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# Evidence of acaricide resistance in different life stages of *Amblyomma mixtum* and *Rhipicephalus microplus* (Acari: Ixodidae) collected from the same farm in the state of Veracruz, Mexico

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ABSTRACT

The objectives of this study were to evaluate the resistance of *Amblyomma mixtum* and *Rhipicephalus microplus* ticks from co-infested bovines from the Veracruz region in Mexico to different acaricide families and to demonstrate the viability of the packet test on different *A. mixtum* instars. The following acaricide families were used: a combination (cypermethrin 15 g + chlorpyrifos 25 g + citronella 1 g + butoxide piperonyl 15 g), amidine (formamidine 12.5 g), pyrethroid (cypermethrin 15 g), and organophosphate (dichlorvos 60 g + chlorpyrifos 20 g). Regarding the packet test in both species, resistance was found for the pyrethroid and amidine families in *A. mixtum* and *R. microplus*, as efficacy did not surpass 40 %, including in immature instars; regarding the adult immersion test in *R. microplus,* the efficacy was 93.3 % for the amidine family and 26.2 % for the pyrethroid family. The proposed methodology is an alternative technique to optimize resistance detection in immature ticks with a heteroxenous life cycle.

## 1. Introduction

In Mexico, there are 82 tick species that parasitize domestic and wild animals. Among these species, *Rhipicephalus microplus* and *Amblyomma mixtum* are known to be responsible for economic losses in bovine production systems due to parasitism (Guglielmone and Nava, 2006; Álvarez and Bonilla, 2007; Hernández et al., 2013; Rodriguez-Vivas et al., 2010; Guzmán-Cornejo et al., 2011; Tapias and Vaca, 2009; Rodriguez-Vivas et al., 2014).

*Amblyomma mixtum* is distributed south of Texas, in Mexico, Panama, Ecuador and some Caribbean Islands (Nava et al., 2014). It is an ectoparasite that presents a heteroxenous biological cycle and is a generalist species that infests birds and mammals, including humans. This species deserves public health attention because it transmits relevant pathogens, i.e., *Rickettsia rickettsii* bacteria, which causes Rocky Mountain spotted fever in humans (Parola et al., 2005).

Tick control is mainly based on the use of acaricides. However, in Mexico, there are reports that *R. microplus* has developed resistance to different chemical products (Guerrero et al., 2002; Rodriguez-Vivas et al., 2006; Fernández-Salas et al., 2012a; Fernandez-Salas et al., 2012b; Rodriguez-Vivas et al., 2013; Perez-Cogollo et al., 2010; Rodriguez-Vivas et al., 2014; Miller et al., 2013). Additionally, little evidence on *A. mixtum* resistance in Mexico exists (Alonso-Diaz et al., 2013).

Due to the current situation, the objective of this study was to evaluate *A. mixtum* and *R. microplus* resistance to different acaricide classes using the larval package test and the adult immersion test, as recommended by the FAO (2004) and Drummond et al. (1973), respectively.

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## 2. Materials and methods

### 2.1. Study location

The study was conducted at the Tick Biology Laboratory, Animal Health Department, Embrapa Beef Cattle, Campo Grande, Mato Grosso do Sul, Brazil.

### 2.2. Sample collection and maintenance

Engorged *A. mixtum* and *R. microplus* females were collected from naturally infested bovines from a farm in Veracruz, Mexico (19°01′01.6″N; 96°08′14.4″W). All the engorged *A. mixtum* and *R. microplus* specimens were kept in BOD incubators (27 °C and 80 % UR) to maintain reproductive parameters (oviposition, egg mass weight and larval hatching). A portion of the engorged *R. microplus* females were used for the adult immersion test (AIT) since there was a higher quantity than that of *A. mixtum* females.

To maintain the *A. mixtum* colony, rabbits of the New Zealand race were used. To obtain three instars of this tick, rabbits were infested using artificial feeding chambers, as described by Szabó et al. (1995).

All procedures using animals were in accordance with the recommendations of the Brazilian National Council for Control of Animal Experimentation (Conselho Nacional de Controle de Experimentação Animal - CONCEA). The study was approved by the Animal Use Ethics Commission (Comissão de Ética no Uso de Animais - CEUA) at the Universidade Federal de Mato Grosso do Sul under protocol no. 699/2015.

### 2.3. Acaricides

Commercial acaricides in emulsifiable concentrate formulations were used and diluted in distilled water. The acaricides were as follows: cypermethrin 15 g + chlorpyriphos 25 g + piperonyl butoxide 15 g + citronella 1 g (1:1000 dilution; Cyperclor® Plus Pulverização; Vetbrands Saude Animal); formamidine 12.5 g (1:500; Clipatic®, Fagra); cypermethrin 15 g (1:1000; Barrage® Pulverização, Zoetis); and dichlorvos 60 g + chlorpyriphos 20 g (1:400; Ectobat® Champion Saúde Animal).

