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Characterization of acaricide resistance in tick isolates collected from Rajasthan, India

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ABSTRACT

Rhipicephalus (Boophilus) microplus and *Hyalomma anatolicum* are the most common tick species infesting milk and meat producing animals throughout the country. The present study was conducted to evaluate the acaricide resistance status of the tick species to deltamethrin, cypermethrin, diazinon collected from 10 districts of Rajasthan. Characterization of resistance was carried out by adult immersion test (AIT) and larval packet test (LPT). In case of *R. (B.) microplus* resistance to deltamethrin at level I (RF = 2.5 – 4.9) in 02 isolates, at level II in 03 isolates (RF = 5.4 – 11.5) and level IV in 02 isolates (RF = 48.1 – 95.7) was detected. The resistance to cypermethrin was detected in 08 isolates of which resistance at level I in 03 isolates (RF = 2.7 - 4.58) and at level II in 05 isolates (RF = 8.05 – 16.2). Diazinon resistance was detected at level II in 06 isolates (RF = 5.8 –22.8), at level III in 01 isolates (RF = 39.0) and level IV in 02 isolates (RF = 65.9 – 66.0). While in case of *H. anatolicum*, the resistance to deltamethrin at level I (RF = 1.79 –2.52) in 03 isolates, to cypermethrin in 03 isolates (RF= 2.0 - 3.95) and to diazinon at level I in 03 isolates (RF = 1.32 –2.18) out of eleven isolates was detected.

A significant correlation between esterase enzyme ratio and resistant factor of tick isolates was observed with correlation coefficient (r) in α- and ß-esterase activity. The coefficient of determination (R^2) for α- and ß-esterase activity indicated that 55.9 and 50.5% data points of *R.(B.) microplus* isolates and 66.7 and 47.2% data points of *H. anatolicum* isolates were very close to the correlation lines.

Analysis of sequence data of 3 targeted positions of the sodium channel gene detected a cytosine (C) to adenine (A) nucleotide substitution (CTC to ATC) at position 190 in domain II S4–5 linker region of para-sodium channel gene in 3 isolates and in reference deltamethrin resistant IVRI-IV line.

The western dry region and central plateau hills region revealed higher density of resistant ticks where intensive crossbred cattle population are reared and synthetic pyrethroids and organophosphate compounds are commonly used. The data shows an urgent need of revisiting the tick control strategy implemented through concerned government/non-government agencies.

Key words: Esterase enzyme, *Hyalomma anatolicum,* Organophosphate, Resistance factor, *Rhipicephalus (Boophilus) microplus,* Sodium channel gene mutation, Synthetic pyrethroids

In India, *Rhipicephalus (Boophilus) microplus* and *Hyalomma anatolicum* are widely prevalent and most economically important ticks infesting dairy animals (Ghosh *et al.* 2007). Direct effects on production are skin damage from tick bites (Biswas 2003, Jongejan and Uilenberg 2004), blood loss, toxicity from the bites, and reduction in weight gain and milk production (Sutherst 1983, Sajid *et al*. 2007); and indirect effects include transmission of tick-borne diseases (Rodríguez-Vivas *et al.* 1998, 2004). The ticks are managed mainly by acaricide, and synthetic pyrethroids, organophosphates and macro cyclic lactones are widely used. In India, limited data on acaricide resistance is currently available (Kumar *et al.* 2011, Sharma *et al.* 2012, Shyma *et al.* 2012).

India with 199.10 million cattle and 105.30 million buffaloes stands first in world bovine population (GOI 2011–12). Rajasthan has 12.12 million cattle and 11.1 million buffaloes. The farmers of Rajasthan raised concern over limited effect of available acaricides on tick stages but concerted efforts to monitor acaricide resistance status have not been initiated. Besides, the possible mechanism of resistance to SP and OP compounds operating in Indian ticks has not been elucidated. The present study was undertaken to characterize tick isolates collected from 10 districts of Rajasthan and to work out the possible mode of developing resistance in ticks to SP and OP compounds.

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MATERIALS AND METHODS

Study area and processing of samples: Two stage stratified sampling method was adopted to collect live engorged female *R. (B.) microplus* and *H. anatolicum* ticks from animals and from the animal sheds. The areas of collection were selected from 2 agro-climatic regions of Rajasthan. Easy accessibility and active participation of veterinary officials of these areas promoted to select these regions. Both cattle and buffalo populations were screened for tick infestations. Both organized and unorganized farms were selected to collect the samples. The organized farms were categorized as per the number of animals maintained, viz. small (up to 25 animals), medium (up to 100 animals), large (up to 500 animals) and very large (more than 500 animals). A questionnaire was formulated to collect data on type of farms (organized, small, medium, large and very large/unorganized), frequency, type and mode of acaricide treatment adopted by the respondents/cattle owners and owners experience about the efficacy of commonly used acaricides.

