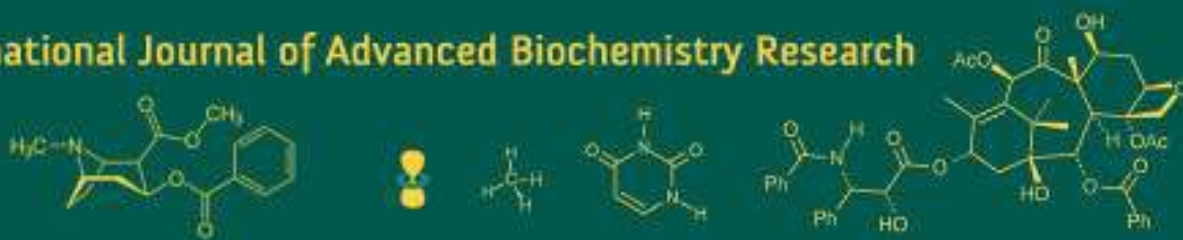


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Acaricide susceptibility status of *Hyalomma marginatum* ticks to organophosphate compounds in Tamil Nadu: Evidence from adult immersion and larval packet tests

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Abstract

Hyalomma ticks are vectors of economically important livestock diseases, including tropical theileriosis. Organophosphate compounds (OPCs) remain widely used for tick control in India, but emerging acaricide resistance threatens their efficacy. Systematic resistance monitoring in *Hyalomma* species remains limited compared to *Rhipicephalus microplus*. This study was conducted to evaluate the susceptibility status of *Hyalomma marginatum* ticks collected from the northeastern agroclimatic zone of Tamil Nadu to three organophosphate compounds, viz., Chlorpyrifos, Malathion, and Diazinon, using standard *in vitro* bioassays. Approximately 200 engorged female *H. marginatum* ticks were collected and subjected to the Adult Immersion Test (AIT) at five concentrations (100, 200, 300, 400, 500 ppm) of each OPC. The Larval Packet Test (LPT) was performed on unfed larvae using established discriminating concentrations. Mortality and tolerance were recorded at 24 h (LPT) and after 7 d (AIT). The results of the adult immersion test revealed 100 per cent of adult ticks were tolerant at 100 ppm for all three compounds. At 200 ppm, 50 per cent were susceptible and 50 per cent tolerant. Complete susceptibility (100 per cent mortality) was observed at 300, 400, and 500 ppm. In LPT, all three compounds induced 100 per cent larval mortality at their respective discriminating concentrations. The identical response profile across all three OPCs suggests potential cross-resistance. The study concluded that adult *H. marginatum* ticks in Tamil Nadu exhibited a dose-dependent response to organophosphates, with partial survival at 200 ppm, suggesting the emergence of acaricide resistance. Larvae remain fully susceptible at established lethal doses. These findings contribute to the limited baseline data on *Hyalomma* resistance in southern India and underscore the need for expanded surveillance and integrated tick management strategies.

Keywords: *Hyalomma marginatum*, organophosphate, acaricide resistance, adult immersion test, larval packet test, Tamil Nadu

1. Introduction

Ticks and tick-borne diseases (TTBDs) are a major problem for livestock production. They infect millions of cattle and cause large economic losses in cattle by deaths, reduced milk yield and treatment costs. Among ixodid ticks, *Hyalomma* species are of particular veterinary and public health significance, serving as vectors of *Theileria annulata* (tropical theileriosis), *Babesia caballi*, and the zoonotic Crimean-Congo haemorrhagic fever virus. In India, *Hyalomma marginatum* and *Hyalomma anatolicum* are widely distributed and frequently implicated in theileriosis outbreaks, resulting in substantial economic losses through mortality, reduced milk yield, and treatment costs.

