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Evaluation of the role of piperonyl butoxide as synergist in deltamethrin resistant *Rhipicephalus sanguineus*[#]

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Abstract

The brown dog tick, Rhipicephalus sanguineus, is an important ectoparasite of dogs and has considerable veterinary and public health significance. Pyrethroid acaricides, particularly deltamethrin, are widely used for their control; however, the development of resistance has increasingly compromised their effectiveness. Deltamethrin resistance in R. sanguineus isolates has already been reported in Kerala. The present study assessed the role of synergist piperonyl butoxide (PBO), a well-known cytochrome P450 inhibitor, in enhancing deltamethrin toxicity against resistant R. sanguineus populations collected from different regions of Kerala. Ten field isolates were evaluated using a modified larval packet test. All isolates tested with deltamethrin exhibited less than 90 per cent mortality at the discriminating dose of 600 ppm and were considered resistant. Piperonyl butoxide, when tested alone, produced dose-dependent larval mortality, achieving complete mortality at concentrations ≥400 ppm. When deltamethrin was combined with a non-lethal dose of PBO (200 ppm), a pronounced reduction in LC_{50} values was observed across all isolates ($p < 0.0001$). Synergistic ratios ranged from 93.60 to 5331.08, indicating strong potentiation of deltamethrin toxicity. These findings suggest that cytochrome P450-mediated metabolic detoxification plays a major role in deltamethrin resistance and that PBO can effectively restore susceptibility in resistant R. sanguineus populations.

Keywords: *Rhipicephalus sanguineus*, acaricide resistance, deltamethrin, piperonyl butoxide, synergist

Acaricide resistance in *Rhipicephalus sanguineus* in India has been reported but is less extensively studied than in *Rhipicephalus microplus*. Field observations, bioassays and molecular studies suggest emerging resistance or reduced susceptibility to commonly used acaricides, including synthetic pyrethroids and macrocyclic lactones. Using larval packet and larval immersion tests on *R. sanguineus* populations from Andhra Pradesh, Sunkara et al. (2022) reported a high prevalence of resistance, with 92.6% of populations resistant to cypermethrin and 42.9% to ivermectin, highlighting a concerning level of resistance in the country. Resistance to acaricides in ticks often arises through metabolic detoxification pathways, where specific enzymes like CYP450 monooxidases and esterases degrade or modify the chemical structure of acaricides before reaching their target site (Waldman et al., 2023). Synergists act as inhibitors of detoxifying

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enzymes, thereby reducing the metabolic degradation of the acaricide, increasing the availability of the active compound at target sites, and restoring acaricide efficacy (Le Gall et al., 2018). Metabolic resistance is particularly concerning because it can evolve rapidly, involve multiple enzyme systems and produce cross-resistance across different chemical classes. In the current scenario, where the development and commercialisation of novel acaricide classes are often economically impractical, the strategic use of existing acaricides in combination warrants greater attention. Understanding the effects of synergists in acaricide resistance is essential for formulating evidence-based integrated tick control strategies at the field level. Previously, deltamethrin resistance has been reported in *R. sanguineus* from Kerala (Amrutha et al., 2021), and a study on amitraz resistance in this species revealed that single nucleotide polymorphisms previously associated with amitraz resistance in *Rhipicephalus microplus* were not detected in highly resistant *R. sanguineus* isolates (Shaik et al., 2024), indicating involvement of alternative resistance mechanisms, potentially metabolic detoxification pathways. In this context, the present study was undertaken with the objective of assessing the synergistic efficacy of piperonyl butoxide (PBO) in mitigating deltamethrin resistance in *R. sanguineus.*

Materials and methods

Ticks were collected from naturally infested dogs from ten locations *viz*, Mannuthy, Therambam, Ollur, Vellanikkara, Kolangattukara, Kodungallur, Triprayar, Ernakulam, Mezhathur and Edappal in Kerala. After identification, engorged female ticks were maintained in a desiccator under controlled laboratory conditions (28±1°C and 85±5 per cent relative humidity) for oviposition and subsequent larval hatching. Healthy larvae aged 12 to 14 days were included in bioassays. Any samples with fungal contamination or desiccation were discarded.

