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Research Article

SYNTHETIC PYRETHROID RESISTANCE IN *RHIPICEPHALUS MICROPLUS* ASSOCIATED WITH METABOLIC DETOXIFICATION MECHANISM

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ABSTRACT: Larval packet test was utilized for detection of resistance against commonly used synthetic pyrethroid acaricides viz. cypermethrin and deltamethrin in cattle tick, *Rhipicephalus microplus* (*R. microplus*) populations from various districts of Punjab, India. The values for the lethal concentrations at 50% mortality (LC50) for cypermethrin and deltamethrin were estimated by probit analysis and the Resistance Factor (RF) was calculated. The RF values for *R. microplus* isolates against cypermethrin ranged from 0.07 to 4.31 indicating susceptibility in five field isolates of western Punjab and level I resistance status in the other three isolates collected from Muktsar, Barnala and Bathinda districts. Similarly, the RF values against deltamethrin ranged from 0.27 to 24.35 with susceptibility in only single isolate from the Bathinda district, level I resistance status in three isolates (Sangrur, Moga and Mansa) and level II status in other four isolates (Ferozepur, Barnala, Muktsar and Fazilka). The α- and β-esterase enzyme activity in the field isolates were estimated as 3.496±0.24 - 5.346±0.75 and 1.801±0.14 - 3.101±0.27 µmol/min/mg protein, respectively and glutathione-S-transferase (GST) enzyme activity was recorded as 0.0265±0.0007 - 0.0300±0.0015 mmol/mg/min in various field isolates. A strong correlation was recorded between enzyme ratios of α-esterase and GST enzymes of the *R. microplus* field isolates and the resistance factor values against deltamethrin which may be playing a major role in developing resistance.

Keywords: Cypermethrin, Deltamethrin, Enzymatic detoxification, *Rhipicephalus microplus*.

INTRODUCTION

The cattle tick, *Rhipicephalus microplus* (Canestrini, 1888) is considered an economically important tick species causing heavy damage to the health and productivity of cattle. The environmental conditions of the Punjab state are very favorable for the development of off-host tick stages during most parts of the year often leading to heavy tick infestations in dairy animals [1]. They suck blood causing anemia, the tick bite marks cause significant depreciation of hide value and also severely decrease the milk yield and weight gain. In India as per a recent report, the annual cumulative loss due to tick infestation and tick-borne diseases has been reported as 61076.46 million rupees or 787.63 million USD [2].

The use of chemical acaricides is most widely used for tick control globally and comprises its repeated and extended use on a large scale [3]. Among the various acaricide classes, synthetic pyrethroids (SPs) *viz.* cypermethrin and deltamethrin have been most widely used in the recent past in India including Punjab [4]. There are several recent reports of SP (cypermethrin and deltamethrin) resistance in cattle ticks from several regions of the country [4, 5, 6] and Punjab [7, 8, 9]. Monitoring acaricide resistance status and understanding the possible resistance development mechanism is very crucial to slowing down the spread and developing region-specific tick control strategy. The esterases are a diverse group of enzymes with multiple functions and key roles in arthropods by aiding in digestion, regulating juvenile hormone levels, influencing reproductive behavior, supporting nervous system functions and also contributing to pesticide resistance [10, 11]. The involvement of esterases [12,

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13, 14] and GST [15, 16, 17] has been demonstrated in the development of resistance against SPs in ticks. Therefore, the current study was undertaken to evaluate the SP resistance status and its development mechanisms in *R. microplus* tick populations of western Punjab, India.

MATERIALS AND METHODS

Collection of ticks

The ticks were collected from randomly chosen locations across eight districts in western Punjab (Bhatinda, Moga, Fazilka, Sangrur, Barnala, Muktsar, Ferozepur and Mansa). Fully engorged dropped-off adult female ticks were gathered from hiding sites in the cattle sheds, placed in vials covered with muslin cloth for air and moisture exchange and immediately transported to the laboratory. The ticks were washed with water, dried and kept in Petri plates inside desiccators placed in an incubator at 28±1ºC and 85±5% relative humidity (RH) for egg laying. After two weeks, the laid eggs were transferred to glass tubes and incubated again at similar conditions for hatching. Unfed larvae aged 10-14 days were utilized in bioassay to detect the resistance status and the rest were stored at -20ºC for later enzyme assays [9].

