RESEARCH ARTICLE

Detection of Acaricidal Resistance in *Rhipicephalus microplus* from Mehsana District, Gujarat, India

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Abstract

A study was conducted in Mehsana district, Gujarat, to assess acaricide resistance in *Rhipicephalus (Boophilus) microplus*, the most common tick species infesting cattle and buffaloes in India. Tick samples were collected from both organized and unorganized farms where treatment failures had been reported. The lethal concentration (LC_{50} and LC_{95}) of fenvalerate and flumethrin was determined by Adult Immersion Test (AIT). Results showed varying resistance levels to fenvalerate, including level I (RF > 5), level II (5.1 < RF < 25), and level IV (RF > 41), while all isolates remained susceptible to flumethrin. Enzymatic assays revealed α- and β-esterase activity ranging from 4.05 ± 0.005 to 17.56 ± 0.182 and 2.34 ± 0.056 to 10.95 ± 0.54 µmol/min/mg protein, respectively. High resistance levels, particularly in Navavas village, correlated with elevated esterase activity, suggesting metabolic resistance. However, some areas showed high enzyme activity despite low resistance, possibly due to exposure to other synthetic pyrethroids. The findings highlight the urgent need for alternative tick control strategies to combat emerging resistance in the region.

Key words: Acaricide, Cattle, Resistance, *Rhipicephalus microplus*.

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Introduction

Rhipicephalus microplus, commonly known as the southern cattle tick, is one of the most economically significant ectoparasites affecting cattle in tropical and subtropical regions, including India. The warm and humid climatic conditions prevalent in the country provide an ideal environment for its survival, proliferation, and year-round activity. Among the major tick-infested regions, Northern Gujarat - part of the Gujarat Plains and Hills agroclimatic zone - has been reported as a hotspot for *R. microplus* infestations (Ghosh *et al*., 2007). Tick infestations and the transmission of tick-borne diseases (TBDs) such as babesiosis, anaplasmosis, and theileriosis have severe impacts on cattle health, productivity, and overall farm economics. Although comprehensive national estimates are lacking, the annual economic losses due to ticks and TBDs in India have been approximated at ₹ 2000 crore (Minjauw and McLeod, 2003). The primary method of tick control remains the application of chemical acaricides, particularly synthetic pyrethroids like fenvalerate and flumethrin, which are widely used due to their perceived efficacy and safety profile. However, the frequent and unregulated use of these compounds has resulted in the development of acaricide resistance (Bhowmik *et al*., 2024). Resistance development poses a serious challenge to livestock management, contributing to treatment failure, increased production costs, environmental contamination, and residues in animal-derived food products. Though sporadic reports from North Gujarat suggest rising treatment failure, systematic data on resistance patterns from this region are largely absent (Singh *et al*., 2015). Given the region's substantial contribution to India's livestock sector, it is imperative to assess the resistance status of *R. microplus* to commonly used acaricides to inform evidence-based management strategies. Therefore, the present study was aimed to evaluate the level of resistance to synthetic pyrethroids, particularly fenvalerate, in field populations of *R. microplus* collected from various locations in Northern Gujarat, using established bioassays and biochemical diagnostic tools.

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Materials and Methods

Collection and Preparation of Ticks

The ticks in the present study were collected from various talukas of Mehsana district of Gujarat. The organized and unorganized farms were selected to collect the ticks (Fig. 1, 2).

The animal farms in this district are having crossbred cattle accompanied with some local cattle and buffalo breeds. While small, unorganized farms primarily let their animals free to graze and are raised by farmers in villages, organized farms adopt a zero-grazing method. A questionnaire survey was done to know the management practices of farm, as well as the acaricides used and frequency of application. Fully engorged adult female ticks were collected from infected animals and their surroundings (cracks and crevices) using forceps without damaging their mouth parts from Kheralu, Satlasana, Unjha, Vadnagar and Visnagar talukas of Mehsana district. Few male ticks were also collected for the identification. The period of collection of ticks was from October 2022 to May 2023. The collected adult ticks were put into clean vials covered with muslin cloth to allow air and moisture exchange and brought to Department of Veterinary Parasitology of the College at Sardarkrushinagar. Ticks collected from the different places were divided into two batches, one for the adult immersion test and second for the biochemical assay.

