J. Parasitol., 98(5), 2012, pp. 1010–1014

RESISTANCE OF *RHIPICEPHALUS MICROPLUS* TO AMITRAZ AND CYPERMETHRIN IN TROPICAL CATTLE FARMS IN VERACRUZ, MEXICO

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ABSTRACT: The objectives of this study were: (1) to determine the prevalence and factors associated with *Rhipicephalus microplus*, resistant to cypermethrin and amitraz, from cattle farms in Veracruz, Mexico, and (2) to determine in vitro mortality percentages of field populations of *R. microplus* exposed to discriminating doses (DD) of cypermethrin and amitraz. Fifty-three populations of *R. microplus* were tested by bioassays using DD of cypermethrin (0.05%) and amitraz (0.0002%). The prevalence of cattle farms with *R. microplus* ticks that were resistant to cypermethrin and amitraz, and co-resistant to both acaricides, was 90.6, 54.7, and 47.2%, respectively. The level of cypermethrin resistance, measured as a survival percentage, was higher as compared to amitraz. Cattle farms with ≤50 animals (odds ratio [OR] = 3.84, 95% confidence interval [CI] = 1.07–13.70, $P = 0.038$) and a stocking density of >1 animal unit per ha (AU/ha) (OR = 4.21, 95% CI = 1.0–17.71, $P = 0.050$) had a higher probability to develop *R. microplus* tick populations co-resistant to both acaricides. In conclusion, there is a high prevalence of *R. microplus* populations on cattle farms in Veracruz, Mexico that are both resistant to cypermethrin and amitraz and co-resistant to both acaricides. The level of cypermethrin resistance is critical, and the exposition variables of ≤50 cattle and a stocking density of >1 AU/ha were factors associated with *R. microplus* co-resistant to both acaricides.

The cattle tick *Rhipicephalus microplus* is one of the major threats to the cattle industry in tropical and subtropical regions due to the direct (blood loss, skin lesions, and injection of toxins) and indirect (transmission of *Anaplasma marginale*, *Babesia bovis*, and *Babesia bigemina*) damage it causes to cattle (Rodríguez-Vivas et al., 2007). In Mexico, the problem has increased due to the presence of *R. microplus* populations resistant to organophosphates (OP), synthetic pyrethroids (SP) (Rodriguez-Vivas, Alonso-Díaz et al., 2006), and amitraz (Rodriguez-Vivas, Rodríguez-Arevalo et al., 2006) as well as multi-resistance to these acaricides (Miller et al., 2007; Fernández-Salas et al., 2012) and endectocides (Perez-Cogollo et al., 2010) used to control them. Nevertheless, these chemicals are still the primary method for tick control in the region.

Veracruz State has the highest number of cattle in Mexico (2,454,171; 10.5% of the national inventory), is the major beef producer (14.7% of the total national production) (INEGI, 2010), and is located in sixth place for milk production (708,230 L/yr) (INEGI, 2010). Likewise, due to its geographical location, Veracruz State is an important transit place for cattle exportation from the south of Mexico and Central and South America to the United States, which increases the risk of spreading resistant populations of ticks. In this area, feeding cattle depend on the direct use of forage through grazing (Castillo-Gallegos et al., 2005) and, consequently, parasite illnesses represent 80% of animal health problems in the region (Garcia-Gutierrez, 2004). *Rhipicephalus microplus* is the main cattle ectoparasite in Veracruz and is present throughout the year in the area (Alonso-Díaz et al., 2007). There are reports by veterinarians and farmers of failures to control this tick. Thus, epizootiological studies are necessary to understand the status of *R. microplus* tick resistance to acaricides, and the factors associated with its presentation, in order to avoid its dispersal and mitigate the negative economic impact on the cattle industry and public health. The objectives of this study were (1) to determine the prevalence and factors associated with *R. microplus*, resistant to cypermethrin and amitraz, in cattle farms from Veracruz, Mexico, and (2) to determine in vitro mortality percentages of field populations of *R. microplus* exposed to discriminating doses (DD) of cypermethrin and amitraz.