Chemical acaricides, including organophosphates (OPs), synthetic pyrethroids, and amidines, have historically formed the cornerstone of tick control programmes. Organophosphate compounds such as Chlorpyrifos, Malathion, and Diazinon exert their acaricidal effect through irreversible inhibition of acetylcholinesterase (AChE), leading to neurotransmitter accumulation and subsequent paralysis and death of the arthropod. Despite their efficacy, the intensive and often indiscriminate use of OPs over several decades has selected for resistant tick populations worldwide (De Rouck *et al.*, 2023; Weng *et al.*, 2024; Zhang, 2024; Kumar *et al.*, 2025; Lempereur *et al.*, 2025) [1-5].

Organophosphate resistance in ticks is mediated through two principal mechanisms. Target site insensitivity involves mutations in the acetylcholinesterase genes (*AChE1*, *AChE2*, *AChE3*), reducing OP binding affinity. Coelho *et al.* (2025)^[6] investigated OP resistance in ten *R. microplus* field strains from São Paulo, Brazil, using an integrated approach combining larval packet tests, biochemical enzyme inhibition assays, and sequencing of *AChE1* and *AChE2*. Their study identified 33 amino acid substitutions, including nine novel variants, distributed across AChE isoforms. Biochemical tests using propoxur demonstrated significantly reduced AChE inhibition in resistant strains, confirming that target-site insensitivity is a primary mechanism in Brazilian populations.

Metabolic detoxification constitutes the second major mechanism, involving enhanced activity of esterases (particularly carboxylesterases), glutathione S-transferases, and monooxygenases. Jyoti *et al.* (2024)^[7] comprehensively reviewed the global status of AChE-mediated OP resistance in *R. microplus*, summarizing that both target site modification and metabolic detoxification contribute to resistance phenotypes. The relative contribution of each mechanism varies geographically and between tick species.

The FAO-recommended bioassays for resistance monitoring include the Larval Packet Test (LPT) and the Adult Immersion Test (AIT). The LPT, originally described by Stone and Haydock (1962)^[8], remains the reference method but is laborious, time-consuming, and requires significant laboratory resources. The AIT, modified from Drummond *et al.* (1973)^[9], offers advantages of simplicity and rapid completion (7 days), making it more accessible for field laboratories.

However, Jonsson *et al.* (2007)^[10] critically evaluated the modified AIT and raised substantial concerns regarding its reliability. Their study, conducted simultaneously in Texas and Queensland using susceptible *B. microplus* strains, demonstrated poor fit to the probit model, unacceptably wide confidence intervals for LC₅₀ and LC₉₉ estimates, and substantial inter-laboratory variation. For coumaphos, the recommended FAO discriminating dose was 0.50 per cent, but their estimates ranged from 0.66 per cent to 130 per cent. The authors concluded that the AIT does not provide a reliable means to discriminate between susceptible and resistant ticks.

Alternative bioassays have been developed to overcome these limitations. Betschart and Sager (2012)^[11] developed the Larval Tarsal Test (LTT), which distributes tick eggs in acaricide-treated microtiter plates and assesses larval mortality post-hatching. The LTT demonstrated satisfactory dose-response curves, sensitivity comparable to the LPT, and required substantially lower acaricide doses. Field validation in Brazil, Argentina, South Africa, and Australia confirmed its utility for resistance monitoring, leading to the detection of the first amitraz resistance cases in Argentina and widespread fipronil resistance in Brazil.

The Food and Agriculture Organization (FAO)^[12] has standardised two *in vitro* bioassays for resistance monitoring in ixodid ticks: the Adult Immersion Test (AIT) and the Larval Packet Test (LPT). While the LPT is considered the reference method, it is laborious and time-consuming, requiring 5-6 weeks to complete. The AIT offers advantages of simplicity, rapid results (7 days), and minimal equipment requirements, although concerns regarding its

reproducibility and discriminating dose validity have been raised.

Rodríguez-Hidalgo *et al.* (2025)^[13] provided a comprehensive framework for understanding acaricide resistance evolution, categorizing resistance into acquired, cross-, and multiresistance. They emphasized the importance of PCR-based assays for rapid resistance detection and advocated for rational acaricide use, vaccination, nutritional management, and genetic resistance in livestock breeds.

