



Research Journal of  
**Parasitology**

ISSN 1816-4943



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## Cytochrome P-450 Monooxygenase Gene Expression Supports a Multifactorial Origin for Acaricide Resistance in *Rhipicephalus microplus*

<sup>1</sup>Raquel Cossío-Bayúgar, <sup>1</sup>Estefan Miranda-Miranda, <sup>2</sup>Alejandrina Ortiz-Nájera,  
<sup>2</sup>Salvador Neri-Orantes and <sup>2</sup>Francisco Olvera-Valencia

<sup>1</sup>Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria,  
Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias INIFAP. Carr. Fed.  
Cuernavaca-Cuautla 8534, Jiutepec, Morelos, CP 62550, México

<sup>2</sup>Departamento de ectoparásitos y dípteros Servicio Nacional de Sanidad,  
Inocuidad y Calidad Agroalimentaria SAGARPA

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**Abstract:** This study was done with the objective of establishing a relationship between cytochrome P-450 gene expression and acaricide resistance levels in Mexican isolates of *R. microplus*, for that purpose the cytochrome P-450 expression levels were measured by real time PCR quantification on acaricide sensitive and resistant strains of ticks, as well as on acaricide resistant sensitive ticks isolated from cattle at the southeastern Mexican state of Chiapas and the northern Mexican State of Tamaulipas. Acaricide susceptible ticks were used as comparison standard P-450 expression level and adjusted as a baseline of 1 Relative Expression Units (REU). A statistically significant ( $p < 0.0001$  unpaired t-test)  $3.47 \pm 0.302$  REU overall increase on the P-450 gene expression level was detected on pyrethroid resistant ticks, we also found a statistically significant ( $p < 0.0001$  unpaired t-test) P-450 expression decrement of  $0.335 \pm 0.054$  REU in multiple acaricide resistant ticks, no significant difference on cytochrome P-450 expression level was detected on the organophosphate acaricide resistant strain. Our results suggest that cytochrome P-450 monooxygenases play a role on at least some types of acaricide resistance phenomenon and the assessment of the data reveals a multifactorial causative for organophosphate and pyrethroid resistant *R. microplus*.

**Key words:** Cytochrome P-450, gene expression, RT-PCR, pyrethroid resistance, organophosphate resistance, cattle tick

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### INTRODUCTION

The presence of the Southern cattle tick *Rhipicephalus (Boophilus) microplus* and tick borne diseases such as babesiosis and anaplasmosis is an obstacle for the development of the cattle industry in tropical and subtropical cattle grazing areas around the world (de Castro, 1997). The basic strategy to prevent monetary losses due to tick infestation of the cattle and control tick transmitted diseases, depends on the chemical treatment of tick infested cattle with several acaricides formulas (de Castro, 1997). Pesticide resistance is widely spread among arthropods including *R. microplus*, this phenomenon neutralizes the control efforts of significant pests originating an adverse economic impact on agriculture and livestock industries (Scott, 1999). The scientific search for the molecular basis underlying this problem lead to the finding that some pesticide resistance mechanisms in arthropods are biochemical related to enhanced activity of metabolic enzymes such as esterases, glutathion-S-

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**Corresponding Author:** Raquel Cossío-Bayugar, Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria, Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP). Carr. Fed. Cuernavaca-Cuautla 8534, Jiutepec, Morelos, CP 62550, México  
Tel: 52 777 319 28 50/119 Fax: 52 777 3192850/129

