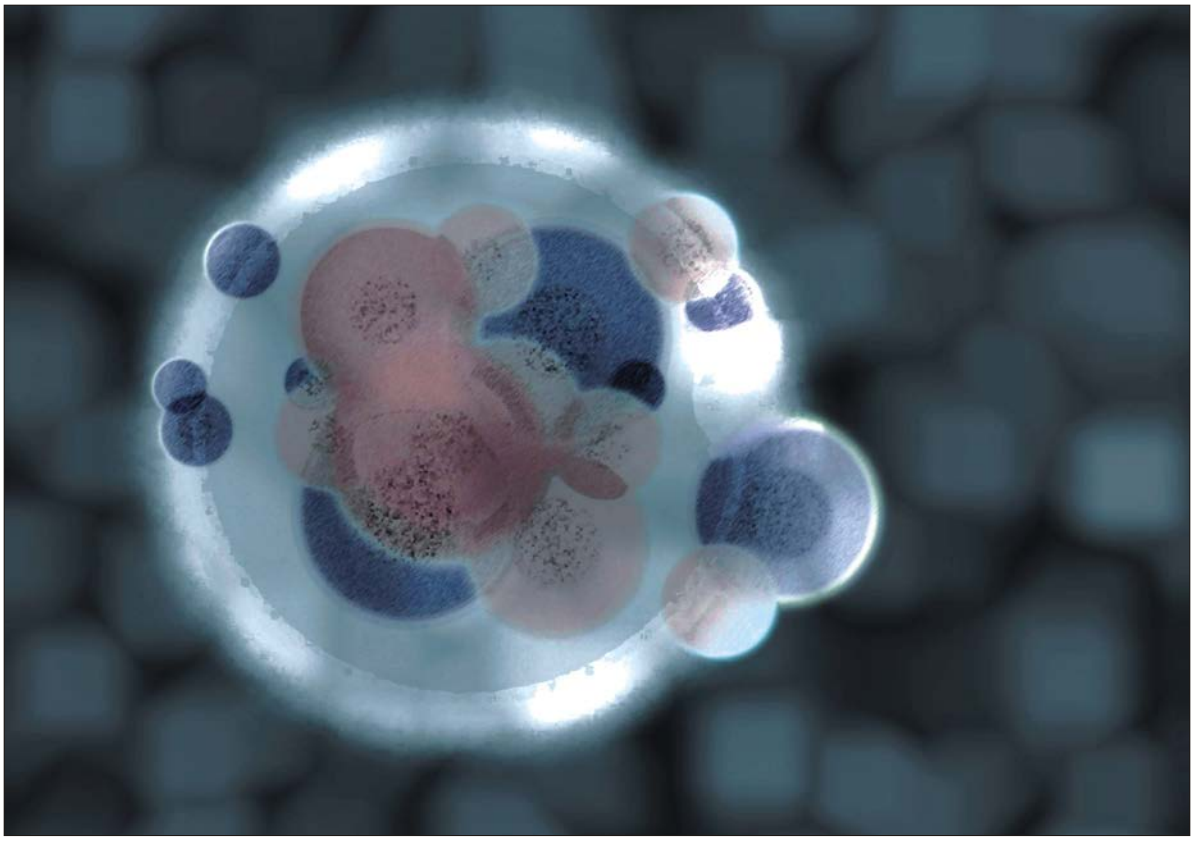

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 8 (2): 404-409, 2008
ISSN 1727-3048

Boophilus microplus Pyrethroid Resistance Associated to Increased Levels of Monooxygenase Enzymatic Activity in Field Isolated Mexican Ticks

[1]Raquel Cossío-Bayúgar, [1]Estefan Miranda-Miranda, [2]Alejandrina Ortiz-Nájera and [2]Salvador Neri-Orantes
[1]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
[2]Departamento de Ectoparásitos y Dípteros Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria Sagarpa, México

