



Acaricide resistance status of *Haemaphysalis* spp. infesting cattle in central Kerala using larval packet test

Rajeshwari¹, Asha Rajagopal¹, Bindu Lakshmanan¹, M.N.Priya¹, Arun George²,
 Surya Sankar³, O.P. Reji Rani⁴ and E.S. Jisni¹

¹Department of Veterinary Parasitology, ³Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur-680 651, ²Department of Clinical Medicine Ethics and Jurisprudence, College of Veterinary and Animal Sciences, Pookode, Wayanad, Kerala Veterinary and Animal Sciences University Kerala, India, ⁴Department of Entomology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala Agricultural University

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Abstract

Ticks are among the most important ectoparasites affecting cattle health and productivity in tropical regions, with chemical acaricides being the primary control strategy. However, indiscriminate and prolonged use of acaricides has resulted in the emergence of resistant tick populations. The present study was undertaken to assess the acaricide susceptibility status of *Haemaphysalis bispinosa* infesting cattle in central Kerala using the Larval packet test (LPT). Fully engorged female ticks were collected from cattle maintained in the Malayoram and Midland agro-climatic zones, reared under controlled laboratory conditions and larval bioassays were conducted on deltamethrin, amitraz, and fipronil following FAO guidelines. In the Malayoram zone, *Haemaphysalis* larvae showed resistance to deltamethrin, with an LC_{50} of 232.59 ppm and LC_{90} of 1007.6 ppm, and survival exceeding 10 per cent at the discriminating dose of 75 ppm. In contrast, the Midland zone population was highly susceptible to deltamethrin, with LC_{50} and LC_{90} values of 4.61 ppm and 24.97 ppm, respectively. Amitraz was effective against tick populations from both zones, with mortality exceeding 90 per cent at higher concentrations; LC_{50} values were 16.15 ppm in the Malayoram zone and 5.35 ppm in the Midland zone. Fipronil exhibited resistance in the Malayoram zone, where mortality at the discriminating dose of 9.6 ppm remained below 90 per cent, while complete susceptibility was observed in the Midland zone. The study reveals zone-specific variation in acaricide resistance among *H. bispinosa* populations in central Kerala and highlights the need for regular resistance monitoring and adoption of region-specific, sustainable tick control strategies.

Keywords: Larval packet test, deltamethrin, amitraz, fipronil

Ticks are obligate blood-feeding ectoparasites of major veterinary and public health importance, responsible for transmitting a wide range of protozoan, bacterial, and viral pathogens to livestock and humans (de Castro, 1997; Balasubramanian et al., 2019). Tick infestation leads to substantial economic losses in cattle through blood loss, skin damage, anaemia, reduced milk yield, lowered body weight gain and increased susceptibility to secondary infections

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^{*}Corresponding author: asha@kvasu.ac.in, Ph. 9446502343

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(Rani et al., 2018; Ghosh and Nagar, 2014). In tropical and subtropical regions such as India, warm temperatures coupled with high humidity provide favourable conditions for the year-round survival, reproduction, and rapid multiplication of ticks (Haque et al., 2014).

Among the various tick genera prevalent in India, *Haemaphysalis* spp. are emerging as ticks of increasing veterinary significance. Species such as *Haemaphysalis (H.) bispinosa* are multi-host ticks commonly infesting cattle and are implicated in the transmission of haemoprotozoan diseases. Despite their growing importance, studies focusing on acaricide susceptibility and resistance in *Haemaphysalis* spp. are comparatively limited, as most resistance surveillance programmes in India have predominantly focused on *Rhipicephalus* and *Hyalomma* species (Shyma et al., 2015; Lenka et al., 2016).

Chemical acaricides remain the primary method for tick control due to their rapid knock-down effect, ease of application, and cost-effectiveness (FAO, 2004). Commonly used acaricides in cattle include synthetic pyrethroids, amidines, organophosphates, macrocyclic lactones and phenylpyrazoles. However, prolonged and indiscriminate use of these compounds has resulted in the widespread development of acaricide resistance in tick populations across different regions of India (Shyma et al., 2012; Singh et al., 2015). Resistance mechanisms in ticks are associated with enhanced metabolic detoxification, reduced penetration of acaricides through the cuticle, and alterations in target site proteins such as sodium channels and octopamine receptors (Guerrero et al., 2012; Fular et al., 2020).

