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First report of a *Rhipicephalus microplus* tick population multi-resistant to acaricides and ivermectin in the Mexican tropics

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ABSTRACT

We document the presence of a *Rhipicephalus microplus* tick population resistant to acaricides (organophosphates (OP), synthetic pyrethroids (SP), amitraz) and macrocyclic lactones (ML) (ivermectin). Engorged females of *R. microplus* were collected from a cattle farm in Veracruz, Mexico, to evaluate acaricide and ivermectin resistance. The modified larval packet test (LPT) was used to detect OP (chlorpiriphos and diazinon) and SP (flumethrin, deltamethrin and cypermethrin) resistance and the larval immersion test (LIT) to detect resistance to amitraz and ivermectin. Both, LPT and LIT were performed twice at different times with different collected samples. Mortality data with ivermectin were subjected to probit analysis to obtain lethal concentrations and resistance ratios (RR) using an ivermectin-susceptible strain (Deutch) as a reference. The *R. microplus* population showed resistance to all acaricides tested, with different mortalities at the discriminate dose: chlorpiriphos (1%), diazinon (24.2%), flumethrin (92.8%), deltamethrin (94.2%), cypermethrin (98.0%) and amitraz (1.5%). The studied tick population also showed resistance to ivermectin with a resistance ratio at 99% of 9.58 and 6.52 in the first and second evaluation, respectively. We report for the first time a *R. microplus* population in Mexico with different levels of resistance to OP, SP, amidines (Am) and ivermectin. The uncontrolled use of these products in the study area may promote the complete failure of tick control within a short period of time.

1. Introduction

Rhipicephalus microplus is the major economic threat to the cattle industry in tropical, subtropical and temperate areas of the world (Rodríguez-Vivas et al., 2010). Currently, tick control is more difficult due to the presence of resistant populations to all major families of acaricides (Rodríguez-Vivas et al., 2006a,b; Cuore et al., 2007), and recently, Klafke et al. (2006) and Perez-Cogollo et al. (2010a) reported populations of *R. microplus* resistant to ivermectin in Brazil and Mexico. There also are reports of populations with diverse forms of resistance to several chemical molecules. There is information on ticks double-resistant to organochlorines and organophosphates (OP) (Aguirre and Santamaría, 1996), synthetic pyrethroids (SP) and OP (Ortiz et al., 1995) and triple-resistant to OP, SP and amidines (Am) (Benavides et al., 2000; Soberanes et al., 2002; Rodríguez-Vivas et al., 2007). To date in Mexico, there are no reports of a *R. microplus* tick population resistant to the three principal acaricide families (OP, SP and Am) and macrocyclic lactones (ML).

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Table 1
Mortality of *Rhipicephalus microplus* after being subjected twice to a discriminating dose of acaricides using the larval packet and the larval immersion tests.

Acaricide	DD %	Mortality (%)	
		First test	Second test
Flumethrin	0.01	6.43	7.81
Deltamethrin	0.09	6.01	5.43
Cypermethrin	0.05	1.89	2.10
Chlorpiriphos	0.5	99.0	99.0
Diazinon	0.8	75.8	*
Amitraz	0.0002	99.0	98%

DD, discriminating dose; *, not determined.

The early detection of resistance in field populations can provide essential information to establish handling procedures to delay its development and to assure sustainable use of acaricides (FAO, 2004). Thus, the objective of this study is to document a *R. microplus* tick population resistant to acaricides (OP, SP and Am) and ML (ivermectin). Information about new populations of multi-resistant ticks may contribute (i) to the design of new control strategies based on the use of novel or scarce chemical molecules, (ii) highlight the necessity for better use of new molecules with the purpose of extending their useful lives on the market (e.g. ML, utilized for controlling gastrointestinal nematodes (GIN), flies and ticks), (iii) to use as a reference population to evaluate and contrast the efficacy of new control alternatives (e.g. entomopathogenic fungi, bioactive compounds of plants and vaccines), and (iv) to design appropriate epidemiological measures to avoid the release of multi-resistant field populations.

