Veterinary Parasitology xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

In vitro diagnosis of the first case of amitraz resistance in *Rhipicephalus microplus* in Santo Tomé (Corrientes), Argentina

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ARTICLE INFO

Article history:
Received 24 May 2012
Received in revised form
27 September 2012
Accepted 14 October 2012

Keywords:
Cattle tick
Rhipicephalus microplus
Amitraz
Resistance
Argentina

ABSTRACT

In Argentina, the cattle tick *Rhipicephalus microplus* has already developed resistance to organophospates and synthetic pyrethroids. However, no cases of amitraz resistance have ever been recorded in this country despite its heavy use. A recent failure of amitraz to control ticks in a farm located in Santo Tomé, province of Corrientes, resulted in the collection of samples for acaricide resistance diagnosis. The modified Drummond adult immersion test (AIT) and the larval tarsal test (LTT) were performed separately in Argentina and Switzerland to evaluate efficacy of amitraz and other acaricides. The AIT showed that oviposition in the Santo Tomé field isolate was not inhibited when it was challenged to 250 and 500 ppm amitraz, and 50 ppm deltamethrin. However, oviposition was reduced by 90.6% when this field isolate was challenged to a combination of 400 ppm ethion and 100 ppm cypermethrin. To confirm the results obtained with the AIT, 2 additional tick samples were collected and shipped to Switzerland for resistance diagnosis of amitraz, cypermethrin and flumethrin, using the LTT. With this bioassay, the resistance ratios of the 2 field isolates were 32.5 and 57.0 for amitraz and between 5.9 and 27.2 for the synthetic pyrethroids. Both *in vitro* bioassays confirmed amitraz and synthetic pyrethroid resistance in the Santo Tomé samples. These results account for the first evidence of amitraz resistance in *R. microplus* in Argentina.

1. Introduction

The cattle tick *Rhipicephalus microplus* can be found in the tropical and subtropical central northeastern regions of Argentina, between 22° and 34° south latitudes (Mattos and Signorini, 1989), associated to the biomes of Chaco and Pampa (Estrada-Peña et al., 2006). In Argentina, a variety of acaricides have already been used and as a consequence, *R. microplus* has developed resistance to organophosphates (Pérez Arrieta et al., 1980) and synthetic pyrethroids (Caracostantogolo et al., 1996) in the province of Corrientes. In this province, the most common way to control tick infestations is the application of amitraz by plunge dipping (Guglielmone et al., 2007). Nevertheless, no cases of amitraz resistance have been yet notified in Argentina. Resistance to amitraz has been already described in other

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http://dx.doi.org/10.1016/j.vetpar.2012.10.014

countries such as Australia (Nolan, 1981), South Africa (Strydom and Peter, 1999), Brazil (Li et al., 2004), Colombia (Benavides et al., 2000) and Mexico (Soberanes et al., 2002).

A recent failure of amitraz to control cattle ticks in the field was observed in a farm located in Santo Tomé, province of Corrientes. Following this observation, all the infested animals were treated twice with amitraz, with a 9-day interval between the treatments. These treatments were supervised by local staff from SENASA (The National Animal Health and Agri-food Quality Service of Argentina) who corroborated the lack of control using this acaricide. In addition, samples of ticks were collected and submitted for *in vitro* testing to a governmental laboratory, as this is usually the case when animal health authorities in Argentina want to confirm suspect cases of resistance. The most common bioassays used to diagnose acaricide resistance are the larval packet test (LPT) (Stone and Haydock, 1962), the larval immersion test (LIT) (Shaw, 1966), and the adult immersion test (AIT) (Drummond et al., 1973). More recently, a new bioassay named larval tarsal test (LTT) has been developed by Lovis et al. (2011). This test is less laborious than the LPT, and compared to the AIT, has the advantage of requiring a small number of engorged ticks. However, 6 weeks are required to obtain the results, as with the LPT. Thus, none of these bioassays are perfect and they all combine advantages and disadvantages in terms of simplicity, sensitivity, accuracy, and promptness to obtain the results. In the present study, the AIT and the LTT were used to document for the first time the presence of *R. microplus* populations in Argentina resistant to amitraz.

2. Materials and methods

Two series of experiments were performed. The first series was carried out in Argentina (The National Institute of Agricultural Technology (INTA)) where efficacy of amitraz, deltamethrin, ethion and cypermethrin against the Santo Tomé field isolate was evaluated in two separate AITs. The second series was performed in Switzerland (Novartis Animal Health Research Centre (CRA)) where resistance to amitraz, cypermethrin, and flumethrin was tested with the LTT.