Abstract: This study was made to assess SDS-PAGE monooxygenases zymograms in the presence of synthetic monooxygenase enzyme substrates, in order to establish a reliable methodology to measure monooxygenase activity levels on acaricide resistant ticks and finding a probable association between monooxygenase enzymatic levels and *B. microplus* acaricide resistance. Monooxygenase activity was found in the zymograms of the analyzed ticks at three polypeptidic bands of 147, 125 and 98 kDa, densitometric measurements of monooxygenases zymograms at these specific enzymatic bands showed a statistically significant monooxygenase activity increase ($p<0.001$ unpaired t-test) on Pyrethroid resistant ticks when compared to the same bands in the acaricide susceptible reference ticks. Present results showed that monooxygenase zymograms combined with densitographic analysis, are useful as an acaricide resistance detection test and suggest a linkage between increased monooxygenase activities and Pyrethroid acaricide resistance in *B. microplus*.

Key words: Cytochrome P-450, zymogram, *Rhipicephalus microplus*, acaricide resistance, monooxygenase

INTRODUCTION

The monooxygenases are ubiquitous enzymes, found in most of the organisms across the phylogenetic tree. This enzymes also known as mixed function oxidases or cytochrome P-450 monooxygenases (Feyereisen, 1999), are involved in endogenous metabolism in insects (Kassai, 2004) an essential activity for the synthesis and degradation of hormones and pheromones. Monooxygenases are also involved in xenobiotics metabolism, this is an important function for the adaptative mechanisms of insects tolerance to toxic chemicals synthesized by plants such as natural pyrethrines (Schuler, 1996). It has been demonstrated that monooxygenases are able to metabolize virtually all known synthetic pesticides leading either to activation of the molecule to a more toxic form, or deactivation of the pesticide toxic action (Berge *et al*., 1998). Many cases of metabolic resistance of insects to insecticides are the result of enhanced monooxygenases activities and arthropods with natural high levels of monooxygenases, are naturally insensitive to Pyrethroids (PS) pesticide formulations, making this chemicals useless for certain type of pest control (Scott, 1999). Extensive analysis of this PS tolerant pests has established that monooxygenases mediated metabolism is implicated in deactivation of pesticides in several PS resistant arthropods, including: *Culex* spp. mosquitoes (Kassai, 2004), the tomato grub *Helicoverpa armiguera* (Chen *et al*., 2005), the kissing bug *Triatoma infestans* (Gonzalez Audino *et al*., 2004), the house fly *Musca* spp. (Kassai and Scott, 2000; Scott and Tomita, 1995) and PS resistant human lice *Pediculus humanus* (Amevigbe *et al*., 2000; Gonzalez Audino *et al*., 2005), among many other reported PS resistant arthropods.

The involvement of monooxygenases enzymes in arthropod PS resistance can be demonstrated by several methods, most commonly used, are *in vivo* tests with resistant arthropods, in the presence of pesticides and monooxygenase inhibitors such as Piperonyl Butoxide

Corresponding Author: Raquel Cossío-Bayúgar, 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

