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Esterase profile of *Rhipicephalus* (*Boophilus*) *microplus* populations collected from Northern India exhibiting varied susceptibility to deltamethrin

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Abstract *Rhipicephalus* (*Boophilus*) *microplus* is an economically important ectoparasite of cattle. Chemical acaricides remain the most practical method for control of these pests. During past two decades there have been increasing reports of resistance development against synthetic pyrethroids in tick populations of this species throughout the world. A study was conducted to determine the level of susceptibility of *R.* (*B.*) *microplus* to deltamethrin collected from different geographical locations of northern India. LPT bioassay results revealed LC_{50} values of deltamethrin ranging from 0.035 to 0.00037 % A.I. Esterase profile of the tick larval extracts using native PAGE, revealed 5 bands of esterase activity designated EST-5 to EST-1A. Inhibitory tests recognized EST-1, EST-2 and EST-3 as Acetylcholinesterases (AchEs), EST-4 and EST-5 as Carboxylesterases (CaEs). The band intensity varied between tick populations of various locations, being more intense in case of the resistant populations. An extra band of esterase activity (EST-1A) was obtained in larval extracts of ticks from 3 locations. This increased esterase activity may be involved in the resistance development in these tick populations. Acaricide resistance is a multi-factorial phenomenon, thus other causes of increased resistance like sodium channel mutation and reduced drug penetration (e.g. cuticle thickening) and behavioural changes (e.g. avoiding the pesticides) are to be tested in future in order to confirm the basic cause of the resistance development in these acaricide resistant tick populations.

Keywords Synthetic pyrethroids · *Rhipicephalus* (*Boophilus*) *microplus* · Resistance · Deltamethrin · Active ingredient (AI) · LC_{50} · Esterase · Acetylcholinesterase · Carboxylesterase

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Introduction

Rhipicephalus (*Boophilus*) *microplus,* the tropical cattle tick is economically the most important ectoparasite of bovines. *R.* (*B.*) *microplus* causes significant losses to the livestock industry from its direct effects on production and more importantly from its transmission of various pathogens such as *Babesia* and *Anaplasma* (Jongejan and Uilenberg 1994). These ticks are distributed throughout the world, particularly in the tropical and subtropical regions with India being no exception. It has been estimated that 80 % of the world's cattle population is exposed to tick infestation (FAO 1984). Tick infestation leads to anorexia, toxicosis, decreased milk yield, meat production, damaged hides and even death of affected animals (Sonenshine 1991; Ocaido et al. 2008). The losses due to TBDs in India alone has been calculated as Rs 20,000 million annually (Minjauw and McLeod 2003).

In India, traditional tick control methods are based on the use of acaricides particularly synthetic pyrethroids. Among synthetic pyrethroids, deltamethrin (Srivastava et al. 1993), cypermethrin (Kumar et al. 2003) and flumethrin (Roy et al. 2005) are commonly used. Because of non availability of alternative control methods, dependence on these chemical acaricides is going to increase, this in turn will exert selection pressure on the tick populations and as such development of acaricide resistance in these parasites (Vatsya and Yadav 2011). The situation is further aggravated by the indiscriminate use of acaricides due to absence of stringent regulations on distribution/purchase of these acaricides.

Resistance against synthetic pyrethroids in *R.* (*B.*) *microplus* is conferred by different mechanisms (Li et al. 2010). Two important mechanisms of resistance development in these ticks are: sodium channel target site insensitivity (Rosario-Cruz et al. 2009; Smirnov and Smirnova 2009) and increased metabolism of the acaricide (Chevillon et al. 2007). Resistance due to increased metabolism of pyrethroids is due to esterases, which represent highly variable and multi-factorial enzymes (Baffi et al. 2008). Studies of acaricide resistant *R.* (*B.*) *microplus* have shown that carboxylesterases (CaEs) and acetylcholinesterases (AchEs) are associated with resistance, involving both increased metabolism i.e. detoxification and target site insensitivity (Chen et al. 2009).

The present study was planned keeping in mind the problem of reduced efficacy of deltamethrin against *R.* (*B.*) *microplus*. The objective of the investigation was (1) to determine variations in susceptibility of various populations of *R.* (*B.*) *microplus* to synthetic pyrethroids collected from different geographical locations of northern India and (2) to evaluate the role of esterases in varied susceptibility of *R. microplus* to synthetic pyrethroids.

