OVER-EXPRESSION OF ESTERASES IN ACARICIDE RESISTANT ISOLATES OF *RHIPICEPHALUS (BOOPHILUS) MICROPLUS*

Gaurav Nagar[1], Kanak Anjali[1], Snehil Gupta[1], Sachin Kumar[1], Saravanan, B.C.[1], Anant Rai[2] and Srikant Ghosh[1]*

[1]Entomology Laboratory, Division of Parasitology, ICAR-Indian Veterinary Research
Institute, Izatnagar, Bareilly – 243122 (UP) India
[2]Mewar University, Chittorgarh-312901(Rajasthan), India
Entomology Laboratory, Division of Parasitology
ICAR-Indian Veterinary Research Institute, Izatnagar -243122, Uttar Pradesh, India
E-mail: sghoshtick@gmail.com (*Corresponding Author*)

Abstract: Monitoring of acaricide resistance and identification of resistance mechanisms are two important facets of sustainable tick control. To identify the role of specific esterases in development of acaricide resistance, a comparative analysis of the esterase (EST) patterns was made in four reference *Rhipicephalus* (*Boophilus*) *microplus* tick populations i.e. susceptible (IVRI-1), diazinon resistant (IVRI-III), deltamethrin resistant (IVRI-IV) and multi-acaricide resistant (IVRI-V) and in different field isolates, using non-denaturing polyacrylamide gel electrophoresis. Comparative profiling of esterases exhibited higher intensities of EST-1, EST-3 and EST-4 bands in resistant lines and in resistant field isolates in comparison to IVRI-I line. Non inhibition of EST-1 with acetylcholinesterase inhibitor, eserine sulphate and increase in EST-1 band intensity with the inhibition of EST-2 in the resistant lines and isolates suggests the possible role of EST-1 in resistance development. The findings of this study highlighted the role of country specific multiple mechanisms in conferring resistance in *R. (B.) microplus* isolates resistant to synthetic pyrethroid (SP) and organophosphate (OP) compounds.
Keywords: acaricide, resistance, esterases, *Rhipicephalus (Boophilus) microplus*.

Introduction

Rhipicephalus (Boophilus) microplus is the most common tick species infesting milk and meat producing animals throughout the world, accountable for heavy damage to livestock industry through weight loss, anemia, transmission of fatal diseases and hide damage. India, is spending more than 498.2 million USD annually for the control of TTBDs (Minnjauw and Mcleod, 2003) while USA is saving 3 billion USD annually by controlling ticks infesting milk producing animals (Guerrero *et al.,* 2012).

Due to the extensive use of acaricides, the cattle tick population has developed resistance to almost all chemicals registered for the control of the economically important tick species and created hurdles in tick control program globally (He *et al.,* 1999, Chen *et al.,*

2009). In India, after the determination of discriminating concentration (DC) of commonly used insecticides, attempts were made to characterize ticks collected from selected states of India and different levels of resistance to SP, OP and initial level of resistance to formamidine compound, amitraz were detected (Kumar *et al.,* 2011, Kumar *et al.,* 2014, Sharma *et al.,* 2012, Singh *et al.,* 2015, Ghosh *et al.,* 2015). To check rapid progression of widespread resistance, it has become necessary to identify factors contributing to resistance development and possible resistance mechanisms operating in ticks against the commonly used compounds. Based on the available information it has been observed that resistance in *R. (B.) microplus*, can be attributed to several mechanisms such as reduction of pesticide absorption, increased metabolization by esterases, oxidases or glutathione-S-transferases, and modification in the target site of pesticides (Coosio-Bayugar *et al.,* 2002).

Esterases are the member of large gene family within α/β hydrolases which play an important role in various physiological and metabolic processes. Esterases metabolize the exposed insecticides, which in majority are structurally ester in nature, prolonging the survival of ticks (Montella *et al.,* 2012). In India, the subject is poorly studied and very little information is available regarding possible mechanism of development of acaricide resistance in Indian tick species. Thus, the present experiments were conducted with an objective to unravel the role of specific esterases in conferring SP and OP resistance in ticks collected from the wide geographical regions of India.