After initial processing of collected samples, these were transported to the Entomology Laboratory, IVRI, Izatnagar and maintained at 28±1°C and 85±5% RH in BOD incubator; 3 to 5 adults were pooled together and maintained as per Ghosh and Azhahianambi (2007). Tick samples were collected from both organized and unorganized farms of Chittorgarh, Pratapgarh, Udaipur, Bhilwara, Banswara, Jaipur, Bharatpur, Alwar, Sikar and Churu districts. In all the areas, animal owners have reported tick infestations as a major problem and synthetic pyrethroids, organophosphates and macrocyclic lactones were commonly used for the tick control and farmers reported very low efficacy of chemicals used for the tick control. The use of acaricides in organized dairy farms was twice or thrice a month. The most common methods of acaricide application were spray, injectable route and swabbing.

Test chemical: Technical grade 100% pure deltamethrin, cypermethrin and diazinon were used. The stock solution of diazinon (50,000 ppm) was prepared in methanol while 10,000 ppm stock solutions of deltamethrin and cypermethrin were prepared in acetone and methanol, respectively. For the experimental bioassay, different concentrations of the acaricide were prepared in distilled water from the stock solutions and tested against *R. (B.) microplus* and *H. anatolicum.*

Reference tick lines: The colony of acaricides susceptible reference IVRI-I line of *R.(B.) microplus* (National registration no. NBAII/IVRI/BM/1/1998) and IVRI-II (NBAII/IVRI/Ha/1/1998) lines of *H. anatolicum* were used as the standard to assess susceptibility/resistance status in tick isolates collected from the study area. These colonies are being maintained in the Entomology Laboratory of Indian Veterinary Research Institute, Izatnagar for the last 15 years and have not been exposed to any acaricides. The biological parameters were continuously monitored and susceptibility status of the colonies was established by periodic testing against several synthetic pyrethroids, organophosphates, organochlorines and formamidine compounds in *in-vitro*- independent bioassays. The genetic homogeneity of the tick lines was established by analysis of the 16 srDNA gene sequences (accession nos. GU222462, GU323287, GU323288, HM176656, HM176657, HM176658) (Kumar R. *et al.* 2011).

The deltamethrin resistant IVRI-IV of *R. (B.) microplus* was originally collected from Danapur in Bihar state. To maintain the resistance level, ticks of each generation was exposed to deltamethrin. The resistance factor of the line was calculated as 194.0 against deltamethrin. Point mutation was detected in the Na-channel gene (GenBank accession no. JQ693154).

Crossbred (*Bos taurus* male × *B. indicus* female) male calves, maintained in tick proof animal houses were maintained as per standard norms. Batches of 6 calves were used to maintain acaricide susceptible IVRI-I, IVRI-II lines and field collected *R. (B.) microplus*. The calves were reared as per the guidelines of statutory Indian body, "Committee for the purpose of control and supervision on experimentation on animals" (CPCSEA).

Bioassays

Adult immersion test (AIT): Adult immersion test was conducted according to Drummond *et al*. (1973) and Benavides *et al.* (1999) with minor modifications (Sharma *et al.* 2012). The following parameters were compared:

(a) Mortality: recorded up to 14 days post treatment (dpt) when normal ticks complete egg laying.
(b) egg masses laid by live ticks.
(c) Reproductive index (RI) = egg mass weight (EW)/ engorged female weight (EFW)
(d) Percentage inhibition of oviposition (IO%) = RI control – RI treated ×100

RI control

Dose response data were analyzed by probit method (Finney 1962) using Graph Pad Prism 4 software. For characterization of field ticks, discriminating dose (DD) was determined as $2\times LC_{95}$ of IVRI-I or IVRI-II lines (Jonsson *et al*. 2007).

Larval packet test (LPT)

In the field situation, it is very common to have condition when getting sufficient number of engorged females for AIT is a major issue. To solve the problems, LPT was standardized in which limited number of engorged females can be sufficient to conduct the test. Briefly, the Whatman filter paper No. 1 were cut in parallelogram shape (5.5 cm × 5 cm) and were impregnated with 0.5–0.6 ml of different concentrations of acaricides with the help of pipette and dried for at least 30 min in incubator at 37°C. Treated and dried parallelograms of paper were folded in half forming equilateral triangular packets and sealed on the sides with adhesive tapes forming an open-ended packet. After insertion of approximately 150 larvae, the open side of each packet was sealed with adhesive tape and the packets were

placed in a desiccator kept in BOD incubator maintained at 28±1°C and 85±5% RH. The packets were removed after 24 h and larval mortality was calculated. The dose-response data of the individual acaricide against the reference susceptible tick (IVRI-II line) were analyzed. The discriminating dose (DD) was determined as in AIT mentioned above.

Resistance characterization of field isolates

Bioassay: Both AIT and LPT as mentioned above were adopted for characterization of field tick isolates. A set of concentrations were prepared by serial dilutions, bracketing the DD. Resistance factors (RF) for field isolates were worked out by the quiescent between LC_{50} of field ticks and LC_{50} of IVRI-I line of *R.(B.) microplus* (Castro-Janer *et al*. 2009). In *H. anatolicum* ticks, LPT was adopted. On the basis of RF, the resistance status in the field population of *R.(B.) microplus* and *H. anatolicum* was classified as susceptible (RF ≤ 1.4), level I (RF = 1.5–5.0), level II (RF = 5.1–25.0), level III (RF = 26–40) and level IV (RF ≥ 41) (Kumar *et al.* 2011).