(PB), this can result in partial or complete loss of resistance, indicating the linkage between resistance and monooxygenases activity (Berge *et al*., 1998). Tick acaricide resistance bioassays are expensive because they require numerous confined cattle in specially designed premises on which the ticks are raised and a lot of hard work is required in the care of experimentally tick infested cattle during three months, in order to complete the ticks life cycle for every reference strain necessary for comparison in every assay, acaricide resistance detection in *B. microplus*, also requires highly qualified laboratory personal to perform the bioassays with several acaricide formulas making this kind of test, one of the most expensive, labor intensive and time consuming pesticide resistance test when compared to similar bioassays in other kind of pesticide resistance arthropods. Therefore some faster and cheaper methods for analyzing the role of monooxygenases on PS resistant arthropods have been proposed. There has been attempts to measure monooxygenases levels by synthetic chromogenic and fluorogenic substrates and NADPH uptake, detected by spectrophotometric and fluorescence measurements in organisms such as pyrethroid resistant *Triatoma infestans* extracts (Gonzalez Audino *et al*., 2004) and *Bacillus subtilis* cloned enzymes (Budde *et al*., 2004), other reports find a valuable methodology on monooxygenases zymograms applied to organophosphate resistant Sawtoothed Grain Beetle *Orizaephilus surinamensis* (Lee and Lees, 2001) and to PS resistant human lice *Pediculus humanus* (Amevigbe *et al*., 2000; Gonzalez Audino *et al*., 2005). In the case of acaricide resistant *B. microplus* Some previous reports found increased mRNA monooxygenase expression in acaricide resistant ticks (Guerrero *et al*., 2007). Although the use of zymograms has proven useful for pesticide resistance detection, there are no previous attempts reported in the literature to identify monooxygenase enzymatic activity zymograms, using SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), combined with monooxygenase synthetic substrate staining and densitometric measurements, on acaricide resistant *B. microplus*. In this study we try to fill the gap and report a SDS-PAGE monooxygenase zymogram analysis applied on sensitive and acaricide resistant ticks in order to establish a probable a role of *B. microplus* monooxygenases 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 *B. microplus* field isolates were collected from tick infested cattle at the Mexican states of Chiapas and Puebla. All ticks isolates have been cultured and tested for acaricide resistance, as part of the cattle tick monitoring programs of the Agriculture Ministry of the Mexican Federal Government. Tick isolates were named after the location of origin and labeled as follows: Las Juntas (LJ), Palenque (Pa), Manantiales (Ma) and Linda Vista (LV), as well as a susceptible reference strain for comparison purposes (Su). Each tick isolate used was cultured by infesting a bovine with 2×10^4 10-15 day old larvae, engorged females were collected 21 days after infestation, placed in Petri dishes for oviposition in groups of ten engorged ticks for each isolate, 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. All the ticks were cultured and maintained at Departamento de Ectoparásitos y Dípteros del Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA-SAGARPA).

Bioassays: Ticks were assayed and selected for their Pyrethroids (PS), Oganophosphorous (OP) toxicological profile demonstrated by acaricide discriminant doses Bioassays (Stone and Haydock, 1962) a modified larval immersion test was used for amitraz bioassays (FAO, 1984) (Table 1). Bioassays were run by using trichloro ethylene diluted acaricides at the following concentration: Coumaphos 0.2%, Chlorphenvinphos 0.2%, Cypermetrine 0.05%, Deltametrine 0.09%. 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 sides with clips, this formed a packet into which approximately 100, 15 days old 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). A 0.0002% discriminant dose was used for Amitraz, with a product exposure of 72 h.

PAGE monooxygenases zymogram determination: One hundred milligram of 15 day old larvae from each tick isolate were macerated in a ceramic mortar in 1 mL of

Phosphate Buffer Solution 0.1 M pH 7.2 (PBS) according to reported protocol (Miranda *et al*., 1995). The protein content of the tick extracts was determined by the Bradford methodology (Bradford, 1976). Seventy five micrograms of protein from each tick extract were applied to separate wells of a 1.5 mm tick 100 mm wide and 82 mm tall Sodium Duodecyl Sulphate Polyacrylamide Electrophoretic Gels (SDS-PAGE), prepared as previously reported (Laemmli, 1970), an electric current of 100 V/15 mA was applied for 80 min. After electrophoretic separation, the SDS-PAGE gels were washed with gentle agitation for 15 min in 50 mL of PBS for three times. Afterwards SDS-PAGE gels were submerged in a solution containing 100 mL of 0.1 M Phosphate buffer pH 7.0, 100 mg of N,N-Dimethyl-p-phenylendiamine and 150 mg of α-Naphtol, according to a previously reported protocol (Manchenko, 2003). The SDS-PAGE gels were incubated at room temperature with gentle agitation until the appearance of blue bands. The gels were washed in water after monooxygenase enzymatic staining and preserved in 3% acetic acid. Zymograms were recorded as digital images using an Epi Chemi® (UVP life sciences USA) image recorder, feeding the LabWorks 4® analysis software in densitometer mode. A Prestained Protein Molecular Mass Standard was commercially obtained (PageRuler™, Fermentas. USA); the densitometer was calibrated with digital images of this mass standard included in every SDS-PAGE zymogram in order to obtain automatically the Mass of each band within the zymogram according to the instructions of the LabWorks 4® software (UVP life sciences. USA). Densitograms of five different experiments were converted to Optical Density (OD) measurements. Means and standard errors of each band registered for the five different experiments were graphically represented and compared against the means and standard errors of susceptible ticks. Statistical comparisons between the monooxygenase OD mean values from the isolates and corresponding monooxygenases OD values from the Su reference tick strain, were made by an unpaired Student's t-test using the GraphPad® Software (GraphPad Software Inc. USA).

