

Genetic basis and impact of tick acaricide resistance

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1. ABSTRACT

Acaricide resistance in *Boophilus microplus* has been studied for the last 20 years from the toxicology, metabolic and genomic points of view, however, only few methods for molecular detection of resistance have been developed. Despite the relatively poor sensitivity for resistance detection, bioassays remain the method of choice for susceptibility evaluation of tick populations, based on their toxicological response after exposure to acaricides. Metabolic detoxification of acaricides is known to be mediated by multigene- families of enzymes such as GST, Esterases and Mixed Function Oxidases (cytochrome P450). In addition, target site insensitivity has been studied on the sodium channel and acetylcholinesterase genes. The use of genomics to understand acaricide resistance in *B. microplus* will play a major role in unraveling the molecular mechanisms of resistance. Advances in genomics, will accelerate the development of new diagnostic and immunoprophylactic tools based on new vaccine candidates, and new molecular targets for acaricide resistance detection and improvement of strategies for the control of ticks and tick-borne diseases in tropical and subtropical areas of Mexico.

2. INTRODUCTION

Insecticide resistance is a major obstacle in the control of agricultural pests and medical and veterinary important insect species. The World Health Organization has recognized this and called insecticide resistance "The biggest single obstacle in the struggle of vector-borne disease" (1). The cattle tick *B. microplus* is one of the most important vectors of cattle diseases (Babesiosis and Anaplasmosis) in Mexico (2). These diseases cause great economic losses if not controlled, and the industry presently relies heavily on the "no ticks/no tick borne diseases" theory of disease control and encourage the heavy use of chemicals to control the tick vectors. In México and other latin american countries, acaricide resistance is becoming a major problem. *B. microplus* resistance to organophosphorous (OP) compounds such as coumaphos, chlorpyrifos, chlorphenvinphos and recently pyrethroids and amitraz have been recorded and in some areas these acaricides are no longer effective (3,4).

In addition to the increasing problem of acaricide resistance, another limiting factor is the reliance on standardized bioassay techniques such as the Larval Packet

(LPT) test (5) for resistance detection. The LPT and other bioassay techniques are both slow to produce results (6 weeks) and time consuming to perform. Therefore, the development of a new resistance detection methods, based on a molecular and biochemical approach, would represent a significant step forward to more rapid and sensitive detection of resistance in ticks.

The presence of ticks resistant to acaricides is a major problem for cattle introduction into the U.S. under the commercial agreement perspective. On the other hand restriction on cattle movement between Mexico and the U.S. due to tick resistance will impact Mexican producers. Biotechnology can be helpful in three major areas: vaccine development for tick control, identification of molecular markers for acaricide resistance detection, and new drugs development, in order to mitigate the problems due to the multiple resistance, already present in different countries around the world.

RNAi technology has currently proven to be the most important tool for massive analysis of potential vaccine candidate genes in a relative short time. Therefore, major contributions of a functional genomic approach, in combination with analysis of expressed sequence tags (ESTs), proteomics and metabolomics can be made in finding new molecular targets for acaricide resistance, necessary for new diagnostic tools that substitute for traditional bioassay, and in the near future probably new drug discovery.

3. EVOLUTIONARY ASPECTS OF PESTICIDES RESISTANCE

Resistance to pesticides is a genetic condition that confers an arthropod population the capability of adaptation, to succeed before a toxic environment, promoted either naturally (due to preadaptative condition of natural selection of genes associated to hydrolysis of allelopathic compounds in nature) or, artificially (by application of pesticides to control pests) (6). Based on the principle that all organisms derive from a common ancestral organism, resistance to xenobiotics imply a chemical evolution and adaptative mechanisms that confers the arthropod the ability to survive the allelopathic compounds produced by the plants they feed on as a result of co-evolution between arthropods and plants.

