**First documentation of ivermectin resistance in *Rhipicephalus sanguineus* sensu lato
(Acari: Ixodidae)**

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ABSTRACT

The brown dog tick, *Rhipicephalus sanguineus* sensu lato (Latreille, 1806), is an ectoparasite and disease vector of significant veterinary and public health importance that is distributed widely around the world. The intensive use of synthetic acaricides for tick control exerts a strong selective pressure for brown dog ticks to become resistant to them. Here, we investigated claims from the field regarding treatment failure associated with the use of veterinary products containing ivermectin (IVM) to control brown dog ticks infesting dogs in Yucatan state, Mexico. Dogs in six state municipalities were inspected to sample 15 *R. sanguineus* s.l. populations. Interviews were conducted with dog owners to gather information on the history of dog treatment with conventional acaricides and IVM. The larval immersion test was used on the progeny of adult female ticks infesting dogs to test for susceptibility to IVM. Dose–mortality regressions, lethal concentrations (LC), their confidence intervals, and slope were estimated by probit analysis. Ten tick populations (66.7%) were classified as resistant compared with the most susceptible population. A high inter–population variation in the phenotypic level of IVM resistance was evident (resistance ratio at LC_{50%} and LC_{99%} ranged from 1.0 to 30.5, and from 1.0 to 458.8, respectively). Tick populations classified as resistant were collected from dogs known to have been treated with IVM. To our knowledge, this is the first report of IVM resistance in *R. sanguineus* s.l. worldwide. Veterinary and pet owner education on integrated tick management practices is required to avoid widespread resistance and increased treatment failure with products containing IVM and other macrocyclic lactones used to control endo– and ectoparasites affecting dogs. Integrated tick management will also help mitigate the burden of brown dog tick-borne diseases on human and animal populations.

Keywords. Ivermectin, Resistance, *Rhipicephalus sanguineus* sensu lato, Brown dog tick, Dog, Mexico.

1. Introduction

The brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae), is an ectoparasite and disease vector of significant veterinary and public health importance (Dantas-Torres et al., 2006; Rodriguez–Vivas et al., 2016a). Results from genetic crosses and molecular experiments indicate that the use of the term *R. sanguineus* sensu lato is preferred over *R. sanguineus* sensu stricto to describe populations of this species complex (Nava et al., 2015). Pathogens known to be transmitted by the brown dog tick include *Rickettsia* species, *Ehrlichia canis*, *Hepatozoon canis*, *Babesia vogeli* and *Anaplasma platys* (Rodriguez–Vivas et al., 2005; Dantas–Torres, et al., 2006; Pat–Nah et al., 2015). Together with its cosmopolitan distribution, the habit of biting dogs, other animals, and humans make the brown dog tick an important vector of zoonotic emerging and re-emerging diseases (Dantas-Torres et al., 2012; Esteve-Gassent et al., 2016).

Chemicals, including organophosphates, pyrethroids, amitraz and macrocyclic lactones are used widely to control *Rhipicephalus* ticks in livestock and pets (Ômura and Crump, 2004; Rodriguez–Vivas et al., 2010; Guerrero et al., 2014). However, the high frequency of acaricide treatments exerts a strong selective force on tick populations to become resistant to them. In the case of *R. sanguineus* s.l., this resulted in the development of resistance to permethrin in the United States (Eiden et al., 2015), to amitraz in Panama (Miller et al., 2001), and to amitraz and cypermethrin in Mexico (Rodriguez–Vivas et al., 2016b). Acaricide resistance is a threat to efforts to mitigate the risk of brown dog tick-borne disease transmission to humans that focus on chemical treatment to control dog infestations.

Ivermectin (IVM) is a semi-synthetic antiparasitic drug from the macrocyclic lactone family that is used extensively in dogs due to its efficacy against susceptible populations of several parasitic nematode and arthropod species (Nolan and Lok, 2012; Campbell, 2016). In some countries IVM is used to control brown dog tick infestations in dogs. Commercial injectable IVM formulations applied subcutaneously to dogs controlled brown dog tick infestations (Morsy and Haridy, 2000; Dias et al., 2005). A pour-on formulation was also effective at controlling severe naturally occurring brown dog tick infestations (Roy and Roy, 2010).

