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Patel MK
Department of Veterinary Parasitology, College of Veterinary Science & A. H., Kamdhenu University, Sardarkrushinagar, Gujarat, India

Parsani HR
Department of Veterinary Parasitology, College of Veterinary Science & A. H., Kamdhenu University, Sardarkrushinagar, Gujarat, India

Shyma KP
Department of Veterinary Parasitology, College of Veterinary Science & A. H., Kamdhenu University, Sardarkrushinagar, Gujarat, India

Corresponding Author:
Patel MK
Department of Veterinary Parasitology, College of Veterinary Science & A. H., Kamdhenu University, Sardarkrushinagar, Gujarat, India

In vitro detection of acaricidal resistance status of *Rhipicephalus microplus* against Fenvalerate and Flumethrin from Banaskantha, Gujarat, India

Patel MK, Parsani HR and Shyma KP

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Abstract
Rhipicephalus microplus is the most common tick species in India infesting cattle and buffaloes and causing significant economic losses to dairy and leather industries by adversely affecting the milk production and quality of hides. A study to evaluate the acaricide resistance status of *Rhipicephalus microplus* to fenvalerate and flumethrin was conducted on the samples collected from organized and unorganized farms of mahesana district from Gujarat state, where treatment failures were reported frequently. Adult Immersion Test (AIT) was conducted using field strain for determination of 50 and 95% lethal concentration of fenvalerate and flumethrin. Results obtained by the AIT showed level I resistance (RF > 5), level II resistance (5.1 < RF < 25) and level IV resistance (RF > 41) has been developed against both fenvalerate. Carboxyl esterase essay was also performed for understanding the mechanism of resistance in *R. microplus* against the synthetic pyrethroid compounds. Quantitative analysis of α and β- esterase enzyme activity was found to be in range of 2.01±0.221 to 13.85±0.141 and 1.79±0.056 to 4.89±0.102 µmol/min/mg of protein, respectively. RF was high (Punjpur village of Danta taluka) having α-esterase and β-esterase were 13.85±0.141 and 4.89±0.102 µmoles/min./mg, respectively which indicated RF correlate with the enzyme activity and also in some villages had the RF was low but enzyme activity was high. This may be due to present strain may be treated with other group of synthetic pyrethroids

Keywords: Acaricide, *Rhipicephalus microplus*, fenvalerate, flumethrin

1. Introduction
The warm, humid climate of tropical nations like India makes *Rhipicephalus microplus* the most common and major tick species there. Although the expenses of tick-borne illnesses have not been thoroughly studied, they are commonly estimated to be around 2000 crore rupees per year in India (Minjauw & McLeod, 2003) [1]. In the Gujarat plains and hills agroclimatic zone, the westernmost subtropical region of the Indian subcontinent, *R. microplus* is the most often found tick species to infest cattle (Ghosh *et al*., 2007) [8]. Chemical control has been the most popular method of managing tick infestations; however, repeated application of these chemicals leads to the development of resistance in the ticks, and the selection of resistant ticks is thought to be the primary obstacle to the success of pest and vector management programmes (Shyma *et al.*, 2013) [25]. Many non-organophosphate pesticide classes have been created; these are safe for the environment, effective against arthropod pests, and, in comparison to organophosphate (OP) compounds, relatively less hazardous to mammals and other non-target animals. According to Graf *et al.* (2004) [11], the use of these chemical acaricides has a limited ability to reduce tick infestations and is often associated with major side effects, such as the growth of resistant ticks, contamination of the environment, and pesticide residues in milk, meat, and dairy products. Among these insecticides, the synthetic pyrethroids flumethrin and fenvalerate are commercially available and are currently the primary acaricides used to control ticks in India (Mathivathani *et al*., 2011; Sharma *et al*., 2012) [16, 22]. Several field strains have reportedly grown resistant to pyrethroids to differing degrees (Andreotti *et al*., 2011; Sharma *et al*., 2012; Shyma *et al*., 2013; Ahanger *et al*., 2015) [2, 22, 25, 1]. Both single-host and multihost tick species have been reported to be resistant to chemical acaricides in ticks collected from North Gujarat (Singh *et al*., 2014; Singh *et al*.,

2015) [26, 27]. With all of this in mind, determining the level of resistance to widely used acaricides in the region that significantly contributes to the nation's livestock wealth and livestock products was extremely difficult.