Materials and methods

Reference susceptible ticks

Ticks collected from Pithoragarh region were designated deltamethrin susceptible. These ticks were found susceptible to deltamethrin and had no history of exposure to any acaricide. They were controlled by manual methods like hand picking and burning or using locally available herbs. Engorged female (F_0 generation) were collected from tick infested cattle and brought to the laboratory and are since then being maintained as colonies without acaricidal pressure.

Collection and rearing of field ticks

Engorged adult *R.* (*B.*) *microplus* female ticks were collected from cattle naturally infested with ticks from eleven different geographical locations {Gadarpur (550 mt above sea level), Nagla Dairy, Sanjay colony, Jawahrnagar, Crop Research Centre (CRC) (243.8 mt above sea level), Rudrapur (208 mt above sea level), Kiccha (293 mt above sea level), Ramnagar (1,729 mt above sea level), Bheemtal (1,375 mt above sea level), Almora (1,638 mt above mean sea level) and Dehradun (700 mt above mean sea level)} of northern India situated between 28°43′N to 31°27′N latitude and 77°34′E to 81°02′E longitudes comprising of Hills with temperate climate and Plains with a subtropical climate. Gadarpur, Rudrapur, Kiccha, CRC, Nagla Dairy, Sanjay colony and Jawaharnagar have a subtropical climate with an average annual rainfall of 573.3–1,607 mm and temperature ranging from 10 to 42 °C. Almora, Dehradun, Ramnagar and Bheemtal have a temperate climate with an average annual rainfall ranging from 205 to 2,073.3 mm and temperature ranging from 10 to 27 °C to below 0 °C in winter months. All the places were at least 100 km^2 apart except CRC, Nagla Dairy, Sanjay colony and Jawaharnagar which were 10–20 km^2 apart. Gadarpur, Rudrapur, Almora, Dehradun, Kiccha and Jawaharnagar had history of deltamethrin being used for last 12 years. In Sanjay colony and CRC, the owners controlled ticks using locally available herbs or the ticks were picked and burned.

A minimum of 50 fully engorged female ticks were collected from a minimum of 10–15 cattle from each area and brought to the laboratory. Immediately upon arrival at the laboratory, engorged female ticks were washed with distilled water and dried using tissue paper. Clean, engorged female ticks were placed in glass vials covered with muslin cloth and rubber band and then incubated in biological oxygen demand (B.O.D.) incubator at temperature 27 (±2 °C) and relative humidity of 85–92 % for oviposition. The larvae of *R. microplus* ticks that hatched out of eggs were preserved in BOD incubator until used in tests. Larvae from each sample were divided into two parts; one was kept in incubator for LPT bioassay and another frozen at −80 °C until esterase profile was conducted.

In vitro laboratory bioassays (Larval Packet Test—LPT)

Deltamethrin susceptibility bioassays were performed as per the procedure described by FAO (2004) with slight modifications i.e. instead of technical grade acaricides, commercially available formulation was used. Deltamethrin was diluted in 2 parts of trichloroethylene and 1 part of olive oil. Serial dilutions of deltamethrin (0.000625, 0.00125, 0.0025, 0.005, 0.010 and 0.020 %) were made in this diluent to generate testing doses. Diluent alone was used as control.

Packets were prepared by depositing 1 ml of testing dose on a 7.5 cm × 8.5 cm piece of Whatman filter paper (Maidstone, England); after this the acaricide impregnated papers were allowed to dry for 2 h to allow trichloroethylene to evaporate. Papers impregnated with diluents only were used as control. Treated papers were folded into half and sealed on the sides with bulldog clips to form packets. About 100 fourteen day old larvae were put into each packet, which were then sealed along the top with additional bulldog clip. The packets were placed in an incubator (27 ± 2 °C and 85–92 % RH) for 24 h. After which the papers were taken out and opened. Control packets were opened first and examined for larval mortality. Then the packets were opened in order of increasing concentration of the acaricide. Each testing dose was tested in triplicate and the average of dead and live larvae was scored. The larval mortality in Larval Packet Test at a given testing dose was expressed as percentage of the total number of larvae.

Statistical analysis

The average larval mortality data were subjected to Probit analysis for calculating LC_{50} (lethal concentration to 50 % tick larvae tested) and LC_{99} (lethal concentration to 99 % tick larvae tested) values, along with 95 % confidence limits and slope of Probit lines. The data were analysed by computer software programme based on Finney (1971). The data were discarded when mortality in the control group were more than 10 %. Abbot's formula (Abbot 1925) was applied when mortality in the control group ranged from 5 to 10 %.

