight populations were submitted to fipronil bioassays; three of them were susceptible to fipronil (U-BEIS, U-CARAG and U-PIL) and the remaining ones were resistant (U-MOR, U-QUE,

1071 and 1073).

### 2.2. Molecular techniques

#### 2.2.1. DNA extraction

The extraction of DNA was performed using the phenol-chloroform method, according to Sambrook et al. (1989), with some modifications. Ticks were homogenized with a disposable plastic pestle in 1.5 mL plastic tubes containing 600 µL of lysis buffer (10 mM Tris-HCl pH 8; 2mM EDTA pH 8; 0.5% SDS) and 3 µL proteinase K (20 mg/mL, Invitrogen, Carlsbad, CA, USA). Homogenates were submitted to phenol-chloroform purification. The DNA was precipitated in an ice-cold absolute ethanol and sodium acetate solution at -20 °C for 12–18 h. The 3M sodium acetate was added to the ice-cold absolute ethanol at a 1:300 dilution (v/v). The DNA pellets were collected by centrifugation (10,000 x g at 4°C for 15 min) and washed two times with 70% ethanol. The final pellet was suspended in 50 µL of Tris-EDTA, pH 8. The genomic DNA was quantified on a spectrophotometer and diluted to 100 ng/µL for PCR.

#### 2.2.2. PCR-RFLP

In order to detect the 868-9 AC/CT mutation, we used the PCR-RFLP technique proposed by Hope et al. (2010) with different primers. Primers were designed using the Primer3web program (version 4.0.0; Untergasser et al., 2012; RmGABA-1F: 5'CGGATTGATCGTGGTTATTT and RmGABA-1R: 5'GTTGCTCTGCAAGTTTTGCC), using as reference the sequence of the GABA-gated chloride channel gene of *R. australis* (GenBank: GQ398111). These primers amplified a fragment of 290 base pairs that included the restriction site of the Tsp45I enzyme, 5'-GTAC. In the dieldrin-resistant ticks, this site has a modification in the last two nucleotides (from AC to CT) and the restriction enzyme loses its anchoring site, which prevents its action. The PCR contained 1x Platinum Taq buffer, 1.5 mM MgCl<sub>2</sub>, 0.4 µM dNTPs, 0.4 µM of each primer, 0.05 units/µl of polymerase (Platinum<sup>®</sup>Taq Polymerase, Invitrogen) and 100–200 ng of DNA template. The mix was amplified in a Veriti<sup>®</sup> thermocycler (Applied Biosystems) with the following conditions: initial step of denaturation was 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s and the final extension was held at 72 °C for 7 min. The amplified products of the PCR were submitted to digestion with the Tsp45I restriction enzyme (New England Biolabs). Ten microliters of a mixture of 3.65 µL of water, 1 µL of reaction buffer, 0.25 µL of Tsp45I, 0.1 µL of bovine serum albumin and 5 µL of PCR product were incubated for 90 min at 65 °C. The final products were visualized in 2% agarose gels stained with ethidium bromide. The presence of two bands of approximately 145 base pairs indicates the absence of the mutations, whereas the presence of a single band at 290 base pairs indicates the existence of the nucleotide substitutions (Hope et al., 2010).

#### 2.2.3. Sequencing

In order to verify and validate the results of the PCR-RFLP technique, the amplicons obtained with the PCR were sequenced. The PCR products were purified with the AMPURE XT kit (Beckham Coulter Inc.). Treated products were then diluted 1/10 and sequenced in both directions using the same forward and reverse primers from the PCR (RmGABA-1F and RmGABA-1R) in a BigDye<sup>®</sup> Terminator v3.1 Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA).

The sequences and the electropherograms were analysed with CLC Main Workbench software (v. 7.0, CLC Bio, 2010). The areas with low confidence values and coverage were eliminated before assembly. The translated amino acid sequences were aligned using the Clustal W algorithm (Chenna et al., 2003) with the sequences of the GABA gene of susceptible (GenBank:GQ398111) and dieldrin-resistant (GenBank: GQ398112) *R. australis*, and the GABA-gated chloride channel *rdl*-S302 of dieldrin-resistant *D. melanogaster* (GenBank: AHE41090).

**Table 1**  
Resistance background to fipronil and lindane of *Rhipicephalus microplus* reference strains and field populations from Brazil and Uruguay analyzed in the present study.