This co-evolution mechanism derived from allelochemical interactions between plants and arthropods has been the major driving force of the chemical evolution of resistance to all kind of allelopathic compounds in arthropods, and has led to different kinds of specialized arthropods. Monophagous arthropods feed from one single plant and therefore are capable of degrading the allelopathic compound produced from that particular plant. Polyphagous arthropods feed on different plant species, and therefore, are capable of degrading a larger number of allelopathic

compounds. These two groups of arthropods are as different as their metabolic defenses against allelopathic compounds. Polyphagous arthropods have impressive metabolic machinery, as they have to deal with many different compounds. In contrast, defenses of monophagous arthropods rely on very specialized mechanisms of detoxication, thus, expression of a single enzyme would suffice to hydrolyze one single compound. Likewise the presence of a single nucleotide polymorphism on a DNA sequence can produce the same effect on the product responsible of the genetic capability to survive the toxic effects of allelochemical compounds. Insects very often depend on a complex of “generic” detoxifying enzymes, to eliminate the potential toxic effects of the plants they feed (7).

In contrast to the slow evolution of resistance to allelopathic compounds, derived from hundreds of millions of years of co-evolution, resistance to synthetic pesticides has arisen extremely fast, probably because arthropods can use the same defense mechanisms developed to degrade allelochemical compounds (8) with very similar structure of synthetic pesticides, including such as pyrethroids molecules, which are analogs of pyrethrins produced by *Chrysanthemum* spp.

Additionally, resistance emerges rapidly when a pesticide is intensively used to control an arthropod population, susceptible individuals are eliminated and the pesticide becomes the most important driving force for appearance of resistance, probably due to mutation on the genes encoding detoxifying enzymes (Esterases, Glutathion-S-Transferases, and Mixed function oxidases) and random variation and selection processes (genetic drift) occurring within sequences of genes also associated with the resistance to xenobiotic compounds.

4. ACARICIDE RESISTANCE IN MEXICO

Livestock production in tropical and subtropical areas of the world is limited by the presence of the cattle tick *B. microplus*, the most important vector of bovine Babesiosis (9). *B. microplus* and *B. annulatus* were eradicated from the United States after an intense eradication program which extended from 1907 to 1960 (10). A similar eradication program was carried out in Mexico, from 1975 to 1985 (11) and Organophosphates (OP) were used extensively and almost exclusively, until the “Tuxpan” strain was reported as the first case of OP resistance in 1981 (12) and later on an OP-organochlorine resistant strain named “Tempoal” (13). The use of Synthetic pyrethroids (SP) and amidines (Am) was authorized for tick control in México by the end of 1985. As a consequence, resistance to SP was detected seven years later in april 1993, in samples from Tamaulipas (“Aldama” Strain resistant to flumethrin), Veracruz (“Coatzacoalcos” Strain, resistant to cypermethrin) and Tabasco (“Mora” Strain highly resistant to SP and moderately resistant to OP) (3). After the reentry of amitraz for tick control in 1994, the first case of amitraz resistance was documented in 2002 (4).

Development of *Boophilus microplus* multiple resistance in Mexico.