2.1. Ticks

Four samples of ticks were collected between July and November 2010 from a farm located in Santo Tomé, province of Corrientes, where lack of efficacy of treatment with amitraz had been observed. The Muñoz strain (Li et al., 2005) was used as susceptible reference strain for the LTT while the INTA A26 strain was used as susceptible reference strain for the AIT. The Muñoz strain used in this study was provided by the Cattle Fever Tick Research Laboratory (CFTRL) in Edinburg, TX, USA, and reared since 2010 at the CRA, St-Aubin, Switzerland, without acaricide selection. This strain was originally collected from an outbreak in 1999 from Zapata County, TX, USA. The INTA A26 strain was originally collected from a farm in Corrientes in 2009, and maintained since then in the laboratory of parasitology at INTA Castelar. Both susceptible strains are free of *Babesia* and *Anaplasma*.

2.2. Acaricides

Commercial acaricides such as Azadieno Plus® (Merial, amitraz 12.5%), Ruster® (Gleba, deltamethrin 2.5%), and Pöhja mix® (Laboratorio Vetué, ethion 40% and cypermethrin 10%) were diluted with 0.1% Triton-X100 (BDH) and used for the AIT. In contrast, for the LTT, technical grade amitraz (Sigma–Aldrich, active ingredient (AI) 99.4%), cypermethrin (Novartis, AI > 90%), and flumethrin (Sigma–Aldrich, AI 97%) were diluted with dimethyl sulfoxide (DMSO, Fluka) and used for testing.

2.3. Adult immersion test

Adult immersion tests were performed in Argentina as described by Drummond et al. (1973) with some modifications related to the immersion time (2 min instead of 30 s) and the acaricide diluent (0.1% Triton-X100 diluted in distilled water instead of 25% water, 65% xylene, and 10% Triton-X100). Engorged female ticks (EFT) were rinsed in tap water, dried on paper towels, and randomised by size to form as many groups as commercial acaricides to be tested. Groups of ticks were immersed 2 min in the different acaricide solutions or in Triton-X100 (control group). Afterwards, ticks were recovered from the immersion solutions, dried on paper towels, and incubated for 14 days at 27–28 °C and 80–85% relative humidity (RH) for subsequent egg collection once oviposition was completed. Effectiveness of a treatment was determined by the reduction in oviposition in the treated groups compared to oviposition in the control group at day 15 according to the formula shown below.

$$\text{Efficacy}(\%) = \left(\frac{\text{Control egg weight mean} - \text{Treated egg weight mean}}{\text{Control egg weight mean}}\right) \times 100$$

Two AITs were performed. In the first AIT, where 10 EFT were used in each group, the inhibition of oviposition at different concentrations of amitraz was compared between the Santo Tomé field isolate and the susceptible reference strain INTA A26. The second AIT assessed the oviposition performance of the Santo Tomé field isolate immersed in 250 ppm amitraz, 50 ppm deltamethrin, and 400 ppm ethion combined with 100 ppm cypermethrin in comparison to a negative control group. Fourteen EFT were used for each group in this second test.

2.4. Larval tarsal test

The LTT was conducted in Switzerland as described by Lovis et al. (2011) using two samples of the Santo Tomé isolate (ST23 and ST24). Briefly, tick eggs were distributed into the wells of 96-well microtitre plates pre-coated with increasing concentrations of acaricides, and DMSO for the control wells. The plates were then sealed and incubated at 28–29 °C and 70–80% RH to allow egg hatching. Three

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Table 1
Relative susceptibility of cattle ticks from Santo Tomé to amitraz using the adult immersion test.

Amitraz concentration (ppm)	Egg weight (g)*	
	Santo Tomé (n = 10)	INTA A26 (n = 10)
0	0.125[a] ± 0.034^	0.150[a] ± 0.032
250	0.089[a] ± 0.044	0 ± 0
500	0.105[a] ± 0.025	0 ± 0

* Egg weight comparison between the Santo Tomé field isolate and the susceptible laboratory strain (INTA A26) after engorged females were immersed for 2 min in 250 ppm and 500 ppm amitraz. Egg weights are expressed as mean ± standard deviation.
^ Equal superscripts in the same row indicate no statistical differences between egg weights among strains (p = 0.11), whereas equal superscripts in the same column indicate no statistical differences between egg weights in Santo Tomé isolate (p = 0.58).

Table 2
Susceptibility of cattle ticks from Santo Tomé to amitraz and other acaricides using the adult immersion test.

Acaricide concentration (ppm)*	Santo Tomé (n = 14)	
	Egg weight (g)	Acaricide efficacy (%)
0	0.112[a] ± 0.018^	–
250 amitraz	0.110[a] ± 0.017	1.5
50 deltamethrin	0.109[a] ± 0.014	3.0
400 ethion + 100 cypermethrin	0.011[b] ± 0.025	90.6

* Engorged females were immersed 2 min in 250 ppm of amitraz, 50 ppm of deltamethrin, and in the combination of 400 ppm of ethion + 100 ppm of cypermethrin. Egg weights are expressed as mean ± standard deviation.
^ Different superscripts indicate statistical differences between egg weights among groups (p < 0.05).

weeks after the distribution of the eggs, larval mortality was assessed in each well by the absence of motility and general appearance of the larvae. Each test was replicated three times, and resistance was determined by the calculation of resistance ratios (RR) (quotient between the concentration inducing 50% mortality (LC_{50}) of the Santo Tomé field isolate and the LC_{50} of the susceptible Muñoz strain).