RESULTS AND DISCUSSION

Reference Susceptible (Su) ticks showed 100% mortality when exposed to all acaricides used (Table 1) and Su strain was considered a comparison reference for both acaricide toxicological test and monooxygenases activity levels. Pa and LJ isolates showed high levels of Pyrethroid (PS) resistance with a 0% mortality when exposed to deltametrine and cypermetrine (Table 1) under acaricide dosages capable of killing a 100% of the reference Su strain. Pa and LJ ticks isolates, also showed an statistically significant increased monooxygenase activity ($p<0.0001$ to $p<0.005$, unpaired t-test) on bands located at 147, 125 and 98 kDa (Fig. 1, 2) when compared against the Su monooxygenase activity at the same bands (Fig. 1, 2). LV and Ma ticks isolates showed low levels of PS resistance and no significative difference was found for monooxygenase activity when compared to the Su reference ticks.

Pesticide resistance in arthropods may have a multifactorial origin, these factors may include an altered pesticide target site (Bull and Ahrens, 1988) and/or increased enzymatic detoxification (Penilla *et al*., 2007), detoxifying enzymes are common in arthropod pesticide resistance mechanism and among some other type of enzymes; monooxygenases participate naturally in enhanced metabolism of drugs and pesticides (Scott, 1999). Monooxygenases-mediated resistance is probably the most frequent type of enhanced 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). The availability of an extensive scientific background on this subject makes a logical choice to search for an increase in monooxygenase activity when a PS resistant pest is detected. During our analysis we did find a significant increase in monooxygenase activity levels ($p<0.001$ to $p<0.005$, unpaired t-test) in the two PS resistant tick isolates Pa and LJ (Table 1), the monooxygenase enzymatic activity was located on bands of 147 125 and 98 kDa (Fig. 1, 2), this high monooxygenases levels suggest a linkage to the

Table 1: Acaricide bioassays results on susceptible strain and different isolates of ticks. Data is represented as percentage of mortality under a standard concentrations of acaricide

	Mortality (%)					
	Chloride	Organophosphates		Pyrethroids		Amidines
Isolate	Lyndane	Coumaphos	Clorfenvinphos	Deltametrine	Cypermetrine	Amitraz
LJ	100.0	98.8	100	0.0	0.0	62.7
LV	100.0	97.6	100	60.9	76.9	92.3
Ma	100.0	100.0	100	82.4	85.8	98.6
Pa	87.9	100.0	100	0.0	0.0	96.0
Su	100.0	100.0	100	100.0	100.0	100.0