Acaricides

Technical grade cypermethrin and deltamethrin (Sigma Aldrich, St. Louis, MO, USA) were used to make stock solutions of 10,000 ppm in methanol and 5000 ppm in acetone, respectively. The working concentrations of cypermethrin (800, 400, 200, 100, and 50 ppm) and deltamethrin (200, 100, 50, 25, and 12.5 ppm) were prepared by serial dilution in distilled water.

Larval packet test (LPT)

The LPT was conducted as per the standardized protocol with minor modifications [9]. Briefly, the filter papers (Whatman No. 1, Whatman, Madstone, UK) cut in a parallelogram shape (7.0 cm by 7.0 cm) were impregnated with 0.5-0.6 ml of different working concentrations of cypermethrin and deltamethrin with a pipette. They were dried for 30 minutes in an incubator at 37°C, diagonally folded and sealed by tape on one side to make a triangular packet with an open end. After placing approximately a hundred 10-14-day-old unfed larvae in each packet, the open end was sealed and kept in a desiccator inside an incubator at 28±1 ºC and 85±5% RH. After 24 hours the packets were opened and the number of live and dead larvae were counted for calculation of larval mortality. For each acaricide concentration, the test was conducted in triplicate with distilled water (diluent) used in the control group.

Estimation of resistance status

The dose-mortality response data was analyzed by probit analysis using GraphPad Prism 4 software. The values of lethal concentration for 50% (LC50) of cypermethrin and deltamethrin for all field isolates of *R. microplus* were estimated by using regression analysis equation of log concentrations of acaricides and the probit transformed mortality data. Resistance factor (RF) or resistance ratio (RR50) was calculated by the quotient between the LC50 values of field isolates and LC50 values of susceptible ticks (IVRI-I) and the resistance status was classified on the basis of RF [4].

Esterase assay

Esterase activities in larval homogenates (µmol of naphthol/min/mg of protein) were determined using 1- and 2-naphthyl acetate as substrates [9]. One-way analysis of variance (ANOVA) was used for statistical analysis along with the Tukey test for group multiple comparisons. The enzyme ratio (ER) was calculated and the correlation coefficient (r) with RFs was determined [9].

Glutathione S-transferase (GST) assay

The GST activities in the field and susceptible larval homogenates (mM/mg/min) were determined [9]. The enzyme ratios (ER) were estimated and their correlations with RFs were determined [9].

RESULTS AND DISCUSSION

Resistance status against cypermethrin and deltamethrin in *R. microplus*

The values of LC50, 95% confidence interval (CI), resistance factor (RF) and resistance level (RL) against cypermethrin and deltamethrin of *R. microplus* isolates of western Punjab were estimated and the details are presented (Table 1). The RF values for *R. microplus* isolates against cypermethrin ranged from 0.07 to 4.31 indicating susceptibility in five field isolates of western Punjab (Sangrur, Mansa, Ferozepur, Moga and Fazilka) and level I resistance status in the other three isolates collected from Muktsar, Barnala and Bathinda districts. Similarly, the RF values against deltamethrin ranged from 0.27 to 24.35 with susceptibility in only single isolate from the Bathinda district, level I resistance status in three isolates (Sangrur, Moga and Mansa), and level II status in other four isolates (Ferozepur, Barnala, Muktsar and Fazilka).

Table 1. Values of LC50, resistance factor (RF), confidence interval (95% CI) and resistance level (RL) in *R. microplus* field isolates by LPT.