Biochemical characterization of resistance

To characterize the tick isolates, esterase activities with the substrates α-and ß-naphthyl acetate were determined according to Hemingway (1998) with some modifications. Deep frozen larvae (20) were homogenized in a precooled pestle mortar in 200 µl of distilled water, spun at 1,100×g at 4°C for 15 min and supernatant was collected for assay. Reaction mixtures contained 20 µl of the supernatant in duplicate adjacent wells of microtitre plate and 200 µl of α-or ß-naphthyl acetate solution (250 µl of 30 mM stock in 25 ml of phosphate buffer 0.02M, pH 7.2), incubated at room temperature for 30 min and then added 50 µl of fast blue solution (0.023 g fast blue salt, dissolved in 2.25 ml distilled water and 5.25 ml of 5% SDS in 0.1 M sodium phosphate buffer, pH 7.2) to each well. The plates were incubated for 5 min at room temperature and absorbance was measured at 570 nm in a microtitre plate reader using Magellan 6 software. The resulting optical densities (OD) were compared with standard curves of ODs for known concentrations of α-and ß naphthols. The esterase activities were expressed as µmole of product formed/min/mg protein. The esterase activities were expressed as enzyme ratio (mean activity of enzyme in resistant isolate/mean activity of enzyme in reference susceptible IVRI-I line).

Molecular characterization of resistance: For molecular characterization of pyrethroid resistance, domain IIIS6 region (encompassing the T2134A mutation site) and domain II S4-5 (encompassing the C190A mutation site) in the voltage-gated sodium channel gene were targeted. The genomic DNA was extracted from 400 mg tick larvae (Sambrook *et al*. 2001) and preserved in 200 µl of TE buffer. The PCR primers to amplify the fragments of sodium channel gene flanking mutation sites were designed from partial sodium channel *R. (B.) microplus* gene sequence (Mexican strain, GenBank accession no. AF134216). Domain IIIS6 (T2134A site) was amplified by primer pair D3F 52 -CTGGTTACATCATATCTAATTGCCAC-32 and D3R 52 -CCAGCCTTCTTCTTTTGTTCATTG-32 and S4-5 linker region (C190A site) in domain-II was amplified by primer pair L2F 52 -TACGTGTGTTCAAGCCTA-32 and L2R 52 -ACTTTCTTCGTAGTTCTTG-32. For amplification of T2134A mutation site, a 25 µl PCR reaction was set up using 2.5 µl of 10× DreamTaq buffer; 5.0 µl genomic DNA (1:5 dilution, 50 ng/µl); 0.3 µM of each primer, 0.2 mM dNTPs and 0.3 µl of DreamTaq DNA polymerase (5 U/µl). The PCR conditions were optimized as 95°C, 2 min, 34 cycles of 95°C,1 min; 55°C, 30 s; 72°C, 30 s and final extension at 72°C for 10 min. For amplification of C190A mutation site, a 25 µl PCR reaction mixture containing 2.5 µl of 10x AccuPrime PCR buffer I, 5.0 µl genomic DNA (1:5 dilution, 50 ng/µl); 0.3 µM of each primer and 0.3 µl of AccuPrime Taq DNA polymerase (5 U/µl). The cycling conditions were 94°C for 2 min, 40 cycles of 94°C, 1 min; 50°C, 30 sec; 68°C, 30 sec and final extension at 68°C for 10 min.

For molecular characterization of organophosphate resistance, acetylcholinesterase 2 (AChE2) gene was taken as target gene. The total RNA was extracted from about 100 mg of 10 to 15 days old larvae of reference IVRI-I, IVRI-III (diazinon resistant, RF =24.9) and IVRI-IV (multiacaricide resistant, RF diazinon = 25.5; RF deltamethrin = 17.5; RF cypermethrin = 15.4) lines and field isolates using Trizol reagent. The integrity of RNA was checked by gel electrophoresis and concentration was determined in spectrophotometer. The cDNA was synthesized from 3 µg of total RNA using the RevertAidTM H minus reverse transcription kit using Oligo dT primer. Primers for the amplification of AChE2 gene were designed from the GenBank accession no. AJ278342. The primer sequences were BmE2 Fwd 52 -ATGTACGTGCGGG TGTCTCTCGT-32 and Bm E2 Rev 52 -TCAGTATAGTTT CAGTTCTTTG-32. The 25 µl PCR reaction mixture contained 2.5 µl of 10× Dream Taq buffer; 5.0 µl cDNA (1:5 dilution, 100 ng/µl); 0.5 µM of each primer; 0.2 mM dNTPs and 0.5 µl of Dream Taq DNA polymerase (5 U/ µl). The cycling conditions for amplifying AChE2 gene were 95°C for 4 min, 35 cycles of 95°C, 45 sec; 58°C, 45 sec; 72°C, 90 sec and finally at 72°C for 10 min.