Received 27 December 2011; revised 19 April 2012; accepted 23 April 2012.
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DOI: 10.1645/GE-3074.1

MATERIALS AND METHODS

Study area

The *R. microplus* tick populations evaluated in this study were collected from cattle farms in 4 municipalities (Misantla, Martínez de la Torre, Nautla, and Vega de Alatorre) in Veracruz, Mexico between September 2010 and October 2011. The climate of the region is tropical humid with a mean annual temperature of 23.4 ± 0.5 C, an annual rainfall of 1,991 ± 392 mm, and a mean relative humidity (RH) of 85% (INEGI, 2008). Tick control is based on the use of chemical acaricides (OPs, SPs, and amidines) applied by dipping, spraying, and pour-on as well as on the use of macrocyclic lactones.

Sample size

From a list of members provided by the Cattleman's Association from each municipality, a sample size of 53 cattle farms was determined using Win Episcope 2.0 software (EPIDECON, Zaragoza, Spain) and considering a 95% confidence level, 10% error, and expected prevalence of 70% based on reports of similar previous studies (Rodríguez-Vivas, Alonso-Díaz et al., 2006; Rodríguez-Vivas, Rodríguez-Arevalo et al., 2006). The number of cattle farms evaluated in each municipality was proportional to the total number of cattle farms in each Cattleman's Association. Selection of cattle farms was carried out by simple random sampling; thus, each farm had the same probability of being chosen.

Tick collection and questionnaire application

From each cattle farm, 10 bovines were chosen from which to collect 20–30 semi-engorged female ticks for classification using morphologic characteristics (Rodriguez-Vivas and Cob-Galera, 2005). Likewise, from these animals 30–50 engorged females of *R. microplus* were collected and then placed into small boxes with holes to allow air to circulate. Engorged females were sent to the Animal Health Laboratory of Centro de Enseñanza Investigación y Extensión en Ganadería Tropical (CEIEGT) of the Facultad de Medicina Veterinaria y Zootecnia—Universidad Nacional Autónoma de México (FMVZ-UNAM). 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 the sampling, a general questionnaire was administered to owners or

TABLE I. Prevalence of cattle farms in Veracruz, Mexico with *Rhipicephalus microplus* ticks resistant to cypermethrin or amitraz, or co-resistant to both acaricides.

Chemical acaricide	Cattle farms among 53	Prevalence (%)
Amitraz	29	54.7
Cypermethrin	48	90.6
Amitraz + cypermethrin	25	47.2
Susceptible*	1	1.9

* Susceptible population to both tested acaricides.

managers about farm management, animal breeds, and acaricides used. Variables considered as possible factors associated to resistance were cattle farm size (≤50 animals; >51 animals), production system (extensive; semi-intensive), stocking density (≤1 AU/ha; >1 AU/ha), frequency of applications (≤15 days; >16 days), criteria for treatment (routine; tick presence), chemical control against flies (yes; no), proximity to another farm (yes; no), recommended dose (recommended; another), and acaricide rotation (yes; no) time using the same acaricide (≤1 yr; >1 yr).

Bioassays

Larval packet test to determine resistance to cypermethrin: The modified larval packet test (Stone and Haydock, 1962) was used to test the in vitro efficacy of cypermethrin on *R. microplus* populations. Briefly, a technical grade acaricide dissolved in a mixture of trichloroethylene and olive oil (2:1 ratio) was used to treat filter papers that were folded into packets held by 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 hr. A DD of cypermethrin (0.05%) was used. After time had elapsed, mortality was determined. Three replicates and a control (filter paper with trichloroethylene and olive oil) were used. Only larvae that had the ability to walk were considered alive.

Larval immersion test to determine resistance to amitraz: The modified larval immersion test was used to test the efficacy of amitraz on *R. microplus* larvae of each population (Soberanes et al., 2002). Briefly, a commercial formulation of amitraz (Taktic® 12.5%, Intervet, Mexico City, Mexico) was diluted in distilled water. DD solutions (0.0002%), 10 ml each, were prepared in Petri dishes (15 mm in diameter) and then approximately 300–500 larvae were placed between 2 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 hr, after which mortality was determined.