Tick isolates	Cypermethrin				Deltamethrin			
	Slope (95% CI)	R^2	LC_{50} (ppm) (95% CI)	RF (RL)	Slope (95% CI)	R^2	LC_{50} (ppm) (95% CI)	RF (RL)
Barnala	1.79 ± 0.16 (1.26-2.31)	0.975	525.80 (540.5-509.1)	2.16 (I)	1.84 ± 0.17 (1.29 - 2.39)	0.974	134.24 (130.5-138.2)	11.3 (II)
Bathinda	1.60 ± 0.09 (1.31-1.89)	0.990	363.97 (351.1-375.4)	1.50 (I)	0.85 ± 0.07 (0.62 - 1.08)	0.978	3.27 (3.1-3.4)	0.27 (S)
Fazilka	1.19 ± 0.23 (0.45-1.93)	0.897	230.77 (220.8-241.1)	0.95 (S)	1.03 ± 0.11 (0.68 - 1.39)	0.966	61.24 (58.1-64.4)	5.18 (II)
Ferozepur	1.45 ± 0.06 (1.26-1.64)	0.994	90.66 (87.4-94.0)	0.37 (S)	1.86 ± 0.08 (1.58 - 2.15)	0.993	66.25 (64.4-68.1)	5.61 (II)
Mansa	2.82 ± 0.34 (1.71-3.94)	0.956	145.66 (145.5-145.9)	0.60 (S)	3.40 ± 0.42 (2.04 - 4.76)	0.954	49.59 (48.8-50.3)	4.20 (I)
Moga	1.96 ± 0.45 (0.53-3.40)	0.864	17.65 (17.3-18.1)	0.07 (S)	1.15 ± 0.17 (0.58 - 1.72)	0.932	41.35 (39.9-42.8)	3.50 (I)
Muktsar	1.11 ± 0.04 (0.97-1.25)	0.995	1046.78 (997.9-1098.1)	4.31 (I)	1.40 ± 0.16 (0.89 - 1.91)	0.962	287.35 (276.8-298.4)	24.4 (II)
Sangrur	1.64 ± 0.26 (0.82-2.45)	0.931	346.73 (335.9-358.1)	1.43 (S)	2.73 ± 0.33 (1.69 - 3.77)	0.958	55.50 (54.4-56.5)	4.70 (I)
Susceptible*	10.23 ± 2.46	0.811	242.4 (241.2-243.6)	1.0	3.42 ± 0.49	0.871	11.8 (11.6-12.0)	1.0

*IVRI-I line [4]

Table 2. The α- and β-esterase activity and Enzyme ratios (ER) in field isolates of *R. Microplus*.

Tick isolates	α-esterase			β-esterase		
	p value	Mean difference (95% CI)	ER	p value	Mean difference (95% CI)	ER
Barnala	< 0.01	2.55 (0.42 - 4.69)	2.06	> 0.05	1.02 (-0.21 - 2.34)	1.58
Bathinda	> 0.05	1.41 (-0.66 - 3.49)	1.58	> 0.05	0.33 (-0.94 - 1.61)	1.19
Fazilka	< 0.01	2.77 (0.70 - 4.85)	2.15	< 0.05	1.35 (0.07 - 2.63)	1.77
Ferozepur	< 0.05	2.07 (0.02 - 4.11)	1.86	> 0.05	0.81 (-0.49 - 2.13)	1.46
Mansa	> 0.05	1.09 (-0.98 - 3.17)	1.45	> 0.05	0.05 (-1.22 - 1.33)	1.03
Moga	< 0.05	2.01 (0.01 - 4.01)	1.83	> 0.05	0.42 (-0.82 - 1.67)	1.24
Muktsar	< 0.001	2.94 (0.94 - 4.94)	2.22	> 0.05	0.99 (-0.25 - 2.24)	1.57
Sangrur	< 0.05	2.05 (0.06 - 4.05)	1.85	> 0.05	0.82 (-0.42 - 2.07)	1.47

The cattle tick, *R. microplus* is the most widely prevalent hard tick parasitizing dairy cattle of Punjab state [1, 18]. The most common approach to controlling ticks is the direct use of topical chemical acaricides on host animals or through parenteral injectables. Among the various commercially available acaricides, SPs particularly cypermethrin and deltamethrin are mostly being used drugs in the state for chemical tick control [4]. A major undesired outcome of using chemical acaricides is the selection for resistance in tick populations which poses great risk to the effectiveness of SPs in controlling these vectors. Thus, monitoring of acaricide resistance is essential to safeguard the cattle population of the state from these ticks as it

Table 3. Correlation between resistance factors and enzyme ratios in *R. microplus*.