Materials and methods

Sampling

All tick isolates used for the study were collected previously following two steps stratified sampling procedure from different agro-climatic regions of India. The ticks collected from an area were pooled and designated as an isolate and maintained for generation of sufficient adults and tested at different grades of DC using adult immersion test (AIT) (Kumar *et al.,* 2011, Sharma *et al.,* 2012) and were categorized on the basis of resistant factor (RF).

Reference tick lines

The laboratory generated reference populations, the susceptible IVRI-I (national registration no. NBAII/IVRI/Bm/1/1998), diazinon resistant IVRI-III (RF = 28.4), deltamethrin resistant IVRI-IV (RF = 194.5) and multi-acaricide resistant IVRI-V (RF = 25.5 against diazinon, 15.4 against cypermethrin and 17.5 against deltamethrin) were used for comparative study.

Qualitative estimation of esterases

Twelve to fourteen days old larvae stored at -80°C were used as starting material. One hundred larvae of IVRI-I, IVRI-III, IVRI-IV, IVRI-V ticks and field isolates of *R.(B.) microplus* were separately homogenized in 200 µl chilled homogenization buffer containing 0.1M sodium phosphate, pH 6.5, 20% sucrose and 0.001M EDTA. The homogenates were centrifuged at 15,000×g, 4°C for 15 min and the collected supernatants were used for characterization of esterase enzymes by native polyacrylamide gel electrophoresis. The total protein concentration of each sample was determined according to dye-binding method of Bradford using a microtiter plate (Axygen, USA).

Each sample was electrophoretically analyzed using 40 µg of total protein in each well of non-denaturing polyacrylamide gel with sample buffer containing sucrose and bromophenol blue. The stacking and resolving gels were 4% and 10% polyacrylamide prepared in 1M Tris–HCl buffer, pH 6.8 and 1.5 M Tris–HCl buffer, pH 8.8, respectively. Electrophoresis was performed in pre-chilled Tris–glycine buffer at 40 mA and 140V for 4 hrs at 4°C. At the end of run, the gels were carefully removed and transferred to 25 ml pre incubation buffer containing 0.1M sodium phosphate buffer, pH 6.5, for 45 min. Following pre incubation, the gels were transferred to gel staining solution (25 ml 0.1 M sodium phosphate buffer containing 23 mg fast blue RR salt, Sigma, USA and 15 mg of α-naphthyl acetate, Sigma, USA, dissolved in 150 µl acetone) for 1 hr at 37°C in dark to determine the esterase activity. After staining, the gels were washed thoroughly with water and reactivity was recorded using Syngene gel documentation system. All the samples were repeated thrice under identical conditions to validate the results.

To identify the specific esterase involved in conferring resistance in field isolates under study, inhibitors, 0.33% TPP and 1.0 mM eserine sulfate were used. After resolving, one half of the gel was kept in pre-incubation buffer containing inhibitor for 45 mins, and then staining protocol was followed as mentioned above. The other half of the gel having identical samples was incubated and stained without inhibitor. The gels were washed with distilled water and kept overnight in water for clearing the background. Reactivity was recorded using Syngene gel documentation system and the results were judged on the basis of comparative band intensity in gels with or without inhibitor.

Results

Resistant status of field isolates

Analysis of AIT data revealed that all the collected tick isolates were resistant in the range of level I-IV with RF from 2.92 to 95.71 against deltamethrin and 4.15 to 57.85 against diazinon (Table 1).

Esterase profile

Biochemical profiling of esterase enzymes detected acetylcholinesterases (EST-1and EST-2) and carboxylesterases (EST-3 and EST-4) as four major esterases in reference IVRI-III, IV, V lines and in field isolates. Except EST-1, all the esterases were present in IVRI-I. However, of the four esterases, the band intensity of EST-3 and EST-4 were higher than EST1 and EST-2 in reference and in all the resistant field isolates. On comparing the band intensity of the tested samples along with IVRI-I, it was observed that amongst the four ESTs, a clear difference of EST-1 band intensity in field isolates (BLW, SUL, DRB, UDP, S-24P, LDH, DNP, SKR) and in reference lines were noticed with the highest band intensity seen in IVRI–III line and in DRB isolate (Fig.1).