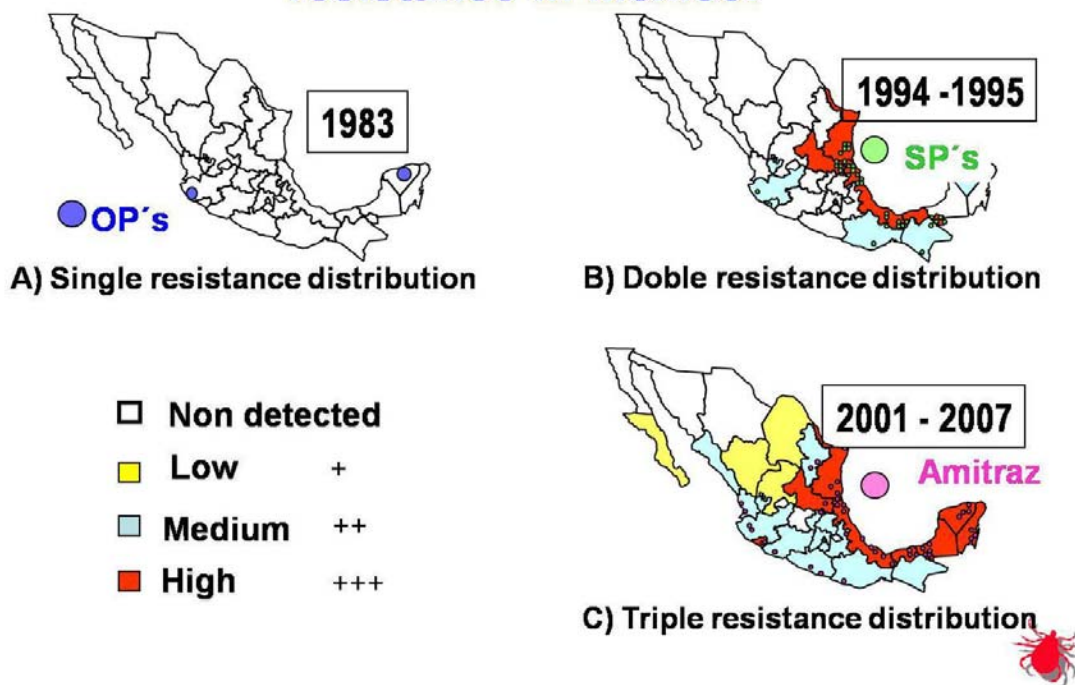


Figure 1. Map of current distribution and development of acaricide resistance in México from 1983 to 2007. (A) Map showing the first cases of resistance to organophosphorous (OP) compounds found in 1983 in México; then, synthetic pyrethroids (SP), were authorized for tick control. (B) Map showing the appearance of double resistance to OP and SP compounds in 1994-1995. (C) Map showing the accumulated distribution from 2001 to 2007 of tick populations resistant to OP, SP and amidines used as acaricides in Mexico. Courtesy of Dr. Fernando Parrodi (SENASICA-SAGARPA México).

Today, several cases of resistance to multiple groups of pesticides have been documented in Mexico (Figure 1).

A quarantine area is currently maintained along the US-Mexico borderline in order to prevent the reentry of *B. microplus* and monitor all imported livestock (10). However, widespread and increasing resistance to organophosphate and pyrethroid acaricides in Mexico is becoming a major concern to the United States, since OP (14) and pyrethroid resistant ticks have been found within the United States (15). In addition, a pyrethroid resistant tick population in the border line State of Coahuila, Mexico, (80% of homozygous resistant individuals) has been detected (16), showing that there is widespread distribution of acaricide resistance in Northern Mexico and the potential risk of resistant *B. microplus* reentry to the USA, due to cattle mobilization and wildlife.

5. MECHANISMS OF ACARICIDE RESISTANCE

Resistance to insecticides is predominantly mediated by metabolic detoxification or changes in the sensitivity of the target site. Metabolic resistance in several different species involves: esterases (17), glutathion-S-

transferases (18) or monooxygenases (19). These three enzymatic systems are regulated by three different molecular mechanisms, overexpression of detoxifying enzyme-encoding genes by DNA amplification (20, 21), overexpression by up-regulation changes, such as in cytochrome P450 genes (22, 23) and structural changes in insecticide-target molecules by point mutations, such as in the GABA receptor (24) and sodium channel genes (25, 26).

Esterases have been recognized, as one of the major detoxifying enzymes involved in insect metabolism of xenobiotics. One mechanism of esterase mediated chemical resistance, thus far identified in several arthropods such as in mosquitoes, aphids and ticks (27, 28, 29, 17) is associated with the massive production of hydrolyzing/sequestering enzymes.

Another esterase-mediated form of resistance involves the presence of point mutations within the active sites of *AChE* gene and this form of resistance was first described in the fruit fly (30, 31).

More recently, it has been documented that two different amino acid substitution within the same esterase gene confer resistance to alternative types of OP insecticides in the sheep blowfly *Lucilia cuprina* (32),

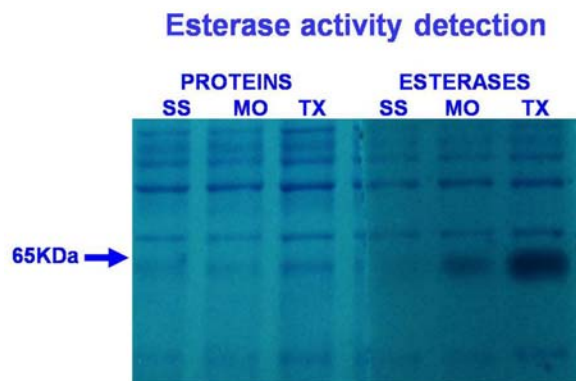


Figure 2. SDS-PAGE of tick larvae total extracts stained for protein with Coomassie Brilliant Blue (Left) and for esterase activity using alfa-Naphtyl acetate in combination with protein staining (Right), showing differences in esterase activity around 65 Kda. SS=Susceptible strain, MO=Mora Strain, and TX=Tuxpan strain, resistant to OP and SP respectively.