Acaricide	Enzyme	r value	Slope (95% CI)	p value	R^2
Cypermethrin	α-esterase	0.559	0.111 ± 0.067 (-0.053 - 0.275)	0.149	0.312
	β-esterase	0.390	0.070 ± 0.067 (-0.095 - 0.235)	0.339	0.152
	GST	0.251	0.0175 ± 0.0134 (-0.0152 - 0.0504)	0.2384	0.222
Deltamethrin	α-esterase	0.691	0.024 ± 0.010 (-0.001 - 0.050)	0.058	0.477
	β-esterase	0.453	0.014 ± 0.011 (-0.014 - 0.043)	0.259	0.205
	GST	0.793	0.0053 ± 0.0016 (0.0012 - 0.0093)	0.0187	0.629

(r: Pearson's correlation coefficient).

helps to slow the spread of resistance and enables the development of region-specific tick control strategies. Although the LPT requires 5-6 weeks for completion, it is a repeatable bioassay that requires few engorged females and thus is appropriate for resistance monitoring in field conditions.

The results of the present study revealed variable levels of resistance status against both the commonly used acaricides (cypermethrin and deltamethrin) in tick isolates from western Punjab which can be probably due to lack of uniformity in the use of chemical acaricide in the state. Resistance development against SPs in *R. microplus* ticks from several geographical areas of the country has been reported in the recent past [4, 5, 6]. Further, in comparison to cypermethrin, more intense and widespread resistance was recorded against deltamethrin probably because of its much prolonged and indiscriminate use as has been reported [4, 8, 9]. The highest resistance factors of 4.31 and 24.4 against cypermethrin and deltamethrin, respectively, were estimated in the Muktsar isolate. Similarly, in the recent past, high RF (95.7) was reported in *R. microplus* from Rajasthan state against deltamethrin [4]. The development of high resistance levels in the region may be due to the increased usage of SPs by the owners due to the predominance of cross-bred cattle population which is highly susceptible to tick infestations leading to more frequent acaricide treatments [4].

Esterase assay

The α- and β-esterase enzyme activity in the field isolates of cattle tick were estimated as 3.496±0.24 - 5.346±0.75 and 1.801±0.14 - 3.101±0.27 µmol/min/mg protein, respectively (Fig. 1). The enzyme ratios (ER) for α- and β-esterases in field isolates, along with multiple pairwise comparisons of their means with the susceptible isolate (IVRI-I) and corresponding significance levels (Tukey, p=0.05), are presented in Table 2. A significant increase ($p<0.05$) in the 1-naphthol produced by ?-esterase activity (via hydrolysis of 1-naphthyl acetate) was observed in six field isolates. In contrast, the increase in β-esterase activity was not significant ($p>0.05$) for all isolates except Fazilka. Also, higher levels of α-esterase activity were strongly correlated with resistance factors. Synthetic pyrethroids are esters that can be broken down by esterases and higher production of the esterases in insects can lead to increased detoxification. It occurs first through sequestration and then through hydrolysis once the inhibited esterase becomes reactivated [18, 19]. Elevated esterase activity is recognized as a key mechanism of SP resistance in cattle ticks globally [11, 12, 13], including in this region [8, 9, 14], due to enhanced detoxification processes.

Glutathione S-transferase assay

The GST activity was recorded as 0.0265±0.0007 - 0.0300±0.0015 mmol/mg/min in various field isolates and 0.0215 ± 0.0003 mmol/mg/min in susceptible isolate (IVRI-I) with enzyme ratio (ER) in range of 1.232 - 1.395. A significant increase ($p<0.05$) of 1.23 - 1.39 times was observed with a strong correlation with resistance factors against deltamethrin. The findings suggest that GST may play a crucial role in the development of resistance to SPs, especially against deltamethrin in the tick isolates of the region as has also been earlier reported [9]. The GSTs have been shown to contribute to the development of acaricide resistance in various tick species [15, 16, 17].

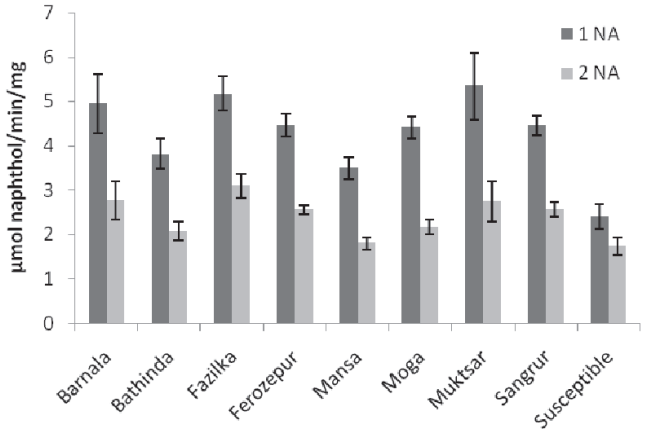

Fig. 1. α- and β-esterase enzyme activity (Mean ± SE) in *R. microplus* field isolates.