Chi-square test was used to estimate deviation of data from linearity i.e. heterogeneity of tick larvae. Differences in LC_{50} estimates were called significant ($P = 0.05$) when their 95 % confidence (CI 95 %) intervals did not overlap (Robertson and Priesler 1992).

The resistance factor (RF) was obtained by comparing the LC_{50} of field ticks relative to the LC_{50} of reference susceptible ticks.

Esterase profile of *Rhipicephalus (Boophilus) microplus*

About 100 tick larvae 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 centrifuged at 15,000×*g*, 4 °C, for 10 min and the supernatant was collected, divided into aliquots and frozen at −80 °C. Total protein concentration of each sample was determined according to the method of Bradford (1976). The total protein concentration was expressed in milligrams of proteins per gram of tick larvae. Individual samples were analyzed for esterase activity profile; electrophoretical analysis was carried out 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 done for 4 h at 4 °C, with a constant current of 40 mA and Tris (0.087 M)-Glycine (0.013 M) running buffer, pH 8.3. 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 α or β-naphthyl acetate and 2.4 mM Fast Blue R/R salt for 60 min, in dark. Inhibitor tests was carried with 1 mM CuSo4, 1 mM p-chloromercurybenzoate (pCMB), 0.4 mM malathion, 1 mM Eserine sulfate and 1 mM Phenyl- methylsulfonyl fluoride (PMSF) for biochemical classification of esterases according to Oakeshott et al. (1993).

Results

LPT bioassay results revealed five tick sample populations from Dehradun (RF 44.92), Jawaharnagar (RF 11.29), Rudrapur (RF 6.80), Nagla Dairy (6.02) and Almora (RF 5.79) to be resistant against deltamethrin ($RF > 5$). Tick samples of Kiccha (RF 4.09) were tolerant ($RF = 3–5$), whereas ticks populations of Gadarpur (RF 1.70), Ramnagar (RF 1.36), Bheemtal (RF 0.88), Sanjay colony (RF 0.76) and CRC (RF 0.48) were susceptible ($RF < 3$) (Table 1).

Esterase profile of tick larval extract revealed 5 bands of esterase activity for each location. These bands were numbered consecutively as EST-5 to EST-1B starting from starting from high molecular mass activity to low molecular mass activity (Fig. 1). Band intensity varied between various sample extracts (Figs. 2, 3).

The results of the assays with inhibitors for the biochemical characterization of these esterases are presented in Table 2, with the degrees of inhibition classified in a decreasing

order of band intensity. None of the esterases detected was affected by $CuSO_4$ and pCMB. Eserine sulfate completely inhibited the activity of EST-1, EST-2, EST-3, which were strongly inhibited by malathion and PMSF and were classified as acetylcholinesterases (AchEs). The enzymes EST-4 and EST-5 were not affected by eserine sulfate but were inhibited by treatment with malation and PMSF and were classified as carboxylesterases (CaEs).

Extra band was observed towards anodic end in case of tick larval extracts of Dehradun, Jawaharnagar and Kiccha and was designated as EST-1A. The band was intense in case of Dehradun, but lighter in case of Kiccha and Jawaharnagar and was recognised as Acetylcholinesterases (AChE) based on the results of inhibitory tests.

Discussion

LPT bioassay revealed wide variations in the susceptibility of *R.* (*B.*) *microplus* to deltamethrin collected from various locations of northern India. The Dehradun ticks showed highest resistance to deltamethrin. This could be due to frequent use of this acaricide for tick control in this region, and it being the most commonly available acaricide in India. Same was the situation in Jawaharnagar, Rudrapur and Nagla Dairy. Vatsya and Yadav (2011) reported the same in the tick populations of this area (RF $>$ 5).

Tick populations of CRC and Sanjay colony were highly susceptible to deltamethrin, the LC_{50} values of tick populations of these regions being lower than the reference susceptible ticks. Tick burdens of animals of these regions being less, the animal owners usually control the infestation by hand picking and burning or by use of herbs. Aguirre et al. (2000) obtained LC_{50} of deltamethrin and cypermethrin estimates for Milagro strain of Argentina as 0.012 (0.010–0.013) and 0.053 (0.050–0.057) respectively, which were lower than the LC50 values of cypermethrin for Brazilian Porto Alegre reference susceptible strain.

In the present study, maximum RF (44.92) was obtained for Dehradun ticks and minimum for CRC (0.48). RF values are relative and depend upon the LC_{50} values of reference susceptible strain. RF values give evidence of the variations in responses of tick populations of different regions to an acaricide. In future, the generated baseline can be employed for confirming reduced susceptibility of ticks to acaricides, if any.