Acaricides

Technical grade drugs (Flumethrin 96.1% and Fenvelarate 99.3%, Sigma Aldrich) were used for detection of acaricide resistance by preparing its stock solutions in methanol and acetone, respectively, and stored at 4°C. For the bioassay, serial concentrations of stock solutions of Flumethrin (80 ppm, 100 ppm, 200 ppm, 400 ppm, 800 ppm) and Fenvelarate (100 ppm, 200 ppm, 400 ppm, 800 ppm, 1600 ppm) were prepared in distilled water as a diluent.

Adult Immersion Test (AIT)

Engorged female ticks, upon arrival at the laboratory, were washed with tap water and thoroughly dried using absorbent paper. The AIT was performed following the protocol of Drummond *et al.* (1973), with slight modifications. The ticks were pre-weighed after washing and drying, then immersed in 10 mL of various concentrations of fenvalerate and flumethrin. The immersion was carried out in 25 mL beakers for 2 min with gentle agitation. Treated ticks were put on petri plate with Whatman filter paper 1 and kept for 24 h at room temperature. After 24 h, ticks were transferred to glass vials, which were covered with muslin cloth. Later stored in desiccators with relative humidity levels of 75 to 85 % and put in BOD incubator at 28 °C upto 15 days. The oviposition and mortality of these ticks were tracked. The egg mass was monitored in a BOD incubator for 15 days. In contrast to the control, the treated ticks' percentage of adult tick mortality and the weight of their eggs were recorded. According to Goncalves *et al.* (2007), the index of egg laying and % inhibition of fecundity was computed using the following formulas. 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
c. $$\text{Reproductive index (RI)} = \frac{\text{Weight of eggs laid (mg)}}{\text{Weight of adult females (mg)}}$$
d. % inhibition of oviposition (IO%) =
$$\frac{\text{RI (control group) - RI (treated group)}}{\text{RI (control group)}} \times 100$$

Biochemical Assay (Esterase assay)

Quantitative analysis of carboxyl esterase enzyme activity in tick larvae by α- and β- naphthyl acetate micro titre plate end point assay was done. Deep frozen 14 days old 20 unfed larvae from each tick were homogenated in 200 µL of distilled water. Homogenated larvae were centrifuged at 2795xg for 15 min at 4°C and supernatant was collected. In a 96 wells microtitre plate 20 µL of the homogenate in triplicates in

Fig. 1: Collected ticks from the animal

Fig. 2: Tick present on animal farm

adjacent wells were added with either 200 µL of α-NA or β-NA working solution, respectively. In the control blank wells, 20 µL of distilled water was added in place of tick homogenate and kept for incubation till 30 min. After that 50 µL of fast blue solution was added to each well and the plates were incubated at room temperature for 5 min. Later absorbance value was measured at 570 nm in an ELISA reader. Homogenate of tick larvae was added to 200 µL of Bradford reagent which was incubated for 10 min and OD value was recorded at 595 nm in ELISA reader (Hemingway, 1998).

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 (Fig. 3).

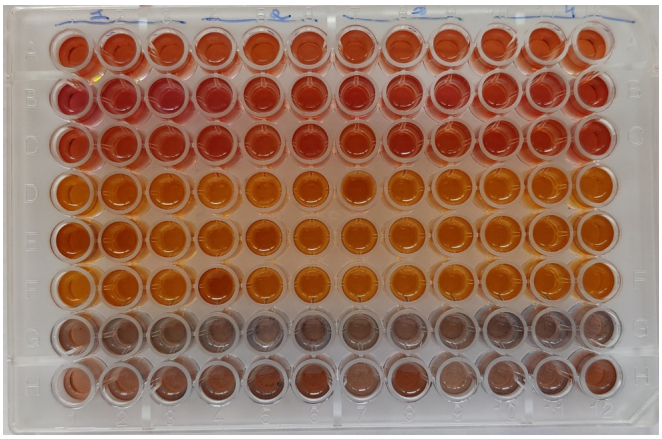

Fig. 3: Base line activity of esterase with substrate α- and β napthyal acetate in field resistant ticks

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. 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* was 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 resistant (RF ≥ 41) (Shyma *et al*., 2015).