The fig. 2 depicts the band pattern of different esterases in the presence of esterase inhibitor, TPP. The band intensity of the esterases indicated significant variations between susceptible IVRI-I and resistant IVRI-III, IV and V. The data obtained in reference lines were comparable to the results obtained in resistant field isolates. It was observed that TPP inhibited EST-2 enzyme activity in susceptible IVRI-I (Fig. 2, lane 1). However, intensity of both the EST-1 and EST-2 bands was higher in all the resistant isolates (BLW, SUL, DRB, UDP, S-24P, LDH, DNP, SKR) and in reference IVRI-III, IVRI-IV, and IVRI-V lines.

Exposure of gels to the acetylcholinesterase (AChE) inhibitor, eserine sulphate, resulted in complete inhibition of EST-2 in all the reference resistant lines and in resistant field isolates irrespective of the resistance status. Although all the field isolates were resistant to deltamethrin and diazinon (as reference line V), the complete inhibition of EST-2 signifies that except EST-2 all the other esterases may be involved in conferring resistance to deltamethrin and diazinon. It was also observed that with the inhibition of EST-2 there was a considerable increase in EST-1 band intensity (Fig. 3). All the three replicates of all samples showed similar gel profile as mentioned above.

Discussion

Acaricide resistance has been recognized globally as the most serious problem linked with the use of chemicals for the control of *R. (B.) microplus*. Although, livestock owners of India

were reporting inefficiency of commonly used acaricides, suitable attention has not been paid to address the problem. Recently, after the standardization and validation of resistance monitoring tools, resistance to diazinon, deltamethrin, cypermethrin and amitraz has been established (Kumar *et al.,* 2011, Kumar *et al.,* 2014, Sharma *et al.,* 2012, Singh *et al.,* 2015). Esterase enzyme mediated metabolic detoxification is a comparatively complex mechanism and in arthropods this class of enzymes is involved in various important physiological activities such as reproductive behavior (Labate *et al.,* 1990), functioning of the nervous system (Villate *et al.,* 2002), and resistance to pesticides [Li *et al.,* 2005). Both carboxylesterases (CES) and acetylcholinesterases (AChE) are reported to be linked with acaricide resistance in *R. (B.) microplus.* Baxter and Barker (2002) correlated OP resistance with increased AChE activity in Australian strains while Baffi *et al.* (2008) characterized esterases involved in OP and pyrethroid resistance in Brazilian population of *R. (B.) microplus.*

In the present study, higher band intensity of acetylcholinesterase (EST-1, Fig.3) and carboxylesterases (EST-3 and 4) were observed in reference and in field isolates and none of the inhibitors could significantly inhibit EST-1, 3 and 4 bands in isolates having high RF to both OP and SP compounds (Fig. 2, lanes 5-14 and Fig. 3, lanes 5-11). Inhibition assay shows that TPP inhibits all the bands to some extent while eserine sulfate exclusively inhibits EST-2 in resistant ticks. The inhibition of EST-2 by eserine sulphate in the resistance isolates and also in diazinon resistant IVRI-III line indicates EST-2 is not responsible for resistance development but EST-1, 3 and 4 enzymes may be involved in the mechanisms of development of resistance in *R.(B.) microplus.* Previously, Baffi *et al.* (2008) reported similar observation while characterizing tolerant and resistant group of Brazilian isolates of *R. (B.) microplus.*

It was observed that the band intensity of EST-1 was enhanced when EST-2 band was inhibited by eserine sulphate (Fig. 3). This finding can be correlated with the studies by Temeyer *et al.* (2013), who evinced multiple, functionally complementary AChEs in ticks. The increased activity of EST-1 following EST-2 inhibition suggests the presence of an enzyme uninhibited by OP acaricides, which may be a possible resistance mechanism in *R.(B.) microplus* from India. Similar results were also obtained during characterization of deltamethrin resistant reference tick colony of *R.(B.) microplus* (Gupta *et al.,* 2016).