### 2.4. Bioassays

#### 2.4.1. Rhipicephalus microplus

To determine *R. microplus* resistance, the adult immersion test and the larval package test were used according to the protocol of the FAO (2004) and adapted for amitraz (Miller et al., 2002).

#### 2.4.2. Adult immersion test (AIT)

Dilutions for each product were prepared in disposable containers that allowed the homogenization of solutions. Each group was composed of 10 engorged *R. microplus* females that were weighed prior to the test and then submerged in each acaricide solution for five minutes. The ticks were then removed, dried, and put in Petri dishes that were maintained in incubators at 28 °C with a relative humidity of 80 % for 14 days. Subsequently, the eggs were weighed and allocated in syringes sealed with cotton and maintained in BODs until the larvae hatched for subsequent evaluation (Drummond et al., 1973).

#### 2.4.3. Larvae packet test (LPT)

The *R. microplus* larvae packet test was used for each treatment according to the FAO (2004) guidelines; three filter papers (85 mm x 75 mm) were impregnated using a 670 µl acaricide solution. After 24 h of impregnation, larvae were put inside the packets and sealed using clips; the packets were subsequently incubated in BODs. After 24 h, mortality was verified. The formula used to identify acaricide efficacy was as follows:

% mortality = [(% mortality of the treated group - % mortality of the control group) / (100 - % mortality of the control group)]

### 2.5. Bioassays with A. mixtum

The same larval packet test used for *R. microplus* larvae was adapted and used to evaluate the resistance of three *A. mixtum* instars (fed and non-fed larvae, fed and non-fed nymphs and non-fed adults), as previously described.

#### 2.5.1. Larval packet test (LPT); nymph packet test (NPT); and adult packet test (APT)

To evaluate acaricide resistance in larvae, nymphs (fed and unfed) and unfed adults, the LPT was adapted using 100 larvae, 30 nymphs and five *A. mixtum* adult pairs for each treatment, with three repetitions each. For each instar, a control group was used. To determine the acaricide efficacy regarding different instars, the following formula was used:

Efficacy = [(% of treated dead ticks - % of control group dead ticks) / (100 - % mortality of control group ticks) * 100]

#### 2.5.2. Engorged larval packet test (ELPT) and engorged nymph packet test (ENPT)

To determine acaricide efficacy on instars of *A. mixtum*, 30 larvae and 30 nymphs were used for each treatment with three repetitions each, according to the methodology. To determine acaricide efficacy regarding different instars that were fed, the following formula was used:

Efficacy = (number of ticks that completed ecdysis / total number of engorged ticks used) * 100

### 2.6. Statistical analysis

The statistical significance of the laboratory treatments was analysed using the Kruskal-Wallis test. Statistical analyses for the packet test, with fed and non-fed larvae and nymphs and with non-fed adults, were calculated using univariate analyses, and a value of $P < 0.05$ was considered statistically significant. The Kruskal-Wallis variance test was performed using the BioEstat 5.0 program.

## 3. Results

### 3.1. Rhipicephalus microplus resistance test

The *Rhipicephalus microplus* larvae submitted to acaricides through the LPT presented different resistance profiles (Table 1). An efficacy of 86.6 % was observed for the combination (cypermethrin 15 g + chlorpyrifos 25 g + citronella 1 g + butoxide piperonyl 15 g), 8.5 % for amidines (formamidine 12.5 g), 10.1 % for pyrethroids (cypermethrin 15 g) and 98.8 % for organophosphates (dichlorvos 60 g + chlorpyrifos 20 g).

For acaricides evaluated using the AIT, engorged *R. microplus* presented increased resistance to pyrethroids (cypermethrin 15 g) and to the combination (cypermethrin 15 g + chlorpyrifos 25 g + citronella 1 g + butoxide piperonyl 15 g), with efficacies of 26.2 % and 62.1 %, respectively. For amidines (formamidine 12.5 g) and organophosphates (dichlorvos 60 g + chlorpyrifos 20 g), the engorged females presented sensitivities of 93.3% and 100%, respectively.

### 3.2. Bioassays with A. mixtum

This tick species was evaluated by the packet test for larvae and

**Table 1**
Larval packet test and adult immersion test efficacy using *Rhipicephalus microplus* strains from the region of Veracruz, Mexico.

| Bioassay | Acaricide efficacy (%) | | | | |
|---|---|---|---|---|---|
| | cypermethrin 15 g + chlorpyrifos 25 g + citronella 1 g + butoxide piperonyl 15 g | formamidine 12.5 g | cypermethrin 15 g | dichlorvos 60 g + chlorpyrifos 20 g | Control |
| ***R. microplus* - LPT** | 86.6% | 8.5% | 10.1% | 98.8% | 2.3% |
| ***R. microplus* -AIT** | 62.1% | 93.3% | 26.2% | 100% | 0 |

LPT: Larval Packet Test; AIT: Adult Immersion Test.