Strain	Mortality at the DC <sup>a</sup> of fipronil (%)		RR fipronil	Fipronil. Resistant?	RR lindane	Lindane. Resistant?
	Adults	Larvae				
<b>Reference strains</b>						
Mozo	100	100	N.A.	No	N.A.	No
Jaguar	86	30.5	11.8	Yes	6.6	Yes
Juarez	50.4	49.3	6.78	Yes	10.5	Yes
RFSan	66	4.8	5.36	Yes	4.4	Yes
<b>Field strains</b>						
U-BEIS	NT	100	1.1	No	N.T.	No
U-CARAG	NT	100	0.6	No	N.T.	Unknown
U-FRIG	NT	100	0.9	No	0.9	No
U-PIL	NT	100	0.4	No	N.T.	Unknown
U-DUR	NT	20	3.3	Yes	4.7	Yes
U-MOR	NT	87	N.T.	Yes	N.T.	Unknown
U-QUE	NT	6	87.7	Yes	N.T.	Unknown
U-AM10	NT	NT	2.1	Yes	1.2	No
LP2081	NT	74.4	1.7	Yes	5.1	Yes
SGF1	100	74	3.3	Yes	1.9	Yes
DES	98	76.9	3.5	Yes	1.6	Yes
1073	63.2	N.T.	N.T.	Yes	N.T.	Unknown
1071	92.3	95	N.T.	Yes	N.T.	Unknown
1232	NT	100	N.T.	No	N.T.	Unknown

<sup>a</sup> DC: discriminating concentration for adults and larvae = fipronil 5 ppm; RR: resistance ratio; N.A. non-applicable; N.T. not tested.

### 3. Results

#### 3.1. PCR-RFLP

In total, DNA samples of 234 adult female ticks from susceptible (Mozo, U-BEIS, U-CARAG, U-FRIG, and U-PIL) and fipronil-resistant strains (Jaguar, RFSan, U-DUR, and U-MOR) were analysed by PCR-RFLP (Table 1). The Tsp45I restriction enzyme cut the PCR products of all the individuals tested (susceptible and fipronil/lindane-resistant populations), indicating the absence of the 868-9 AC/CT mutations among the ticks analysed.

#### 3.2. Genomic DNA sequencing

Fig. 1 shows the sequences of 290 base pair fragments of DNA corresponding to the transmembrane domain 2 of the GABA-Cl gene of individuals from five fipronil- and lindane-resistant tick strains (Jaguar, Juarez, RFSan, U-DUR, and LP2081), one population susceptible to both acaricides (U-FRIG) and two populations resistant to fipronil and susceptible to lindane (SGF1, DES). It also included a Mozo susceptible reference strain, three fipronil-susceptible (U-BEIS, U-CARAG, U-PIL) and three fipronil-resistant field populations, but they were not tested to lindane (U-MOR, U-QUE, 1071). The sequences obtained were aligned with the complete translated gene sequence of wild-type and dieldrin-resistant *R. australis* and dieldrin-resistant *D. melanogaster*.

Ten fipronil-resistant populations and seven presented individuals with amino acid changes were analysed. We observed 11 synonymous and 10 non-synonymous nucleotide substitutions associated with amino acid changes. Seven amino acid changes were detected: S281T (in the transition region between domain TM1 and TM2), A286S/L (domain TM2), V317I, T328A and A329S (domain TM3). The amino acid change A286S was never detected alone; it was only detected simultaneously with S281T, V317I, T328A and A329S amino acid alterations. The amino acid change A286L was detected alone without these other amino acid alterations. Table 2 shows the summarized amino acid

alterations. The 868-9 AC/CT nucleotide substitutions in the transmembrane domain 2 (TM2) of the GABA-Cl gene, associated with dieldrin-resistant *R. australis* ticks (Hope et al., 2010), were not detected in the 166 sequenced individuals, including fipronil- and lindane-resistant *R. microplus* tick populations from Uruguay and Brazil. Among the samples herein, the amino acid alterations in the TM2 (A286S/L) were observed only in resistant populations (Jaguar, Juarez, RFSan, U-MOR, SGF1, 1071 and 1073). The other alterations (S281 T, V317I, T328 A and A329S; Fig. 1) were always present with the A284S substitution in the TM2.