2. Materials and methods

2.1. Study area

During 2010, under field conditions, acaricides and ivermectin treatment failures had been reported by veterinarians and farmers in Veracruz, Mexico. In order to determine the susceptibility of field populations of *R. microplus* to acaricides and ivermectin, a dual-purpose cattle farm (beef and milk) was studied. The cattle farm is located in the municipality of Martinez de la Torre, Veracruz, Mexico (24°4′N, 97°03′W). The regional climate is humid tropical, with a mean annual temperature of 23.4 ± 0.5 °C, annual rainfall of 1991 ± 392 mm and a relative humidity of 85% (INEGI, 2008). Over the last ten years, tick and fly control on the cattle farm has been based on the use of acaricides or acaricide mixtures (used ten to twelve times per year) and ML have been routinely used for GIN control (six applications per year) mainly in calves.

2.2. Ticks

On the cattle farm, two tick sample collections were made at different times (July 20, 2010, and October 17, 2010). The sample consisted of 40-50 engorged female ticks of *R. microplus* collected from at least 10 animals from the study farm. Ticks were placed in Petri dishes, with the cover perforated to allow ventilation and then transported to the Animal Health Laboratory at the Centro de Enseñanza, Investigación y Extensión en Ganadería Tropical (CEIEGT) of the Facultad de Medicina Veterinaria y Zootecnia-Universidad Nacional Autónoma de Mexico (FMVZ-UNAM). Upon arrival, engorged ticks were washed and immediately incubated under laboratory conditions at 27 ± 1.5 °C and 70–80% relative humidity (RH) (Cen-Aguilar et al., 1998) to allow for egg laying and hatching. Live larvae of 14–21 days of age were used for resistance bioassays.

2.3. Bioassays

2.3.1. Larval packet test to determine resistance to organophosphates and synthetic pyretrhroids

The modified larval packet test (Stone & Haydock, 1962) was used to test *in vitro* resistance of OP (chlorpiriphos and diazinon) and SP (flumethrin, deltamethrin and cypermethrin). Briefly, a technical grade acaricide dissolved in a mixture of trichloroethylene and olive oil (2:1 ratio) was used to treat filter papers which were folded into packets using bulldog clips. Approximately 100 *R. microplus* larvae were placed into each treated filter paper packet, which was then sealed with additional bulldog clips and placed in an incubator (27 °C and 85–86% RH) for 24 h. A discriminating dose (DD) of the technical grade acaricide (Table 1) was used and was calculated by doubling the mean lethal dose 99.9% derived from the series of tests conducted with a susceptible strain (Kemp et al., 1998). After 24 h had elapsed, mortality was determined. Three replicates and a control (filter paper with trichloroethylene and olive oil) for each acaricide were used. Only larvae that had the ability to walk were considered alive.

2.3.2. Larval immersion test to determine resistance to amitraz

The modified larval immersion test was used to test the susceptibility of *R. microplus* larvae to amitraz (Soberanes et al., 2002). Briefly, a commercial formulation of amitraz (Taktic® 12.5%, Intervet, Mexico) was diluted in distilled water. DD solutions, 10 mL each, were prepared in Petri dishes (15 mm in diameter), and then approximately 300–500 larvae were placed between two Whatman No. 1 papers and immersed in each solution for 10 min. Three replicates of the acaricide dilution and a control (distilled water) were used. Approximately 100 larvae from the treated and control solutions were transferred to clean filter paper packets, and kept in an incubator (27 ± 1.5 °C, 80–90% RH) for 72 h, after which mortality was determined.