Statistical analysis

Questionnaire information was analyzed using descriptive statistics to estimate frequencies of the variables found on each cattle farm. The prevalence of cattle farms with *R. microplus* that was resistant to amitraz and cypermethrin, or both, was calculated using the formula of Leaverton (1991).

A univariate analysis was performed using 2 × 2 contingency tables of exposure variables (Epi Info 3.5.3; CDC 2008) to estimate the association level with the response variables (resistant, susceptible, and co-resistant to both acaricides). A chi square test was used to estimate the significance level for each association. Exposure variables with *P*-values ≤0.20 were included in a logistic-binomial regression model (multivariate analysis) using Statgraphics® Centurion 15.2.06 (StatPoint Technologies, Inc., 2007). Exact regression estimates, 95% confidence intervals (95% CI), odds ratios (OR), *P*-values, and beta values (regression coefficient) were obtained. A *P*-value ≤0.05 was considered significant.

RESULTS

Questionnaire

The questionnaire survey described the management practices of cattle farmers from Veracruz, Mexico. The percentage of cattle farms using the crossbreeds *Bos taurus* × *Bos indicus* was 90.6% (48/53) while the rest used *Bos indicus*. The percentage of farmers using an extensive grazing system was 92.5% (49/53) while 7.5% (4/53) used a semi-intensive system. The percentage of cattle farms having double-purpose (beef and milk) cattle production was 75.5% (40/53) and the rest had only beef production. Overall, 100% of the cattle producers used acaricides as the principal method to control ticks, with amitraz being the most widely utilized (58.5%; 31/53) followed by chemical mixtures (26.4%; 14/53), SPs (11.3%; 6/53), and ivermectin and fipronil (3.8%; 2/53). All cattle producers used chemicals 6 or more times per year; 69.8% (37/53) applied acaricides every 12–15 days during the season of high tick infestation, 17.0% (9/53) every 21 days, and only 13.2% (7/53) every 30 days or more. The percentage of cattle producers using the recommended dose indicated by manufacturers was 54.7% (29/53) while 45.3% (24/53) applied incorrect doses. The percentage of producers applying acaricides by aspersion (hand spraying) was 96.2% (51/53) while 3.8% (2/53) applied them as a pour-on. In 94.3% (50/53) of the cattle farms, a cohabitation of *R. microplus* (a 1-host tick) and *Amblyomma* spp. (a 3-host tick) was found.

Prevalence and mortality percentages

The prevalence of cattle farms with *R. microplus* that were resistant to cypermethrin, amitraz, or co-resistant to both acaricides is shown in Table I. Mortality percentages of *R. microplus* after having been exposed to a DD of cypermethrin and amitraz are shown in Table II.

Factors associated with resistance

The independent variables that had *P*-values ≤0.20 in the univariate analysis, and which were included in the logistic regression, were: (1) for amitraz resistance, cattle farm size and production system; (2) for cypermethrin resistance, treatment

TABLE II. Larval mortality of 53 *Rhipicephalus microplus* populations tested using the modified larval immersion test (discriminating dose of amitraz 0.0002%) and larval packet test (discriminating dose of cypermethrin 0.05%) from cattle farms in the State of Veracruz, Mexico.

		Resistant					
	Susceptible	Mortality					
Cattle farms	100%	99–95%	94–70%	69–50%	49–20%	<20%	Total
Cattle farms resistant to cypermethrin (frequency)	5 (9.4)	10 (18.8)	11 (20.7)	8 (15.1)	10 (18.8)	9 (17.0)	53
Cattle farms resistant to amitraz (frequency)	24 (45.3)	16 (30.2)	9 (17.0)	2 (3.7)	0	2 (3.7)	53

TABLE III. Logistic regression analysis to identify factors associated with farms in Veracruz, Mexico having *Rhipicephalus microplus* ticks co-resistant to amitraz and cypermethrin*.