Conclusion

Native gel electrophoresis followed by inhibitor assay clearly substantiates the possible involvement of esterases in resistance development in tested tick isolates. Non-inhibition of general esterase activity by TPP further proves the involvement of esterase in pyrethroids and OP resistance. A positive correlation between high RF to deltamethrin and diazinon, mutation in sodium channel gene and involvement of over-expressed EST-3, EST-4 and EST-1 indicates that multiple mechanism of resistance are operating in Indian field isolates.

References

[1] Minjauw, B. and McLeod, A. (2003). Tick-borne diseases and poverty: The impact of ticks and tick-borne diseases on the livelihood of small scale and marginal livestock owners in India and eastern and southern Africa. Research Report, DFID-AHP. UK: Centre for tropical Veterinary Medicine, University of Edinburgh, p. 116.

[2] Guerrero, F.D., Lovis, L. and Martins, J.R. (2012) Acaricide resistance mechanisms in *Rhipicephalus* (*Boophilus*) *microplus*. Rev. Bras. Parasitol. Vet. 21: 1–6.

[3] He, H., Chen, A.C., Davey, R.B., Ivie, G.W. and George, J.E. (1999) Identification of a point mutation in the para–type sodium channel gene from a pyrethroid–resistant cattle tick. Biochem. Biophys. Res. Commun. 261: 558–561.

[4] Chen, A.C., He, H., Temeyer, K.B., Jones, S., Green, P. and Barker, S.C. (2009) A survey of *Rhipicephalus microplus* populations for mutations associated with pyrethroid resistance. J. Econ. Entomol. 102: 373–380.

[5] Kumar, S., Paul, S., Sharma, A.K., Kumar, R., Tewari, S.S., Chaudhuri, P., Ray, D.D., Rawat, A.K.S. and Ghosh, S. (2011) Diazinon resistant status in *Rhipicephalus (Boophilus) microplus* collected from different agro–climatic zones of India. Vet. Parasitol. 181: 274–281.

[6] Kumar, S., Sharma, A.K., Ray, D.D. and Ghosh, S. (2014) Determination of discriminating dose and evaluation of amitraz resistance status in different field isolates of *Rhipicephalus (Boophilus) microplus* in India. Exp. App. Acarol. 63: 413–422.

[7] Sharma, A.K., Kumar, R., Kumar, S., Nagar, G., Singh, N.K., Rawat, S.S., Dhakad, M.L., Rawat, A.K., Ray, D.D. and Ghosh, S. (2012) Deltamethrin and cypermethrin resistance status of *Rhipicephalus (Boophilus) microplus* collected from six agro–climatic regions of India. Vet. Parasitol. 188: 337–345.

[8] Singh, N.K., Gelot, I.S., Jyoti, Singh, V., Rath, S.S. (2015) Detection of amitraz resistance in *Rhipicephalus (Boophilus) microplus* from North Gujarat, India. J. Parasit. Dis. 39: 49–52.

[9] Ghosh, S., Kumar, R., Nagar, G., Kumar, S., Sharma, A.K., Srivastava, A., Kumar, S., Ajith Kumar, K.G. and Saravanan, B.C. (2015) Survey of acaricides resistance status of *Rhipiciphalus (Boophilus) microplus collected from selected places of Bihar, an eastern state of India. Ticks Tick Borne Dis. 6: 668–675.*

[10] Coosio–Bayugar, R., Rola, B., Burghardt, R.C., Wagner, G.G. and Holman, P.J. (2002) Basal cellular alterations of esterase, glutathione, glutathione *S*–transferase, intracellular calcium, and membrane potentials in coumaphos–resistant *Boophilus microplus* (Acari Ixodidae) cell lines. Pestic. Biochem. Physiol. 72: 1–9.

