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Ivermectin resistance status and factors associated in *Rhipicephalus microplus* (Acari: Ixodidae) populations from Veracruz, Mexico

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ABSTRACT

The objectives of the present study were to (1) determine the status of resistance or susceptibility to ivermectin (IVM) in *Rhipicephalus microplus* tick populations in Veracruz, Mexico, (2) determine the level of resistance (resistance ratios [RR] and lethal concentrations for 50% [LC_{50}] and 99% [LC_{99}]) mortality in each *R. microplus* population, and (3) identify factors associated with resistance. Populations of *R. microplus* were sampled from 53 cattle farms to evaluate their resistance using the larval immersion test. Mortality data were subjected to probit analysis to calculate LC_{50} and LC_{99}. Resistance ratios were calculated in relation to a susceptible reference strain. A logistic regression model was used to evaluate the relation between resistance and possible associated factors. Thirteen tick populations were susceptible to ivermectin, eighteen had incipient resistance and twenty-two had significant resistance. RR_{50} of the susceptible tick populations varied from 0.59 to 1.07. The populations that showed the highest level of resistance were: ANTE ($RR_{50}=8.21$; $RR_{99}=46.0$), PALO ($RR_{50}=6.25$; $RR_{99}=35.47$), P.VIE ($RR_{50}=5.89$; $RR_{99}=180.3$), AURO ($RR_{50}=5.36$; $RR_{99}=13.82$ and CEDR ($RR_{50}=4.11$; $RR_{99}=26.47$). Cattle farms that used macrocyclic lactones ≥4 times per year were more likely to develop *R. microplus* resistant to ivermectin (OR = 13.0; $p=0.0028$). In conclusion, more than two-thirds of the farms sampled in Veracruz, Mexico, showed some level of ivermectin-resistant *R. microplus* populations and the number of ML applications per year is factor associated with the resistance of *R. microplus* to IVM.

1. Introduction

Rhipicephalus microplus is the major threat to the cattle industry in tropical, subtropical and temperate areas of the world. Chemical acaricides have played an essential role in its control, but their intensive use has favored the development of resistant populations (Rodríguez-Vivas et al., 2006a,b). Macrocyclic lactones (MLs) have emerged as an alternative to mitigate the negative effects of ticks, including tick populations resistant to most acaricides (Lanusse et al., 1997). Macrocyclic lactones are endectocides derived from the actinomycetes *Streptomyces avermitilis* (avermectins) and *S. cyaneogriseus* (milbemicins) that are used for the control of gastrointestinal nematodes (GINs) and ectoparasites (Lifschitz et al., 2002; Sumano and Ocampo, 2006). In Mexico, the pharmaceutical industry reported that IVM is the preferred anthelmintic to control GINs in ruminants and it is also used to control cattle ticks (Soberanes, 2010). However, as an effect from its intensive use, reports of *R. microplus* resistant to IVM have been documented in Latin America.

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The first cases of *R. microplus* populations resistant to IVM were reported in Brazil (Martins and Furlong, 2001; Klafke et al., 2006) and Uruguay (Castro-Janer et al., 2011).

In Mexico, the first tick populations resistant to IVM were reported by Pérez-Cogollo et al. (2010a,b). Epidemiological studies to evaluate the spatial distribution of resistance to IVM, the classification of phenotypic resistance of *R. microplus* to IVM, and the factors involved in resistance development are still needed. This information would help in the design of strategies to delay the development of resistance to IVM and, thus, to preserve the efficacy of this chemical for parasite control.

Veracruz is the Mexican state with the highest cattle productivity (INEGI, 2010) and parasitic illnesses are the main threat to grazing bovines in this region (Alonso-Díaz et al., 2007). Studies to evaluate the resistance of *R. microplus* to IVM in the area have been not conducted, hence, the objectives of the present study were (1) to determine the status of resistance or susceptibility to IVM in 53 populations of *R. microplus* collected in Veracruz, Mexico, (2) to determine the level of resistance (resistance ratios [RR] and lethal concentrations for 50% [LC_{50}] and 99% [LC_{99}]) mortality in each *R. microplus* population, and 3) to identify factors associated with resistance to IVM in the tick populations.