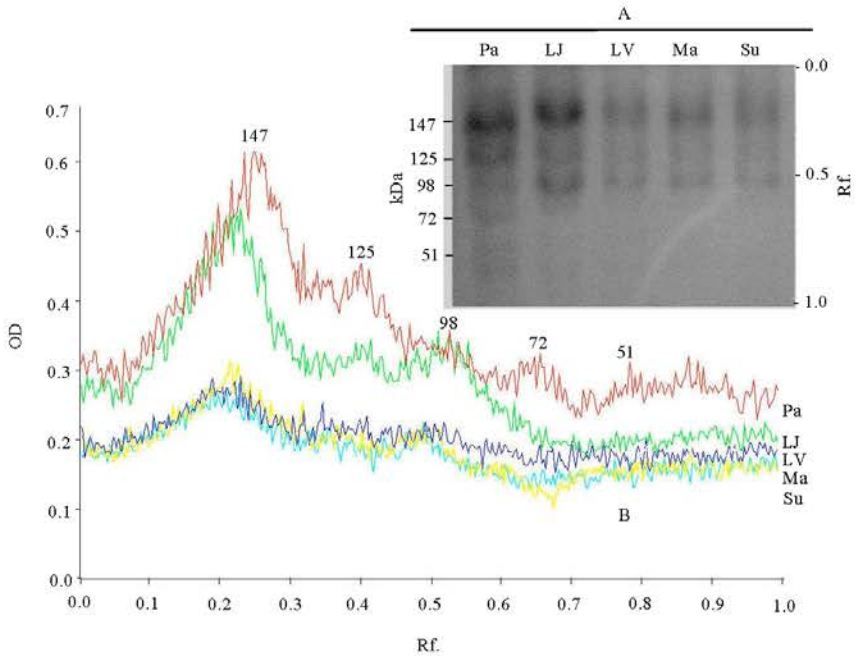

Fig. 1: Example of a typical SDS-PAGE monooxygenase Zymogram (A) and its correspondent densitogram (B). Zymograms were obtained by specific monooxygenase synthetic substrate staining; enzymatic bands were subjected to densitometric measurement and transformed to Optical Density (OD) data. X-axis in the graph represents the relative electrophoretic motility of the bands (Rf) within the zymogram

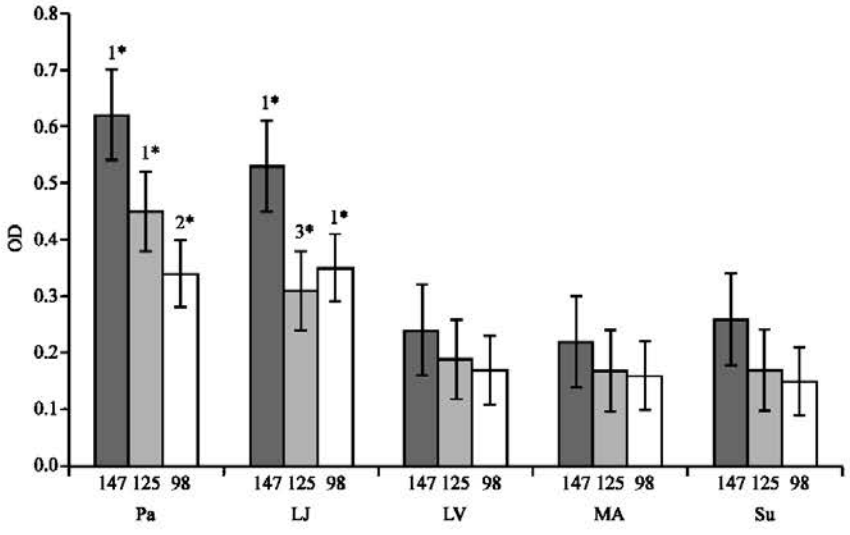

Fig. 2: Graphical comparisons of the Optical Density (OD) of monooxygenase bands. Means and standard errors of densitometric values were obtained as Optical Density (OD) from five independent experiments. OD values for 147, 125 and 98 kDa. Bands from PS ticks isolates were graphically and statistically compared against acaricide susceptible ticks. Obtained p-values (unpaired t-test) were indicated on statistically significant OD values as: 1* $p<0.0001$., 2* $p<0.0005$., 3* $p<0.005$. X axis represents the mass of analyzed band in Kilo Daltons (kDa) and its corresponding tick isolate

high levels of resistance found on this ticks to both pyrethroid formulations used on the toxicological bioassays (Table 1). These results are consistent with a monooxygenase enhanced metabolic detoxification conferring PS resistance in *R. microplus*. LV and Ma isolates showed a mild PS resistance and monooxygenases levels detected by zymogram analysis showed no significative differences; these result suggest a non-linear relationship between monooxygenase levels and PS resistance levels, another possible explanation for this non-linear relationship may be 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 varying survival rates under the astringent laboratory conditions. Nonetheless there is the possibility for another type of PS resistance based on an altered target site recently reported (Guerrero *et al*., 2002) which is consistent with the low levels of monooxygenases and mild PS resistant.