2.3.3. Modified larval immersion test to determine resistance to ivermectin

The modified larval immersion test using 11 concentrations (Klafke et al., 2006) was used to test *in vitro* resistance to ivermectin in this *R. microplus* population. Technical grade ivermectin (22, 23-dihydroavermectin B1, Sigma–Aldrich, USA) was used to prepare a 1% ivermectin stock solution in absolute ethanol. An ethanol solution with 2% Triton X-100 (Sigma–Aldrich, USA) was diluted to 1% in distilled water (Eth-TX 1%). Then, the top immersion solution of ivermectin (0.01%) was prepared in Eth-TX 1%. In order to prepare the immersion

Table 2
Lethal concentration estimates at 50% and 99% for ivermectin resistance in *Rhipicephalus microplus* subjected twice to the larval immersion test.

Population	Slope	50%				99%			
		LC_{50}	CL95%	RR_{50}	CL95%	LC_{99}	CL95%	RR_{99}	CL95%
CLAR*	2.22	0.00150	0.0010–0.0020	2.67	1.92–3.33	0.0163	0.0081–0.0856	9.58	5.4–40.7
CLAR**	2.83	0.00170	0.0014–0.0020	3.03	2.69–3.33	0.0111	0.0074–0.0218	6.52	4.9–10.3
Deutch	4.72	0.00056	0.00052–0.00060	NA	NA	0.0017	0.0015–0.0021	NA	NA

Deutch, susceptible reference strain (SRS); LC, lethal concentration; CL, confidence limits; RR, resistance ratio (LC from the population studied divided by the LC from the SRS); NA, not applicable; *, first sample, **, second sample.

solutions, eleven different concentrations of ivermectin were obtained through 30% serial dilutions from the top 0.01% solution. Concentrations (%) of immersion solutions were: 0.01, 0.007, 0.0049, 0.00343, 0.0024, 0.00168, 0.00117, 0.00082, 0.00057, 0.0004 and 0.00028. Eth-TX1% was used as a control solution. Immersion solutions, 0.5 mL of each concentration, were transferred into 1.5 mL microcentrifuge tubes (three repetitions for each solution) and approximately 300 larvae were added using a paintbrush. The larvae were immersed for 10 min. Then the tubes were opened and approximately 100 larvae were transferred with another paintbrush to a filter paper (850 mm × 750 mm) that was folded and closed with "bulldog" clips forming a packet. The packets were incubated at 27–28 °C and 80–90% RH for 24 h, after which mortality was determined.

2.4. Mortality data analysis

If one or more larvae were found alive after exposure to DD of OP, SP and Am, the tick population was considered resistant, because the population was exposed to a double dose of acaricide that usually would kill up to 99.99% of the individuals in a susceptible population.

To determine *R. microplus* resistance to ivermectin the lethal concentrations (LC) to kill 50% and 99% of the population and their respective confidence limits of 95% (CL95%) were calculated by probit analysis using the POLO PLUS software (LeOra Software, 2003). Resistance ratios (RR) at 50% and 99% were calculated in relation to the Deutch reference strain (USDA, Cattle Fever Tick Research Laboratory, Edinburg, TX, USA) and the difference was considered significant if the CL95% of the tested population were not included in the CL95% of the reference strain.

3. Results

The *R. microplus* population in this study showed resistance to all SP evaluated, with mortality percentages between 1.8 and 7.8%. When the tick population was evaluated for amitraz resistance, the population showed low resistance to this acaricide (mortality 98–99%) (Table 1). The tested population showed different behavior (levels) of resistance to OP; chlorpiriphos (99% of mortality) and diazinon (75.8% of mortality) (Table 1). The slope, LC, to kill 50% and 99%, and RR to ivermectin and their respective CL95% in the tested population are shown in Table 2. The tested tick population showed significantly higher LC_{50}/LC_{99} estimates than the reference susceptible Deutch strain.