The positive amplification of AChE2 and sodium channel gene fragments was visualized by electrophoresis of the product in ethidium bromide stained 1 and 3% agarose gel, respectively. The PCR products were purified using gel extraction kit. The purified PCR product of AChE2 gene (1692 bp) was ligated with the T/A cloning vector pTZ57R/ T and recombinant plasmids were transformed into *E. coli* DH5α cells. Insert positive clones were verified by restriction enzyme digestion before sequencing. The positive clones and purified PCR product of T2134A mutation site (193 bp) and C190A mutation site (167 bp) were outsourced to DNA sequencing facility at University of Delhi, South Campus for double stranded sequencing.

To screen mutations, the forward and reverse sequence

data were aligned and analyzed by multiple sequence alignment using Clustal W method in Lasergene software and BTI software and compared with homologues in GenBank using BLAST (NCBI). Sequence information of at least five PCR products/clones from each isolate was analyzed.

RESULTS AND DISCUSSION

The problem of ticks and tick-borne diseases is highly relevant in India because of the congenial environmental conditions for tick survival throughout most parts of the year and maintenance of susceptible crossbred animals to improve the production of milk and other animal products. The tick population has immense potential for developing resistance rapidly to commonly used chemicals due to their biological and behavioral characteristics and resistance in ticks to different active ingredients has been reported in almost all countries including India (Alonso-Diaz *et al*. 2006, Kumar *et al*. 2011, Sharma *et al*. 2012, Shyma *et al*. 2012).

To protect the huge animal assets of Rajasthan from the attack of ticks, the first step is to work out an acaricide resistance monitoring system. Monitoring of ticks is crucial to diagnose resistance at an early stage, to slow down the process of spreading of resistance and to obtain knowledge of the distribution of resistance. To address the objectives, robust bioassays, viz. AIT, LPT need to be standardized. In the present study, AIT was standardized to establish discriminating doses of acaricides for characterization of field isolates. A 100% mortality of reference IVRI-I line of *R.(B.) microplus* was recorded at 30 ppm deltamethrin, 350 ppm cypermethrin and 650 ppm diazinon. The egg masses, RI and IO% of survived ticks were also found to be significantly inhibited in dose dependent manner. The details of mortality slope, R^2, LC_{50}, LC_{95} and confidence limit are elaborated in Table 1. The discriminating doses (DD) of deltamethrin, cypermethrin and diazinon were determined as 59.2, 698.2 and 1270.4, respectively. In LPT, 100% mortality of reference IVRI-II line of *Hyalomma anatolicum* was obtained at 34.9 ppm deltamethrin, 350.9 ppm cypermethrin and 650.0 ppm diazinon. The discriminating doses (DD) of deltamethrin, cypermethrin and diazinon, were calculated as 69.8, 701.8 and 1300.0, respectively (Table 2).

Both AIT and LPT were recommended by different workers for evaluation of resistance to acaricides (Castro-Janer *et al*. 2010, Klafke *et al*. 2012). The bioassay can be conducted with ease and data can be generated within 2 weeks time. However, Jonsson *et al*. (2007) reported non reproducibility of AIT data and the slope for dose–response curve was very low. They were of the opinion that the results of the experiment could be improved by increasing the number of replications and number of ticks per replication. In the present experiments, the predictive value of the AIT has been improved by increasing the number of ticks used in each trial and on an average 250–300 field ticks were

Table 1. LC_{50} and LC_{95} with 95% confidence limit of deltamethrin, cypermethrin and diazinon obtained by AIT for IVRI-I line of *R. (B.) microplus*

Acaricide	Variables	Mortality Slope±SE	R^2	LC_{50} (ppm) (95% CI)	LC_{95} (ppm) (95% CI)	DD (ppm)
Deltamethrin	Mortality	4.51±0.28	0.99	13.4 (12.4–14.5)	29.6 (27.7–31.7)	59.2
	Egg mass	–69.89±29.39	0.65			
	RI	–0.52±0.35	0.42			
	% IO	162.6±67.42	0.66			
Cypermethrin	Mortality	4.08±1.14	0.76	138.5 (134.5–142.6)	349.1 (323.2–377.0)	698.2
	Egg mass	–60.0±19.97	0.69			
	RI	–0.56±0.22	0.62			
	% IO	139.3±54.63	0.62			
Diazinon	Mortality	7.06±0.54	0.74	372.0 (341.28–405.28)	635.2 (582.75–692.37)	1270.0
	Egg mass	–29.65±7.88	0.64			
	RI	–0.32±0.06	0.75			
	% IO	0.11±0.02	0.73			

Table 2. LC_{50} and LC_{95} with 95% confidence interval of larvae of IVRI-II line of *H. anatolicum* against deltamethrin, cypermethrin and diazinon in larval packet test