[11] Temeyer, K.B., Olafson, P.U., Brake, D.K., Tuckow, A.P., Li, A.Y. and Pérez de León, A.A. (2013) Acetylcholinesterase of *Rhipicephalus (Boophilus) microplus* and *Phlebotomus papatasi*: gene identification, expression, and biochemical properties of recombinant proteins. Pestic. Biochem. Physiol. 106: 118–123.

[12] Montella, I.R., Schama, R. and Valle, D. (2012) The classification of esterases: an important gene family involved in insecticide resistance – A Review. Mem. Inst. Oswaldo Cruz. 107: 437–449.

[13] Baffi, M.A., de Souza, G.R.L., de Sousa, C.S., Ceron, C.R., Bonetti, A.M. (2008) Esterase enzymes involved in pyrethroid and organophosphate resistance in a Brazilian population of *Riphicephallus* (*Boophilus*) *microplus* (Acari: Ixodidae). Mol. Biochem. Parasitol. 160: 70–73.

[14] Gupta, S., Ajith, Kumar, K.G., Sharma, A.K., Nagar, G., Kumar, S., Saravanan, B.C., Ravikumar, G. and Ghosh, S. (2016) Esterase mediated resistance in deltamethrin resistant reference tick colony of *Rhipicephalus (Boophilus) microplus*. Exp. App. Acarol. 69: 239–248.

[15] Labate, B., Game, Y., Cooke, H., and Oakeshott J.G. (1990) The number and distribution of esterase 6 alleles in populations of *Drosophila melanogaster*. Heredity. 63: 203–208.

[16] Villatte, F.F. and Bachmann, T.T. (2002) How many genes encode cholinesterase in arthropods? Pestic. Biochem. Physiol. 73: 122–129.

[17] Li, A.Y., Pruett, J.H., Davey, R.B. and George, J.E. (2005) Toxicological and biochemical characterization of coumaphos resistance in the San Roman strain of *Boophilus microplus* (Acari, Ixodidae). Pestic. Biochem. Physiol. 81: 145–153.

[18] Baxter, G.D. and Barker, S.C. (2002) Analysis of the sequence and expression of a second putative acetylcholinesterase cDNA from organophosphate–susceptible and resistant cattle ticks. Insect Biochem. Mol. Biol. 32: 815–820.

Table 1. LC50 value, RF and level of deltamethrin and diazinon resistance in field tick isolates and in reference tick lines

Tick isolates	LC50 values (ppm)		Resistance Factor		Level of resistance‡	
	deltamethrin	diazinon	deltamethrin	diazinon	deltamethrin	diazinon
BLW	114.04	8474.5	8.51	22.78	II	II
MKTW	39.2	11048.4	2.92	29.7	I	III
SUL	467.1	21522.3	34.86	57.85	III	IV
DRB	46.1	5381.9	3.44	14.48	I	II
UDP	153.6	14476.9	11.46	38.92	II	III
S-24P	158.0	5255.3	11.79	14.13	II	II
LDH	90.0	1543.8	6.72	4.15	II	I
DNP	55.9	14059.1	4.17	37.79	I	III
SKR	1282.5	6065.5	95.71	16.30	IV	II
MKT	71.7	5212.6	5.35	14.01	II	II
IVRI-I[†]	13.4	372.0	1.0	1.0	S	S
IVRI-III[†]	-	10564.8	-	28.4	-	III
IVRI-IV[†]	2606.3	-	194.5	-	IV	-
IVRI-V[†]	234.5	9486.0	17.5	25.5	II	III

