Explor Anim Med Res,
Vol.9, Issue - 1, 2019, p. 47-53

ISSN 2277- 470X (Print), ISSN 2319-247X (Online)
Website: www.animalmedicalresearch.org

Research Article

ENZYMATIC DETOXIFICATION MEDIATED DELTAMETHRIN RESISTANCE IN *HYALOMMA ANATOLICUM* (ACARI: IXODIDAE) POPULATIONS OF WESTERN PUNJAB

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Received 03 June 2019, revised 10 June 2019

ABSTRACT: Resistance status against deltamethrin was assessed in *Hyalomma anatolicum* ticks collected from seven districts of western zone, Punjab, India using Larval Packet Test (LPT). The regression graphs of probit mortality of larval ticks plotted against log values of increasing concentrations of deltamethrin were utilized for the determination of slope of mortality, lethal concentration for 50% (LC_{50}) and 95% (LC_{95}) with 95% confidence interval (CI), and resistance ratios (RR_{50} and RR_{95}). The LC_{50} and LC_{95} values ranged from 2.36–18.72 and 19.28–59.9 ppm in various field isolates whereas, in susceptible ticks (Fazilka isolate) were 1.47 and 9.33 ppm, respectively. The values of RR_{50} and RR_{95} ranged from 1.60 to 12.73 and 2.06 to 6.42, respectively, confirming the resistance status of all field populations. On the basis of RR_{50} values, level I resistance status was recorded in three field isolates and level II in four isolates. Esterase profile of the tick larval extracts using native PAGE, revealed 5 bands of esterase activity designated EST-1 to EST-5. Inhibitory tests recognized EST-1 and EST-2 as acetylcholine esterases, EST-3, EST-4 and EST-5 as carboxylesterases. Quantitative analysis of general esterase activity revealed a range of 3.249 ± 0.31 to 4.523 ± 0.59 and 1.747 ± 0.11 to 2.296 ± 0.20 µmol/min/mg protein for α and β-esterase activity, respectively in different field isolates whereas, glutathione-S-transferase (GST) activity was in range of 0.0127 ± 0.0007 to 0.0289 ± 0.0017 mM/mg/min. A strong correlation was recorded between the resistance ratios against deltamethrin and enzyme ratios (∝-esterase and GST) of various field isolates of *H. anatolicum* indicating their possible role in resistance development.

Key words: Deltamethrin, Esterase, glutathione S-transferase, *Hyalomma anatolicum*, Punjab.

INTRODUCTION

Hyalomma anatolicum (Koch, 1844) (Acari: Ixodidae), a multi-host tick, is widely distributed over most geographical regions in India including Punjab state (Singh and Rath 2013). They are voracious bloodsuckers causing heavy blood losses, bite marks results in low-quality hides and severely lower productivity in terms of weight gain and milk yield. Among various available tick control measures *viz*. chemical acaricides, tick vaccines, biological control and tick-resistant animals, chemical control is by far the most important and widely used strategy. Synthetic pyrethroids (SPs) particularly deltamethrin has been the most extensively used acaricide employed for tick control in recent past in India including the state of Punjab (Sharma *et al*. 2012).

Recent studies report development of resistance against several acaricides in *H. anatolicum* from Indian subcontinent (Shyma *et al*. 2012, 2013; Singh *et al*. 2014a), particularly in Punjab state (Singh *et al*. 2013, Jyoti *et al*. 2015, Nandi *et al*. 2015). Acaricide resistance is a multi-factorial phenomenon arising through several mechanisms like target site insensitivity, increased metabolic detoxification by esterases, oxidases or glutathione-S-transferases and reduced cuticular penetration. Esterases represent a group of highly variable and multifunctional hydrolytic enzymes. In arthropods, these enzymes are involved with various physiological activities and resistance to pesticides. The involvement of esterases (Jamroz *et al*. 2000, Baffi *et al*. 2008, Li *et al*. 2013), and Glutathione S-transferases (Enayati *et al*.