4. Discussion

This study is the first report of *R. microplus* tick population resistant to four families of chemical products used for their control. We diagnosed resistance to OP, SP and Am (Table 1), as well as to ivermectin (Table 2). Previous studies have reported tick strains with diverse resistance to two or three acaricide families. For example, Aguirre and Santamaría (1996) mention a *R. microplus* strain resistant to organochlorines and OP, Ortiz et al. (1995) to OP and SP, Kunz & Kemp (1994) to SP and Am and Benavides et al. (2000) to OP, SP and Am. Recent epidemiological studies have found tick populations with diverse resistance to acaricides (OP, SP and/or Am) (Rodríguez-Vivas et al., 2006a,b; Mendes et al., 2011) and have contributed to the understanding of different perspectives on the evolution of resistance in diverse ecological niches, and supports the presence of ticks resistant to more than one acaricide, making it difficult to control *R. microplus*. The development of resistance in a tick population is due to factors related with acaricide use, ecological niches and the genus of ticks involved (Kunz and Kemp, 1994; Rodríguez-Vivas et al., 2006a,b; Alonso-Díaz et al., 2006). It is known that the application of a chemical product more than six times a year can contribute to the development of resistant populations (Rodríguez-Vivas et al., 2006a; Mendes et al., 2011). During the last decades, the tick population in this study has been subjected to frequent applications of OP, SP and Am for its control, and this has led to selection of resistant individuals.

ML are chemical molecules that have emerged as an efficient alternative for *R. microplus* control (Lanusse et al., 1997). In Mexico, over the last ten years, the ML have been utilized frequently for control of endoparasites and ectoparasites (Rodríguez-Vivas et al., 2006a), resulting in the first reported cases of *R. microplus* resistant to this molecule (Martins and Furlong, 2001; Klafke et al., 2006; Perez-Cogollo et al., 2010b). In this study, the tick population had a resistance ratio 50 (RR_{50}) to ivermectin of 2.67 and 3.03 and RR_{99} of 9.59 and 6.52 at the first and second test, respectively (Table 2). These results are similar to those reported by Perez-Cogollo et al. (2010a) who reported RR_{50} of 1.70–3.97 and RR_{99} of 2.10–9.92 to ivermectin in 18 of 30 *R. microplus* populations, and with Klafke et al. (2006) who reported RR_{50} of 1.09 and 3.78 to ivermectin in two *R. microplus* populations. ML are fat-soluble molecules that are widely distributed in the intestinal lumen, fat and skin of animals after its application (Entrocasso et al., 1996; Rodríguez-Vivas et al., 2010). The pharmacokinetic properties of these products

are characterized by a period of declining drug concentration, meaning that ML may stay in tissues at sub-lethal doses, favoring the development of resistant individuals. Thus, as drug profiles decline over time, there will invariably be a period where resistant individuals are able to establish but susceptible individuals cannot (Leathwick & Sutherland, 2002). However, there are other factors (e.g. the use of generic versions of ML or high frequency of treatments) that can influence the development of *R. microplus* resistance to ML in the field (Perez-Cogollo et al., 2010a,b).

The main use of ML on cattle farms is for GIN control in young animals. Arnaud and Alonso-Díaz (2010) mentioned that 50% of cattle farmers in the area utilize ML for GIN control in calves and over 75% used them improperly. For example, they do not weigh the animals to formulate a correct application of product, and recent studies mention a strong relation between the efficacy of a drug and its therapeutic concentration (Köhler, 2001; Van Zeveren et al., 2007). On the other hand, parasitosis in tropical and subtropical regions involves different parasites such as flies and ticks, which can be controlled with either SP or OP, or ticks and GIN with ML. This situation increases the probability of developing parasites that are resistant and multi-resistant on cattle farms (Nari, 2005). As well, Alonso-Díaz et al. (2007) have documented the presence of *R. microplus* throughout the year in the study area, increasing the frequency of treatments and favoring the selection pressure for resistant individuals.

In conclusion, we report for the first time a *R. microplus* population in Mexico with different levels of resistance to SP, OP, Am and ivermectin. The uncontrolled use of these products in the study area may promote the complete failure of tick control within a short period of time.

Conflict of interest statement

The authors of this manuscript have no financial or personal relationships with other people or organizations that could inappropriately influence or bias the content of the paper.

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