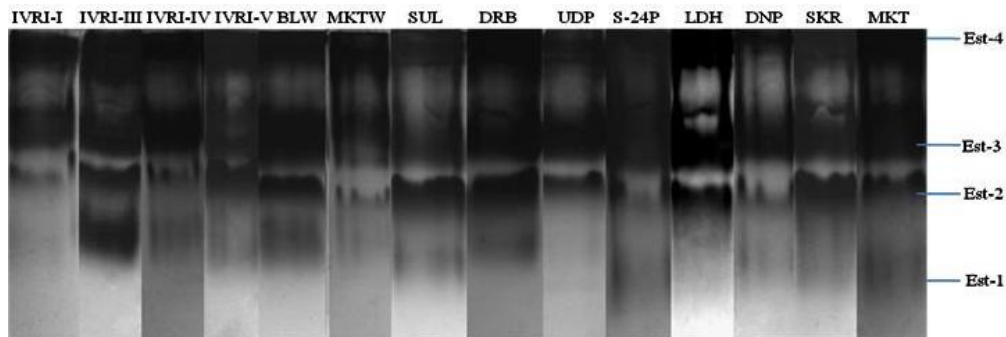[†] reference tick lines; ‡ Susceptible = RF<1.4; level I = 1.5<RF<5; level II=5.1<RF<25; level III = 26<RF<40; level IV = RF>41; S=susceptible
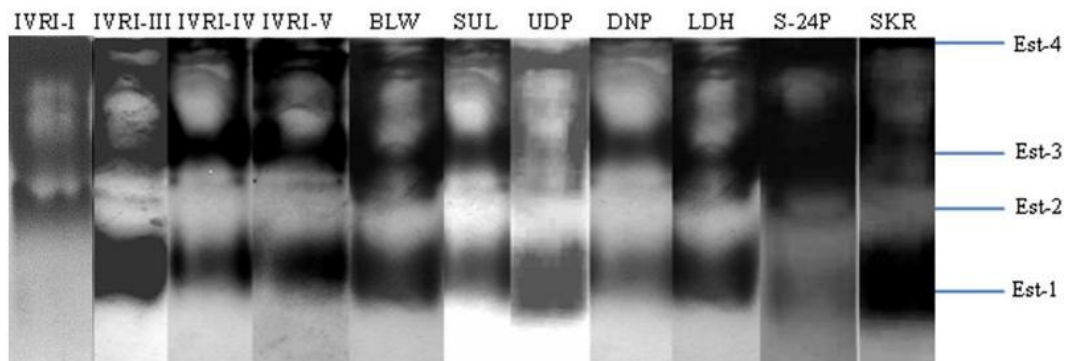
Figures
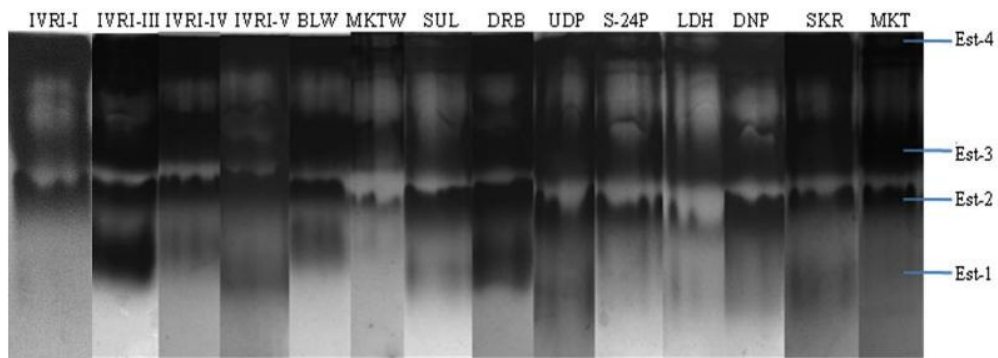
Fig. 1. Reactivity pattern of general esterases in different field isolates and in reference tick lines.

Fig. 2. Reactivity pattern of general esterases in different field isolates and in reference tick lines after probing with TPP.

Fig. 3. The pattern of eserine sulphate induced inhibition of acetylcholinesterase (EST1 and 2) in different field isolates. Lane 1 was not probed with eserine sulphate and shows the corresponding position of EST2 band.