2.5. Statistical analyses

Statistical analysis of the AIT data was performed with Statistix 8 (Analytical Software, 2003). A Kruskal–Wallis test was used to compare Santo Tomé egg weight means between the different treatment groups in the first and second AIT. Oviposition in the non-treated Santo Tomé and INTA A26 ticks was compared with a Wilcoxon Rank Sum Test. Data analysis of the LTT results was performed on the R software (version 2.12.0) using the drc package (version 2.0-1), specific for modelling dose–response curves (Ritz and Streibig, 2005). Lethal doses inducing 50% mortality, RR and their 95% confidence intervals (CI) were calculated as previously described by Lovis et al. (2011).

3. Results

3.1. Adult immersion test

Results of the comparison of the egg weights between the Santo Tomé field isolate and the susceptible INTA A26 strain after immersion in increasing concentrations of amitraz are summarised in Table 1. Both the field isolate and the susceptible strain laid eggs in absence of amitraz, and there were no significant differences between them (p = 0.11). Oviposition in the Santo Tomé ticks was not affected by 250 and 500 ppm amitraz whereas it was completely inhibited in the INTA A26 ticks by the two amitraz concentrations. There were no statistical differences (p = 0.58) among egg weights of the Santo Tomé isolate in the first AIT.

In the second AIT (Table 2), where the Santo Tomé field isolate was challenged with several acaricides, 250 ppm amitraz and 50 ppm deltamethrin provided efficacies of 1.5% and 3.0% in preventing oviposition, respectively. In contrast, an efficacy slightly over 90% was obtained when testing the combination product containing organophosphate and synthetic pyrethroid acaricides.

3.2. Larval tarsal test

A full dose–response curve was obtained with the LTT for the 2 Santo Tomé samples. Both samples demonstrated resistance to amitraz with RR of 57.0 (95% CI: 41.9–72.0) and 32.5 (95% CI: 24.1–40.8) (Table 3). Resistance to both cypermethrin and flumethrin was also observed with RR between 5.9 (95% CI: 3.0–8.8) and 27.2 (95% CI: 15.4–39.1).

4. Discussion

We diagnosed amitraz and synthetic pyrethroid resistance in the Santo Tomé field isolate using two different *in vitro* assays: the AIT and the LTT. These two tests diagnose resistance using different parasitic stages. Practical features of the AIT include its ease of use and relatively rapid turn around time to provide a diagnosis of resistance within two weeks, while the LTT requires six weeks before results are available. On the other hand, a drawback of the AIT is the difficulty to obtain sufficient numbers of engorged females to carry out the test. This limitation applies to the number of field ticks and laboratory susceptible ticks required for testing. To overcome this limitation, the AIT was recommended to screen for resistance using a single dose, the discriminating dose (DD) (Food and Agriculture Organization of the United Nations (FAO), 2004), which would perfectly differentiate between susceptible and resistant individuals by killing only the susceptible individuals (ffrench-Constant and Roush, 1990). The DD is determined as $2 \times LC_{99.9}$ or $2 \times LC_{99}$ of a susceptible reference strain to a specific compound (FAO, 2004; Jonsson et al., 2007). However, due to the difficulty to estimate the DD, because of natural biological variation for example (Robertson et al., 2007), it is preferable to test several doses to establish a full dose–response curve and to calculate lethal concentrations (LC_{50}, LC_{90}) and their corresponding resistance ratios whenever possible.

The use of DD has been questioned by Jonsson et al. (2007) when they evaluated the performance of the AIT as an acaricide resistance screening test. In their experiments,

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Table 3
Relative susceptibility of cattle ticks from Santo Tomé to acaricides using the Larval Tarsal Test.