Confidence interval (CI) and Chi-square values give an idea about the composition of the sample population i.e. whether it is a homogenous population, which gives narrow CI and low Chi square values or a heterogeneous population for which CI is wide and Chi square values are high. In the present study CI values are wide and Chi square values are high, indicating a heterogeneous population of ticks in each location.

These sensitivity/resistance profiles of *R.* (*B.*) *microplus* to synthetic pyrethroids are indicative of emerging acaricide resistance and requires more frequent monitoring of synthetic pyrethroid resistance in the ticks.

Esterases represent a group of highly variable and multifactorial enzymes. In arthropods, these enzymes are involved in various physiological activities such as regulation of juvenile hormone levels (Hidayat and Goodmann 1994), digestive processes (Argentine and James 1995), reproductive behaviour (Labate et al. 1990), functioning of nervous system (Villate and Bachmann 2002) and resistance to pesticides (Hemingway et al. 2002; Li et al. 2005).

Non-denaturing PAGE of tick larval extracts revealed 5 bands of esterase activity designated EST-5 to EST-1, according to their migration from high molecular mass

Table 1 Efficacy of deltamethrin against *Rhipicephalus (Boophilus)* collected from various geographical locations of northern India

Location	Larvae	Slope	χ^2	Standard error (SE)	LC_{50}	CI (95 %)	LC_{99}	CI (95 %)	Resistance Factor (RF)
Plains									
Nagla Dairy	2100	1.89	130.73	0.14247	0.00464	0.00213–0.00532	0.28231	0.11245–0.32164	6.03
CRC	2100	1.42	34.13	0.12024	0.00037	0.00029–0.00048	0.01618	0.01004–0.02607	0.48
Sanjay colony	2100	2.03	374.12	0.11660	0.00059	0.00049–0.00072	0.00829	0.00599–0.01147	0.77
Jawaharnagar	2100	1.18	36.81	0.05553	0.00877	0.00762–0.01009	0.82491	0.49651–1.37082	11.30
Rudrapur	2100	1.52	75.86	0.05704	0.00524	0.00477–0.00576	0.17972	0.13302–0.24263	6.81
Gadarpur	2100	1.77	90.56	0.08645	0.00131	0.00117–0.00145	0.02678	0.02029–0.03534	1.70
Kiccha	2100	1.59	97.14	0.05718	0.00315	0.00289–0.00344	0.09151	0.07125–0.11750	4.09
Ramnagar	2100	1.60	84.96	0.06522	0.00105	0.00094–0.00119	0.03019	0.02394–0.03807	1.36
Bheemtal	2100	1.76	42.86	0.12452	0.00068	0.00058–0.00080	0.01455	0.00995–0.02099	0.88
Almora	2100	1.30	40.22	0.05471	0.00446	0.00401–0.00497	0.27219	0.18758–0.39450	5.79
Dehradun	2100	1.82	195.28	0.05736	0.03459	0.03206–0.03733	0.65405	0.53131–0.80515	44.92
Pithoragarh	2100	1.58	63.08	0.08813	0.00077	0.00070–0.00089	0.02283	0.01672 –0.03118	**1**