Several “similar” (generic) products have entered the veterinary market worldwide since IVM was first launched to protect animal health in 1981 (Ömura, 2008). More than 60 different IVM formulations are currently registered for use in veterinary medicine (Suarez et al., 2013). In Mexico, IVM products have been used to control intestinal nematodes and ticks affecting cattle (Aguilar-Tipacamu and Rodriguez-Vivas, 2003; Rodriguez-Vivas et al., 2006). In some countries the use of commercial injectable IVM products is promoted as “vaccination” against brown dog tick infestation in dogs (Junquera, 2016). Commercial IVM products labeled for use in cattle and pigs are known to be administered to dogs subcutaneously or orally depending on the formulation type (Rodriguez-Vivas pers. comm.).

Resistance to macrocyclic lactones has been documented in several species of gastrointestinal nematodes (Felippelli et al., 2014; Alegria-Lopez et al., 2015), and *R. microplus* ticks (Klafke et al., 2010; Pérez-Cogollo et al., 2010a, b). Failure of IVM treatment against *R. sanguineus* s.l. infestation in dogs has been reported by veterinarians and dog owners in Mexico. The main objective of this study was to assess the veracity of

those reports from the field. Here, we demonstrate for the first time that *R. sanguineus* s.l. populations are resistant to IVM.

2. Materials and methods

2.1. Study area

This study was conducted in the state of Yucatan, Mexico, located between 19° 30' and 21°35'N latitude and 90° 24' W longitude. The climate in Yucatan is sub-humid tropical with a summer rainy season. The monthly mean temperature is 26.6 °C, and relative humidity (RH) varies from 65 to 100% (mean 80%). Annual rainfall across the state can vary from 415 mm to 1290 mm (INEGI, 2002).

2.2. Ticks and questionnaire

Engorged female *R. sanguineus* s.l. were collected from dogs in six municipalities of Yucatan, Mexico (Panaba, Merida, Tizimin, Muna, Calotmul, and Valladolid). The number of *R. sanguineus* s.l. populations sampled according to municipality and collection site is shown in Table 1.

Ticks were manually removed with the aid of fine-point forceps as close to the dog's skin as possible to avoid compromising their mouthparts (Gammon and Salam, 2002). Specimens were taken to the Veterinary Parasitology Laboratory at the Campus of Ciencias Biológicas y Agropecuarias of Universidad Autónoma of Yucatán (CCBA–

UADY) for taxonomic classification, which was accomplished with the aid of taxonomic keys described by Rodríguez-Vivas and Cob-Galera (2005), and morphological comparison with available images.

Engorged females were placed in Petri dishes, with the cover perforated to allow ventilation, and then transported to the Laboratory of Veterinary Parasitology at CCBA-UADY. Upon arrival, engorged ticks were washed and immediately incubated under laboratory conditions at 27 ± 1.5 °C, 70–80% relative humidity (Cen-Aguilar et al., 1998), and photoperiod (LD 12 : 12 h) to allow for egg laying and hatching. Live larvae of 14–21 days of age were used for resistance bioassays.

Interviews were conducted with dog owners at residential homes, visiting veterinary clinics, and at cattle farms where *R. sanguineus* s.l. engorged female ticks were collected to gather information on places where the dogs lived (municipalities), and on conventional acaricide and IVM used in the last five years trying to control brown dog tick populations (Table 1).

2.3. Dose-response bioassays

The larval immersion test was used to test IVM resistance *in vitro* of *R. sanguineus* s.l. according to the method described by Rodríguez-Vivas et al. (2014). 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 with 2% Triton X-100 (Sigma-Aldrich, USA) was diluted at 1% in distilled water (Eth-TX 1%). The IVM stock and Eth-TX solutions were then mixed. Eleven doses of IVM in Eth-TX were obtained through 50% serial dilutions from the top 0.32% concentration. The immersion solution concentrations

(%) tested were: 0.32, 0.16, 0.08, 0.04, 0.02, 0.01, 0.005, 0.0025, 0.00125, 0.0006 and 0.0003. Eth-TX 1% was used as the control solution. An aliquot (0.75 mL) of each test concentration was 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 and periodically agitated. The tubes were then opened and approximately 100 larvae were transferred with another paintbrush to a filter paper 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. Thereafter, mortality was determined.