however, the presence of SNP's within *B. microplus* esterase gene sequences for OP resistance have not yet been found, even though, there is evidence that this group of enzymes is involved (Figure 2). The major advance for diagnostic assays has been a SNP found within the *B. microplus para*-type sodium channel gene, associated with a target site modification mechanism conferring resistance to pyrethroids (33, 34).

5.1. Metabolic detoxification

Intensive use of acaricides during the last 40 years has been a major driving force in selecting for resistance in the cattle tick *Boophilus microplus*. Most of the currently used acaricides are esters of substituted phosphoric or carbamic acids and consequently subject to hydrolysis by esterases (35). The overproduction of esterases by arthropods may result in the increased detoxification of insecticide esters by hydrolysis or sequestration (36). Increased esterase activity has been implicated with OP resistance in Mexican *B. microplus* tick strains, when compared with a susceptible reference strain (17) (Figure 2). Similarly, pyrethroid hydrolyzing esterases have also been identified in Australian *B. microplus* tick strains (37). Recently, a point mutation on an esterase gene has been identified in the Mexican Coatzacoalcos strain and a strong signal was detected by ribonuclease protection assay indicating at least fivefold higher mRNA esterase concentration compared with a susceptible strain, suggesting that esterases are an important mechanism of resistance (38, 39). Miller *et al*, (14) found that triphenylphosphate (TPP), an esterase inhibitor, increases the toxicity of pesticides indicating an esterase-based mechanism of resistance. Further studies on Coatzacoalcos strain confirmed an enhancement of general esterase activity, permethrin hydrolysis and permethrin synergism by TPP (40, 41), indicating that metabolic detoxification of pyrethroids by carboxylesterases is an important resistance mechanism in this strain. Pyrethroid resistance in Mexican tick wild populations however, do

not correlate with esterase activity, rather, correlate with the sodium channel mutations as strong correlations were found between mortality rates and resistant allele frequencies (42), apparently esterases are more related to OP resistance (Figure2).

5.2. Target site modification

Among the various molecules involved in target site modification as a mechanism of resistance to acaricides in the cattle tick *B. microplus*: AChE and the sodium channel are better characterized to date. AChE and sodium channels, are the conventional targets of two of the most widely used classes of pesticides in Mexico, OP's and synthetic pyrethroids respectively.

Insensitivity of AChE was proposed as an important mechanism of resistance in Mexican tick strains by Wright and Ahrens in 1988 (43); however there was not a physical evidence of structural modification, since the genes were not sequenced. Currently three putative sequences of *AChE* from *B. microplus* have been reported (44,45,46). However, only a biochemical method based on AChE activity on total extracts has been developed for the rapid diagnosis of OP-insensitivity and assigning of homozygous resistant (RR), heterozygous resistant (RS), and homozygous susceptible (SS) genotypes to individual ticks, males or females. *BmAChE3* gene has been for the first time expressed in a baculovirus vector, showing sensitivity comparable with that of an adult OP susceptible neural AChE (47), but there is no physical evidence of a target site insensitivity mechanism based on *AChE* gene modifications in cattle tick populations.

On the other hand, as mentioned above, pyrethroids are known to exert their insecticidal effect by altering the function of voltage-sensitive sodium channels in nerve membranes (48, 49). "Knockdown resistance" (Kdr) is the generic term applied to the resistance to pyrethroids associated with sodium channel mutations, one of the two major types of resistance to pyrethroids (50,51).

A sodium channel mutation associated with a target site insensitivity mechanism has been found in *B. microplus* (33) and a new molecular diagnostic method was developed based on the detection of the point mutation using a PCR allele specific assay (PASA) for genotyping of single individual larvae (34). PASA has been used for genotyping reference and field strains have shown a statistically significant correlation between the presence of the mutation and survival rate to the pyrethroids as a class, corroborating the central dogma of resistance to pyrethroids by the target site insensitivity mechanism (40, 42). To date this mutation has only been detected in ticks from Mexico. Australia and Brazil contain tick populations with similar pyrethroid resistance profiles as the Mexican ticks (Miller, Unpublished data). However, detection of the point mutation contained in Mexican ticks with PCR has been unsuccessful. It is very likely that other mutations of the Na channel occur in pyrethroid resistant populations in Australia, Brazil, and elsewhere.