Acaricide	Slope±SE	R^2	LC_{50} (95% CL)	LC_{95} (95% CL)	DD(ppm)
Deltamethrin	3.45±0.30	0.947	11.7 (11.5–11.9)	34.9 (34.2–35.6)	69.8
Cypermethrin	10.63±2.02	0.874	245.91 (245.2–246.6)	350.91 (347.4–354.4)	701.8
Diazinon	3.872±0.80	0.794	246.1 (243.7–248.6)	650.0 (639.21–665.04)	1300.0

tested for each isolate. This was possible because colonization of the field ticks was performed to enhance the sample size of the adult females whenever necessary. Besides number, the age and condition of ticks prior to AIT are also likely to play an important role in variability of results (Jonsson *et al*. 2007). These factors were standardized in repeated laboratory experiments and consistent results were obtained. Food and Agriculture Organization (FAO 2004) recommended and provided standardized protocols for 2 bioassays to evaluate tick resistance, the larval packet test (LPT), originally described by Stone and Haydock (1962), and the adult immersion test (AIT), originally developed by Drummond *et al.* (1973). However, LPT takes 5–6 weeks to complete, is laborious and requires significant laboratory resources to conduct the test routinely. While AIT can be conducted with ease and data can be generated within 2 weeks. However, in multihost tick the availability of significant number of adults for conducting AIT is not always possible and for the same LPT can be very useful tool. In the present case, DD's were worked out for all the 3 acaricides using both the tests, and the data were comparable (Tables 1, 2).

The data on LC_{50} value of deltamethrin, cypermethrin, diazinon, slope value, RF values and the level of resistance in the field isolates of *R. (B.) microplus* are shown in Table 3. In AIT, resistance to deltamethrin was detected in 07 isolates (RF = 2.5 to 95.7). Out of which, level I was detected in 2 isolates (RF = 2.5 – 4.8), 03 isolates at level II in (RF = 5.4 to 11.5) and 02 isolates at level IV in (RF = 48.1 to 95.7). Three isolates showed RF below 1.5 and were designated as susceptible populations. The SKR isolate collected from western dry region was characterized as highly resistant (level IV; RF=95.7). The resistance to cypermethrin was detected in 08 isolates, of which level I was in 03 isolates (RF = 2.7 to 4.58) while at level II in 05 isolates (RF = 8.0 to 16.2). Two isolates (JPR and BHT)

Table 3. The result of adult immersion test (AIT) performed on *R. (B.) microplus* collected from different places of Rajasthan against deltamethrin, cypermethrin and diazinon

Tick isolates	Deltamethrin				Cypermethrin				Diazinon			
	Mortality Slope±SE	LC_{50} values (95% CI)	RF*	RL#	Mortality Slope±SE	LC_{50} values 95% CI)	RF*	RL#	Mortality Slope±SE	LC_{50} values (95% CI)	RF*	RL#
COR	3.44±0.87	71.9 (67.8-76.2)	5.4	II	2.78±0.42	634.14 (587.8–685.6)	4.58	I	1.18±0.95	3655.6 (3384.8–3948.0)	9.8	II
PRT	1.37±0.32	33.5 (28.9–38.9)	2.5	I	2.23±0.70	479.8 (440.2–523.0)	3.46	I	1.38±0.47	24504.8 (21308–281805)	65.9	IV
UDP	2.96±0.42	153.6 (143.5–164.3)	11.5	II	4.94±2.09	1145 (1101.0–1190.8)	8.3	II	1.47±0.38	14476.9 (12699–16503)	39	III
BLW	5.09±1.03	114.04 (109.6–118.6)	8.5	II	4.83±1.30	1115.03 (1072.1–1159.6)	8.05	II	2.18±0.96	8474.5 (7813–9283)	22.8	II
BSW	0.55±0.22	0.27 (0.18–0.39)	0.02	S	2.33±1.14	373.67 (342.8–407.3)	2.7	I	1.38±0.47	24545.9 (21722.0 – 27736.9)	66	IV
JPR	1.08±0.43	6.1 (5.04–7.37)	0.45	S	0.57±0.22	3.21 (2.21–4.64)	0.02	S	1.81±0.31	6377.5 (5594.3–7270.3)	17.1	II
BHT	3.30±0.76	65.9 (53.7–60.3)	4.9	I	1.22±0.19	133.97 (113.5–158.1)	0.97	S	4.70±0.93	2161.5 (2078.4–2247.9)	6.5	II
ALW	0.68±0.27	3.33 (2.19–5.06)	0.25	S	5.31±0.85	1270.3 (1198.4–1346.5)	9.2	II	2.73±0.80	2150.9 (2000.5–2333.3)	5.8	II
SKR	1.27±0.42	1282.5 (1089.4–1516.9)	95.7	IV	3.84±0.66	1369.11 (1291.6–1451.2)	9.88	II	3.26±1.73	6065.5 (5668.7–6490.1)	16.3	II
CHU	1.418±0.3005	644.7 (560.6–741.4)	48.1	IV	2.955±0.2528	2245.4 (2118–2380)	16.2	II	5.713±1.17	2276.4 (2210–2344)	6.1	II

*RF, Resistance Factor; #RL , Resistance level; Susceptible (S) , RF<1.4; level I, 1.5<RF<5; level II, 5.1<RF<25; level III, 26<RF<40; level IV, RF>41.