transferases and mixed function oxidases including cytochrome P-450-dependent monooxygenases (Hemingway and Karunaratne, 1998). Cytochrome P-450 monooxygenase enzymes are ubiquitous among living organisms across the phylogenetic tree, they belong to a wide enzymatic family involved in the metabolism of xenobiotics compounds (Scott, 1999), these enzymes play a role in the regulation of endogenous bioactive molecules like hormones as well as in the metabolism and detoxification of cell damaging chemicals such as plant toxins, drugs and pesticides in a large variety of arthropods (Scott, 1999). As a result of this metabolic activity mediated by monooxygenases, some arthropods with natural high levels of cytochrome P-450 are naturally insensitive to some Pyrethroid (PS) formulations, making these chemicals useless for certain type of pest control (Scott, 1999). Extensive analysis of these highly PS tolerant pests has established that cytochrome P-450 mediated metabolism is implicated in deactivation on pyrethroid pesticides in several PS resistant arthropods including *Culex* sp. mosquitoes (Kassai, 2004), the tomato grub *Helicoverpa armigera* (Chen *et al.*, 2005) and the house fly *Musca* sp. (Kassai and Scott, 2000; Scott and Tomita, 1995). The role of cytochrome P-450 in *R. microplus* PS resistance and organophosphate OP resistance has been implicated by using the pesticide synergist piperonyl butoxide which inhibits mixed function oxidases altering the acaricide detoxification metabolism thus enhancing toxicity for pesticide (OP or PS) tolerant and/or resistant ticks (Li *et al.*, 2003; Miller *et al.*, 1999). Some other researches rely on the quantification of tagged cytochrome P-450 mRNA expression in organophosphorous resistant ticks (Guerrero *et al.*, 2007). Oxidases enzymatic function may deactivate some forms of PS acaricides and PS toxicity has been reported to be neutralized by the action of cytochrome P-450 in *R. microplus* (Miller *et al.*, 1999). OP pesticides on the other hand require a totally different approach since this family of pesticides have been designed to be metabolized by cytochrome P-450 enzymatic pathway in order to get bioactivated to a more toxic form of pesticide (Parkinson, 1996) such is the case with OP acaricides coumaphos and diazinon, for which *R. microplus* tolerance or resistance has been linked to a lower expression or enzymatic function of cytochrome P-450, in order to avoid the acaricide metabolic transformation to a toxic biotransformant (Li *et al.*, 2003). In this study we analyze cytochrome P-450 expression levels by measuring specific constitutive mRNA, using real time PCR on several sensitive and acaricide resistant ticks in order to establish a probable a role of *R. microplus* cytochrome oxidases on the acaricide resistance phenomenon.

## MATERIALS AND METHODS

This study was carried out in Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria, INIFAP, México and the Departamento de Ectoparásitos y Dípteros del Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA-SAGARPA) México during the months of November 2006 to April 2007.

### Ticks

Five *R. microplus* acaricide resistant reference ticks with known acaricide resistance or susceptibility were used (Table 1); an acaricide susceptible reference strain (Sus) was used for acaricide resistance comparison and P-450 expression baseline adjustment. An Organophosphorous (OP) resistant strain named Tuxpan (Tx); a multiple resistant strain for OP and Pyrethroids (PS) and Amidines identified as San Alfonso (SA) and an OP and PS resistant strain named Mora (MO) (Table 1). All reference tick strains have been cultured for several generations under constant exposure to acaricides and used for routine comparison against field isolated ticks on acaricide resistance monitoring programs of the Mexican Federal Government. Several tick isolates were selected for experimental purposes which included Isolate 83 (I-83) susceptible to all the acaricides used in this work; Isolate 76 (I-76) with double resistance to OP and PS; Isolates 10, 77 and 821 (I-10, I-77, I-821) with PS resistance

Table 1: Acaricide Bioassays results on different strains and isolates of ticks

Tick sample	Percentage of mortality							
	Organophosphorous				Pyrethroids			Amidines
	Chlorfenvinphos	Coumaphos	Diazinon	Chlorpiriphos	Cypermethrine	Deltamethrine	Flumethrine	
Sus	100	100	100	100	100	100	100	100
MO	0	0	0	0	0	0	0	100
SA	15	20	0	0	0	0	0	0
Tx	0	0	0	16	100	100	100	100
Isolate 10	100	100	100	100	78	80	73	100
Isolate 76	100	0	60	100	60	25	60	100
Isolate 77	100	100	94	93	0	0	0	100
Isolate 83	100	100	100	100	100	100	98	100
Isolate 821	94	98	87	99	0	0	0	100

Data is represented as percentage of mortality under a standard concentration of acaricide

(Table 1). Tick isolates were collected from cattle at locations in the northern Mexican State of Tamaulipas (I-83, I-77, I-76 and I-821) and the Southern Mexican state of Chiapas (I-10), tick isolates were cultured for one generation only and resistance levels were determined without any further culture and/or acaricide pressure or selection. All the ticks were cultured and maintained at: Departamento de Ectoparásitos y Dípteros del Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA-SAGARPA).

Each reference strain used was cultured by infesting a bovine with  $2 \times 10^4$  10-15 day old tick larvae, engorged females were collected 21 days after infestation, placed in Petri dishes for oviposition in groups of ten ticks for each strain, while incubating at 28°C in 80% relative moisture until complete oviposition. The eggs were collected, weighted and aliquoted in vials with 200 mg each until eclosion and kept at 28°C in 80% relative moisture.