Larval Packet Test (LPT) for deltamethrin

Larval susceptibility to deltamethrin was assessed using LPT, following FAO (2004) guidelines with minor modifications. Filter papers were treated with graded concentrations of deltamethrin (0.2 to 4000 ppm), air-dried and folded into packets. Approximately 100 larvae were introduced into each packet, which was then sealed and incubated for 24 h. Control packets were treated with methanol alone.

Dose-standardisation of piperonyl butoxide

Piperonyl butoxide was tested independently at concentrations ranging from 200 to 5000 ppm to determine lethal and non-lethal doses. Based on preliminary bioassays, 200 ppm was selected as the non-lethal concentration for synergist studies.

Synergist bioassay

For synergist assays, each deltamethrin concentration was tested in combination with PBO at a fixed dose of 200 ppm. Impregnation, larval exposure and incubation were done similar to those used in the deltamethrin-alone assays.

Mortality assessment and statistical analysis

Larvae unable to walk or showing only appendage movements were considered dead. Mortality percentages were calculated 24 h post-exposure. Dose-response data were analysed using non-linear regression in GraphPad Prism 8.0.2 to estimate LC_{50} values. Synergistic ratios (SR) were calculated as the ratio of LC_{50} values for deltamethrin alone to those for deltamethrin combined with PBO (Eiden et al., 2017). Statistical significance was considered at $p < 0.05$.

Table 1. Dose-response data of piperonyl butoxide across different tick isolates of Kerala

Tick isolate	Concentration of PBO for attaining		Mean LC_{50} ± Mean Standard Error (SEM) (ppm)
	Zero per cent mortality (ppm)	100 per cent mortality (ppm)	
Mannuthy	200	400	294.3 ± 49.3
Therambam	200	400	260.5 ± 43.6
Ernakulam	200	500	359.3 ± 57.53
Edappal	200	4500	1382.0 ± 304.2
Kodungallur	200	5000	1995.0± 39.0
Triprayar	200	1000	452.1 ± 88.5
Palakkad	200	2400	919.8 ± 183.8
Kulangattukara	200	4000	1185.0 ± 260.8
Ollur	200	3800	1483.0 ± 329.5
Vellanikkara	200	2600	1216.0±260.43

Results and discussion

Deltamethrin resistance in field isolates

Although previous studies on amitraz resistance in *R. sanguineus* populations from Kerala have determined a discriminating dose (DD) of 1200 ppm, the absence of a known susceptible population for deltamethrin prevented the determination of an appropriate DD in the present study. The FAO (2004) established a diagnostic dose of 600 ppm for susceptible *R. microplus* strains. All isolates in this study exhibited less than 90 per cent mortality at the discriminating dose of 600 ppm and were considered resistant, consistent with the resistance criteria defined by Amrutha et al. (2021) and Lovis et al. (2012). Besides, the recommended effective concentration range of deltamethrin is 1.25 to 100 ppm (Sandhu, 2006). In the present study, the lethal concentration causing 95 per cent larval mortality (LC95) values for deltamethrin ranged from 478.63 to 3467.37 ppm, which were substantially higher than the recommended dosage. The developed resistance is likely due to the result of prolonged selection pressure arising from repeated pyrethroid exposure. These findings align with the report of Amrutha et al. (2021) that revealed 60 per cent of the tested isolates exhibited resistance or tolerance, with resistance factors ranging from 1 to 53.7 in *R. sanguineus* in Kerala.