Obtained zymograms showed other bands on Pa PS resistant ticks with a lower peptidic mass than 98 kDa with probable monooxygenases activity (Fig. 1). These bands located at 51 and 72 kDa showed a progression of increasing polypeptidic mass that may suggest peptidic oligomeric arrangements for *B. microplus* monooxygenases, there are no previous reports on the location of monooxygenases zymograms on SDS-PAGE gels obtained from the cattle tick but we do know from other organisms, that monooxygenases form oligomeric and complex structures that include a large prosthetic group and three or more peptides (Cheng *et al*., 1984). Although the presence of this low mass monooxygenase peptides in one of the PS resistant tick, seems to be related with the resistance phenomenon, this should be investigated under more rigorous experiments.

Present results support two distinctive monooxygenase phenotypes in *B. microplus*: One phenotype with a normal level of monooxygenases activity for the acaricide sensitive ticks and a second phenotype showing increased monooxygenase activity levels at 147, 125 and 98 kDa on PS resistant ticks, these hypothetical phenotypes for monooxygenases expression in *B. microplus* should be tested in coming field surveys and acaricide resistance field monitoring, in order to establish definitive trends and biochemical linkage relationship between acaricide resistance and monooxygenases levels in the cattle tick, further studies on this issue should help to establish a diagnostic predictive parameter based on zymograms of monooxygenases and other detoxifying enzymes, useful for the management of the persistent and ever increasing problem of acaricide resistance in *B. microplus*.

REFERENCES

Amevigbe, M.D., A. Ferrer, S. Champorie, N. Monteny, J. Deunff and D. Richard-Lenoble, 2000. Isoenzymes of human lice: *Pediculus humanus* and *P. capitis*. Med. Vet. Entomol., 14 (4): 419-425.

Berge, J.B., R. Feyereisen and M. Amichot, 1998. Cytochrome P450 monooxygenases and insecticide resistance in insects. Philos. Trans. R. Soc. Lond. B. Biol. Sci., 353 (1376): 1701-1705.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Budde, M., S.C. Maurer, R.D. Schmid and V.B. Urlacher, 2004. Cloning, expression and characterization of CYP102A2, a self-sufficient P-450 monooxygenase from *Bacillus subtilis*. Applied Microbiol. Biotechnol., 66: 180-186.

Bull, D.L. and E.H. Ahrens, 1988. Metabolism of coumaphos in susceptible and resistant strains of *Boophilus microplus* (Acari: Ixodidae). J. Med. Entomol., 25 (2): 94-98.

Chen, S., Y. Yang and Y. Wu, 2005. Correlation between fenvalerate resistance and cytochrome P450-mediated O-demethylation activity in *Helicoverpa armigera* (Lepidoptera: Noctuidae). J. Econ. Entomol., 98 (3): 943-946.

Cheng, K.C., H.V. Gelboin, B.J. Song, S.S. Park and F.K. Friedman, 1984. Detection and purification of cytochromes P-450 in animal tissues with monoclonal antibodies. J. Biol. Chem., 259 (19): 12279-12284.

FAO, 1984. Acaricide Resistance. Tick and Tick-Born Disease Control. A Practical Field Manual/Tick Control, Vol. 1. FAO, Rome.

Feyereisen, R., 1999. Insect P450 enzymes. Ann. Rev. Entomol., 44: 507-533.

Gonzalez Audino, P., C. Vassena, S. Barrios, E. Zerba and M.I. Picollo, 2004. Role of enhanced detoxication in a deltamethrin-resistant population of *Triatoma infestans* (Hemiptera, Reduviidae) from Argentina. Mem. Inst. Oswaldo Cruz, 99 (3): 335-339.