The Larval Packet Test (LPT), originally described by Stone and Haydock (1962) and later standardised by the Food and Agriculture Organization (FAO, 2004; Morgan et al., 2009), is a widely accepted laboratory bioassay for detecting and quantifying acaricide resistance in ticks. This method is valued for its reproducibility, simplicity, and ability to generate dose–response data, including lethal concentration estimates. While LPT has been extensively applied for resistance monitoring in one-host ticks, its application to multi-host ticks such as *Haemaphysalis* spp. remains limited.

Kerala has a well-developed dairy sector, where tick infestations continue to pose a serious constraint to cattle health and productivity. Reports of acaricide resistance in ticks from neighbouring regions further emphasise the need for regular resistance surveillance. In this context, the present study was undertaken to assess the acaricide susceptibility status of *Haemaphysalis* spp. infesting cattle in central Kerala against deltamethrin, amitraz, and fipronil using the Larval Packet Test, with the objective of generating baseline data to support sustainable and region-specific tick control strategies.

Materials and methods

Study area and tick collection

Fully engorged female ticks were collected from cattle maintained in organised farms and smallholder production systems located in Chirakkcode and Ollur regions in central Kerala, belonging to the Malayoram and Midland agro-climatic zones, respectively. The collected ticks were examined under a microscope and identified to the species level using the taxonomic keys of Sen and Fletcher (1962) and Walker et al. (2013). The predominant species identified was *Haemaphysalis bispinosa*. Individual ticks were placed in labelled glass vials and maintained under controlled laboratory conditions at $28 \pm 1^\circ\text{C}$ temperature and 85 ± 5 per cent relative humidity to facilitate oviposition.

Rearing of tick larvae

Egg masses laid by engorged females were incubated under the same controlled conditions until hatching. Larvae aged between 12 and 21 days were used for bioassay experiments to ensure uniform susceptibility.

Larval packet test (LPT)

The Larval Packet Test was performed following FAO-recommended procedures with minor modifications. Technical-grade deltamethrin, amitraz, and fipronil were used for bioassays. Stock solutions of deltamethrin and amitraz were prepared in methanol, while fipronil was dissolved in acetone. Working concentrations were prepared by appropriate dilution using distilled water.

The concentrations tested in the present study included deltamethrin at 15, 30, 60, 120, 240 and 600 ppm; amitraz at 15.62, 31.25, 62.5, 125, 250, 500 and 750 ppm; and fipronil at 5, 10, 20 and 40 ppm. Distilled water was used as the control treatment.

Whatman No. 1 filter papers were impregnated with the respective acaricide solutions, air-dried, folded into packets, and sealed on three sides. Approximately 100 larvae were introduced into each packet, which was then sealed completely and incubated for 24 hours at $28 \pm 1^\circ\text{C}$ and 85 ± 5 per cent relative humidity. Each concentration was tested in triplicate.

Assessment of mortality

After 24 hours of exposure, packets were opened and larvae were examined. Larvae showing no movement were considered dead. Percentage mortality was calculated for each concentration.

Statistical analysis

Mortality data were expressed as mean $\pm$ standard error of mean (SEM). One-way analysis of

variance (ANOVA) followed by Duncan's multiple range test was used to compare mortality between concentrations. Probit analysis was performed to estimate median lethal concentration (LC_{50} and LC_{90}) values. Resistance or susceptibility status was determined based on mortality at the discriminating dose (DD). Statistical significance was considered at $p < 0.05$.