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2010, Nandi *et al*. 2015) in pyrethroid resistance has been demonstrated in many species of ticks. Enhanced carboxylesterase mediated metabolic detoxification has been indicated in pyrethroid resistance in *H. anatolicum* (Shyma *et al*. 2012, Nandi *et al*. 2015). Biochemical characterization of esterases of *H. anatolicum* ticks by inhibition tests is still not available. Hence, the aim of the present study was to determine the deltamethrin resistance status and its underlying detoxification mechanisms in *H. anatolicum* tick of western districts of Punjab.

MATERIALS AND METHODS

Collection of ticks

The areas of collection were selected from seven different districts (Barnala, Bhatinda, Ferozepur, Mansa, Moga, Muktsar and Sangrur) of western Punjab, India. Engorged female ticks were collected from cattle sheds, placed in separate vials, closed with muslin cloth to allow air and moisture exchange and brought to the Entomology Laboratory of the department. The ticks were kept individually in labelled plastic tubes covered with muslin cloth in desiccators which were placed in an incubator maintained at 28±1 ºC and 85±5% relative humidity (RH) for oviposition. The eggs laid were allowed to hatch under similar incubation conditions and 10-14 days old unfed larvae were used for larval packet test to detect the resistance status and rest were stored at -80 ºC for further use.

Acaricide susceptible ticks

The *H. anatolicum* ticks collected from village Amarpura, district Fazilka, Punjab were susceptible to the recommended concentration of deltamethrin and were used as field susceptible isolate for estimation of resistance status (Prerna 2015).

Acaricides

Technical grade deltamethrin (AccuStandard® Inc., USA) was used to prepare the stock solutions of 10,000 ppm in acetone. Different working concentrations of deltamethrin was prepared in distilled water from the stock solutions and tested against the various field isolates of *H. anatolicum*.

Larval packet test (LPT)

The LPT was conducted according to FAO (1971) guidelines with minor modifications. Briefly, 0.5 ml of different concentrations of deltamethrin (12.5, 25, 50, 100 and 200 ppm) were used to impregnate 7.0 cm by 7.0 cm filter paper (541 Whatman). The aqueous solutions of acaricides were dried by keeping the filter papers for 30 min in incubator at 37 ºC. The filter papers were then folded in half diagonally and sealed on one side with adhesive tape, forming an open-ended triangular packet to place tick larvae. After insertion of approximately 100 larvae, the open end of each packet was sealed with adhesive tape, kept in a desiccator and placed in an incubator maintained at 28±1 ºC and 85±5% RH. For each concentration of acaricide the test was conducted in triplicate and in control group distilled water was used. The packets were removed after 24 h, and larval mortality was recorded.

Estimation of resistance status

Dose response data were analyzed by probit method (Finney 1962) using Graph Pad Prism 4 software. This analysis included probit transformation of percentage mortality and natural logarithm transformation of concentration. The lethal concentrations at 50% (LC_{50}) and 95% (LC_{95}) with 95% confidence interval (CI) were estimated. Resistance ratios (RRs) were worked out by the quotient between LC_{50}/LC_{95} of field isolates and LC_{50}/LC_{95} of susceptible ticks (Fazilka isolate). On the basis of RR, the resistance status was classified as susceptible ($RR < 1.5$), level I ($1.5 < RR < 5$), level II ($5 < RR < 25$), level III ($25 < RR < 40$) and level IV ($RR > 40.0$) (Sharma *et al.* 2012).

Esterase profiling of larval ticks

Approximately 100 deep frozen larvae stored at - 80 ºC of each isolate were macerated in liquid nitrogen and homogenized in 0.01 sodium phosphate buffer, pH 6.5, containing 20 % sucrose, 0.01 M EDTA and 0.5 % Triton X-100. The homogenates were spun at 15000 g in a refrigerated centrifuge at 4 °C for 10 min and the resulting supernatant was collected, divided into aliquots and frozen at - 80°C. Protein concentration of the larval homogenates was determined as per the method of Bradford (1976). Individual samples were analyzed for esterase activity profile by electrophoretical analysis using 100 microgram of total protein per sample in non-denaturing polyacrylamide gel. The gel system was prepared with 4 % stacking gel and 12 % separating gel. The vertical electrophoresis was carried out for 4 h at 4 °C, with a constant current of 40 mA using Tris (0.087 M)-Glycine (0.013 M) running buffer, pH 8.3 (Laemmli 1970). The esterases were identified by pre-incubation of gel in 0.1 M sodium phosphate buffer, pH 6.5, for 30 min at 37 °C followed by incubation in 0.1 M sodium phosphate buffer

pH 6.5 containing 3.2 mM 1- or 2-naphthyl acetate and 2.4 mM Fast Blue R/R salt for 60 min, in dark.