Variables	OR	CI (95%)	β	SE (β)	*P*
Cattle farm size					
>50 animals	1	NA	NA	NA	NA
≤50 animals	3.84	1.07–13.70	1.34	0.65	0.038
Stocking density					
≤1 AU/ha	1	NA	NA	NA	NA
>1 AU/ha	4.21	1.00–17.71	1.43	0.73	0.050
Acaricide rotation					
Yes	1	NA	NA	NA	NA
No	3.71	0.76–18.07	1.31	0.80	0.104

* OR, odds ratio; CI, confidence interval; β, beta value; SE (β), standard error of beta; AU/ha, animal unit per hectare; *P*, probability, NA, not applicable.

interval and recommended dose, and (3) for co-resistance to both acaricides, cattle farm size, stocking density, and acaricide rotation. The cattle farms with ≤50 animals (OR = 3.84, 95% CI = 1.07–13.70, $P = 0.038$) and a stocking density of >1 animal unit per ha (AU/ha) (OR = 4.21, 95% CI = 1.0–17.71, $P = 0.050$) had a higher probability of developing *R. microplus* populations that were co-resistant to both acaricides (Table III). There were no factors associated with resistance to amitraz and cypermethrin.

DISCUSSION

In Mexico, the main method of controlling *R. microplus* is through the use of OP, SP, and amitraz (Rodríguez-Vivas, Alonso-Díaz et al., 2006; Rodríguez-Vivas, Rodríguez-Arevalo et al., 2006 ; Rodríguez-Vivas et al., 2007). Nevertheless, their efficacy has been reduced due to the presence of *R. microplus* that are resistant to these chemicals (Alonso-Díaz et al., 2006; Cabrera-Jimenez et al., 2008). Although Veracruz State has the largest population of cattle in Mexico, to our knowledge epizootiological studies about the prevalence of *R. microplus* tick populations resistant to acaricides, and the factors associated with its presence, have not been reported. These observational studies are necessary to understand the status of *R. microplus* resistance to acaricides, and the factors associated with its presentation, in order to avoid its dispersal and mitigate the negative economic impact on the cattle industry and public health.

In the present study, the prevalence of cattle farms with *R. microplus* resistant to cypermethrin (90.6%; 48/53) was similar to that reported by Mendes et al. (2011) (83.6% of populations resistant to cypermethrin) in Sao Paulo, Brazil but higher than the prevalence reported by Rodríguez-Vivas, Alonso-Díaz et al. (2006) and Villarroel et al. (2006), with 59.3% and 75.9% of cattle farms having *R. microplus* resistant to cypermethrin in Yucatan, Mexico and Santa Cruz, Bolivia, respectively. The prevalence of resistance to acaricides varies between regions depending mainly on factors such as ecological niches, cattle management, and use of acaricides (Jonsson, 1997). For example, in Veracruz State cattle producers apply acaricides more than 10 times per year while cattle producers in Yucatan, Mexico apply acaricides less than 6 times per year (Rodríguez-Vivas, Alonso-Díaz et al., 2006). Jonsson et al. (2000) mentioned that more than 6 acaricide applications per year create a risk for the development of resistant ticks because it imparts different selective pressures for *R. microplus* resistance.

In Mexico, at the beginning of 1993, amitraz began to be used intensively to control *R. microplus* tick resistance to OP and SP (Rosado-Aguilar et al., 2008). In the present study, the prevalence of cattle farms with *R. microplus* ticks that were resistant to amitraz was 54.7% (29/53). This prevalence was higher than that reported by Jonsson et al. (2000), who indicated a prevalence of cattle farms with *R. microplus* ticks resistant to amitraz of 10%, and Rodríguez-Vivas, Rodríguez-Arevalo et al. (2006) indicated 19% in Queensland, Australia and Yucatan, Mexico. These differences may be due to climatic conditions, management practices, and ecological conditions that are different between Queensland and Yucatan. Jonsson et al. (2000) and Roulston et al. (1981) suggested that the probabilities of occurrence of resistant ticks differed between regions. For example, higher resistance may be due to the short generation time seen in cattle ticks in Veracruz (Alonso-Díaz et al., 2007), which possibly results in the selection for more generations of ticks as well as creating a need for more frequent applications of acaricides (Jonsson et al., 2000).