2.4. Mortality data analysis

To estimate 50% and 99% lethal concentrations (LC), with their respective confidence intervals of 95% (CI_{95%}), bioassay mortality data were submitted for probit analysis using the POLO PLUS software (LeOra Software, 2004). Given the unavailability of a reference susceptible strain, the field population herein referred to as “Mer-PA” with the lowest LC_{50%} and LC_{99%} was used to estimate the resistance ratio (RR). Differences between LC_{50%} and LC_{99%} estimates of the tested tick population and the “Mer-PA” population were designated as statistically significant if their LC_{95%} did not overlap (Robertson and Preisler, 1992).

Susceptibility or resistance status to IVM for every *R. sanguineus* s.l. population sampled was determined if both RR at 50% and 99% were statistical different from the Mer-PA population.

3. Results

Fifteen brown dog tick populations were sampled by collecting specimens from dogs at residential homes (5), veterinary clinics (7), and cattle farms (3) (Table 1). Tick product use information provided by the individuals responsible for the dogs revealed that the ticks had been exposed to acaricides (amitraz, coumaphos, cypermethrin, or fipronil). Sixty percent (9/15) of the locations reported treatment of the dogs with commercial products containing IVM.

The slopes, LC estimates of technical grade IVM to kill 50 and 99% of *R. sanguineus* s.l. representatives of the 15 populations together, and their respective LC_{95%} are shown in Table 2. Ten tick populations (66.7%) were classified as resistant compared with the Mer-PA population. A high inter-population variation in the phenotypic level of IVM resistance was evident (RR at LC_{50%} and LC_{99%} ranged from 1.0 to 30.5, and from 1.0 to 458.8, respectively).

The most IVM resistant tick population (M-A) was found in the municipality of Muna. These ticks were collected from dogs at a veterinary clinic, and had been treated with IVM, amitraz, coumaphos, and fipronil (Table 1). All the tick populations classified as resistant were reported to receive IVM treatment in the last five years. Only one (1/5) population classified as susceptible (Vallad) was reported to be treated with IVM.

4. Discussion

Our results showed phenotypic resistance to IVM in *R. sanguineus* s.l. infesting dogs in Yucatan state, Mexico. To our knowledge, and based on previous reports in the

scientific literature (Cafarchia et al., 2015), this is the first documentation of resistance to IVM in the brown dog tick worldwide. The efficacy of IVM at approved concentrations for dogs against ticks like the brown dog tick was considered to be determined (Nolan and Lok, 2012). Doses of IVM applied subcutaneously reaching 0.6 mg/kg were reported efficacious against demodicosis (Mueller et al., 2012; Walther et al., 2015). But, such relatively high doses may pose safety risks when used in IVM-sensitive dogs with the MDR1 mutation (Firdova et al., 2016).

The exact mechanisms of IVM action and resistance remain to be elucidated and appear to vary between organisms. IVM inhibits signal transmission at the neuromuscular junction of some arthropods by stimulating release of the inhibitory neurotransmitter gamma-aminobutyric acid from presynaptic nerve terminals. However, IVM was found to bind selectively and with high affinity to glutamate-gated chloride channels, commonly found in invertebrate nerve and muscle cells (Burkhart, 2000). Resultant increased cell membrane permeability with respect to chloride ions causes hyperpolarisation of the cells, resulting in paralysis and death of the parasite (Turner and Schaeffer, 1989). Compounds of this class may interact with other ligand-gated chloride channels, such as those gated by the neurotransmitter gamma-amino-butyric acid. Furthermore, Pohl et al. (2011) demonstrated that the ABC transporter efflux pump is a defense mechanism against IVM in *R. microplus*.