**Table 2**
Different acaricide family efficacy in different *Amblyomma mixtum* instars from the region of Veracruz, Mexico.

| Instar | Acaricides (Packet Test) | | | | |
|---|---|---|---|---|---|
| | cypermethrin 15 g + chlorpyrifos 25 g + citronella 1 g + butoxide piperonyl 15 g | formamidine 12.5 g | cypermethrin 15 g | dichlorvos 60 g + chlorpyrifos 20 g | Control |
| Larvae | 87.4%[a] | 8.6%[b,c] | 9.8% [b] | 98.8% [a] | 1.9% [b,c] |
| Engorged larvae | 100.0% [a] | 12.0% [b,c] | 2.2% [b] | 100.0% [a] | 2.3% [b,c] |
| Nymphs | 100.0% [a] | 7.5% [b,c] | 31.0% [b] | 100.0% [a] | 2.5% [b,c] |
| Engorged nymphs | 97.8% [a] | 24.4% [b,c] | 26.7% [b] | 100.0% [a] | 4.4% [b,c] |
| Non-fed adults | 100.0% [a] | 0.0 [b,c] | 40.0% [b] | 100.0% [a] | 0.0 [b,c] |

*PBO: piperonyl butoxide; the same letters among values represent a nonsignificant difference ($p > 0.05$). Statistical comparisons were made between the acaricide groups.

nymphs (fed and unfed) and non-fed adults. The combination (cypermethrin 15 g + chlorpyrifos 25 g + citronella 1 g + butoxide piperonyl 15 g) presented 87.4 % efficacy for unfed larvae and 97.8 % efficacy for fed nymphs, while for the other instars, the efficacy was 100%. For amidines (formamidine 12.5 g), the adult tick was completely resistant (0 % efficacy), and the highest efficacy observed for fed nymphs was 24.4 %. For pyrethroids (cypermethrin 15 g), the highest efficacy, 40 %, was observed in adults, and the lowest, at only 2.2 %, was observed in fed larvae. The organophosphates (dichlorvos 60 g + chlorpyrifos 20 g) presented an efficacy of 98.8 % in unfed larvae and 100% in the other instars of this tick (Table 2).

## 4. Discussion

One of the great challenges for the global bovine supply chain is tick parasitism. In Mexico, *R microplus* and *A. mixtum* cause losses and control challenges since acaricide treatments are developed without distinction among the species (González-Cerón et al., 2009; Almazán et al., 2016). It is important to note that these two tick species present different parasitic cycles and a marked difference in seasonal fluctuation (Alonso-Díaz et al., 2007; Almazán et al., 2016), making it difficult to establish specific control methods for concomitant species.

Concomitant infestations are common in approximately 86 % of farms in this locality (Alonso-Diaz et al., 2013), and not unlike the global situation, resistance against almost all the main chemical compounds used for control has been observed, and there are reports of strains resistant to many acaricides from different regions (Fernandez-Salas et al., 2012b; Klafke et al., 2017). Due to this reality, it was possible to determine the resistance of these two tick species to acaricides, with the goal of creating an efficient control programme for these ectoparasites.

In Mexico, the *R. microplus* resistance profile has been previously described and includes organophosphates, pyrethroids, macrocyclic lactones and fipronil (Miller et al., 1999; Rodriguez-Vivas et al., 2006, 2007; Li et al., 2007; Rosado-Aguilar et al., 2008; Perez-Cogollo et al., 2010; Miller et al., 2013). However, for *A. mixtum* (former *A. cajennense*), Alonso-Diaz et al. (2013) reported resistance to the amidine, organophosphate and fipronil families, confirming the results obtained in this study, in which we reported the resistance of this tick to the amidine family.

It was shown in this study that for *A. mixtum,* the organophosphate family, which is recommended by the FAO (> 95 %), had a satisfactory effect. This chemical base is globally used for *R. microplus* control; however, there have been resistance reports for this species since the 1980s in Mexico, as well as in other countries (Aguirre and Santamaría, 1996; Rodriguez-Vivas et al., 2006; 2007; Klafke et al., 2017).

For *R. microplus* control, the organophosphate family has been used in different regions in Mexico since the 1980s. Tick resistance reports from the southeastern region are related to this product and suggest an association between the acaricide time period and resistance development (Rodriguez-Vivas et al., 2007). In this study, *R. microplus* was found not to be resistant to this acaricide family; however, this may have been due to the formulation used herein (dichlorvos 60 g + chlorpyrifos 20 g). This formulation was used for *A. mixtum* (all instars) and presented an efficacy greater than 98.8 % for all instars subjected to treatment, showing that there was no resistance in all instars of this tick species.

