Veterinary Parasitology 197 (2013) 288–296

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

In vitro and *in vivo* evaluation of cypermethrin, amitraz, and piperonyl butoxide mixtures for the control of resistant *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) in the Mexican tropics☆

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ARTICLE INFO

Article history:
Received 12 April 2013
Received in revised form 8 July 2013
Accepted 11 July 2013

Keywords:
Rhipicephlaus (*Boophilus*) *microplus*
Acaricide
Pyrethroid
Formamidine
Synergist
Mixture
Resistance
Tick control

ABSTRACT

A study was conducted to evaluate the efficacy of cypermethrin, amitraz, and piperonyl butoxide (PBO) mixtures, through *in vitro* laboratory bioassays and *in vivo* on-animal efficacy trials, for the control of resistant *Rhipicephalus* (*Boophilus*) *microplus* on cattle in the Mexican tropics. Also, to examine mechanisms of resistance to cypermethrin in this tick population, the frequency of a mutated sodium channel gene (F1550I) was determined using a PCR assay. Results of laboratory bioassays using modified larval packet tests revealed that cypermethrin toxicity was synergized by PBO (from 46.6–57.0% to 83.7–85.0% larval mortality; $P < 0.05$). The cypermethrin and amitraz mixture showed an additive effect (from 46.6–57.0% to 56.0–74.3% larval mortality). Strong synergism was observed with the mixture of cypermethrin + amitraz + PBO and this mixture was the most effective killing resistant tick larvae *in vitro* (96.7–100% of larval mortality). Tick larvae surviving exposure to cypermethrin or mixtures either with amitraz and PBO *in vitro* showed 2.9–49.6 higher probability to present the mutated allele than those killed by acaricide treatment ($P < 0.05$). In the *in vivo* trial, the mixtures containing cypermethrin + PBO (80.6–97.3%), and cypermethrin + amitraz (87.0–89.7%) were more efficacious than cypermethrin alone (76.3–80.5%). The highest level of efficacy was obtained with the mixture of cypermethrin + amitraz + PBO, which yielded >95% control that persisted for 28 days post-treatment against *R. microplus* infesting cattle when tested under field conditions in the Mexican tropics. Although this mixture is a potentially useful tool to combat pyrethroid resistance, a product based on an acaricide mixture like the one tested in this study has to be used rationally.

☆ This study was conducted under a Cooperative Research Agreement between University of Yucatan and USDA, ARS, Knipling-Bushland U.S. Livestock Insects Research Laboratory (agreement 58-6205-1-060FN and UADY registration: FMVZ-2012-0005). This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or a recommendation by the USDA for its use. The USDA is an equal opportunity provider and employer.

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0304-4017/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.vetpar.2013.07.018

1. Introduction

Rhipicephalus (*Boophilus*) *microplus* (Canestrini) is a haematophagous arachnid (Acari: Ixodidae) recognized globally as an economically important ectoparasite of cattle in tropical and subtropical agroecosystems (Rodriguez-Vivas et al., 2012). This tick species inflicts significant economic loss to livestock production by direct effects associated with its obligate blood feeding habit and secondarily through the infectious agents it can transmit, principally *Babesia bovis*, *B. bigemina*, and *Anaplasma marginale*, during infestation, which can kill cattle (Solorio et al., 1999; Rodriguez-Vivas et al., 2004). Acaricides have played a pivotal role in the control of *R. microplus*. However, populations of this invasive tick species around the world have developed resistance to all major classes of acaricides due to the sustained use of these pest management tools (Rodriguez-Vivas et al., 2006b, 2011; Chevillon et al., 2007; Perez-Cogollo et al., 2010).

Resistance to synthetic pyrethroids is one of the most serious problems in tick control worldwide, particularly in parts of the world where *R. microplus* is endemic now such as Australia, Africa, North America and South America (Jonsson et al., 2000; Rodriguez-Vivas et al., 2007; Andreotti et al., 2011). Once established in a given tick population, resistance becomes a serious obstacle to the continued and effective use of acaricides including pyrethroids (Eisler et al., 2003; Ahmed and Matsumura, 2012). It is very important to formulate effective resistance management strategies to avoid or delay the development of pyrethroid resistance among ticks.