The development of acaricide resistance in a tick population is dependent on the frequency of occurrence of resistant individuals in the population and the intensity of chemical selection pressure (Kunz and Kemp, 1994). IVM insensitivity or differential responsiveness has emerged as a major problem in a number of intestinal nematodes of goats, sheep, and cattle, with most cases being related to intensive IVM use and its detection taking months to years depending on surveillance efforts (Sangster and Gill,

1999; Alegria–Lopez et al., 2015). *R. microplus* resistant to IVM has been reported in Brazil (Klafke et al., 2006, 2010; Oliveira, 2015), Uruguay (Castro–Janer et al., 2011), India (Singh et al., 2015), and Mexico (Perez–Cogollo et al., 2010a, b; Fernández–Salas et al., 2012; Rodriguez–Vivas et al., 2014).

Products containing macrocyclic lactones, have been used widely to control endo– and ectoparasites on cattle (IVM, moxidectin, doramectin) and dogs (IVM, selamectin) in Mexico (Rodriguez–Vivas et al., 2006). Resistance to treatment in a tick population is a risk associated with the intense use of a product containing a particular acaricide, endectocide in the case of macrocyclic lactones, as the active ingredient(s), or other products with chemically related acaricides sharing the mode of action. IVM is administered in dogs at different concentration, orally or subcutaneous as single or repeated treatments (Walther et al., 2015); some veterinarians may choose to administer IVM at higher “off-label” doses to treat dogs for endo– and ectoparasites (i.e. 0.2–0.6 mg/kg for sarcoptic mange, Mueller, 2004; 0.2 mg/kg for microfilaria of *Dirofilaria immitis*, Bolio-Gonzalez et al., 2007). There has also been “off-label” use of the macrocyclic lactones to remove adult worms – so called ‘slow-kill’ (M. Bolio-Gonzalez, pers. comm.), which involves multiple drug administrations. These drug regimens could also place selective pressure for IVM resistance in *R. sanguineus* s.l. infesting dogs treated for helminth infections. A similar situation was reported in cattle and red deer where IVM treatment against gastrointestinal nematode infections was a predisposing factor for resistance to that endectocide in the cattle tick *R. microplus* (Rodriguez-Vivas et al., 2014; Alegria–López et al., 2015). Thus, the resistance to IVM detected in the six municipalities of Yucatan is not unexpected. Sixty seven percent of the studied tick populations and a substantial inter–population variation in the phenotypic level of resistance was evident.

In the present study, all tick populations classified as resistant were reported to receive IVM treatment in the last five years. The antiparasitic broad spectrum of IVM, together with the increasingly affordable pricing of generic products containing IVM, and “off-label” doses to treat other parasites, has promoted its intensive use on dogs in Mexico. In Yucatan, Mexico, this intensive use of IVM in the last decades may have contributed to the selection of resistance to this endectocide in *R. sanguineus* s.l. populations as it has already happened with the cattle tick *R. microplus* (Pérez–Cogollo et al., 2010a, b). Early detection of resistance is necessary in order to avoid further selection of resistant ticks using the same active ingredient and to delay the spread of resistance. Further surveys are urgently needed because we lack detailed information on the epidemiology of IVM resistance in brown dog tick populations infesting dogs in Mexico and other parts of the world.

In conclusion, populations of *R. sanguineus* s.l. in Yucatan state, Mexico show phenotypic resistance to IVM. Dogs were infested with *R. sanguineus* s.l. populations that have substantial inter–population variation in the phenotypic level of resistance. Veterinary and pet owner education on integrated tick management practices is required to avoid widespread resistance and increased treatment failure with products containing IVM and other macrocyclic lactones used to control endo– and ectoparasites affecting dogs (Pérez de León et al., 2014). Integrated tick management will also help mitigate the burden of brown dog tick-borne diseases on human and animal populations (Dantas-Torres et al., 2012; Esteve-Gassent et al., 2016).

Acknowledgements

The USDA is an equal opportunity provider and employer.

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Table 1. Populations of *Rhipicephalus sanguineus* sensu lato infesting dogs collected in six municipalities from Yucatan, Mexico.