Table 4. The result of larval packet test performed (LPT) on *H. anatolicum* collected from different places of Rajasthan against deltamethrin, cypermethrin and diazinon

Tick isolates	Deltamethrin				Cypermethrin				Diazinon			
	Mortality Slope±SE	LC_{50} values (95% CI)	RF*	RL#	Mortality Slope±SE	LC_{50} values 95% CI)	RF*	RL#	Mortality Slope±SE	LC_{50} values (95% CI)	RF*	RL#
COR	1.1±0.50	14.4 (12.5–16.5)	1.23	S	1.17±0.20	28.9 (24.7–33.8)	0.12	S	0.91±0.18	55.4 (44.6–68.6)	0.22	S
PRT	0.91±0.18	3 (2.7–3.2)	0.25	S	1.92±0.28	110 100.1–121.2)	0.44	S	2.35±1.40	295.5 (273–319)	1.2	S
UDP	2.43±0.34	23 (21.2–24.8)	1.96	I	2.64±0.66	503.5 (470.5–538.7)	2.05	I	1.55±0.19	423.3 (371–482)	1.72	I
BLW	1.64±1.12	14 (12.5–15.6)	1.2	S	2.15±0.95	335.4 (304.8–368.8)	1.36	S	1.50±1.0	220 (193–250)	0.89	S
BSW	2.07±0.46	29.6 (27.1–32.2)	2.52	I	2.18±1.05	291.3 (267.2–317.5)	1.18	S	1.85±0.34	537.7 (484–596)	2.18	I
JPR	2.22±0.31	21 (19.2–22.8)	1.79	I	3.17±0.35	972 (925.7–1020)	3.95	I	1.65±0.33	501.8 (448–562)	2.04	I
BHT	1.24±0.20	11.5 (9.7–13.3)	0.98	S	1.34±0.39	132.9 (115.5–152.8)	0.54	S	1.44±0.17	324.9 (282–373)	1.32	S
ALW	1.85±0.01	11.6 (10.4–12.8)	0.99	S	3.68±0.56	492.1 (468.6–516.7)	2	I	2.20±0.12	335.4 (307–365)	1.36	S
SKR	1.33±0.12	4.4 (3.73–4.94)	0.37	S	1.66±0.36	76.2 (68.0–85.3)	0.31	S	1.24±0.35	205.8 (177–238)	0.84	S
DGR	2.14±0.52	17.3	1.48	S	1.87±1.28	142.3	0.58	S	2.14±0.52	322	1.3	S

RF*, Resistance Factor; RL*, Resistance Level; Susceptible (S), RF<1.4; Level I, 1.5<RF<5; level II, 5.1<RF<25; level III, 26<RF<40; level IV, RF>41.

were susceptible. The level of resistance to diazinon was found very high in all the isolates, viz. at level II in 07 isolates (RF= 5.8 to 22.8) and at level IV in 02 isolates. Two ticks isolates (PRT & BSW) were highly resistant to diazinon. In case of *H. anatolicum,* the data on LC_{50} value of deltamethrin, cypermethrin, diazinon, slope value, RF values and the level of resistance are given in Table 4**.** The resistance to deltamethrin, cypermethrin and diazinon was detected at level I in 03 isolates (RF = 1.7 to 3.95).

The level of resistance was comparatively higher in 1 host tick, *R.(B.) microplus* than that in the 3 host tick, *H. anatolicum,* which corroborates the observations made on global basis (Wharton and Roulston 1970). A much larger fraction of the total population of 1 host ticks remains under chemical challenge more than multi-host ticks because of their shorter life cycle and host specificity to domestic cattle of the former. However, off the host ticks of genus *Hyalomma* may come in contact with environmental residues of the insecticides increasing the selection pressure. The detection of acaricide resistance in *Hyalomma* ticks is of particular importance in planning a control programme because of the continuous introduction of these ticks from Rajasthan through the migrating cattle herds and involvement of these ticks in transmission of *Theileria annulata* infection. The results indicated that there is a need of continuous monitoring of acaricide resistance in field situation for strategic application of available acaricides and for maintaining the life span of the product.

The α- and ß-esterase enzyme activity in terms of enzyme ratio in field isolates of *R.(B.) microplus* and *H. anatolicum* is summarized in Table 5. The enzyme ratio

Table 5. Esterase activity in Indian isolates of *R. (B.) microplus* and *H. anatolicum* collected from different places.

Tick species	Tick isolates	Resistance Factor	α- esterase ratio	ß-esterase ratio
R. (B.) microplus	IVRI-I	1.0	1.0	1.0
	IVRI-IV	194.0	3.07	1.77
	COR	5.4	1.44	1.13
	PRT	2.5	1.84	1.4
	UDP	11.5	3.21	2.46
	BLW	8.5	3.47	2.24
	BSW	0.02	1.2	1.03
	JPR	0.45	1.08	1.0
	BHT	4.9	2.33	2.13
	ALW	0.25	1.12	1.07
	SKR	95.7	4.06	2.9
	CHU	48.1	3.10	2.27
H. anatolicum	IVRI-II line	1.0	1	1
	COR	1.23	0.69	0.71
	PRT	0.25	0.68	0.79
	UDP	1.96	1.35	1.35
	BLW	1.2	0.89	0.86
	BSW	2.52	1.65	1.58
	JPR	1.79	1.24	1.14
	BHT	0.98	0.74	0.64
	ALW	0.99	0.67	0.7
	SKR	0.37	0.65	0.62
	DGR	1.48	0.55	0.28
	CHU	0.7	0.67	0.29

Table 6. Correlation between RF and enzyme activity in collected Indian isolates of *R.(B.) microplus* and *H. anatolicum*