Inhibition tests for the biochemical characterization of esterases used 1.0 mM copper sulphate, 1.0 mM p-chloromercurybenzoate (pCMB), 0.4 mM malathion and 1.0 mM eserine sulphate as per Oakeshott *et al.* (1993). Gels were pre-incubated for 30 min in dark in 0.1 M sodium phosphate buffer (pH 6.5) containing inhibitor and then stained for esterase activity in the presence of inhibitor. Technical grade malathion was dissolved in 1 ml methanol and pCMB in 1 ml of 0.1 M NaOH, pH 8.0, prior to use. Eserine sulphate and copper sulphate were added directly to the pre-soaking and staining solution.

Esterase assay

Esterase activities in larval homogenates were estimated as per the method of Nandi *et al*. (2015). The assays for each tick isolate was repeated with 10 different larval homogenates for analysis of statistical significance using a one way analysis of variance (ANOVA) with group multiple comparisons made using the Tukey test (GraphPad Prism 4 software). The correlation between RR_{50} and RR_{95} against deltamethrin and enzyme ratios (α- and β-esterase) of various field isolates was studied and correlation coefficient (r) was determined.

Glutathione S-transferase (GST) assay

The GST activity in larval homogenates were estimated as per the method of Nandi *et al.* (2015). The GST activity (mM/mg/min) in the sample was calculated as O.D./4.39 $mM^{-1} \times$ 6 mm $\times$ total protein in 10 ml homogenate $\times$ 20 min $\times$ 1000. The enzyme ratios were determined and its correlation with RR_{50} and RR_{95} against deltamethrin was determined. The data were statistically analyzed using ANOVA with group multiple comparisons by Tukey test (GraphPad Prism 4).

RESULTS AND DISCUSSION

Deltamethrin resistance in *H. anatolicum*

The LC_{50}, LC_{95}, 95% confidence interval (CI), resistance ratios (RR_{50}, RR_{95}) and resistance level (RL) values of deltamethrin in different field isolates of *H.*

Table 1. LC_{50}, LC_{95}, resistance ratios (RR_{50} and RR_{95}) and resistance level (RL) values for deltamethrin in field isolates of *H. anatolicum* from western Punjab.

Tick isolates	Slope (95% CI)	R^2	LC_{50} (ppm) (95% CI)	LC_{95} (ppm) (95% CI)	$^aRR_{50}$ (RL)	$^bRR_{95}$ (RL)
Barnala	2.35 ± 0.46 (0.89 to 3.82)	0.89	8.32 (8.14-8.59)	39.81 (38.27-41.40)	5.65 (II)	4.26 (I)
Bathinda	3.24 ± 0.58 (1.39 to 5.09)	0.91	18.72 (18.42-19.01)	59.90 (57.98-61.87)	12.73 (II)	6.42 (II)
Ferozepur	1.80 ± 0.54 (0.06 to 3.54)	0.84	2.36 (2.30-2.43)	19.28 (18.09-20.56)	1.60 (I)	2.06 (I)
Mansa	2.24 ± 0.13 (1.82 to 2.65)	0.99	9.09 (8.87-9.30)	49.09 (46.57-51.74)	6.18 (II)	5.26 (II)
Moga	2.55 ± 0.49 (0.97 to 4.14)	0.89	10.60 (10.40-10.83)	46.40 (44.36-48.54)	7.21 (II)	4.97 (I)
Muktsar	2.46 ± 0.71 (0.19 to 4.73)	0.79	6.67 (6.53-6.81)	30.87 (29.48-32.33)	4.53 (I)	3.30 (I)
Sangrur	2.33 ± 0.70 (0.09 to 4.57)	0.79	5.46 (5.33-5.58)	27.59 (26.28-28.95)	3.71 (I)	2.95 (I)
Fazilka*	2.18 ± 0.27 (1.43 to 2.92)	0.94	1.47 (1.54-1.39)	9.33 (9.80-8.87)	1.0 (S)	1.0 (S)

$^aRR_{50}$: LC_{50} of field isolate/LC_{50} of susceptible; $^bRR_{95}$: LC_{95} of field isolate/LC_{95} of susceptible; *Susceptible isolate.