Result and discussion

Susceptibility to deltamethrin

The bioassay was performed using serial dilutions of technical grade deltamethrin, ranging from 600 ppm to 15 ppm. The proportion of ticks surviving at a specific concentration, designated as the discriminating dose (DD), was interpreted as the percentage resistance to the acaricide. According to FAO (2004), the DD for susceptible Yeerongpilly (Australia) strains of *Rhipicephalus (R.) microplus* was established at 600 ppm. However, studies using IVRI susceptible lines of the same species reported considerably lower DD values, including 59.2 ppm (Sharma et al., 2012) and 71 ppm (Shyma et al., 2013). Similarly, Rani et al. (2018) proposed an approximate DD of 75 ppm for *Haemaphysalis* spp. Furthermore, Lovis et al. (2012) suggested that a tick population may be classified as resistant when the survival rate at the discriminating dose exceeds 10 per cent.

Larval packet test carried out in the Malayoram zone demonstrated a gradual and concentration-dependent increase in mortality following deltamethrin exposure

(Table 1). No mortality was recorded in the control group or at the lowest concentration of 15 ppm. Mortality increased to 6.00 ± 0.58 per cent at 30 ppm and 12.00 ± 0.58 per cent at 60 ppm. At higher concentrations of 120 ppm and 240 ppm, mortalities rose to 23.67 ± 1.86 per cent and 53.67 ± 0.88 per cent, respectively. Probit analysis of the mortality data generated a slope value of 2.013 ± 0.221 , with a chi-square statistic of 3.631 ($p = 0.304$), indicating that the data fitted the probit model well. The LC_{50} and LC_{90} values were estimated at 232.59 ppm and 1007.6 ppm, respectively. This population exhibited clear resistance to deltamethrin, as indicated by high larval survivability at DD of 75 ppm, as proposed by Rani et al. (2018). Resistance has been reported in *Haemaphysalis* spp. of goats in Kerala by Kajal et al. (2019), who documented LC_{50} and LC_{90} values of 80.09 ppm and 270.61 ppm, respectively, in a resistant Vadakkanchery isolate. High resistance levels have also been described in *R. microplus* (LC_{50} : 223.87 ppm; LC_{95} : 602.56 ppm) and in *Rhipicephalus (R.) sanguineus*, where LC_{95} values exceeded the recommended dose range (Anand et al., 2021). Patel et al. (2020) reported elevated LC_{50} values (93.92–297.29 ppm) in *R. microplus* populations from Gujarat, categorizing resistance as level II–III.

Midland zone showed higher larval mortality at 15 ppm (83.33 ± 0.88 per cent), which increased further to 90.33 ± 1.20 per cent at 30 ppm (Table 2). At 60 ppm, mortality reached 96.67 ± 0.33 per cent, and complete mortality (100 per cent) was recorded at 120 ppm and 240 ppm. Probit analysis yielded a slope of 1.747 ± 0.352 ,

Table 1. Results of LPT in malayoram zone with deltamethrin

Concentration (ppm)	Per cent mortality (Mean $\pm$ SE)	Probit analysis				
		Slope $\pm$ SE	χ^2 value	P-value	LC_{50} (ppm)	LC_{90} (ppm)
Control	$0^e \pm 0$	2.013 ± 0.221	3.631 ^{ns}	0.304	232.59	1007.6
15	$0^e \pm 0$					
30	$6.00^d \pm 0.58$					
60	$12.00^c \pm 0.58$					
120	$23.67^b \pm 1.86$					
240	$53.67^a \pm 0.88$					

ns non-significant; Means having different letter as superscript differ significantly in per cent mortality

Table 2. Results of LPT in midland zone with deltamethrin

Concentration (ppm)	Per cent mortality (Mean $\pm$ SE)	Probit analysis				
		Slope $\pm$ SE	χ^2 value	P-value	LC_{50} (ppm)	LC_{90} (ppm)
Control	$0^e \pm 0$	1.747 ± 0.352	1.773 ^{ns}	0.621	4.611	24.969
15	$83.33^d \pm 0.88$					
30	$90.33^c \pm 1.2$					
60	$96.67^b \pm 0.33$					
120	$100^a \pm 0$					
240	$100^a \pm 0$					

ns non-significant; Means having different letter as superscript differ significantly in per cent mortality