### Bioassays

Ticks isolated from infested cattle at the Mexican states of Tamaulipas and Chiapas were assayed and selected for their PS and OP resistant toxicological profile demonstrated by acaricide discriminant doses Bioassays (Stone and Haydock, 1962) (Table 1). The modified larval immersion test was used for amitraz bioassays (FAO, 1984). Bioassays were run by using trichloro ethylene diluted acaricides at the following concentration: Coumaphos 0.2%, Chlorphenvinphos 0.2%, Diazinon 0.08%, Chlorpiriphos 0.2%, Cypermethrine 0.05%, Deltamethrine 0.09%, Flumethrine 0.01% and Amitraz 0.0002%. One milliliter of each dilution was applied evenly to a 7 by 9 cm piece of filter paper. The trichloroethylene was allowed to evaporate from the filter paper for 2 h. The treated papers were then folded in half and sealed on the slides with clips, this formed a packet into which approximately 100 larvae were placed and then the top of the packet was sealed with another clip. The packets were kept at 27°C 92% relative humidity for 24 h, after that the packets were removed from incubation and opened, live and death larva were counted and the data was processes as percentage of mortality for each tick group under every acaricide concentration (Table 1).

### Relative Quantitation of P-450 Gene Expression

Total RNA was isolated from each *R. microplus* tick sample following the manufacturer's instructions (Totally RNA kit™, Ambion, TX, USA) and treated with DNase according to manufacturer's instructions (Turbo DNA- free™, Ambion, TX, USA). The RNA was transcribed to cDNA using random decamer primers following the instructions of a commercial kit (RETROscript™ Kit Ambion).

Cytochrome P-450 relative gene expression quantitation was made by using Real Time PCR with Taqman Fluorescent probes specifically designed for P-450 *R. microplus* gene sequence (GenBank access AAD54000). Measurement of P-450 mRNA in acaricide susceptible and acaricide resistant ticks

by real-time PCR was carried out using a fluorogenic 5' nuclease assay (TaqMan® system) on an ABI Prism 7300 Sequence Detector (Applied Biosystems, CA, USA). The reactions included 900 nM of each primer, 300nM probe, 1X TaqMan® Universal Master Mix (Applied Biosystems). The real-time PCR thermal program consisted of one cycle at 50°C for 2 min and one cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 15 S and 60°C for 1 min. Cytochrome P-450 gene-specific PCR primers and the TaqMan® probe (Applied Biosystems) labeled with 6-carboxyfluorescein (FAM)/MGB® designed for cytochrome P-450 *R. microplus* gene sequence were as follows: forward primer P-450F (5'- CAAGCTGGTTGCTCTACATTATCGA -3'), reverse primer P-450R (5'- TTGGCCTCAGGACGAGTTC -3') and TaqMan® probe 5'- CCATGACATGAATCTTG -3' (Fig. 1). The 18S rRNA (Applied Biosystems) probe served as endogenous controls and was labeled with VIC® /MGB® (Applied Biosystems).

The number of RNA transcripts detected for each strain and field isolate was determined by the 7300 SDS Software v1.2.2 (Applied Biosystems); quantitative measurements were normalized by measuring the expression levels of 18S ribosomal RNA as internal control. The susceptible strain was considered the basal level of cytochrome P-450 expression for the reference strains and assigned a relative value of 1. The susceptible isolate (I-83) was considered the basal level of cytochrome P-450 expression for the isolates strains and assigned a relative value of 1.

### Statistical Analysis

Means and dispersion measurements of relative cytochrome P-450 gene expression in REU from acaricide resistant strains were compared against expression means and dispersion measurements of susceptible strains by an unpaired Student's t-test using the GraphPad® Software (GraphPad Software Inc. CA, USA)