Table 2. Biochemical characterization of *H. anatolicum* esterase by different inhibitors.

Esterases*	$CuSO_4$	pCMB	Malathion	Eserine sulfate	Classification
EST-1	-	-	+	+	AChE
EST-2	-	-	+	+	AChE
EST-3	-	-	+	-	CaE
EST-4	-	-	+	-	CaE
EST-5	-	-	+	-	CaE

* Esterases numbered EST-1 through EST-5, starting from anodic end of the gel.
$CuSO_4$- copper sulfate; pCMB- p-chloromercurybenzoate; AChE- acetylcholinesterase,
CaE- carboxylesterase
(-) absence of inhibition; (+) presence of inhibition.

Table 3. Correlation between resistance ratios (RRs) against deltamethrin and enzyme ratios in different isolates of *H. anatolicum* .

Enzyme	RR	r value	Slope (95% CI)	p value	R^2
∝-esterase	RR_{50}	0.71	0.03 ± 0.01 (0.001 to 0.05)	0.046	0.51
	RR_{95}	0.79	0.06 ± 0.02 (0.01 to 0.11)	0.023	0.61
β-esterase	RR_{50}	0.55	0.02 ± 0.01 (-0.009 to 0.04)	0.157	0.30
	RR_{95}	0.69	0.05 ± 0.02 (-0.003 to 0.09)	0.060	0.47
GST	RR_{50}	0.89	0.09 ± 0.02 (0.05 to 0.13)	0.002	0.81
	RR_{95}	0.83	0.17 ± 0.05 (0.05 to 0.29)	0.011	0.68

RR- resistance ratio; r- Pearson's correlation coefficient; CI- confidence interval.

anatolicum collected from the western districts of Punjab state, India were determined using LPT assay and are presented in Table 1. Values of the coefficient of determination (R^2) for LPT ranged from 0.79 to 0.99 indicating the statistical model a good fit. The LC_{50} and LC_{95} values ranged from 2.36 to 18.72 and 19.28 to 59.9 ppm in various field isolates whereas, in susceptible Fazilka isolate were 1.47 and 9.33 ppm, respectively. The values of RR_{50} and RR_{95} ranged from 1.60 to 12.73 and 2.06 to 6.42, respectively, confirming the deltamethrin resistance status of all field populations. On the basis of RR_{50} values, level I resistance status was recorded in three field isolates (Ferozepur, Muktsar and Sangrur) and level II in four isolates (Barnala, Bhatinda, Mansa and Moga). However, level I resistance status was recorded in five field isolates (Barnala, Ferozepur, Mansa, Muktsar and Sangrur) and level II in two isolates (Bathinda and Mansa) on the basis of RR_{95} values against deltamethrin.

Esterase profile

The esterases were detected in native polyacrylamide gel followed by specific staining using 1- and 2-naphthyl acetate. The patterns of esterase activity in different tick isolates were quite similar and same esterase pattern was

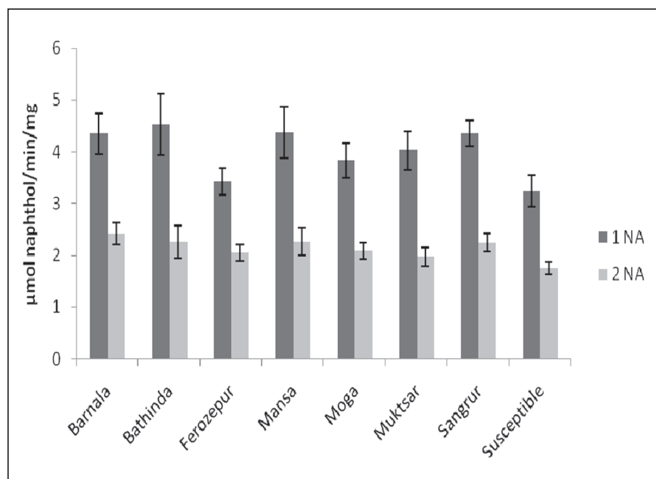

Fig. 1. 1- and 2-Naphthyl acetate hydrolysis activity (Mean ± SE) in *H. anatolicum* isolates.