Tick isolates	Enzyme activity	Pearson's correlation coefficient (r) (95% CL)	P value	R^2
R.(B.) microplus	a-esterase	0.748 (0.3045 to 0.9248)	0.0052[b]	0.559
	ß-esterase	0.710 (0.2302 to 0.9124)	0.0096[b]	0.505
H. anatolicum	a-esterase	0.817(0.4574 to 0.9469)	0.0012[b]	0.667
	ß-esterase	0.687(0.1874 to 0.9045)	0.0135[a]	0.472

[a] Significant at $P < 0.05$, [b] Significant at $P < 0.01$, [c] Significant at $P < 0.001$.

and RF of tick isolates were observed significantly (P<0.01) correlated with correlation coefficient (r) in α- and ß-esterase activities. The correlation coefficient (r) indicated the real correlation between both the variables which tend to increase or decrease together when r exists between 0–1. The coefficient of determination (R^2) for α- and ß-esterase activity indicated that 55.9 and 50.5 data points of field isolates of *R. (B.) microplus* and 66.7 and 47.2% data points of field isolates of *H. anatolicum* were very close to the correlation lines (Figs 1, 2) . However, the correlation was more pronounced with α-esterase than ß-esterase. When RF and enzyme activity was compared, a significant correlation between α- and ß-esterase activities with RF was detected (Table 6).

The biochemical nature of acaricide resistance involving esterase, glutathione S-transferase, monoxygenase and altered AChE are well documented in *R. (B.) microplus* (Baxter *et al.* 1999, Hemingway and Ranson 2000, Jamroz *et al*. 2000, Villarino *et al.* 2003). Earlier, significant correlation between resistant factor and α-esterase activity was reported involving ticks collected from wide geographical areas (Shyma *et al*. 2012, Kumar R. *et al.* 2013).

The PCR amplification of the domain IIIS6 transmembrane segment of the sodium channel gene from the susceptible and resistant isolates showed a clear band at 193 bp. Following multiple sequence alignment of DIII

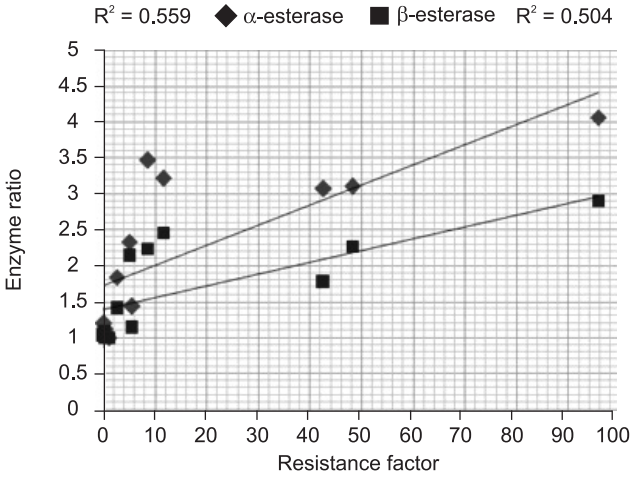

Fig.1. Correlation between RF and α and ß-esterase enzyme activity in different isolates of *R. (B.) microplus.*

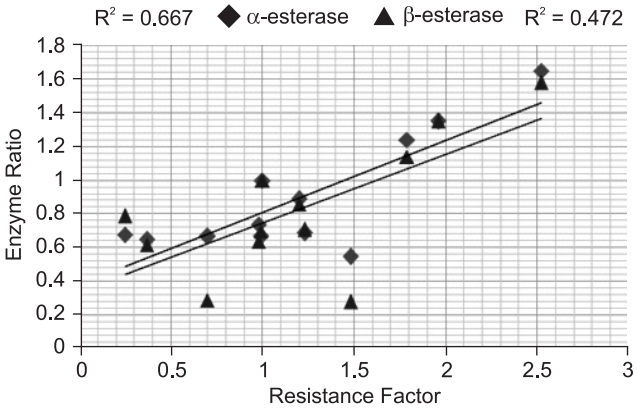

Fig. 2. Correlation between RF and α and ß-esterase enzyme activity in different isolates of *H. anatolicum*.

```
TTCGGCTCCTTCTTCACCTTGAATCTAT Mexico, Susceptible
TTCGGCTCCTTCATCACCTTGAATCTAT Mexico, Resistant
TTCGGCTCCTTCTTCACCTTGAATCTAT IVRI-I (HQ157236
TTCGGCTCCTTCTTCACCTTGAATCTAT SKR (JQ693155)
TTCGGCTCCTTCTTCACCTTGAATCTAT UDP (JQ693156)
TTCGGCTCCTTCTTCACCTTGAATCTAT IVRI-IV (JQ693158)
```