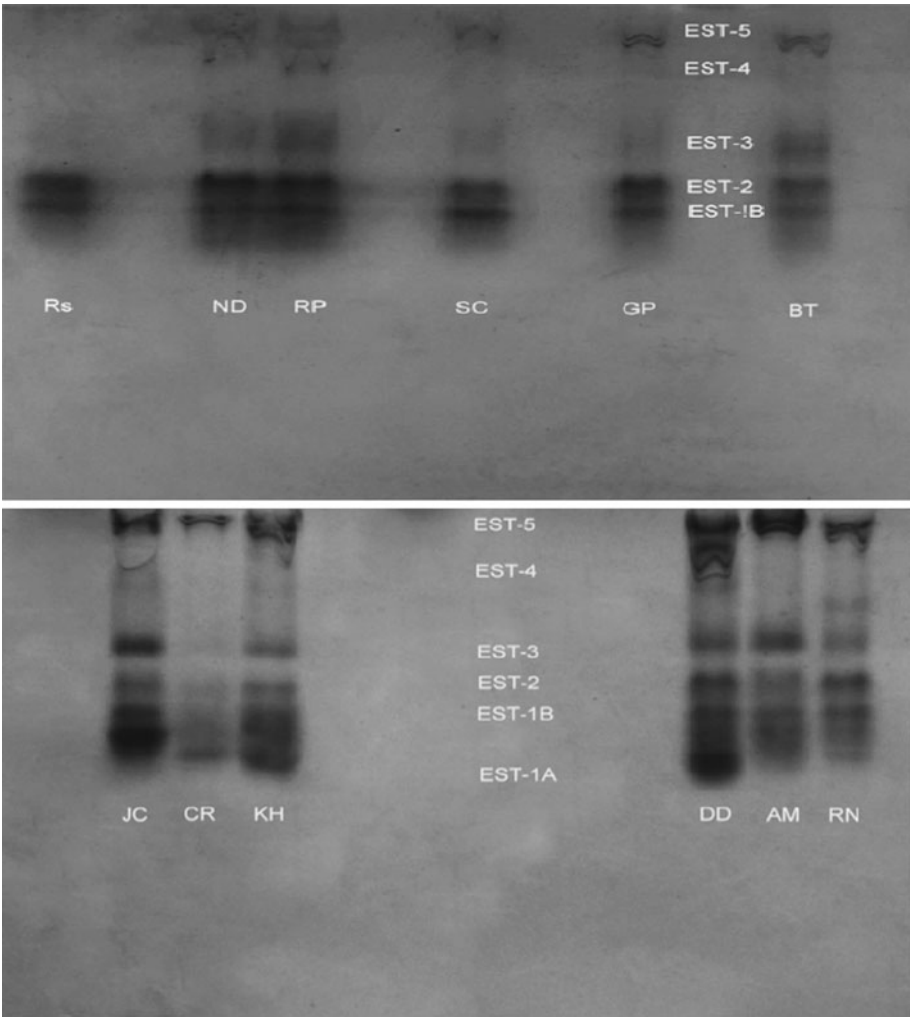

Fig. 1 Esterase profile of tick larval extracts collected from various geographical locations of northern India. *Rs* reference susceptible, *JC* Jawaharnagar (Pithoragarh), *CR* Crop Reseach Centre, *ND* Nagla dairy KH Kiccha, *RP* Rudrapur, DD Dehradun, *SC* Sanjay colony, *AM* Almora, *GP* Gadarpur, *RN* Ramnagar, *BT* Bheemtal

activity to low molecular mass activity. Baffi et al. (2007) reported same banding pattern during the electrophoresis of protein extracts of larval stages of *R.* (*B.*) *microplus*.

Action of inhibitors on these esterases revealed 3 enzymes- EsT-1, EsT-2 and EsT-3 as AchEs, whereas EsT-4 and EsT-5 were recognised as CaEs. Same kind of classification was obtained by Baffi et al. (2007) and Miranda et al. (2009) during the esterase profiling of various developmental stages of *R. microplus*.

Inhibition of esterases by $CuSO_4$ and pCMB did not occur, both being arylesterase inhibitors. Eserine sulphate, an AchE inhibitor inhibited only EsT-1, EsT-2 and EsT-3, leaving EsT-4 and EsT-5 unaffected, which were designated as CaEs. All the 5 bands were inhibited by malathion, an organophosphate. Miranda et al. (2009), Baffi et al. (2005) and

Fig. 2 Complete inhibition of esterase enzymes in presence of Malathion

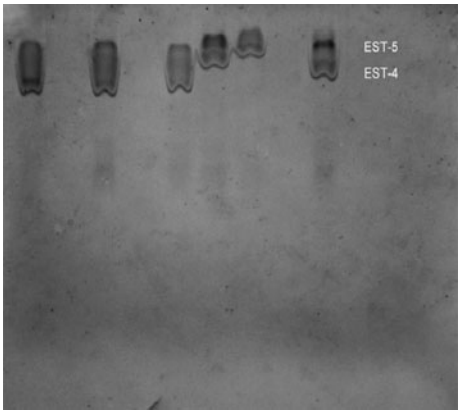

Fig. 3 Esterase enzyme profile in presence of Eserine sulphate

Table 2 Effects of different inhibitors on esterase enzymes used during enzyme characterisation

Esterases*	$CuSO_4$	pCMB	Malathion	Eserine	PMSF	Classification[1]
EST-1A**	–	–	+++	+++	+++	AChE
EST-1B	–	–	+++	+++	+++	AChE
EST-2	–	–	+++	+++	+++	AChE
EST-3	–	–	+++	+++	+++	AChE
EST-4	–	–	+++	–	++	CaE
EST-5	–	–	+++	–	++	CaE

* Esterases numbered EST-1 through EST-5, starting from anodic end of the gel

** Present only in tick populations of Dehradun, Jawaharnagar and Kiccha

[1] *AchE* acetylcholinesterase, *CaE* carboxylesterase; (−) absence of inhibition: (+, ++, +++) increasing level of inhibition

Baffi et al. (2007) reported same kind of inhibitory results during the esterase profiling of *R. microplus.*

Native PAGE studies revealed that there was no difference in the banding pattern between the various susceptible populations. Baffi et al. (2008) reported similar esterase

pattern between the sensitive Mozzo reference susceptible strain and two other cypermethrin susceptible strains.