Dose-standardisation of piperonyl butoxide

To evaluate the role of PBO in deltamethrin resistance, a synergist bioassay was conducted in which PBO was added at a constant, non-lethal concentration to all deltamethrin doses in the LPT. Initial trials using the recommended PBO concentrations of one to two per cent resulted in complete larval mortality, indicating isolate-specific sensitivity and the need for dose standardisation. Consequently, PBO concentrations ranging from 200 to 5000 ppm were evaluated across all isolates, and 200 ppm was identified as the non-lethal concentration. The dose-response data of PBO are presented in Table 1. The synergist bioassay was then performed by combining PBO at this fixed concentration (200 ppm) with each tested dose of deltamethrin. Notably, PBO was used in commercial veterinary products at concentrations ranging from 1000 to 50,000 ppm. However, this study revealed that complete larval mortality could be achieved at concentrations below 5000 ppm, which was substantially lower than the concentrations commonly used. This indicated that lower doses of PBO might be sufficient for synergistic applications and the present study suggested the need for dose-optimisation and re-evaluation of PBO concentration in acaricide formulations to achieve effective synergism while minimising excessive exposure.

Table 2. Mean LC_{50} ± SEM (Lethal concentration 50 ± Mean Standard Error) values of treatment groups

Treatment groups	Mean LC_{50} ± SEM (ppm)
PBO alone	260.5 ± 43.6 to 1995 ± 394^{B}
Deltamethrin alone	157.8±15.23 to 757.2±45B^{B}
Deltamethrin+PBO	0.03 ± 0.01 to 5.03 ± 0.75A^{A}

Values with different letter as superscript differ significantly within in the column ($p< 0.001$).

Synergistic effect of PBO with deltamethrin

The mean LC_{50} values for the treatment groups are presented in Table 2. Responses were compared among larvae treated with deltamethrin alone, PBO alone, and the deltamethrin+PBO combination. Pairwise comparisons were performed using one-way ANOVA followed by Tukey's post hoc test.

The addition of PBO to deltamethrin resulted in a significant reduction in LC_{50} values across all isolates. Specifically, LC_{50} values decreased from 157.8 to 757.2 ppm for deltamethrin alone to 0.03 to 5.03 ppm for the deltamethrin+PBO combination ($p < 0.0001$). The markedly lower LC_{50} values observed with the combination, compared with deltamethrin or PBO tested individually, clearly demonstrated the synergistic effect of PBO on deltamethrin toxicity. Statistically, no significant difference was observed between the LC_{50} values of deltamethrin and PBO when tested alone, suggesting a high level of deltamethrin resistance in the field isolates evaluated. Comparable reductions in deltamethrin LC_{50} values following the use of deltamethrin + PBO combinations have been reported in *Rhipicephalus (Boophilus)* spp. by Shakya et al. (2022) and Vatsya & Yadav (2011), findings that align with the present study and further accentuate the role of PBO in enhancing deltamethrin efficacy against field tick populations.

Additionally, the calculated synergistic ratios ranged from 93.60 to 5331.08, indicating a pronounced enhancement of deltamethrin toxicity by PBO across all field isolates. Shakya et al. (2022) reported comparatively lower synergistic ratios (1.3 to 18) for the deltamethrin+PBO combination in field populations of *R. microplus*, suggesting the differential susceptibility of tick populations to the treatment applied.

Conclusion

The highest non-lethal concentration of PBO was determined as 200 ppm using bioassay. Piperonyl butoxide enhanced deltamethrin efficacy against resistant *R. sanguineus* isolates, which was evident by the marked reduction in LC_{50} values observed in larvae treated with the deltamethrin + PBO combination compared to those with deltamethrin alone or PBO alone. The findings of the present study suggest that cytochrome P450-mediated metabolic detoxification pathways might contribute to deltamethrin resistance, a finding that warrants further confirmation through enzyme quantification assays. Incorporating optimised doses of PBO into deltamethrin-based formulations might provide a practical and sustainable approach to managing resistant populations of *R. sanguineus*.

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Conflict of interest

We report no conflicts of interest among the authors.

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