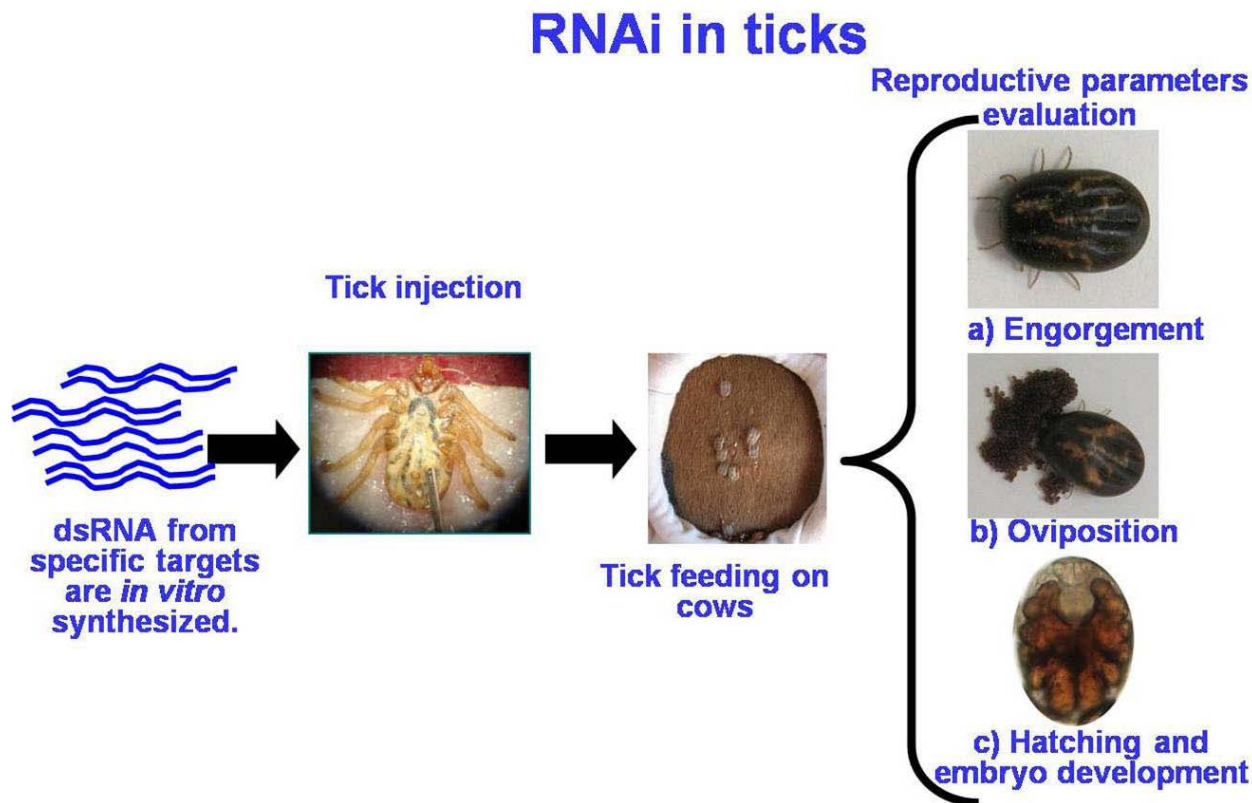


Figure 3. Experimental procedure for RNAi in *B. microplus* ticks. *B. microplus* metanymphs are detached from hosts after 14-16 days of feeding and allowed to molt *in vitro*. After 1-2 days of molting, adult females are injected with 0.2-0.5 ds RNA (1×10^{11} molecules/ μ l) and feed on cells attached to a cow until repletion is completed. Once ticks detach by themselves are weighted and kept in humidity chambers to evaluate the oviposition and hatching. Embryo development is evaluated based on morphological changes compared with controls.