Results and Discussion

AIT of *R. microplus* against Fenvelarate

Ticks isolates collected from different places of Mehsana district were subjected to AIT for detection of resistance or susceptibility. The dose dependent mortality against different concentration of fenvalerate was determined in all tick isolates. Further, log concentrations of drugs were plotted against probit mortality to determine the LC_{50} and LC_{95} through log dosage probit mortality analysis. Results of dose dependent mortality of *R. microplus* tick populations against fenvalerate in different places under study are given in Table 1.

Among five talukas surveyed during the research work, tick populations from Ralisana and Umta village of Visnagar taluka showed level I resistance with resistant factor 1.68 and 3.79, respectively. Ticks of Dasaj village of Unjha taluka had level II resistance with resistant factor 13.15, while those of Navavas village of Satlasana taluka had level IV resistance with resistant factor 72.68. The present study highlights varying levels of fenvalerate resistance in *R.microplus* tick populations across different regions of Mehsana (Gujarat), India. A high level of resistance observed in Navavas village (RF 72.68), compared well with the findings of Jyoti *et al*. (2014) in Kapurthala (RF 54.34). Moderate resistance (Level II) in Dasaj (RF 13.15) also aligned with observations of Jyoti *et al*. (2014) from Punjab. Similarly, Level I resistance found in Ralisana and Umta corresponds with both Jyoti *et al*. (2014), Nisa *et al*. (2021) and Patel *et al*. (2024), who reported low resistance levels in other parts of India. The likely cause of this resistance trend is the repeated and indiscriminate use of fenvalerate-based acaricides by farmers, which promotes

Table 1: Slope, R^2, LC_{50}, LC_{95}, RF_{95} resistant factor and resistance level of *R. microplus* against fenvalerate of Mehsana district

Taluka	Places	Slope	R^2	LC_{95}	LC_{50}	RF	RL
Kheralu	Ambavada	1.20	0.92	31021.78	1333.52	1.10	S
	Chansol	1.42	0.95	12548.49	878.33	0.73	S
Satlasana	Mumanvas	1.14	0.91	38055.69	1402.33	1.16	S
	Navavas	1.42	0.95	3821673	87549.47	72.68	IV
Unjha	Dasaj	1.31	0.95	691831	15848.93	13.15	II
Vadnagar	Babipura	1.25	0.95	24660.39	1202.26	0.99	S
	Sundhiya	1.14	0.91	38700.74	1409.68	1.17	S
Visnagar	Ralisana	1.12	0.95	59633.73	2035.10	1.68	I
	Umta	1.06	0.99	199699.7	4574.85	3.79	I

the selection of resistant tick populations. These findings underscore the need for integrated tick management and resistance monitoring strategies.

Tick population from Ambavada and Chansol villages of Kheralu taluka, Mumanvas village of Satlasana Taluka, Babipura and Sundhiya villages of Vadnagar taluka were found susceptible to fenvalerate. The current findings, where tick populations from multiple villages across Mehasana were found susceptible to fenvalerate, aligned with previous reports by Kumar *et al*. (2015) and Nisa *et al*. (2021). Kumar *et al*. (2021) identified susceptibility in MKT, MOG, and COR field isolates with low resistance factors (RF 0.04, 0.29, and 0.29), while Nisa *et al*. (2021) reported susceptibility in Poonch and Doda districts with RF values of 1.06 and 1.0, respectively. These consistent results suggest minimal or no selection pressure in these areas, likely due to limited or judicious use of fenvalerate. Continued monitoring and responsible acaricide usage are essential to maintain this susceptibility and prevent resistance development.