2. Materials and Methods

2.1 Collection and preparation of ticks

Ticks were gathered from several talukas in Gujarat's Banaskantha district. For the collection of the ticks, both organised and disorganised farms were used. These districts' animal farms have certain local cattle and buffalo breeds in addition to cross-bred livestock. Animals in small, unorganised farms are mostly reared by farmers in villages and are allowed to graze freely, while organised farms adopt a zero grazing regime. Using forceps, ticks were removed without causing any damage to their mouth parts. In the Banaskantha district, ticks were gathered from the talukas of Deesa, Danta, Dantiwada, Dhanera, and Palanpur. Adult female ticks that were fully engorged were gathered from the bodies of sick animals as well as from cracks and crevices around the cow shed. A small number of male ticks were collected as well for identification. Ticks were collected between October 2022 to May 2023. The mature ticks were collected and placed in muslin cloth-covered, clean vials to facilitate moisture and air exchange. These vials were then transported to the Department of Veterinary Parasitology, College of Veterinary Science and Animal Husbandry at Kamdhenu University, Sardarkrushinagar. Two batches of ticks are separated—one for the adult immersion test and the other for the biochemical assay—after they are collected from the various locations.

2.2 Acaricides

Fenvelarate and flumethrin, two technical-grade drugs, were used to identify acaricide resistance. Technical grade Fenvelarate (99.3%) and Flumethrin (96.1%) from Sigma Aldrich were utilised to prepare stock solutions in acetone and methanol, respectively, and were then kept at 4o C. Flumethrin (80 ppm, 100 ppm, 200 ppm, 400 ppm, 800 ppm) and Fenvelarate (100 ppm, 200 ppm, 400 ppm, 800 ppm, 1600 ppm) stock solutions were produced in distilled water as a diluent for the bioassay.

2.3 Adult immersion test

Engorged female ticks on arrival in the laboratory were washed in tap water and then dried on an absorbent paper properly. The Adult Immersion Test (AIT) was carried out with only slight modifications to the technique outlined by Drummond *et al.* (1973) [6]. The pre-weighed ticks were submerged in 10 millilitres of various chemical acaricide concentrations—80, 100, 200, 400, and 800 parts per millilitre of flumethrin and 100, 200, 400, 800, and 1600 parts per millilitre of fenvelarate—for two minutes in a 25 millilitre beaker with mild stirring. The medium in the control group was distilled water. Subsequently, the ticks were placed on petri plates covered with Whatman filter paper number 1. Every petri plate containing treated ticks was kept at room temperature for a full day. After 24 hours, the ticks were moved to glass vials with muslin cloth covers. They were then kept in desiccators with relative humidity values ranging from 75 to 85% and placed in a BOD incubator at 28 °C. Each replica (n = 15) contained five ticks, and each concentration was utilised three times. For a maximum of fifteen days, the oviposition and mortality of these ticks were monitored. Egg mass was tracked in a BOD incubator using the same incubation parameters for the next fifteen days. The weight of the treated ticks' eggs and the percentage of adult tick mortality were recorded in comparison to the control. The following formulas were used to calculate the index of egg laying and the percentage of fecundity inhibition, according to Gonçalves *et al.* (2007) [10]. The proportion of hatched eggs was calculated visually after the eggs were incubated under the identical conditions. To calculate dose dependent response the following parameters were recorded:

(a) Mortality: Engorged females that oviposited were considered as live and females that did not oviposit were considered as dead.

(b) Egg masses on day 14 post AIT

$$\text{Reproductive index (RI)} = \frac{\text{Weight of eggs laid (mg)}}{\text{Weight of adult females (mg)}}$$

$$\text{Percentage inhibition of oviposition (I0\%)} = \frac{\text{RI (control group) - RI (treated group)}}{\text{RI (control group)}} \times 100$$

2.4 Biochemical Assay (Esterase assay)

2.4.1 Sample preparation

200 µl of ice-cold distilled water was used to homogenise pre-counted deep freeze larvae kept at -20 °C in a mortar and pestle that had been previously chilled. For every strain, the esterase enzyme activity profiles were computed with an equivalent number of 12- to 14-day-old, unfed larvae. The suggested microplate enzyme assay was performed with a few minor modifications. Twenty larvae each tick, stored at a temperature of -20 °C, were mixed and homogenised in 200 µl of distilled water. The mixture was centrifuged at 5000 rpm for 15 minutes at 4 °C, and the supernatant was then collected.