Different pesticide resistance management strategies, including rotation of pesticides and use of pesticide mixtures or PBO-synergised pesticides, have shown to be effective in controlling resistant arthropods (Curtis, 1985; Corbel et al., 2003; Li et al., 2004, 2007). However, acaricide rotation may no longer be a good option for tick control in some areas of Mexico, as many tick populations have developed resistance to multiple classes of acaricides (Rodriguez-Vivas et al., 2007; Miller et al., 2013).

It is often assumed that acaricides typically act in synergy (*e.g.* Li et al., 2007; Barré et al., 2008); however, it is important to note that synergies are one specific type of non-additive interaction, which occurs when the combined impact of several acaricides is greater than the algebraic sum of the effects of individual acaricides (Darling and Cote, 2008). Knowles (1982) noted that the formamidine pesticides chlordimeform and amitraz can act as a synergist of organophosphate, organochlorine, carbamate, and pyrethroid insecticides. Subsequent publications confirmed the synergism between amitraz, and pyrethroids in insects and ticks (Usmani and Knowles, 2001; Li et al., 2007). Recent studies also demonstrated synergism between amitraz and fipronil against ticks, and pyrethroids and neonicotinoids against mosquitoes (Prullage et al., 2011; Ahmed and Matsumura, 2012). Li et al. (2007) showed under laboratory conditions that adding amitraz to permethrin led to a dramatic increase in larval mortality of a highly pyrethroid-resistant strain of *R. microplus*. The synergism between deltamethrin and amitraz was subsequently confirmed in a field trial at a dairy farm in New Caledonia (Barré et al., 2008). However, the efficacy of pyrethroids with synergists to control natural infestation of pyrethroid-resistant *R. microplus* populations has not been evaluated in the Mexican tropics.

Metabolic enzyme defense systems like the cytochrome P450 monooxygenases (P450s) and esterases are present at a ‘baseline level’ in arthropods. In resistant arthropods, their activity can be elevated to detoxify pesticides (Hemingway and Ranson, 2000; Young et al., 2006). Piperonyl butoxide (PBO) is a synergist that inhibits these metabolic enzyme systems, which enhances the toxicity of several pesticides, including pyrethroids (Bingham et al., 2008). A clearer inhibitory effect of PBO on the enhanced metabolic enzyme systems is noted in resistant arthropods (Moores et al., 2009). Li et al. (2010) showed *in vitro* that adding PBO to permethrin and amitraz markedly increased larval mortality in multiresistant Mexican strains of *R. microplus*. The main mechanism of resistance to pyrethroid acaricides in Mexican populations of *R. microplus* is a mutation (F1550I, He et al., 1999) in the S6 transmembrane segment of the domain III of the sodium channel (*Na+*) gene (Rosario-Cruz et al., 2009; Rodriguez-Vivas et al., 2012; Guerrero et al., 2012). Although, other mutations corresponding to the domain II S4-5 linker region (L64I and G72V) have been identified and associated with pyrethroid resistance in populations of *R. microplus* in Australia (Jonsson et al., 2010), this mutation has not been identified in Mexican isolates.

It was speculated that amitraz may act as a target site synergist by enhancing the binding of pyrethroid acaricides to the *Na+* channel, which is the known target of pyrethroid action (Barré et al., 2008). If amitraz indeed works as a target site synergist, the amitraz-synergized pyrethroid acaricide formulation may help to eliminate resistant individuals of the RR genotype. To test such hypothesis, *in vitro* toxicity bioassay experiments with PCR assays were conducted to test the relationship between the *Na+* channel F1550I mutation frequency and tick mortality. Toxicities of cypermetrhin, amitraz, and PBO alone or in mixtures were evaluated against tick larvae in laboratory bioassays. To corroborate the synergist effect found *in vitro*, an *in vivo* field study was carried out to control natural infestation of *R. microplus* ticks on cattle in the Mexican tropics.

2. Materials and methods

2.1. In vitro study

2.1.1. Study area

The present study was carried out in the State of Yucatan, Mexico, which is located between 19°30′ and 21°35′ N latitude and 90°24′ W longitude of the Greenwich meridian. Yucatan has a sub-humid tropical climate with a rainy season, from June to October, and a dry season, from November to May (INEGI, 2002). The monthly maximum temperature varies between 35 and 40 °C (mean 26.6 °C). Relative humidity (RH) varies from 65% to 100% (mean 80%) and the annual rainfall varies from 415 to 1290 mm depending on the location.