2. Materials and methods

2.1. Study area

An epidemiological study of resistance to IVM in *R. microplus* was carried out between September 2010 and November 2011 in four municipalities (Misantla, Martínez de la Torre, Nautla, and Vega de Alatorre) in Veracruz, Mexico. The climate of the region is tropical humid, with a mean annual temperature of 23.4 ± 0.5 °C, an annual rainfall of 1991 ± 392 mm and a mean relative humidity (RH) of 85% (INEGI, 2008). According to a list of members provided by the Cattleman's Association from each municipality, the number of cattle farms in the study area is about of 1200. The main cattle production method in the area is the dual-purpose system (beef and milk) based on extensive grazing systems. The preferred methods of tick control incorporate are the use of chemical acaricides (organophosphates, synthetic pyrethroids, and amidines) and MLs (Alonso-Díaz et al., 2007; Fernández-Salas, 2011).

2.2. Tick collection

Fifty-three cattle farms were selected using a simple random sampling model. Engorged female ticks of *R. microplus* ($n = 30$–50) were collected from each cattle farm of at least 10 bovines and were placed into small boxes with holes to allow for air circulation, and then sent to the Animal Health Laboratory at the Centro de Enseñanza Investigación y Extensión en Ganadería Tropical (CEIEGT) in the Facultad de Medicina Veterinaria y Zootecnia – Universidad Nacional Autónoma de México (FMVZ-UNAM). Engorged females were collected at least 35 days after any acaricide treatment. Upon arrival, engorged ticks were washed with distilled water and immediately incubated under laboratory conditions at 27 ± 1.5 °C and 70–80% RH (Cen-Aguilar et al., 1998) to allow for egg-laying and egg-hatching. Live larvae of 14–21 days of age were used for resistance bioassays. During sampling, a questionnaire about the history of ML use was administered to farm owners or managers.

2.3. Bioassays

The modified larval immersion test (LIT) was used to test *in vitro* resistance to IVM (Klafke et al., 2006). Technical grade IVM (22,23-dihydroavermectin B1, Sigma–Aldrich, USA) was used to prepare a 1% IVM stock solution in absolute ethanol. An ethanol solution of 2% Triton X-100 (Sigma–Aldrich, USA) was diluted to 1% in distilled water (Eth-TX 1%). Then, the top immersion solution of IVM (0.01%) was prepared in Eth-TX 1%. In order to prepare the immersion solutions, eleven different concentrations of IVM 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-TX 1% 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 "bull-dog" 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. Statistical analysis

Questionnaire information was analyzed using descriptive statistics to estimate frequencies of the variables studied on each cattle farm.

To determine the lethal concentrations to kill 50% (LC_{50}) and 99% (LC_{99}) of tick populations and their respective 95% confidence intervals (CI 95%), a probit analysis was used with the POLO PLUS software (LeOra Software, 2003). Resistance ratios of 50% (RR_{50}) and 99% (RR_{99}) were calculated in relation to the Deutch susceptible reference strain (USDA, Cattle Fever Tick Research Laboratory, Edinburgh, TX, USA) and the difference was considered significant when the CI 95% of the tested populations were not included in the CI 95% of the reference strain. RR indicates the magnitude of the difference between the LC of the susceptible strain and the LC of the evaluated strain.

Diagnosis of resistance of the tick populations was classified according to three categories proposed by Castro-Janer et al. (2011): (1) susceptible, when the LC_{50} (CI 95%) of the field population is not statistically different from the reference strain; (2) incipient resistance, when the LC_{50} (CI 95%) of the field population is statistically different from the reference strain and $RR_{50} < 2$ and 3) resistant, when the LC_{50} (CI 95%) of the field population is statistically different from the reference strain with $RR_{50} \geq 2$.