2.4.2 Titration of esterase

The α- and β-NA 30 mM stock solutions were prepared in acetone and stored separately at 4° C in bottles with tight stoppers. A working solution of α-NA and β-NA was prepared by mixing 120 µl of 30 mM α-NA and β-NA with 12 ml of 0.02 M phosphate buffer (pH 7.2). To make a fresh quick blue stain solution, 0.023 g of fast blue was combined with 2.25 ml of distilled water and 5.25 ml of 5% SDS in 0.1 M sodium phosphate buffer pH 7. 200 µl of the α-NA or β-NA working solution and 20 µl of the homogenate were added in duplicate to each of the adjacent wells of a 96-well microtitre plate. 20 µl of distilled water were added to the tick homogenate in the control blank wells. The reaction mixtures were allowed to sit at room temperature for half an hour. After adding 50 µl of fast blue solution to each well, the plates were allowed to change colour for five minutes at room temperature. The absorbance was then measured in an ELISA reader at 570 nm. The resulting optical densities (OD) were compared with comparable standard curves of known product concentrations in order to translate the absorbance to product (α or β naphthol) concentrations. The absorbance readings from the samples were converted to nanomoles of α- and β-naphthol using the relevant reference curves. The amount of time that the substrate and homogenate were incubated before the stain was added divided by the naphthol production values. The protein levels in grammes for 20 microliters of homogenate were calculated using a protein standard curve. The protein value was split again and then multiplied by 1000

in the computation of the amount of naphthol produced per minute that was previously indicated. Esterase activity was measured in micromoles of product generated per minute per milligramme of protein (Hemingway, 1998) [13].

2.4.3 Estimation of total protein of tick larvae

The total protein content of the homogenate of larvae was determined using a 96-well plate approach and the standard Bradford Protein assay procedure (Bradford, 1976) [3]. A standard curve was produced using a BSA (1 mg/ml) stock solution that was serially diluted. Two hundred microliters of a Bradford reagent 1x solution were mixed with ten microliters of BSA. The OD at 595 nm was recorded using an ELISA reader following a 10-minute incubation period at room temperature. A standard curve was generated using a linear regression analysis of the data. 200 µl of Bradford reagent was combined with tick larvae homogenate, incubated for 10 minutes, and the optical density (OD) was recorded. Utilising the BSA standard curve, the homogenate's protein content was determined.

2.4.4 Visual observation of the assay

Individuals with non-elevated levels of esterase activity showed pale blue or pink colour with α-NA or β- NA respectively. Individuals with elevated esterase activity showed an intense blue/black or pink/red colour with α- NA or β- NA, respectively.

2.5 Statistical analysis

The LC_{50} was estimated from the dose response curves and compared to a susceptible reference strain. Resistance factor were calculated relative to the susceptible reference strain.

$$\text{Resistance factor} = \frac{LC_{50} \text{ of tested field tick isolate}}{LC_{50} \text{ of susceptible reference strain}}$$

LC_{50} value of flumethrin and fenvelarate against *R. microplus* was calculated by regression curve of probit mortality plotted against values of log acaricide concentrations by log dosage probit mortality analysis (Finney, 1971) [7]. Resistance factors (RF) for field tick isolates were worked out by the quotient between LC_{50} of field isolates and LC_{50} reference of susceptible line against flumethrin and fenvelarate.

On the basis of RF, the resistance status in the field population of *R. microplus* were classified as susceptible (RF ≤ 1.4), level I resistant (RF = 1.5-5.0), level II resistant (RF = 5.1-25.0), level III resistant (RF 26-40) and level IV resistance (RF ≥ 41) (Shyma *et al*., 2015) [24].

3. Results

3.1 Adult Immersion Test (AIT) of *R. microplus* against fenvelarate

All tick isolates were tested for dose-dependent mortality against varying concentrations of fenvalerate. To ascertain the LC_{50} and LC_{95} via log dose probit mortality analysis, drug concentration logs were plotted against probit mortality. Table 3.1 displays the dose-dependent mortality results of *R. microplus* tick populations against fenvalerate in the various study locations. The resistant factor was computed by comparing the LC_{50} values of susceptible IVRI-I strain of *R. microplus*, which is not exposed to any acaricides, with those of field isolates. The LC_{50} values obtained from data published by Sharma *et al.* (2012) [22] and Shyma *et al.* (2013) [25] were applied to *R. microplus* sensitive strains against fenvalerate and flumethrin.

Tick populations from Punjpur village of Danta taluka with level II resistance with resistant factor 17.39. Tick population from Baiwada, Jorapura, Motakpra and Vasda villages of Deesa taluka, Pethapur village of Danta taluka, Bhakhar and Dhaneri villages of Dantiwada taluka, Khimat village of Dhanera taluka, Jagana and Laxmipura villages of Palanpur taluka were found susceptible to fenvalerate.