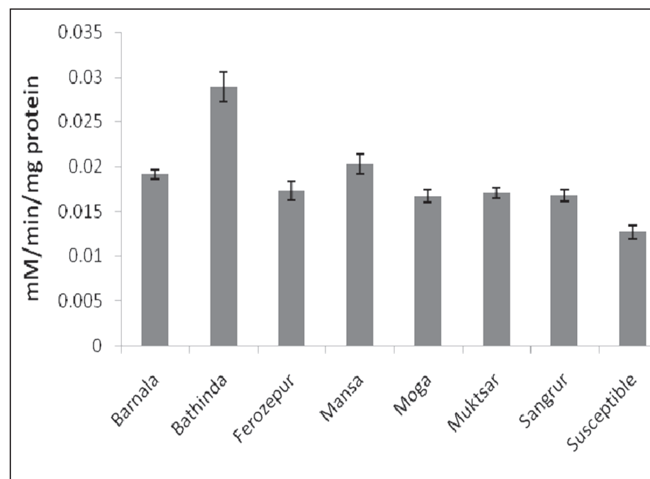

Fig. 2. Glutathione S-transferase (GST) activity (Mean ± SE) in *H. anatolicum* isolates.

observed with 1- and 2-naphthyl acetate. Although, five regions of esterase activity were detected in the susceptible (Fazilka) as well as all seven field isolates, variation in band intensity between isolates was evident. These esterases were numbered EST-1 to EST-5, starting from anodic end of the gel from low molecular mass activity to high molecular mass activity. These esterases were classified using various inhibitors and details of the results of the inhibition assays are presented in Table 2. Copper sulfate and pCMB had no inhibitory effect on these esterases. EST-1 and EST- 2 were inhibited by eserine sulfate and malathion, suggesting that they are acetylcholine esterases (AChEs) whereas, EST-3, EST-4 and EST-5 were affected by malathion and were classified as carboxyl esterases (CaEs).

Esterase assay

Quantitative analysis of general esterase activity revealed a range of 3.425 ± 0.26 to 4.523 ± 0.59 and 1.977 ± 0.18 to 2.424 ± 0.21 µmol/min/mg protein for ∝- and β-esterase activity, respectively, in different field isolates of *H. anatolicum* (Fig. 1). The ∝- and β-esterase activity of susceptible isolate were determined as 3.249 ± 0.31 and 1.747 ± 0.11 µmol/min/mg and was taken as baseline value for comparison. A non-significant ($p > 0.05$) elevation in mean amount of 1-naphthol produced by hydrolysis of 1-naphthyl acetate by ∝-esterase activity was recorded in all field isolates in comparison to susceptible. Also, non-significant ($p > 0.05$) increase in the β-esterase enzyme activity was recorded.

Glutathione S-transferase assay

The GST activity in susceptible isolate was determined as 0.0127 ± 0.0007 mM/ min/mg protein whereas, in field isolates ranged from 0.0167 ± 0.0007 to 0.0289 ± 0.0017 mM/ min/mg, corresponding to ER between 1.314 and 2.275. Statistically significant ($p<0.05$) elevation in GST activity was recorded in Barnala, Bathinda and Mansa isolates of *H. anatolicum* in comparison to susceptible (Fazilka) isolate (Fig. 2).

Correlation between various enzyme activities and resistance ratios

The correlation data between resistance ratios (RR_{50} and RR_{95}) against deltamethrin and enzyme activities in term of enzyme ratios (∝-, β-esterase and GST) in various field isolates of *H. anatolicum* are presented in Table 3. The enzyme ratios of ∝-esterase and GST were correlated ($p<0.05$) with RR_{50} and RR_{95} against deltamethrin of *H. anatolicum* isolates with high correlation coefficient (r). The correlation coefficient indicates the real correlation between both the variables which tend to increase or decrease together and exists between 0 and 1. However, non-significant ($p>0.05$) correlation between RR_{50} and RR_{95} against deltamethrin and β-esterase enzyme ratios were observed with lower r values.