In India, Kadam *et al.* (2025)^[14] conducted a comprehensive survey of acaricide resistance in the Marathwada region of Maharashtra, examining both *R. microplus* and *H. anatolicum*. Their results demonstrated high resistance levels to deltamethrin in *R. microplus* (resistance factors ranging from level II to IV), while *H. anatolicum* exhibited only type I resistance in select districts. Notably, all isolates of both tick species remained susceptible to the organophosphate coumaphos at the baseline concentration, suggesting that OPs may still retain efficacy in this region. Biochemical assays revealed elevated α - and β -esterase activity in resistant *R. microplus* isolates, although no significant correlation was found between enzyme activities and resistance ratios, indicating the presence of alternative resistance mechanisms. However, systematic resistance monitoring in *Hyalomma* species remains critically limited.

The literature survey revealed a critical knowledge gap: while acaricide resistance in *R. microplus* has been extensively characterized in India, systematic data on *Hyalomma* species, particularly *H. marginatum* remain sparse. Furthermore, no published studies have examined OP susceptibility in *Hyalomma* populations from Tamil Nadu, a state with diverse agro-climatic zones and high livestock density. The present study addresses this gap by providing the baseline data on OP susceptibility in *H. marginatum* from Tamil Nadu, utilizing both adult and larval bioassays.

The northeastern zone of Tamil Nadu is characterised by semi-arid conditions and a high density of smallholder dairy farms, where organophosphates are frequently used for tick control. To date, no published data exist on the susceptibility of *H. marginatum* to OPs in this specific region. The present study was therefore undertaken with the following objectives: (i) to assess the *in vitro* susceptibility of adult *H. marginatum* ticks collected from Northeastern zone of Tamil Nadu to three commonly used organophosphate compounds (Chlorpyrifos, Malathion, Diazinon) using the Adult Immersion Test; (ii) to evaluate larval susceptibility through the Larval Packet Test at established discriminating doses; and (iii) to provide baseline data for evidence-based resistance management strategies in the region.

2. Materials and Methods

2.1 Study Area and Tick Collection

Engorged adult female *Hyalomma marginatum* ticks were collected from cattle maintained under smallholder management systems in the northeastern zone of Tamil Nadu. Approximately 200 visibly healthy, fully engorged females were handpicked, placed in ventilated plastic vials with moistened filter paper, and transported to the laboratory within 4 h of collection. Ticks were identified to species level using standard morphological keys under a stereo zoom microscope.

2.2 Acaricide Formulations and Dilutions

Three commercial organophosphate formulations *viz.*, Chlorpyrifos 50% EC, Malathion 50% EC, and Diazinon 50% EC, were procured from a local veterinary supplier. Stock solutions were prepared in distilled water, and serial dilutions were made to obtain final test concentrations of 100, 200, 300, 400, and 500 ppm (active ingredient) for the Adult Immersion Test.

For the Larval Packet Test, discriminating concentrations of 227 ppm for Chlorpyrifos, 248 ppm for Malathion, and 283 ppm for Diazinon were employed. These concentrations represent the established LD₅₀ values for susceptible tick strains as recommended by FAO guidelines.

2.3 Adult Immersion Test (AIT)

The AIT was performed following the modified Drummond method described in FAO (2004)^[12]. For each concentration of each compound, twenty engorged female ticks were tested. Five millilitres of the acaricide solution were dispensed into a 5 mL disposable tea cup. The ticks were immersed for exactly 1 minute, then retrieved, dried on filter paper, and placed individually into labelled glass vials. Twenty ticks immersed in distilled water served as negative controls.