and the chi-square value of 1.773 ($p = 0.621$) indicating a satisfactory model fit. The LC_{50} (4.61 ppm) and LC_{90} (24.97 ppm) were markedly below the DD, confirming susceptibility. Comparable low LC_{50} values have been reported in susceptible isolates of *Rhipicephalus* (*R.*) *annulatus* (2.11 ppm) and *R. microplus* (2.15 ppm) (Jyothimol et al., 2014; Lenka et al., 2016). Similarly, Gupta et al. (2024) observed predominantly susceptible field isolates of *Hyalomma* (*H.*) *anatolicum* from Haryana, with resistance factors between 0.17 and 0.42. Similarly, Rani et al. (2018) reported that deltamethrin induced resistance in multiple tick species in Karnataka, including *H. bispinosa*, *Haemaphysalis intermedia*, *Haemaphysalis kutchensis*, *Hyalomma marginatum issaci*, *Hyalomma anatolicum anatolicum*, *Rhipicephalus haemaphysaloides*, and *R. sanguineus*, with resistance categorized as level II in most species, while *H. intermedia* exhibited only level-I resistance.

Susceptibility to amitraz

The bioassay was carried out using serial dilutions of technical grade amitraz, ranging from 1000 ppm to 15.62 ppm. For amitraz DD has been variably reported for susceptible Australian strains of *R. microplus*, with values of 4000 ppm, 1000 ppm, and 250 ppm as per FAO (2004). Lovis et al. (2011) further indicated that a single DD may

not be appropriate for amitraz and recommended the use of three concentrations in accordance with FAO guidelines. In Indian isolates of *Haemaphysalis* spp., Rani et al. (2018) estimated the DD of amitraz to be 2500 ppm.

Amitraz in the Malayoram zone revealed a clear dose-response trend (Table 3). Mortality at the lowest concentration of 16 ppm was 50.67 ± 0.33 per cent, which increased progressively to 61.67 ± 0.67 per cent at 31 ppm, 69.67 ± 0.88 per cent at 63 ppm, and 74.67 ± 0.33 per cent at 125 ppm. Higher concentrations resulted in further increase in mortality, with 84.67 ± 0.67 per cent at 250 ppm, 90.33 ± 0.88 per cent at 500 ppm and 95.67 ± 0.33 per cent at 750 ppm. Probit analysis produced a slope of 0.891 ± 0.100 and the chi-square statistic (2.167, $p = 0.826$) confirmed an adequate fit. LC_{50} and LC_{90} values were 16.154 ppm and 443.946 ppm, respectively. At 750 ppm larval mortality exceeded 90 per cent, indicating susceptibility.

In the Midland zone, amitraz produced consistently high mortality across the tested concentrations (Table 4). At 16 ppm, mortality was 86.33 ± 0.88 per cent, increasing slightly to 90.67 ± 0.33 per cent at 31 ppm. Complete mortality (100 per cent) was observed from 63 ppm onwards, which confirmed susceptibility. Probit analysis yielded a slope of 2.105 ± 0.475 , with a chi-square statistic

Table 3. Results of LPT in malayoram zone with amitraz

Concentration (ppm)	Per cent mortality (Mean $\pm$ SE)	Probit analysis				
		Slope $\pm$ SE	χ^2 value	P-value	LC50 (ppm)	LC90 (ppm)
Control	0 ^h $\pm$ 0	0.891 $\pm$ 0.100	2.167 ^{ns}	0.826	16.154	443.946
15.62	50.67 ^g $\pm$ 0.33					
31.28	61.67 ^f $\pm$ 0.67					
62.5	69.67 ^e $\pm$ 0.88					
125	74.67 ^d $\pm$ 0.33					
250	84.67 ^c $\pm$ 0.67					
500	90.33 ^b $\pm$ 0.88					
750	95.67 ^a $\pm$ 0.33					

ns non-significant; Means having different letter as superscript differ significantly in per cent mortality