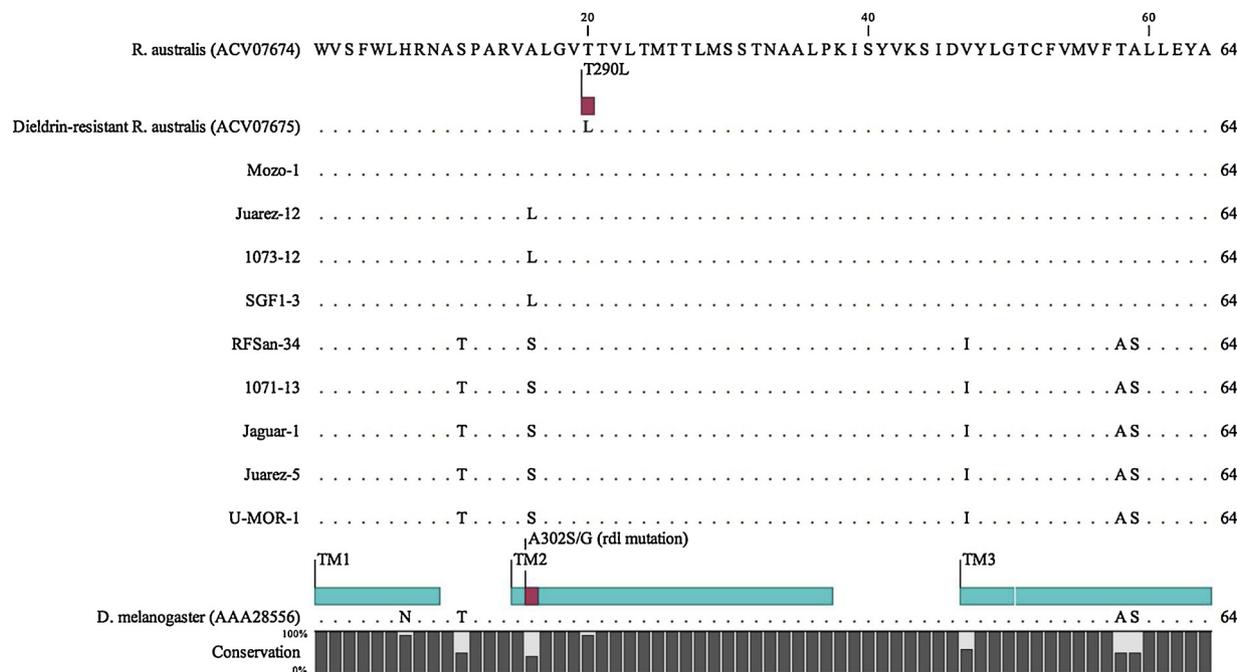
The fragments of DNA sequences obtained for the wild-type and mutated genotypes are available at the GenBank, accession numbers MH801932, MH801933 and MH801934.

### 4. Discussion

The T290 L mutation observed in ticks resistant to dieldrin in Australian cattle tick populations (Hope et al., 2010) was not present in fipronil- and lindane-resistant *R. microplus* ticks from Uruguay and Brazil. Therefore, several amino acid substitutions in the RDL region were registered. The substitutions in the domain TM2 (A286S/L) were observed only in fipronil-resistant populations (Jaguar, Juarez, RFSan, U-MOR, SGF1, 1071 and 1073) and they corresponded to the same alanine residue in the position 302 substituted by a serine or glycine (A302S/G) identified in *D. melanogaster* and other insects resistant to cyclodienes and fipronil (french-Constant et al., 2004).

It should be noted that the mutations associated with lindane- and cyclodiene-resistant planthoppers in Japan (Nakao, 2017) corresponding to A302S/G would produce low levels of cross-resistance to fipronil. The same author found an association of the A302 N with high levels of resistance to fipronil. Therefore, we can hypothesize that the mutations found in the present study (A286S or L) can be associated to cross-resistance pre-selected by cyclodienes, as this mutation was not found in lindane-susceptible populations but in populations resistant to fipronil. In the case of populations resistant to both chemicals, both substitutions were found, A to S and A to L. According to Remnant et al. (2014), phenylpyrazole resistance levels in *D. melanogaster* depended on the altered amino acid in the position 301 of the *Gaba-Cl* gene. These authors demonstrated that the replacement of alanine (301) by glycine resulted in more survivors to phenylpyrazole treatment than the replacement by a serine residue. In the present study, the A286 L substitution was detected as a single mutation in the TM2 and the A286S mutation was in combination with other four mutations. Maybe this fact could be related to different levels of resistance. In *Nilaparvata lugens* (Hemiptera: Delphacidae), a pest of rice crops, a single point mutation (A301S) in the RDL confers low levels of resistance to fipronil; however, a combination with a Q359E mutation was associated with higher levels of resistance (Garrood et al., 2017). Le Goff et al. (2005), working with fipronil-resistant *Drosophila simulans*, detected an A301 G replacement that, when found in combination with a T350M substitution, conferred a higher level of fipronil resistance than when found alone. In *D. melanogaster*, the combination of A301S with Q359E resulted in higher levels of resistance to ethiprole, a phenylpyrazole insecticide (Zhang et al., 2016). The same was observed in *Sogatella furcifera* (Hemiptera: Delphacidae) when presenting A301N and R340Q (between TM3 and TM4; Nakao et al., 2012).