Direct observation of band intensity of various esterases indicated variations in the intensity of bands between susceptible and resistant tick populations. The bands appeared more intense in resistant strains when compared to reference susceptible and resistant tick strain band intensities. Same variations in band intensities were reported by Baffi et al. (2008); they obtained more intense staining in the resistant group, which was associated with pyrethroid resistance. Similarly Cossio-Bayugar et al. (2009) indicated increased levels of AchE and CaE expression in resistant strains as compared to susceptible strain by employing quantitative PCR for this purpose. Increased CaE activity was reported by Miranda et al. (2009). They observed significant difference between band intensities of the resistant and susceptible strains.

Esterase profile of Dehradun, Kiccha, Jawaharnagar revealed an extra band towards the low molecular mass activity, which was designated as EsT-1A. All the above three tick populations showed resistance against synthetic pyrethroids and thus the extra band may be involved in the development of this resistance. An extra band of esterase activity was reported in the esterase profile of cypermethrin resistant adult tick extracts by Baffi et al. (2005) and (2008). In resistant tick populations of Rudrapur, Nagla Dairy and Almora no such extra band was obtained in their esterase profiles. Miller et al. (1999) used synergist bioassays to characterize mechanisms of resistance to pyrethroid acaricides. Triphenylphosphate (TPP) synergism ratio was found to be significantly higher in a pyrethroid resistant strain (*Cz*), with 166-fold resistance to permethrin, than in the reference susceptible strain, suggesting an enhanced esterase activity. Biochemical studies resulted in the isolation and identification of an over-expressed esterase, *CzEst9*, which hydrolyzes pyrethroids (Pruett et al. 2002). Further molecular study also indicated that the transcript of *CzEst9* gene was more abundantly expressed in the *Cz* strain than any other tick strains (Guerrero et al. 2002; Hernandez et al. 2002). In contrast, synergists failed to synergize pyrethroid toxicity in two Mexican strains of *B. microplus* that had more than 1000-fold resistance to pyrethroids, suggesting the existence of a target site insensitivity mechanism.

Vatsya (2009) reported multifactorial and wide spread resistance against deltamethrin in *R. microplus* populations of various geographical regions of Uttarakhand. Genotyping of the tick larvae confirmed existence of mutant sodium channel genes in Kashipur, New Tehri and Pantnagar Colony populations of ticks. Pantnagar Dairy, Kashipur and Dehradun resistant phenotypes of tick populations exhibited different patterns of resistance mechanisms. Synergist studies indicated the role of esterase enzymes in the resistance development in Dehradun ticks.

Esterase enzyme profile of the tick larval extracts gives information about the level of esterase enzyme activity in a tick population, but the enzyme profile of the tick larval population taken include both resistant and susceptible larvae and thus the profile obtained is for the whole population and the phenotype of a single larva i.e. whether resistant or susceptible cannot be determined, also the proportion of resistant and susceptible ticks in the whole population cannot be ascertained. Similarly, it cannot be determined with certainty whether the increased esterase enzyme levels are wholly and solely responsible for the increased resistance in the tick population. Acaricide resistance is a multi-factorial phenomenon, thus other causes of increased resistance like sodium channel mutation and reduced drug penetration (e.g. cuticle thickening) and behavioural changes (e.g. avoiding the pesticides) are to be tested in future in order to confirm the basic cause of the resistance development in these acaricide resistant tick populations.

India has varied climatic zones and thus ecology and epidemiology of *R.* (*B.*) *microplus* varies from region to region. Similarly, animal rearing practices vary from place to place. Animal owners of various regions of the country employ different methods and measures of tick control. Control methods involve use of chemical acaricides and other non chemical means. Within chemical control different types of chemical acaricides and drug formulations are utilised. All these factors are to be kept in mind for planning of control methods of ticks and during investigation of acaricide resistance in a tick population.

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