All vials were kept in a tray lined with moistened cotton and incubated at 28 °C with 85% relative humidity. Parameters

recorded over 7 days included mortality (defined as the absence of movement and leg retraction upon stimulation) and oviposition. After 7 days, ticks that had died or ceased oviposition were discarded; surviving, ovipositing ticks were retained until completion of egg laying and hatching, although these latter data were not systematically quantified for the present analysis due to resource constraints.

2.4 Larval Packet Test (LPT)

The LPT was conducted according to FAO (2004)^[12] guidelines. Whatman No. 1 filter paper sheets were cut into 7 × 12 cm rectangles and folded to form packets sealed on three sides with adhesive tape. Each packet was impregnated with 0.7 mL of the respective acaricide dilution at the discriminating concentration. Control packets received 0.7 mL of distilled water. Packets were dried horizontally for 2 h in a laminar flow hood.

Approximately 50 unfed larvae (14-21 days old, progeny of the field-collected adults) were introduced into each packet using a fine camel hair brush, and the open side was sealed. Packets were incubated at 25 °C with 85% relative humidity in a BOD incubator. After 24 h, the packets were opened, and the numbers of live and dead larvae were counted under a stereomicroscope. Larvae exhibiting any movement were considered alive; those with no movement upon gentle prodding were considered dead.

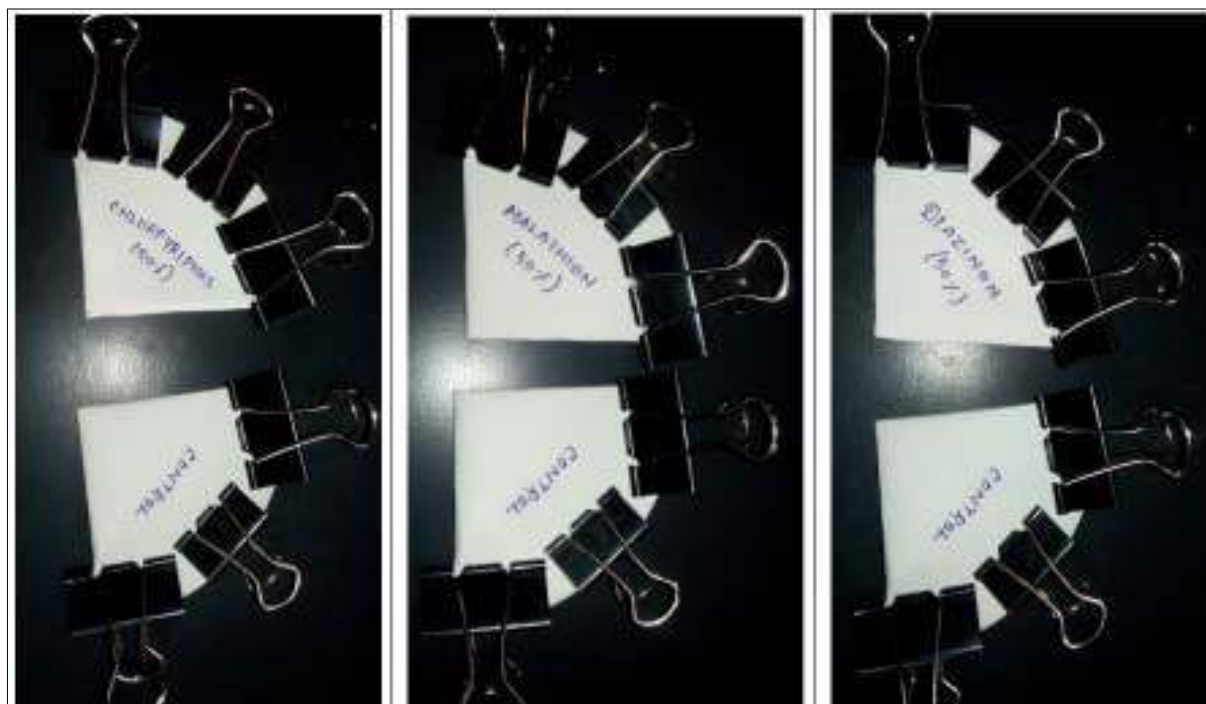


Fig 1: Larval Packet Test (LPT) with three different compounds

2.5 Data Analysis

The data obtained were subjected to descriptive statistics (percentages). For the AIT, the proportion of susceptible (dead) and tolerant (alive) ticks at each concentration was determined. For the LPT, mortality percentage was calculated as (number dead/total larvae exposed)×100.