Acaricide	Tick[a]	LC_{50} (95% CI)	RR (95% CI)
Amitraz	Muñoz	1.09 (0.89–1.29)	–
	ST23	62.06 (45.55–78.57)	57.0 (41.9–72.0)
	ST24	35.40 (27.17–43.64)	32.5 (24.1–40.8)
Cypermethrin	Muñoz	0.51 (0.42–0.60)	–
	ST23	6.62 (2.92–10.33)	13.0 (6.6–19.4)
	ST24	13.78 (8.62–18.94)	27.2 (15.4–39.1)
Flumethrin	Muñoz	0.012 (0.01–0.014)	–
	ST23	0.072 (0.036–0.108)	5.9 (3.0–8.8)
	ST24	0.268 (0.186–0.35)	21.8 (14.3–29.2)

[a] Comparison of LC_{50} values and RR for amitraz, cypermethrin and flumethrin between the susceptible reference strain (Muñoz) and the two Santo Tomé isolates (ST23, first collection; ST24, second collection). Concentrations are in mg/m^2.

in Australia and USA, they found impossible to apply any of the DDs suggested by the FAO (2004), especially for amitraz. Similarly, Miller et al. (2007), who evaluated the ability of three larval bioassays ('Soberanes', 'Miller' and 'White' techniques) to determine amitraz susceptibility, showed the difficulty to obtain a satisfactory dose–response relationship to amitraz and to determine a DD that perfectly discriminates between susceptible and resistant individuals in these bioassays. Despite their limitations, the AIT and larval bioassays are still valuable tools to detect resistance in tick isolates.

The intrinsic simplicity of the AIT may paradoxically counteract its utility. As the technique was adapted for the evaluation of different acaricides and was modified by several authors, there is yet no AIT standard protocol. Modifications are usually related to the nature of the acaricide (technical grade or commercial), the immersion time (from 30 s (Drummond et al., 1973) to 30 min (FAO, 2004)), and the agents (Gonçalves et al., 2007) used to dissolve the acaricides. While Oliveira et al. (2000) determined 5 min as the minimal immersion time to establish the LC_{50} for technical amitraz diluted in 40% acetone, Soberanes et al. (2002) was able to detect resistance to amitraz by immersing engorged females in water-diluted commercial amitraz (12.5%) for 1 min. More recently, Kumar et al. (2011) found no significant differences in mortality when they compared immersion times varying from 2 to 30 min for diazinon, cypermethrin and malathion. This contrasts with Sabatini et al. (2001) who found that the mortality increased proportionally to the immersion time from 30 s to 30 min when diluting commercial abamectin in water. The present study provides evidence of significant acaricide effects using 2 min as the immersion time for amitraz, deltamethrin and a combination of an organophosphate and synthetic pyrethroid. All the examples cited above account for the need of a standardisation of the variables that affect the performance of the AIT.

In the present study, no DD based on the susceptible INTA A26 was available. Therefore, in the first AIT, amitraz was tested at the recommended label concentration (250 ppm) and its double (500 ppm) to corroborate the failure of amitraz observed in the farm. The production of eggs at these two concentrations by the field isolate but not by the susceptible ticks was a clear indication of resistance in this assay. For the second AIT, in which various acaricides (including amitraz) were tested at their recommended label concentration, no susceptible INTA A26 ticks were available for testing. Hence, efficacy of the acaricides was tested only on the field isolate and compared to a control group. As expected, and in agreement with the result of the first AIT, the efficacy of amitraz was very low. The egg masses laid by the Santo Tomé ticks immersed in 250 ppm amitraz in the first and second AIT showed no statistical differences (0.089 ± 0.044 *vs.* 0.110 ± 0.017; $p = 0.26$). Similarly to amitraz, the inefficacy of 50 ppm deltamethrin to inhibit oviposition in the field isolate was also evident. Only the acaricide combination of an organophosphate and a synthetic pyrethroid constituted an acceptable alternative of treatment reducing oviposition by 90%.

To confirm the AIT results, two additional samples of ticks were collected in the Santo Tomé farm and shipped to Switzerland where the LTT was performed (Table 3). Amitraz and synthetic pyrethroids resistance was diagnosed in the two Santo Tomé field isolates, corroborating the AIT results. The significant difference observed between the LC_{50} of the two samples for amitraz and flumethrin probably results from the natural biological variation of the samples collected in the farm.

The combined results of the AIT and LTT confirmed amitraz and synthetic pyrethroid resistance in the Santo Tomé field isolate. These bioassay studies were triggered by the apparent resistance to the treatment with commercial acaricides in the field. SENASA used the *in vivo* and *in vitro* results as the basis to implement an Integrated Control Programme (ICP) in the Santo Tomé farm in combination with information on the seasonal population dynamics of *R. microplus* (Ivancovich et al., 1984). This programme comprises the monitoring of a strategic rotational treatment scheme between the pour-on application of fluazuron, a chitin synthesis inhibitor, and plunge dipping in a mixture of ethion and cypermethrin. The emergence of amitraz resistance in Santo Tomé, Corrientes, is of concern for the cattle industry in Argentina. As a result, SENASA plans to conduct an extended survey of farms in the area to further investigate this situation and the possible spread of resistance to neighbouring farms.

Acknowledgments

We thank Dr. Robert Miller from the Cattle Fever Tick Research Laboratory (CFTRL) of the USDA-ARS, Edinburg,

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TX, USA for providing us with the susceptible Muñoz strain. The LTT data are part of the PhD dissertation of Léonore Lovis.

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