## RESULTS

Sensitive reference strain and I-83 showed 100% mortality when exposed to all acaricides, both groups of ticks were considered as reference acaricide sensitive expression and their cytochrome P-450 gene expression values were adjusted as 1 Relative Expression Unit (REU). I-77 and I-821 collected from field infested cattle at the Mexican northern State of Tamaulipas showed high levels of PS resistance with 0% mortality when exposed to all PS acaricides dosages capable of killing a 100% of the reference susceptible strain, these two group of ticks also showed a statistically significant increased ( $p < 0.005$ ) P-450 expression levels of 2.44 and 3.22 REU, respectively compare to susceptible ticks (Table 2, Fig. 1). I-10 showed PS resistance of 78, 80 and 73% mortality to cypermethrin, deltamethrin and flumethrin, respectively with a statistically significant ( $p < 0.0005$ ) increased P-450 expression levels of 4.7 REU (Table 2, Fig. 1). Multiple resistant ticks included in MO strain and SA strain showed high levels of acaricide resistance, with mortality close to 0% to OP and PS acaricides (Table 1), this two strains of ticks also showed a statistically significant ( $p < 0.005$ ) decreased expression of 0.189 and 0.239 REU, respectively (Fig. 1). The I-76 showed multiple resistance with a 60, 25 and 60% mortality when exposed to cypermethrin, deltamethrin and flumethrin, respectively and 0% and 60% mortality toward coumaphos and diazinon, respectively, I-76 P-450 expression levels showed a statistically significant ( $p < 0.05$ ) decrement of 0.577 REU. The OP resistant Tx strain showed 0% mortality to all OP acaricides and a similar P-450 expression levels to the susceptible ticks. Regrouping the data into categories of acaricide resistance yielded a statistically significant ( $p < 0.0001$ ) 3.47 REU overall increase in the cytochrome P-450 gene expression level detected on pyrethroid resistant ticks (Table 2) and a statistically significant ( $p < 0.0001$ ) decrement of 0.335 REU detected in multiple acaricide resistant ticks (Table 2).

Table 2: Statistical analysis of relative quantitation of P-450 expression levels on regrouped ticks by phenotypes

Tick sample	N	Mean	SE	SD	df <sup>a</sup>	t-ratio	p-value
Susceptible	10	1.00	0.134	0.389	23	0.00	1.00
OP-resistant	5	1.102	0.170	0.398	23	0.5665	0.5866
Multiple-resistant	15	0.335	0.054	0.211	23	7.3112	0.0001
PS-resistant	15	3.470	0.302	1.170	23	6.5402	0.0001

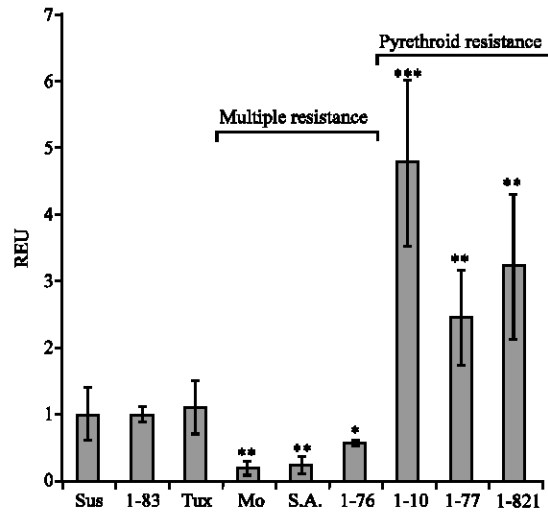


Fig. 1: Real-time relative quantification of the cytochrome P-450 gene from resistant susceptible *R. microplus*. Data is represented as Relative Expression Units (REU). Results are normalized against 18S rRNA as endogenous control. Mean and SD are shown, n = 5. Statistically significant by unpaired t-test \*: p<0.05, \*\*: p<0.005, \*\*\*: p<0.0005

## DISCUSSION

Pesticide resistance in arthropods is defined as the hereditary capacity of certain pest arthropods to survive under a certain dosage of a pesticide (WHO, 1957), this definition underlies a genetic mechanism that may involve one or several genes in order to achieve an inheritable pesticide resistance phenomenon, the phenotypes conferred by this genes may include an altered pesticide target site (Bull and Ahrens, 1988) and/or increased enzymatic detoxification (Perilla *et al.*, 2007). Detoxifying enzymes are common in arthropod resistance mechanism and among other type of enzymes, cytochrome oxidases participates naturally in enhanced metabolism of drugs and pesticides (Scott, 1999). Monooxygenases-mediated resistance is probably the most frequent type of metabolism based arthropod pesticide resistance (He *et al.*, 2002), as a result certain pyrethroid pesticides have limited use in controlling some pest species with high natural levels of monooxygenases (Sawicki, 1962). This extensive scientific background makes a logical choice to search for an increase in cytochrome P-450 activity and/or expression when a PS resistant pest is detected. During our analysis we did find a significant increase (p<0.005) of P-450 expression levels in two Tamaulipas tick field isolates I-77 and I-821 with 2.44 and 3.22 REU, respectively (Fig. 1), this high cytochrome P-450 monooxygenases levels corresponded with high levels of resistance to all pyrethroid formulations and no cross resistance was detected to any other formulation (Table 1), this results are consistent with a cytochrome P-450 monooxygenase detoxification conferring PS resistance also suggesting that increased levels of the monooxygenases do not create cross resistance to acaricide formulations other