AIT of *R. microplus* against Flumethrin

The tick populations collected from Ambavada and Chansol villages in Kheralu taluka, Mumanvas and Navavas in Satlasana taluka, Dasaj in Unjha taluka, Babipura and Sundhiya in Vadnagar taluka, and Ralisana and Umta in Visnagar taluka of Mehsana district showed 100% mortality of *Rhipicephalus (Boophilus) microplus* when exposed to flumethrin, indicating that the tick populations in these areas are susceptible to the chemical. The observed effectiveness of flumethrin may be attributed to its relatively recent introduction into the Indian market. However, due to the absence of data on a reference tick line, it was not possible to calculate the resistance factor for flumethrin. Tick resistance to synthetic pyrethroids is typically described in terms of family resistance, where ticks simultaneously develop resistance to multiple compounds within the group (Shyma *et al.,* 2012). However, data on reference tick lines for flumethrin were available for other countries. Kumar *et al.* (2015) asserted that country-specific discriminating concentration for various acaricides is a mandatory requirement to monitor the level of resistance in ticks because a variety of factors, such as geographic location, climate, the economic standing of the farmers, dose and frequency of acaricide application, and breed of animal, can contribute to the development of resistance.

Biochemical Assay for α- and β-Esterase Enzyme Activity

The enzyme activity of α-esterase and β-esterase found significantly higher than the value of field susceptible IVRI-I line. α-esterase and β-esterase enzyme activity in ticks under study was found to be in the range of 4.05 to 17.56 µmole/min/mg of protein and 2.34 to 10.95 µmole/min/mg of protein, respectively (Table 2). There is positive correlation between resistance factor and enzyme activity.

Multiple studies have demonstrated a strong correlation between esterase enzyme activity and resistance to pyrethroid acaricides in *R. microplus*. Esterase assays, often used alongside AIT and Larval Packet Tests (LPT), consistently revealed elevated α- and β-esterase activity in resistant tick populations. For example, Jyothimol *et al*. (2014) and Nandi *et al*. (2015) all reported significantly increased esterase activity in resistant isolates, confirming enzymatic detoxification as a key resistance mechanism. Kumar *et al.* (2016, 2021) and Godara *et al*. (2019) also noted a 3-4 fold or higher increase in enzyme activity in resistant strains compared to the susceptible IVRI-I strain. Fular *et al*. (2020) and Ziapour *et al*. (2016) further supported this trend, reporting multi-acaricide resistance with elevated esterase and other detoxifying enzymes like P450 and GST. These findings collectively reinforce that biochemical assays, especially esterase activity measurements, are reliable indicators of resistance and should be integrated into regular tick resistance surveillance programs.

Conclusion

The present study revealed varying levels of fenvalerate resistance in tick populations across Mehsana district, with one site showing high resistance. However, all isolates were susceptible to flumethrin. Elevated α- and β-esterase activities correlated positively with resistance levels, indicating their role in resistance development among *R. microplus* ticks in the region.

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

Taluka	Tick isolates	Fenvalerate RF	α-esterase (µmoles/min./mg of protein)	Enzyme ratio	β-esterase (µmoles/min./mg of protein)	Enzyme ratio
Kheralu	Ambavada	1.10	4.25 ± 0.18	1.72	2.52 ± 0.075	2.06
	Chansol	0.73	4.05 ± 0.059	1.64	2.34 ± 0.056	1.92
Satlasana	Mumanvas	1.16	4.18 ± 0.15	1.69	2.52 ± 0.075	2.06
	Navavas	72.68	17.56 ± 0.182	7.10	10.95 ± 0.54	8.97
Unjha	Dasaj	13.15	11.49 ± 0.12	4.65	4.16 ± 0.081	3.41
Vadnagar	Babipura	0.99	4.88 ± 0.045	1.97	2.61 ± 0.054	2.14
	Sundhiya	1.17	4.29 ± 0.045	1.74	2.71 ± 0.062	2.22
Visnagar	Ralisana	1.68	4.52 ± 0.16	1.83	2.87 ± 0.11	2.35
	Umta	3.79	5.89 ± 0.175	2.38	3.25 ± 0.16	2.66
IVRI-I susceptible		1.0	2.47 ± 0.008	1	1.22 ± 0.006	1

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