Table 1: Slope, R^2, LC_{50}, LC_{95}, RF_{95} Resistant Factor and Resistance Level of *R. microplus* against fenvalerate of Banaskantha district

Sr. no.	Taluka	Places	Slope	R^2	LC_{95}	LC_{50}	RF	RL
1.	Deesa	Baiwada	1.40	0.91	11029.43	757.58	0.62	S
		Jorapura	1.46	0.87	9241.73	695.77	0.57	S
		Motakapra	1.25	0.95	24660.39	1202.26	0.99	S
		Vasda	1.36	0.93	14719.17	934.98	0.77	S
2.	Danta	Pethapur	1.79	0.97	4671.37	573.23	0.47	S
		Punjpur	1.36	0.93	914466.7	20949.22	17.39	II
3.	Dantiwada	Bhakhar	1.53	0.93	8737.11	746.38	0.61	S
		Dhaneri	1.47	0.95	10445.87	811.59	0.67	S
4.	Dhanera	Khimat	1.25	0.95	1904.55	269.18	0.22	S
5.	Palanpur	Jagana	1.53	0.93	8737.11	746.38	0.61	S
		Laxmipura	1.53	0.93	8483.42	730.52	0.60	S

Susceptible = RF ≤ 1.4; Level I = 1.5≤ RF ≥ 5; Level II= 5.1 ≤ RF ≤ 25 Level III = 26 ≤ RF ≤ 40; Level IV = RF ≥ 41

3.2 Adult Immersion Test (AIT) of *R. microplus* against flumethrin

The LC_{50} and LC_{95} of Baiwada and Pethapur were calculated as 1366.93 and 22799.34, respectively. The LC_{50} and LC_{95} of Jorapura, Motakapra, Bhakhar, Dhaneri, Jagana and Laxmipura were calculated as 393.76 and 3727.59, respectively.

Tick Population from Vasda villages of Deesa taluka, Punjpur village of Danta taluka, Khimat village of Dhanera taluka with 100% mortality of *R. microplus* ticks against flumethin itindicated that the flumethrin is susceptible in research area.

3.3 Spectrophotometric interpretation of the assay

The mean activities of α- and β- esterase enzyme in tick isolates collected from Banaskantha district of Gujarat were determined from the naphthol standard curves. The results of α- and β- esterase activities were summarized in Table 2. The α- and β- esterase enzyme activity in IVRI -I reference ticks were reported as 2.47±0.008 and 1.22±0.006 nmoles/min/mg of protein, respectively (Ghosh *et al*., 2017) [9]. This value was considered as the base line activity of esterase enzyme and used for comparing field resistant ticks to determine the enzyme ratio. The α - and β- esterase enzyme activity in field susceptible ticks were recorded in the range of 2.014 to 13.85 µmole/min/mg of protein and 1.79 to 4.89 µmole/min/mg of protein, respectively (Table 2).

Table 2: Resistance factor along with α- and β-Esterase enzyme activity and enzyme ratio (ER) in different field isolates from Banaskantha district of *R. microplus*

Sr. no.	Taluka	Tick isolates	Fenvalerate RF	α-esterase (µmoles/min./mg of protein)	Enzyme ratio	β-esterase (µmoles/min./mg of protein)	Enzyme ratio
1.	Deesa	Baiwada	0.62	3.165±0.214	1.28	2.41±0.021	1.97
		Jorapura	0.57	3.10±0.064	1.25	2.21±0.056	1.81
		Motakapra	0.99	4.74±0.021	1.92	2.92±0.078	2.39
		Vasda	0.77	4.15±0.114	1.68	3.75±0.120	3.07
2.	Danta	Pethapur	0.47	2.56±0.024	1.03	2.75±0.021	2.25
		Punjpur	17.39	13.85±0.141	5.60	4.89±0.102	4.00
3.	Dantiwada	Bhakhar	0.61	3.065±0.0414	1.24	2.82±0.012	2.31
		Dhaneri	0.67	3.15±0.012	1.27	2.86±0.22	2.34
4.	Dhanera	Khimat	0.22	2.014±0.221	0.82	1.79±0.056	1.47
5.	Palanpur	Jagana	0.61	4.255±0.046	1.72	2.06±0.025	1.69
		Laxmipura	0.60	3.33±0.056	1.35	1.98±0.089	1.62
6.		IVRI I susceptible	1.0	2.47±0.008	1	1.22±0.006	1