than PS. Chiapas state I-10 showed a mild PS resistance, yet I-10 cytochrome P-450 expression levels were the highest detected during our experiments with a significant difference ( $p < 0.0005$ ) of 4.756 REU, these result is only partially consistent with the expected results, suggesting a non-linear relationship between cytochrome P-450 expression levels and PS resistance levels, another possible explanation for this non-linear relationship may lay on the fact that ticks Isolates are by definition heterogeneous for pesticide resistance genes and/or susceptibility phenotype, pesticide resistance values of tick isolates reflect this heterogeneity and therefore we can expect lower survival rates under the astringent laboratory conditions, in any case this result also suggest that high a level of cytochrome oxidases expression precedes the PS resistance on the field, this is an important observation and may be a useful acaricide resistant predictive parameter to corroborate in a subsequent research.

If we consider OP acaricide resistance we should expect a low level of cytochrome P-450 activity or expression to induce tolerance and/or resistance, this is due to cytochrome oxidase enzymatic bioactivation required for the OP pesticide toxic form, multiple resistant reference strains SA and Mo showed high levels of OP resistance (Table 1) and a statistically significant ( $p < 0.005$ ) low level of 0.239 and 0.189 REU, respectively for cytochrome P-450 gene expression. Isolate 76 also showed multiple resistance with a mild OP tolerance and a statistically significant ( $p < 0.05$ ) low level of 0.577 REU for cytochrome P-450 expression. This results are consistent with a scenario were low enzyme activity also limits the amount of toxic OP acaricide bioactivated by cytochrome oxidase however SA and MO strains are also highly resistant to PS and therefore low levels of cytochrome P-450 expression is incompatible with monooxygenases playing a role on PS resistance on multiple resistance strains, this data suggest a different PS mechanism resistance in multiple resistant ticks, this alternative PS resistance mechanism should be unrelated to cytochrome oxidases, as opposed to those ticks showing high levels PS resistance as well as high levels of cytochrome P-450 expression. There is a likely candidate for an alternative P-450 independent *R. microplus* PS resistance previously reported in the literature as a sodium channel mutation which is thought to be PS acaricides target site (Guerrero *et al.*, 2002) if this sodium channel mutation based PS resistance mechanism is confirmed, we may have at least two different genes conferring acaricide resistance involving an enhanced metabolic detoxification and an altered target site leading to a multifactorial PS resistance in *R. microplus*.

Cytochrome P-450 expression measurement failed to detect a significant difference on the expression levels in our OP reference resistant strain Tx when compared to susceptible strains, although this result is incompatible with our assumption that hypothesizes that low levels of cytochrome P-450 expression are required for OP resistance it is consistent with previous research that attributes OP resistance of the Tx strain to enhanced hydrolases metabolism (Miranda *et al.*, 1995; Miranda-Miranda *et al.*, 2005). This result suggests once again a multifactorial causative for OP resistance in *R. microplus* requiring at least two different genes codifying for two different enhanced metabolic detoxification mechanisms.

Regrouping the data into three different group of acaricide resistance statistically reinforces the role of cytochrome P-450 in acaricide resistance phenomenon (Table 2), supported by our results and a more robust confidence interval ( $p < 0.0001$ ), we are in position to propose three hypothetically distinctive cytochrome P-450 expression phenotypes in *R. microplus*: one phenotype with a normal level of cytochrome P-450 expression for the acaricide sensitive ticks and OP Tx type resistant ticks; a second phenotype showing increased cytochrome P-450 expression levels leading to PS resistant ticks; a third low level cytochrome P-450 expression phenotype identifying multiple acaricide resistant ticks. This hypothesis for cytochrome P-450 expression phenotypes in *R. microplus* should be tested in coming field surveys and acaricide resistance field monitoring in order to establish definitive trends and biochemical relationship between acaricide resistance and monooxygenases levels in the cattle tick, further studies on this issue should help to establish a diagnostic predictive parameter useful for the management of pesticide resistance.

## ACKNOWLEDGMENT

This research was partially supported by SEP-CONACYT Grant J39756-Z.

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