The chemical compounds in the organophosphate family were efficacious; this was probably due to the high chemical compound concentrations. Similar situations have been reported in different Brazilian regions by Higa et al. (2016), where the susceptibility of *R. microplus* to this formulation (dichlorvos 60 g + chlorpyrifos 20 g) was verified, even though resistance to this chemical compound has been widely recognized (Higa et al., 2015). Natala et al. (2005) found similar results when working with *Amblyomma variegatum*, showing that a clofenvinfos and dioxathion combination was more effective than clofenvinphos alone.

The combination of acaricides from different families (cypermethrin 15 g + chlorpyrifos 25 g + citronella 1 g + butoxide piperonyl 15 g) presented 86.6 % efficacy against *R. microplus* in the LPT; in *A. mixtum* fed larvae and nymphs and non-fed adults, the efficacy was greater than 95 %. This is the first time that such results with this formulation are reported. It is important to note that in the tropical regions of Mexico, the acaricide formulation differs. The combination of chemical products (cimiazole + cypermethrin) was used in only 3.10 % of 98 farms studied by Rodriguez-Vivas et al. (2006). Soberanes et al. (2002) reported that combinations of chemical products (cimiazole + cypermethrin high cis; permethrin + clofenvinphos) had efficacies of 55.6 % and 35 %, respectively.

For *R. microplus,* resistance to pyrethroid and amidine chemical

groups has been reported, with efficacies of 67.11 % and 23.03 %, respectively. Resistance to pyrethroids has been reported since the 1990s in Mexico (Ortiz et al., 1995; Rosario-Cruz et al., 2009) since these acaricides are commonly used in the country, according to Rodriguez-Vivas et al. (2006) and Perez-Cogollo et al. (2010). Resistance has been reported for different acaricide families, i.e., amidine, pyrethroid and organophosphate families. Soberanes et al. (2002) reported an efficacy of 22.8 %, 29 % and 24.1 % for amidines, pyrethroids and organophosphates, respectively, for *R. microplus*.

In this work, regarding *A. mixtum*, the amidine family compounds presented the highest efficacy, at 24.4 %, for fed nymphs, while adults were not sensitive. For the pyrethroid family, the highest resistance was in fed larvae, with an efficacy of only 2.2 %, while in adults, the efficacy was 40 %. Alonso-Diaz et al. (2013), using the larvae immersion technique for *A. mixtum*, reported a resistance profile of 12.5 % for the pyrethroid family, in which the resistance level was 72.6 %; for the organophosphate family, the resistance rates were 100%, 91.7% and 12.5% for diazinon, chlorpyriphos and coumaphos, respectively. This finding, in addition to the ones found herein, shows that *A. mixtum* and *R. microplus* are susceptible to developing resistance to these acaricides.

In the present study, in the evaluation of the amidine family, the lowest efficacy was observed in unfed nymphs (7.5 %), and the highest efficacy was observed in fed nymphs (24.4 %). This result confirms that reported by Natala et al. (2005), who evaluated the susceptibility of *Ambylomma variegatum* using the larval immersion test in fed and unfed ticks and found low efficacy in all instars.

Wharton and Roulston (1970) reported that ticks that parasitize bovines are more susceptible to acaricide exposure and therefore are more prone to developing resistance. In this study, it was possible to verify *A. mixtum* resistance to pyrethroids and amitraz, showing the capacity of this species to develop resistance in three instar periods whether fed or not fed.

The evaluated ticks in this study were collected from animals infested with both *A. mixtum* and *R. microplus* species; the ticks were consequently submitted to the same acaricide products in the field. However, there was a higher resistance rate in *R. microplus* than in *A. mixtum*, in agreement with Mekonnen et al. (2002), who reported increased resistance in ticks with monoxene cycles. It is possible that resistance is increased because *R. microplus* remain on the host for a relatively long period of time and are therefore extensively exposed to the products, consequently suffering a higher selection pressure, which is known to be a factor that contributes to the development of resistance.

This study also suggested the use of other instars of trioxene (nymphs, fed or not fed, and non-fed adults) ticks as a viable and efficient model for bioassays, and the packet test is a fast and reliable alternative to detect resistance in economically important or public health-relevant tick species.

## 5. Conclusions

According to this study, the use of a combination of acaricide products belonging to the organophosphate class (between organophosphates) is recommended as an option for tick control. It is important to note that the acaricide efficacy profile was similar in both of the studied species, which is of great relevance to resistance investigations by bioassays in *R. microplus* and *A. mixtum*. This information could be used in further studies to mitigate resistance development or optimize acaricide control with integrated tick management.

## Declaration of Competing Interest

The authors declare they have no conflicts of interest.

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