Tick population from Punjpur village of Danta taluka exhibited the highest α- esterase and β- esterase activity *i.e.*, 13.85 µmole/min/mg of protein and 4.89 µmole/min/mg of protein respectively, amongst all the isolates assayed. The α- and β- esterase activity of the ticks having high RF factor were significantly higher than susceptible isolates. The ratio of mean activities of each enzyme of the resistant populations to those of susceptible ones (enzyme ratio -ER) are also presented in Table 2. The α-esterase enzyme activity was elevated up to 5.60 fold in the resistant isolates as compared to the susceptible isolates. The β- esterase enzyme activity was elevated up to 4.00 fold in the resistant isolates. A significant elevated levels of both α-esterase and β -esterase were detected in tick samples collected from all nine isolates which were resistant to fenvalerate.

4. Discussions

In the present study, RF was high (Punjpur village of Danta taluka) having α-esterase and β-esterase were 13.85±0.141 and 4.89±0.102 µmoles/min./mg, respectively which indicated RF correlate with the enzyme activity and also in some villages had the RF was low but enzyme activity was high. This may be due to present strain may be treated with other group of synthetic pyrethroids. Esterase based resistance has been demonstrated to be one of the mechanisms for SPs in *R. microplus*. However, the specific mechanism through which resistance is conferred has not been suitably elucidated.

Due to its delayed entry into the Indian market, flumethrin appears to be somewhat effective against *R. microplus*. The ticks' flumethrin resistance factor could not be ascertained due to incomplete data on the reference tick line. On the other hand, family resistance—in which ticks concurrently acquire resistance to several substances within the group—is the usual description of tick resistance to synthetic pyrethroids (Miller, 1988 [18]. For other countries, however, data on reference tick lines for flumethrin were available. According to Kumar *et al.* (2015) [15], monitoring the level of resistance in ticks requires a country-specific discriminating concentration for different acaricides because a number of factors, including geographic location, climate, farmers' economic status, acaricide application dose and frequency, and animal breed, can lead to the development of resistance.

The study was carried out in response to reports of treatment failure in North Gujarat and Kutch, which led researchers to look for safer alternatives to chemical acaricides like flumethrin and fenvalerate. There have been concerns expressed regarding the indiscriminate use of these pesticides resulting in tick populations developing resistance, especially to synthetic pyrethroids like fenvalerate, which could jeopardize their effectiveness (Shyma *et al.*, 2014) [23].

Higher slope values were noted in the study, which highlighted the Adult Immersion Test (AIT) applicability for tracking resistance in field isolates (Sharma *et al.*, 2012; Andreotti *et al.*, 2011; Castro-janer *et al.*, 2010; Kumar *et al.*, 2015; Mendes *et al.*, 2007; Camillo *et al.*, 2009) [22, 2, 5, 15, 17, 4]. The findings indicated the inefficiency of the present dosages used in the field and suggested that larger concentrations of fenvalerate and flumethrin may be needed to produce considerable mortality.

Compared to analytical grade acaricides, the study's use of commercially available acaricides allowed for a more accurate evaluation of their efficacy (Haque *et al.*, 2014) [12]. The necessity for better tick control procedures and the prudent application of acaricides is highlighted by the development of resistance in *R. microplus* to chemical acaricides. In order to handle this new concern, it is imperative that resistance be tracked and that prudent acaricide use be encouraged.

Herbal acaricides have demonstrated potential in lowering tick resistance to chemical acaricides and managing tick populations. Tick populations can be prevented from developing resistance by implementing integrated tick management techniques, including as rotational grazing, pasture management, and targeted acaricide application. This multifaceted strategy encourages sustainable livestock management practices in addition to helping to reduce tick infestations (Singh *et al.*, 2014) [28].

5. Conclusion

The study conducted in Banaskantha district of Gujarat highlighted the presence of varying levels of resistance in *Rhipicephalus microplus* tick populations against fenvalerate and flumethrin. The resistance factor (RF), as well as the elevated levels of α- and β- esterase enzyme activities in resistant isolates, indicated the possible mechanisms behind the development of resistance. It is crucial to monitor resistance levels in tick populations to ensure effective tick control strategies and to prevent the spread of resistance to other acaricides. Integrated tick management approaches, including the use of herbal acaricides and sustainable livestock management practices, can play a significant role in managing tick populations and reducing the development of resistance. Continued research and monitoring efforts are needed to address the challenges posed by tick resistance and to promote the responsible use of acaricides in tick control programmes.

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