Table 4. Results of LPT in Midland zone with amitraz

Sl. No.	Concentration (ppm)	Per cent mortality (Mean $\pm$ SE)	Probit analysis				
			Slope $\pm$ SE	χ^2 value	P-value	LC50 (ppm)	LC90 (ppm)
1	Control	0 ^c $\pm$ 0	2.105 $\pm$ 0.475	5.194 ^{ns}	0.393	5.352	21.739
2	15.62	86.33 ^b $\pm$ 0.88					
3	31.28	90.67 ^b $\pm$ 0.33					
4	62.5	100 ^a $\pm$ 0					
5	125	100 ^a $\pm$ 0					
6	250	100 ^a $\pm$ 0					
7	500	100 ^a $\pm$ 0					
8	750	100 ^a $\pm$ 0					

ns non-significant; Means having different letter as superscript differ significantly in per cent mortality

of 5.194 ($p = 0.393$). The LC_{50} and LC_{90} were 5.352 ppm and 21.739 ppm, respectively.

The results are in agreement with Rani et al. (2018), who reported complete mortality of *Haemaphysalis* spp. at the recommended amitraz concentrations in Karnataka and Jyoti et al. (2019), who observed mostly low-level resistance or susceptibility in *H. anaticum* ticks from various districts of Punjab using LPT. However, emerging amitraz resistance has been documented in other tick genera. Kumar et al. (2014) reported resistance factors up to 27.3 in *R. microplus*, while Shaik et al. (2024) identified amitraz resistance in *R. sanguineus* from Kerala using bioassays and genotyping. The findings emphasized the need for cautious use of amitraz despite its current effectiveness against *Haemaphysalis* spp. Martínez-Ibañez et al. (2026) reported amitraz resistance in seven per cent of *Rhipicephalus sanguineus* sensu lato (s.l.) populations across Mexico. The findings emphasised the need for cautious use of amitraz despite its current effectiveness against *Haemaphysalis* spp.

Susceptibility to fipronil

The bioassay was conducted using serial dilutions of technical-grade fipronil, ranging from 40 ppm to 5 ppm. The DD for fipronil was 9.6 ppm, as recommended by Kumar et al. (2015) for the susceptible IVRI-I line of *R. microplus*.

Larval packet test with fipronil, the Malayoram zone showed high levels of larval mortality even at low concentrations (Table 5). At 5 ppm, mortality was 74.33 ± 0.88 per cent, increasing to 86.00 ± 0.58 per cent at 10 ppm, 92.00 ± 0.58 per cent at 20 ppm and 97.67 ± 0.33

per cent at 40 ppm. Probit analysis provided a slope value of 1.386 ± 0.278 , with a chi-square statistic of 0.300 ($p = 0.861$), confirming a good fit. The LC_{50} and LC_{90} values were 1.704 ppm and 14.334 ppm, respectively. Larval mortality at DD of 9.6 ppm remained below 90 per cent, thereby classifying the population as resistant. The LC_{50} (1.70 ppm) and LC_{90} (14.33 ppm) values, although relatively low, suggested reduced field effectiveness. Similar early indications of fipronil resistance have been reported by Shyma et al. (2015), who observed level I resistance in *R. microplus* using both LPT and AIT, with resistance factors of 2.36 and 3.78, respectively. Likewise, Shakya et al. (2020) detected resistance factors ranging from 1.56 to 10.9 in sixteen isolates of *R. microplus*. Further supporting these observations, Gupta et al. (2021) reported comparatively lower resistance factors for *H. anaticum* (0.23–3.8), and notable higher resistance factors for *R. microplus* (2.4 to 28.2), indicating species-specific differences in the development and intensity of fipronil resistance. Bhagat et al. (2026) reported that, among 15 populations of *R. microplus* from the Jammu region of the north-western Himalayas, eight were susceptible (RR_{50} : 0.75–1.38), while seven showed low-level (Level I) resistance (RR_{50} : 1.5–1.98), with higher resistance observed in lower-altitude areas, likely due to intensive livestock rearing and frequent acaricide use. Four per cent of *Rhipicephalus sanguineus* sensu lato (s.l.) populations examined across Mexico were found to be resistant to fipronil (Martínez-Ibañez et al., 2026).