Fig. 3. Sequence analysis of domain III S-6 region. Partial nucleotide sequence alignment of the domain III S-6 region of sodium channel gene in Indian and Mexican isolates of *R. (B.) microplus*. Mexican resistant isolate showed T to A nucleotide change while no T to A nucleotide changes were recorded in Indian deltamethrin resistant isolates. The position of mutation is 2134 in the reference sequence of sodium channel gene (accession no. AF134216).

```
ACCATCGGTGCCCTCGGGAACTTGACCT Australian susceptible
ACCATCGGTGCCATCGGGAACTTGACCT Aus tralian resistant
ACCATCGGTGCCCTCGGGAACTTGACCT IVRI-I (HM579820)
ACCATCGGTGCCCTCGGGAACTTGACCT ALW (JX262011)
ACCATCGGTGCCCTCGGGAACTTGACCT BSW (JX262012
ACCATCGGTGCCATCGGGAACTTGACCT BLW (JX262013)
ACCATCGGTGCCATCGGGAACTTGACCT SKR (JQ693152)
ACCATCGGTGCCATCGGGAACTTGACCT UDP (JQ693153)
ACCATCGGTGCC ATCGGGAACTTGACCT IVRI -IV (JQ693154)
```

Fig. 4. Sequence analysis of domain II S4-5 linker region. Partial nucleotide sequence alignment of the domain II S4-5 linker region of para-sodium channel gene of different isolates of *R. (B.) microplus* showing C to A mutation in isolates having high RF and in deltamethrin resistant IVRI-IV line. This position is 190 in the reference sequence, accession no. AF134216.

S6 sequences (GenBank accession nos. HQ157236, JQ693155; JQ693156; JQ693158) no mutation was detected at position 2134 (T to A) in resistant field isolates and also in reference IVRI-IV line (Fig. 3) despite of varying degree of resistance status (Table 3). Sequence analysis of the S4-

5 linker region (GenBank accession nos.HM579820, JX262011, JX262012, JX262013, JQ693152, JQ693153, JQ693154) led to the identification of a cytosine (C) to adenine (A) nucleotide substitution (CTC to ATC) at position 190 in 3 isolates (BLW, UDP, and SKR) having high RF in the range of 8.5–95.7 (Fig. 4). *In silico* translation of this nucleotide substitution causes an amino acid change from leucine in the susceptible isolate to isoleucine (L64I) in resistant isolates. However, gene mutation in domain II S4-5 linker of sodium channel gene was not detected in any of the *H. anatolicum* isolates.

The PCR amplification of the AChE2 gene showed a single band of 1692 bp. The multiple sequence alignments (GenBank accession nos. GU944959, GU944955, HQ184946, HQ184946, HQ184945, JN624772, JN624773) between the susceptible and resistant field isolates along with IVRI-I and OP resistant reference IVRI-III line, led to the identification of four nucleotide substitutions in five resistant isolates (BLW, BSW, PRT, SKR, UDP) as well as in IVRI-III line. In silico translation of these nucleotide changes cause V297I, S364T, H412Y and R468K amino acid substitutions in AChE2.

One major mechanism of resistance targeting the sodium channel gene is resulting from one or more point mutations in domain IIIS6 transmembrane segment and was detected in San Felipe and Corrales isolates of *R. (B.) microplus* in Mexico that were extremely resistant to permethrin (He *et al.* 1999). To date, this mutation in domain IIIS6 has been detected in many tick isolates from North America (Guerrero *et al.* 2002, Rosario-Cruz *et al.* 2005, Miller *et al*. 2007, Chen *et al*. 2009, Aguirre *et al*. 2010, Rodriguez-Vivas *et al*. 2012). However, in the present study, no such mutation was detected in any of the resistant tick isolates. The absence of T2134A mutation was also reported in various pyrethroid resistant tick isolates from Australia and Brazil (Li *et al*. 2007, Chen *et al.* 2009, Rosario-Cruz *et al*. 2009, Andreotti *et al.* 2011). A mutation, which included substitution of adenine (A) by cytosine (C) (CTC to ATC) was reported at position 190 in the domain II S4-5 linker of the sodium channel gene of Parkhurst isolate of *R. (B.) microplus* from Australia, which was resistant to all pyrethroids including flumethrin, cyhalothrin and deltamethrin (Morgan *et al*. 2009). In the present investigations, a mutation in the domain II S4-5 linker region of the sodium channel gene was detected in 3 isolates having high resistance factors. The present information gives a significant clue to the Indian researcher involved in characterization of pyrethroids resistance in Indian ticks.

The principle target of organophosphates is the acetylcholinesterase protein and thus the gene coding for acetylcholinesterases in different organisms was studied extensively for characterizing molecular mechanism of OP resistance (Mutero *et al.* 1994, Weill *et al*. 2003, Nabeshima *et al.* 2003). However, in ticks no such resistance conferring mutations could be detected (Baxter and Barker 2002, Hernandz *et al*. 1999) until recently; Temeyer *et al*. (2013) found amino acid substitutions in AChE1, AChE2 and AChE3 genes associated with OP resistance in *R. (B.) microplus*. Recently, four nucleotide substitutions were identified in AChE2 gene of OP resistant ticks (Ghosh et al. 2015). In the present study, similar substitutions in the resistant isolates were recorded. The finding of these mutation in resistant Indian isolates of R. (B.) microplus indicates a probable mechanism of OP resistance, however the extent to which these substitutions contribute in resistance development is to be further studied.

This is the first comprehensive study on acaricide resistantce status in ticks of Rajasthan and will help in formulating tick control strategy at the administrative level.

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