Municipality	Geographic coordinates	Tick population ID	Collection site	History of acaricide and IVM use
Merida	20°98'40"N, 89°63'95"W	Mer-G	Residential home	IVM, amitraz, fipronil
		Mer-E	Residential home	Amitraz
		Kan	Residential home	IVM, amitraz, fipronil, coumaphos
		Mer-EM	Veterinary clinic	Amitraz, coumaphos
		Mer-PA	Veterinary clinic	Amitraz, coumaphos, fipronil
		Mer-MA	Veterinary clinic	Amitraz, coumaphos
		V-YB	Veterinary clinic	Amitraz, coumaphos, cypermethrin
		V-YA	Veterinary clinic	Amitraz, coumaphos
		Caucel	Residential home	IVM, amitraz
Valladolid	20°41'22"N, 88°12'06"W	Vallad	Residential home	IVM, amitraz
Muna	20°45'32"N, 89°73'82"W	M-A	Veterinary clinic	IVM, amitraz, coumaphos, fipronil
		M-B	Veterinary clinic	IVM, amitraz, coumaphos, fipronil
Tizimin	21°25'72"N, 87°84'78"W	Tiz	Cattle farm	IVM, amitraz, cypermethrin
Calotmul	21°01'08"N, 88°10'37"W	Calot	Cattle farm	IVM, amitraz, cypermethrin
Panaba	21°17'47"N, 88°16'14"W	Yal	Cattle farm	IVM, amitraz, coumaphos, fipronil

ID= Identification, IVM= ivermectin

Table 2. Fifty % and 99% lethal concentration estimates for ivermectin resistance ratios at 50% and 99% of *Rhipicephalus sanguineus* sensu lato populations sampled from dogs residing at different municipalities in Yucatan state, Mexico.

Tick Population*	Municipality	Slope	LC _{50%} (CI _{95%})	LC _{99%} (CI _{95%})	RR _{50%} **	RR _{99%}
M-A	Muna	0.82	0.061 (0.48–0.078)	41.3 (12.8–242.6)	30.5b	458.8b
Caucel	Merida	1.32	0.052 (0.041–0.065)	2.97 (1.46–8.47)	26b	33b
Tiz	Tizimin	1.95	0.036 (0.030–0.028)	0.56 (0.38–0.95)	18b	6.2b
Calot	Calotmul	1.70	0.033 (0.022–0.046)	0.76 (0.33–4.39)	16.5b	8.4b
M-B	Muna	1.08	0.029 (0.021–0.038)	4.14 (1.8–14.2)	14.5b	46b
V-YB	Merida	1.43	0.022 (0.014–0.030)	0.92 (0.35–6.93)	11b	10.2b
Kan	Kanasin	1.67	0.020 (0.013–0.026)	0.48 (0.26–1.30)	10b	5.3b
Mer-EM	Merida	1.92	0.017 (0.015–0.020)	0.28 (0.18–0.49)	8.5b	3.1b
Yal	Panaba	1.70	0.009 (0.006–0.012)	0.20 (0.143–0.387)	4.5b	2.2b
Mer-G	Merida	0.75	0.007 (0.004–0.010)	8.26 (3.25–34.0)	3.5b	91.7b
Vallad	Valladolid	1.12	0.006 (0.0050–0.0080)	0.74 (0.38–1.81)	3b	8.2a
Mer-E	Merida	1.78	0.006 (0.003–0.009)	0.12 (0.077–0.29)	3b	1.3a
V-YA	Merida	2.77	0.004 (0.0033–0.0049)	0.027 (0.017–0.054)	2b	0.3a
Mer-MA	Merida	1.36	0.003 (0.002–0.005)	0.17 (0.10–0.37)	1.5a	1.8a
Mer-PA	Merida	1.00	0.002 (0.0016–0.0025)	0.09 (0.038–0.43)	NA	NA

NA: Not applicable, LC= Lethal concentration, CI= confidence interval, *See Table 1 for more information on the tick population source. **Different letters in the column show statistical differences. Tick populations were considered statistically different ($p < 0.05$) when the CI_{95%} of the LC_{50%} did not overlap with the Mer-PA population.