Gonzalez Audino, P., S. Barrios, C. Vassena, G. Mougabure Cueto, E. Zerba and M.I. Picollo, 2005. Increased monooxygenase activity associated with resistance to permethrin in *Pediculus humanus capitis* (Anoplura: Pediculidae) from Argentina. J. Med. Entomol., 42 (3): 342-345.

Guerrero, F.D., A.Y. Li and R. Hernandez, 2002. Molecular diagnosis of pyrethroid resistance in Mexican strains of *Boophilus microplus* (Acari: Ixodidae). J. Med. Entomol., 39 (5): 770-776.

Guerrero, F.D., K.G. Bendele, A.C. Chen, A.Y. Li, R.J. Miller, E. Pleasance, R. Varhol, M.E. Rousseau and V.M. Nene, 2007. Serial analysis of gene expression in the southern cattle tick following acaricide treatment of larvae from organophosphate resistant and susceptible strains. Insect Mol. Biol., 16 (1): 49-60.

He, H., A.C. Chen, R.B. Davey and G.W. Ivie, 2002. Molecular cloning and nucleotide sequence of a new P-450 gene, CYP319A1, from the cattle tick, *Boophilus microplus*. Insect Biochem. Mol. Biol., 32 (3): 303-309.

Kassai, S. and J.G. Scott, 2000. Overexpression of cytochrome P-450 CYP6D1 is associated with monooxygenase-mediated pyrethroid resistance in house flies from Georgia. Pestic. Biochem. Physiol., 68 (1): 34-41.

Kassai, S., 2004. Role of cytochrome P450 in mechanism of pyrethroid resistance. J. Pestic. Sci., 29 (3): 220-221.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227 (5259): 680-685.

Lee, S.E. and E.M. Lees, 2001. Biochemical mechanisms of resistance in strains of *Oryzaephilus surinamensis* (Coleoptera: Silvanidae) resistant to malathion and chlorpyrifos-methyl. J. Econ. Entomol., 94 (3): 706-713.

Manchenko, G.P., 2003. Handbook of Detection of Enzymes on Electrophoretic Gels. 2nd Edn. CRC Press, Boca Raton, Florida.

Miranda, E., R. Cossío-Bayúgar, M.R. Téllez-Alanís, Z. García-Vázquez, R. Rosario-Cruz and M. Ortiz-Estrada, 1995. An enzymatic marker for ixodicide resistance detection in the cattle tick *Boophilus microplus*. Adv. Agric. Res., 4 (3): 001-008.

Penilla, P.R., A.D. Rodriguez, J. Hemingway, A. Trejo, A.D. Lopez and M.H. Rodriguez, 2007. Cytochrome P-450-based resistance mechanism and pyrethroid resistance in field *Anopheles albimanus* resistance management trial. Pestic. Biochem. Physiol., 89 (2): 111-117.

Sawicki, R.M., 1962. Insecticidal activity of pyrethrum extract and its four insecticidal constituents against house flies. III. Knock-down and recovery of flies treated with pyrethrum extract with and without piperonyl buroxide. J. Sci. Food Agric., 13 (5): 283-292.

Schuler, M.A., 1996. The role of cytochrome P-450 monooxygenases in plant-insect interactions. Plant Physiol., 112 (4): 1411-1419.

Scott, J.G. and T. Tomita, 1995. Characterization of a cytochrome P450 responsible for pyrethroid resistance in the house fly. Pestic. Sci., 43 (3): 244-245.

Scott, J.G., 1999. Cytochromes P450 and insecticide resistance. Insect Biochem. Mol. Biol., 29 (9): 757-777.

Stone, B.F. and K.P. Haydock, 1962. A method for measuring the acaricide-susceptibility of the cattle tick *Boophilus microplus* (Canestrini). Bull. Entomol. Res., 53: 563-578.