Conversely, Midland zone exhibited larval mortality of 77.00 ± 1.00 per cent at 5 ppm (Table 6), increasing to 91.33 ± 0.88 per cent at 10 ppm, with complete mortality (100 per cent) recorded at 20 ppm and 40 ppm. Probit analysis produced a slope of 2.832

Table 5. Results of LPT in Malayoram zone with fipronil

Sl. No.	Concentration (ppm)	Per cent mortality (Mean $\pm$ SE)	Probit analysis				
			Slope $\pm$ SE	χ^2 value	P-value	LC_{50} (ppm)	LC_{90} (ppm)
1	Control	$0^e \pm 0$	1.386 ± 0.278	0.300 ^{ns}	0.861	1.704	14.334
2	5	$74.33^d \pm 0.88$					
3	10	$86.00^c \pm 0.58$					
4	20	$92.00^b \pm 0.58$					
5	40	$97.67^a \pm 0.33$					

ns non-significant; Means having different letter as superscript differ significantly in per cent mortality

Table 6. Results of LPT in Midland zone with Fipronil

Sl. No.	Concentration (ppm)	Per cent mortality (Mean $\pm$ SE)	Probit analysis				
			Slope $\pm$ SE	χ^2 value	P-value	LC_{50} (ppm)	LC_{90} (ppm)
1	Control	$0^e \pm 0$	2.832 ± 0.549	2.105 ^{ns}	0.349	2.86	8.107
2	5	$77.00^d \pm 1.00$					
3	10	$91.33^c \pm 0.88$					
4	20	$100^b \pm 0$					
5	40	$100^a \pm 0$					

ns non-significant; Means having different letter as superscript differ significantly in per cent mortality

± 0.549 , and the chi-square statistic (2.105, $p = 0.349$) confirmed the appropriateness of the model. The LC_{50} and LC_{90} values were 2.86 ppm and 8.107 ppm, respectively. Larval mortality at DD of 9.6 ppm was approximately 90.19 per cent (>90 per cent), thereby classifying the population as susceptible. The findings are consistent with Vilela et al. (2020), who reported absence of fipronil resistance in *R. microplus* populations despite resistance to multiple other acaricides in Brazil.

The differential resistance patterns observed highlight the importance of region-specific acaricide resistance monitoring. Resistance to deltamethrin and emerging fipronil resistance in the Malayoram zone may be linked to frequent acaricide application, under-dosing, and lack of rotation, as suggested by Patel et al. (2020) and Kumar et al. (2021). The continued susceptibility of ticks in the Midland zone indicates comparatively rational acaricide use resulting in lower selection pressure. Similarly, susceptibility to amitraz in both zones might be attributed to the less frequent use of this drug for tick control in cattle in these areas.

Conclusion

The present study clearly demonstrates the existence of agro-climatic zone-dependent variation in acaricide susceptibility of *H. bispinosa* infesting cattle in central Kerala. Resistance to deltamethrin and fipronil was evident in tick populations from the Malayoram zone, as indicated by elevated LC_{50} and LC_{90} values and survival beyond DD. In contrast, tick populations from the Midland zone remained highly susceptible to all three acaricides tested.

Amitraz emerged as the most consistently effective acaricide across both zones, suggesting its continued utility for tick control in the region. However, the comparatively higher LC_{90} value observed in the Malayoram population signals the need for cautious and judicious use to prevent the development of resistance. The observed resistance patterns are likely influenced by differences in acaricide usage practices, frequency of application, and farm management systems.

The findings emphasize the importance of routine acaricide resistance surveillance using standardized bioassays such as the Larval Packet Test. Adoption of integrated tick management approaches, including acaricide rotation, correct dosing, and farmer awareness programmes, is essential to delay resistance development and ensure sustainable control of *Haemaphysalis* spp. ticks in cattle production systems of Kerala. In addition, alternative control strategies such as semiochemicals, entomopathogenic fungi, herbal acaricides, biological control agents including natural predators and other eco-friendly approaches should be explored as part of integrated pest management programmes. These strategies can reduce reliance on chemical acaricides and contribute to long-term sustainable tick control.

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Conflict of Interest

There are no potential conflicts of interest

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