3. Results

3.1 Adult Immersion Test

The mortality responses of engorged adult *H. marginatum* ticks to increasing concentrations of Chlorpyrifos, Malathion, and Diazinon are summarised

in Table 1. The response pattern was identical for all three organophosphate compounds.

All ticks in the control group (distilled water immersion) survived and commenced oviposition within the 7-day observation period. At 100 ppm, all treated ticks survived and appeared behaviourally normal, indicating complete tolerance. At 200 ppm, ten of the twenty ticks died (or became moribund) for each compound, yielding 50% susceptibility. Complete mortality was achieved at 300 ppm and all higher concentrations. No concentration-dependent variation in mortality was observed among the three compounds; all elicited identical responses at each dose.

Table 1: Mortality of *Hyalomma marginatum* adult ticks exposed to organophosphate compounds in the Adult Immersion Test.

Concentration (ppm)	Ticks tested (n)	Ticks susceptible (dead)	Ticks tolerant (alive)	Susceptibility (%)
100	20	0	20	0
200	20	10	10	50
300	20	20	0	100
400	20	20	0	100
500	20	20	0	100

Note: Identical results were obtained for Chlorpyrifos, Malathion, and Diazinon

3.2 Larval Packet Test

The results of the Larval Packet Test are presented in Table 2. Exposure of unfed larvae to the discriminating concentrations of Chlorpyrifos (227 ppm), Malathion

(248 ppm), and Diazinon (283 ppm) for 24 h resulted in 100% mortality for all three compounds. Control packets (distilled water) exhibited negligible mortality (<2%), confirming assay validity.

Table 2: Larval mortality in response to organophosphate compounds at discriminating concentrations.

Compound	Concentration (ppm)	Larvae exposed (n)	Mortality (n)	Mortality (%)
Chlorpyrifos	227	~50	50	100
Malathion	248	~50	50	100
Diazinon	283	~50	50	100
Control (water)	0	~50	0-1	0-2

4. Discussion

Acaricide resistance is defined as the heritable ability of a tick population to survive a dose of acaricide that would be lethal to the majority of individuals in a susceptible population of the same species. The global scenario of OP resistance development in cattle ticks has been extensively reviewed, with resistance documented across Latin America, Africa, Asia, and Australia (Dzemo *et al.*, 2022; Kumar *et al.*, 2025)^[15,4].

4.1 Evidence of Emerging Resistance in Adult Ticks

The present study provides the first empirical data on acaricide susceptibility in *Hyalomma marginatum* from the northeastern region of Tamil Nadu. The Adult Immersion Test revealed a clear dose-dependent mortality response: complete tolerance at 100 ppm, 50% mortality at 200 ppm, and complete mortality at concentrations ≥ 300 ppm. This pattern was identical across the three tested organophosphates, suggesting a shared resistance mechanism or cross-resistance.

The survival of 50% of adult ticks at 200 ppm is particularly noteworthy. Although 200 ppm is not a formally established discriminating dose for *Hyalomma* in India, it is substantially lower than the LD₅₀ values reported for susceptible reference strains of other ixodid ticks. The observation that half of the tested adults survived this concentration while the remainder succumbed implies heterogeneous susceptibility within the local tick population, a hallmark of ongoing selection for resistance.