It is possible that when there is an association of mutations, one compensates for the deleterious effects of the other, as was observed in *Nilaparvata lugens* (Zhang et al., 2016). Low levels of fipronil resistance are associated with the A302S substitution. When combined with R300Q (TM2), the resistance level increased. The R300Q mutation is never found alone and it increases the resistance to fipronil. In this case, the A302S mutations would have a compensatory effect over the deleterious effect on the function of the GABA receptor caused by the R300Q mutation. Perhaps the S281 T substitution detected in the present study could be associated with deleterious effects on GABA



**Fig. 1.** Predicted amino acid sequence of the GABA-gated chloride channel from *Rhipicephalus microplus* resistant (Juarez, Jaguar, RFSan, 1073, 1071, and U-MOR) and susceptible (Mozo) to fipronil and lindane, aligned with susceptible *R. australis* (ACV7674), dieldrin-resistant *R. australis* (ACV07675) and *Drosophila melanogaster* (AAA28556) GABA-Cl sequences. Red shows the amino acid residue mutations found in *R. australis* (T290 L) and in *D. melanogaster* (A302S/G) associated to dieldrin resistance. Identical amino acid residues are represented by dots. Transmembrane domains are shown in blue (TM1, TM 2 and TM 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

**Table 2**

Number of engorged females with amino acid changes at the resistance to dieldrin (*rdl*) site in the Gaba-Cl gene of fipronil- and lindane-resistant and susceptible *Rhipicephalus microplus* from Uruguay and Brazil.

Strain	Resistance	N	Genotype at <i>rdl</i> -mutation site		
			Wild-type	A286L	A286S
Mozo	Sus	10	10	0	0
Jaguar	Fip/Lind	10	3	1	6
Juarez	Fip/Lind	10	2	3	5
RFSan	Fip/Lind	16	5	0	11
U-BEIS	Sus	5	5	0	0
U-CARAG	Sus	5	5	0	0
U-FRIG	Sus	2	2	0	0
U-PIL	Sus	4	4	0	0
U-DUR	Fip/Lind	24	24	0	0
U-MOR	Fip	33	22	0	11
U-QUE	Fip	8	8	0	0
U-Am10	Fip	6	6	0	0
LP2081	Fip/Lind	2	2	0	0
SGF1	Fip/Lind	23	22	1	0
DES	Fip/Lind	6	6	0	0
1073	Fip	4	3	1	0
1071	Fip	5	4	0	1
1232	Sus	8	8	0	0

Sus: Susceptible; Fip/Lind: resistant to fipronil and lindane; Fip: resistant to fipronil; N: number of genotyped individuals.

receptor function due to its very low frequency and it was never detected alone in field populations. This mutation does not appear to have the same impact that R300Q has on *N. lugens* because other mutations are present.

The four other mutations (S281T, V317I, T328A and A329S) were not detected in the absence of the A286S. This could suggest that when all of them are present, there would be a tri-dimensional structural modification that would interfere with the binding of the insecticide and produce different levels of resistance. It should be noted that the RFSan population, with a very high percentage of survival at the

discriminating concentration, always presented the four mutations and that the Jaguar and Juarez strains, with a survival rate between 30 and 49%, respectively, presented the two mutation patterns, the A286 L and the A286S, plus the four mutations. The U-MOR population, with a lower survival percentage than these last two populations, presented only the pattern of four mutations with A286S. Perhaps this situation is similar to that described previously by Zhang et al. (2016) for Brown planthopper, but it is not possible to elucidate the synergistic or compensatory effects of the different mutations.

Fipronil insensitivity could be due to multiple mutations in the *Rdl* as demonstrated in *D. melanogaster* by Remnant et al. (2014). The authors identified three fipronil-resistant strains (A301S + T350S, A301S + T360I and A/S301 + M/I360). In the present study, the mutation A286S detected in *R. microplus* could be analogous to 302 in *D. melanogaster* and to 296 in *Anopheles funestus* resistance to fipronil, and the V317I to 327 in both strains. In some fipronil-resistant populations, no mutations in the *rdl* loci were detected. Of the 16 fipronil resistant populations, seven had amino acid alterations. This may be due to the low number of individuals analysed, which may coincide with a low allelic resistance frequency in the population. In the present study, it is difficult to correlate mutations to different levels of resistance and we cannot discard the presence of a different mechanism of resistance, such as metabolic detoxification (Punyawattho et al., 2013; Elzaki et al., 2015) or gene duplication as observed in resistant individuals of *Myzus persicae* and *D. melanogaster* (Anthony et al., 1998; Remnant et al., 2013). Further studies on modelling, gene expression and cloning should be carried out in order to elucidate these hypotheses. Expression of recombinant RDL receptors could help to elucidate the synergistic or compensatory effects of the different point mutations.

### 5. Conclusions

The mutation T290L present in the Gaba-Cl of dieldrin-resistant *R. australis* tick strains from Australia is not present in the tick populations from Brazil and Uruguay analysed in this study. Other mutations in the

transmembrane 2 domain of Gaba-Cl were detected in fipronil- and lindane-resistant ticks. The amino acid change A286S/L could be related to the same alanine residue replaced by serine (A302S) described in insects resistant to cyclodiene and phenylpyrazoles.

### Conflicts of interest

The authors declare no conflicts of interest.

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