6. TICK GENOMICS AND ACARICIDE RESISTANCE

Gene cloning provided an excellent tool for better understanding of acaricide resistance. Sequencing of cloned genes associated to acaricide resistance is a very precise method for analysis of mutations potentially associated to acaricide-resistant phenotypes. The study of single nucleotide polymorphisms (SNP) potentially associated to the acaricide resistance phenomenon is one of the most popular approaches in trying to find genetic markers for diagnostic purposes. However, development of PCR based technology for detection of SNP's relies on the cloning, sequence analysis and an exhaustive screening of the target genes, before a new diagnostic tool, or tick vaccine can be developed.

Availability of genome sequences and development of bioinformatic tools has created new disciplines based on reverse approaches such as reverse vaccinology and reverse genetics. Both disciplines have provided powerful tools for vaccine development, based on *in silico* analysis of genome information and the disruption of gene expression with RNA interference (RNAi).

RNAi, or the induction of sequence specific gene silencing by double stranded RNA (dsRNA), is

accomplished when expression of double-stranded RNA leads to specific decreases in the abundance of cognate mRNAs (52). dsRNAs can be delivered in a variety of ways, including introduction of large or small dsRNA directly, and through expression from appropriate expression vectors following transfection (53).

The first application of RNAi in ticks was reported by Aljamali and coworkers in 2003 (54). A dsRNA from an *A. americanum* histamine binding protein was cloned and incubated with tick salivary glands. Results showed a lower histamine binding ability, suggesting that RNAi might be a tool for target encoded gene proteins (54). Experiments *in vivo* can be performed (55) in which molecules of interest may be injected into the hemolymph in order to interrupt vital functions of the tick (Figure 3). Using this methodology, de la Fuente *et al*, in 2005 (56), demonstrated that RNAi, can be used for the screening, identification and characterization of tick protective antigens and tick genetic manipulations (57,58). Recently Nijhof and *et al*, (35) evaluated the effect of gene silencing in the progeny larvae when RNAi was induced by introducing dsRNA to *B. microplus* eggs through injection of engorged females. RNAi is currently the most important tool for massive analysis of potential vaccine candidate genes in a relative short time and with a minimal use of

laboratory animals. The use of this functional genomic approach, in combination with analysis of expressed sequence tags (ESTs), proteomics and metabolomics will be of great interest to find new molecular targets for acaricide resistance detection, the identification on new tick and tick-borne vaccine candidates, and the discovery of new drugs against ticks, despite the fact that only minimal information about a significant fraction of the tick genome is available (59). Currently, great efforts are being developed to highlight the insights in tick biology that could be derived from a tick genome sequencing project (60).

7. PERSPECTIVES

The presence of resistant *Boophilus microplus* on both sides of the border between Mexico and USA, will require the political and scientific cooperation of both countries in order to solve the problems generated by multiple-resistance tick populations in Mexico and the cattle movement within commercial agreements (North America Free Trade Agreement). Isolated efforts will not guarantee a sustainable eradication program or pesticide resistance management program for ticks resistant to acaricides and will place the U.S. eradication effort at serious risk.

The Mexican cattle industry exports an average of 1.3 millions/year of cattle heads to the U.S. and the presence of ticks resistant to acaricides will be a major problem for cattle introduction into the U.S. Restrictions on cattle movement, to avoid the re-entry of resistant ticks into the U.S., will impact Mexican producers.

Another problem associated with acaricide resistance and the increasing use of acaricides used to control ticks, is the environmental impact, human health and food safety problems derived from these activities.

Therefore, the application of genomics, proteomics and metabolomics will have to play a multifunctional role to solve the problems associated with the ticks, and tick borne diseases. The use of biotechnology will be a key instrument through tick-vaccine development, new target drugs discovery, and discovery of new molecular targets for acaricide resistance, in order to mitigate the tick resistance to acaricides problems and improvement of the control programs for ticks and tick-borne diseases in Mexico and all tropical and subtropical areas of the world.

Our scientific understanding on the molecular basis of acaricide resistance and tick vaccine development is moving forward and will have an immediate impact on parasites control solutions, however, the major challenge, in the short and long term will be the translation from scientific information to generation of suitable technologies to enhance the potential of farming systems, joint efforts of scientists and countries will be needed.

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