The results of the present study revealed presence of widespread low levels of deltamethrin resistance in *H. anatolicum* collected from western Punjab, India with maximum RR_{50} of 12.73 in Bathinda isolate. Similar low levels of rresistance to deltamethrin has been earlier reported in *H. anatolicum* from Haryana state (Shyma *et al*. 2013), trans-gangetic plain region in the state of Punjab, Haryana, Rajasthan and Uttar Pradesh (Shyma *et al*. 2012) and central plain zone of Punjab state (Nandi

et al. 2015). Development of acaricide resistance has much slower rate in multi-host ticks as smaller fraction of the total population is under chemical challenge than one-host ticks. Also, longer generation period and feeding of immature stages often on other hosts could lead to decreased exposure to chemicals and thus reduction in selection pressure of multi-host ticks (Singh *et al*. 2014b).

The ∝- and β-esterases have been extensively studied in insects and are known to be involved in development of resistance to insecticides. The inhibition tests for biochemical characterization of esterases in insects have been important to decipher the different physiological processes in which esterases act on. Native PAGE studies revealed that there was no difference in the banding pattern between the various populations of *H. anatolicum*. Although, till date no data is available regarding the esterase pattern of *H. anatolicum* ticks, our results showed similarity with the banding pattern of *R.* (*B.*) *microplus* esterases reported earlier by several authors (Jamroz *et al*. 2000, Baffi *et al*. 2005). The band intensity of esterases detected showed variations in the intensity between susceptible and various deltamethrin resistant tick populations and appeared more intense in resistant strains, similar to earlier reports in *R.* (*B.*) *microplus* (Baffi *et al*. 2008).

For SPs resistance development in ticks, sodium channel target site insensitivity and increased metabolism of acaricide by esterase enzymes are the two major mechanisms. In the current study increased levels of ∝- and β-esterase were recorded in field isolates of *H. anatolicum* along with a significant correlation in deltamethrin resistance status and ∝- esterase activity. Increased esterase activity has been established as one of the primary mechanism of SP resistance due to increased detoxification in *R.* (*B.*) *microplus* ticks (Jamroz *et al*. 2000, Li *et al*. 2013, Singh and Rath 2014) and *H. anatolicum* (Shyma *et al*. 2012, Nandi *et al*. 2015).

Glutathione S-transferases (GSTs) catalyze the nucleophilic attack of reduced glutathione (GSH) on the electrophilic centers of lipophilic compounds and have been demonstrated to play a role in acaricide resistance development in many species of ticks (Li *et al*. 2003, Enayati *et al*. 2010, Nandi *et al*. 2015). Involvement of an increased GST activity in conferring OP resistance in Pesqueria (Mexican) resistant *R.* (*B.*) *microplus* strain (Li *et al*. 2003) and SPs resistance in *R. bursa* (Enayati *et al*. 2010), *R.* (*B.*) *microplus* (Nandi *et al*. 2015) and *H. anatolicum* ticks (Nandi *et al*. 2015) has been documented. In the present study a significant ($p<0.05$) elevation in GST activity with 1.314 and 2.275 fold increase was recorded along with a positive correlation with deltamethrin resistance status in *H. anatolicum*. These results indicate that GST may play a significant role in conferring SPs resistance, particularly deltamethrin, in the Indian isolates of *H. anatolicum*.

CONCLUSION

The present work seems to be the first study describing esterase banding pattern in deltamethrin resistance *H. anatolicum* ticks. In the current study increased levels of detoxification enzymes (∝-, β-esterase, GST) were recorded in field isolates of *H. anatolicum* along with a positive correlation with deltamethrin resistance status. The results indicate that ∝-esterase and GST may play a significant role in conferring resistance against SPs particularly deltamethrin in the Indian isolates of *H. anatolicum*.

ACKNOWLEDGEMENT

Authors are thankful to the Director of Research, GADVASU, Ludhiana for providing facilities to carry out the research work. This work was supported by University Grants Commission, New Delhi through Project No. F.No. 42-633/2013 (SR).

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***Cite this article as:** Prerna M, Singh NK, Jyoti, Singh H, Rath SS (2019) Enzymatic detoxification mediated deltamethrin resistance in *Hyalomma anatolicum* (Acari: Ixodidae) population of western Punjab. Explor Anim Med Res 9(1): 47-53.