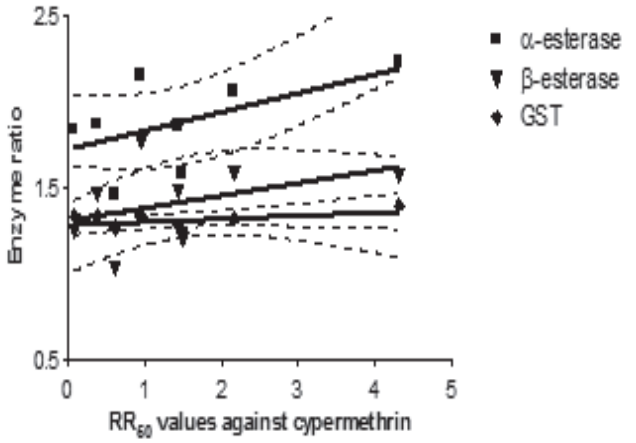

Fig. 2. Correlation between enzyme ratios (α-, β-esterase, and GST) and RR_{50} against cypermethrin.

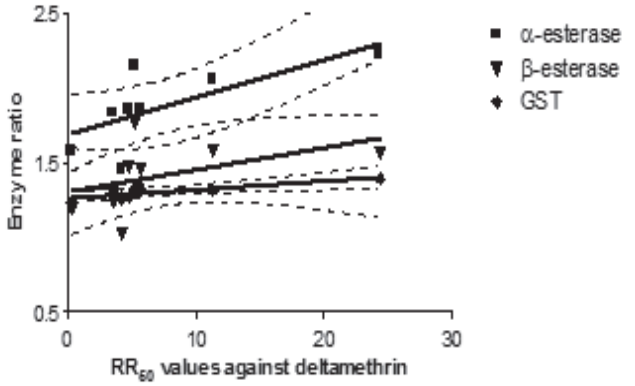

Fig. 3. Correlation between enzyme ratios (α-, β-esterase, and GST) and RR_{50} against deltamethrin.

Correlation between various enzyme activities and resistance ratio

The correlation of resistance factors (against cypermethrin and deltamethrin) and the enzyme ratios (α-, β-esterase, and GST) in the field isolates of *R. microplus* are presented (Table 3 and Figs. 2-3). The ERs of α-esterase were strongly correlated (r=0.559) with RF against cypermethrin. The correlation coefficient (r) represents the true correlation between two variables, indicating how they tend to increase or decrease together with a value ranging between 0 and 1. Further, a stronger correlation with higher r values was observed between α- and β-esterase ERs and RF values deltamethrin. A significant ($p<0.05$) correlation between RF values against deltamethrin and GST ERs was recorded (r=0.793). Thus, increased levels of detoxification enzymes (α-, β-esterase, and GST) and their correlation with resistance factors against SPs indicate that the α-esterase and GST enzyme levels may have a significant role in resistance development in the isolates of the region.

CONCLUSION

A susceptible status was reported against cypermethrin from the majority of cattle ticks of western Punjab while for deltamethrin, the trend was reversed and the majority of isolates showed resistance status. The quantitative enzyme analysis revealed elevated levels of α-, β-esterase, and GST in the field tick isolates. Furthermore, a significant correlation of activities of β-esterase and GST, with SP, particularly, deltamethrin, was recorded indicating their possible role in resistance development in *R. microplus*. It is evident that a single mechanism is unlikely to be sufficient in providing resistance to pyrethroids. Multiple enzymes are involved in the process of detoxifying pesticides, which is accompanied by many genetic alterations occurring at various loci. Although it is challenging to establish a direct correlation between these independent characteristics and an individual's capacity to withstand pyrethroid pesticides, it is probable that a mix of processes contributes to the observed resistance in *R. microplus* of western Punjab. The present work, thereby, adds to the underlying mechanism of resistance development in ixodid ticks and the data generated will be helpful to formulate better control strategies to delay the development of SP resistance.

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