The levels of resistance to cypermethrin found in this study, measured as survival percentage, were high. In 17.0% of cattle farms, 80–100% of the ticks survived after contact with a DD of this compound (Table II). However, the survival levels of those that were amitraz resistant were low; 45.3% of tick populations were susceptible and 30.2% of the populations had only 1 to 5% of ticks survive amitraz contact (Table II). The knowledge of resistance levels among compounds in the same family, or between families, might help to re-design strategies of tick control (Fernández-Salas et al., 2012). It is suggested that when the levels of resistance to a specific compound are greater than 80%, the use of this chemical family should be avoided. In contrast, when the levels of resistance are 30% or lower, resistance may be reversible. In fact, Rosado-Aguilar et al. (2008) suggested that resistance to amitraz decreased sharply without selection pressure within a few generations.

In the present study, a higher level of resistance, measured as survival percentage, and the prevalence of cattle farms with *R. microplus* ticks resistant to cypermethrin rather than amitraz, were found. Some possible explanations for these differences include: (1) SPs were available on the Mexican market 10 yr before amitraz, (2) SPs are applied in several forms (immersion, aspersion, and pour-on), (3) SPs have a higher residual effect than amitraz (10–12 days vs. 3–5 days), and (4) SPs are used to control both ticks and flies. Furthermore, in Mexico, several chemical compounds from the SP family are used to control arthropods on cattle (cypermethrin, deltamethrin, and flumethrin) (Rodríguez-Vivas, Alonso-Díaz et al., 2006), and cross-resistance to SPs has been found in *R. microplus* (Nolan et al., 1989), selecting for higher numbers of tick populations resistant to these chemical compounds.

Natural populations of species respond to changes that alter their communities and the natural environment. This response is an evolutionary adaptation that can be fast and complex, helping species to tolerate adverse conditions and still have opportunities for establishment (Hoffmann and Sgrò, 2011). *Rhipicephalus microplus* is a species that has the ability to accumulate different mechanisms of resistance to some acaricide families (Rodríguez-Vivas et al., 2007). In the present study, the prevalence of cattle farms with *R. microplus* ticks resistant to both cypermethrin and amitraz was 43.8%. These results agree with Mendes et al. (2011), who reported 50% of cattle farms with *R. microplus* co-resistance to SP and OP in Sao Paulo, Brazil. The multi-resistant behavior of *R. microplus* populations in farms from Veracruz State compromises the efficacy of this previously successful class of acaricides.

Several factors associated with increased probability of resistance to different acaricides were reported by Jonsson et al. (2000). The risk factors differed among the acaricides tested, i.e., farm localization, type of application, frequency of application, fly control, and grazing management. Here, farms with ≤50 animals (OR = 3.84, $P = 0.038$) and a stocking density of >1 AU/ha (OR = 4.21, $P = 0.050$) showed a higher probability of developing *R. microplus* co-resistance to both acaricides. To our knowledge, the influence of animal populations on farms (≤50 animals) has not been previously related with a higher probability of developing acaricide resistance in *R. microplus* populations. However, Bianchi et al. (2003) mentioned that this might be a factor, perhaps because those farms tend to apply more acaricide treatments and generally use aspersion systems with the risk of applying inadequate dosing of animals. However, stocking density has been considered as a possible risk factor for the development of acaricide resistance in many studies (Bianchi et al., 2003; Rodríguez-Vivas, Alonso-Díaz et al., 2006; Rodríguez-Vivas, Rodríguez-Arevalo et al., 2006), but there is no evidence to confirm this suggestion. High stocking density on a farm provides more probabilities that ticks will complete their biological cycle; this may have epidemiological implications due to the larger number of generations per year.

In conclusion, there is a high prevalence of *R. microplus* populations on cattle farms from Veracruz, Mexico that are resistant to amitraz and cypermethrin and co-resistant to both acaricides. The level of cypermethrin resistance is critical and farms with ≤50 of cattle or a stocking density of >1 AU/ha had a higher probability of developing *R. microplus* tick populations that were co-resistant to both acaricides.

ACKNOWLEDGMENTS

We are very grateful to CONACYT–Mexico for supporting Agustín Fernández Salas in his Masters Degree at FMVZ-UADY. This project was funded by Fundación Produce Veracruz (FUNPROVER) and partially by CONACYT-Fomix (YUC-2008-C06-108773). Thanks also to Dr. Felipe de Jesus Torres Acosta and Genny T. Ramirez Cruz for their comments and review of the manuscript.

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