Findings of this study are broadly consistent with those of Kadam *et al.* (2025)^[4], who reported that *H. anatolicum* populations in Maharashtra remained susceptible to the organophosphate coumaphos. However, direct comparison is complicated by differences in compound (coumaphos vs. chlorpyrifos/malathion/diazinon), tick species (*H. anatolicum* vs. *H. marginatum*), and geographical location. The partial survival at 200 ppm observed in this study may represent an earlier stage of resistance selection compared to the fully susceptible Marathwada populations.

Complete mortality at 300 ppm and above indicates that resistance, if present, is not yet fixed in the population and can be overcome by higher doses. This finding has practical

implications: increasing application concentrations may temporarily restore efficacy, but this strategy accelerates resistance selection and is economically and environmentally unsustainable.

4.2 Larval Susceptibility Contrasts with Adult Tolerance

In marked contrast to the adult bioassay, the Larval Packet Test demonstrated 100% mortality at the established discriminating concentrations for all three compounds. This finding suggests that the larval stage of *H. marginatum* in this population remains fully susceptible to organophosphates.

Discrepancies in acaricide susceptibility between life stages have been reported previously. They may arise from several factors, viz., differential expression of detoxification enzymes during ontogeny, greater cuticular penetration in larvae due to a higher surface area-to-volume ratio, absence of prior selection pressure in larvae if acaricide applications are timed to target adults, and inherent physiological differences in acetylcholinesterase sensitivity between life stages.

Coelho *et al.* (2025)^[6] demonstrated that OP resistance in Brazilian *R. microplus* is primarily linked to target-site insensitivity mediated by AChE mutations. If similar mechanisms operate in *H. marginatum*, the differential susceptibility between adults and larvae could reflect stage-specific expression of AChE isoforms or differential penetration of acaricides. Alternatively, if resistance is primarily metabolic (e.g., elevated esterase activity), larvae may not have developed the full complement of detoxification enzymes present in adults.

From a practical perspective, the preserved susceptibility of larvae is encouraging, and it implies that appropriately timed applications of organophosphates at recommended field concentrations should still effectively suppress tick recruitment. However, continued reliance on a single chemical class while adult resistance is emerging will inevitably select for resistant larvae over successive generations.

4.3 Implications for Acaricide Resistance Management

The co-occurrence of partial adult tolerance and complete larval susceptibility carries important implications for

resistance management in the northeastern agroclimatic zone of Tamil Nadu. First, the observation underscores the value of using both adult and larval bioassays in resistance surveillance; reliance on a single life stage could lead to underestimation or overestimation of resistance.

Second, the identical response profile across three different OPs suggests that rotation between Chlorpyrifos, Malathion, and Diazinon is unlikely to mitigate resistance, as all belong to the same chemical class and share a common mode of action. Rotation should involve compounds from different acaricide classes (e.g., synthetic pyrethroids, amidines, macrocyclic lactones, isoxazolines) and be integrated with non-chemical strategies.

Third, these findings support the urgent need for establishing species-specific discriminating doses for *Hyalomma* species in India. Currently, resistance monitoring in *Hyalomma* relies on doses validated for *R. microplus*, which may not be appropriate given interspecific differences in acaricide pharmacokinetics and pharmacodynamics. The recent development of the Larval Tarsal Test offers a promising, high-throughput alternative that could facilitate large-scale resistance surveillance across multiple tick species.

De la Fournière *et al.* (2023) [16] demonstrated transovarial transmission of *Anaplasma marginale* in *R. microplus*, with significant implications for acaricide resistance management. Their finding that infected larvae are competent vectors underscores the need for integrated strategies beyond adult tick control.

The increasing prevalence of acaricide resistance has catalyzed recognition that sole reliance on chemical control is unsustainable. Integrated Tick Management (ITM) combines chemical acaricides with complementary strategies, including biological control, vaccination, pasture spelling, breeding of resistant cattle, and botanical acaricides.

Krishnamurthy *et al.* (2025) [17] emphasized that ITM is the only suitable option for TTBD control in India, given the predominance of smallholder farming systems. They highlighted the importance of eco-friendly, cost-effective herbal acaricides as sustainable alternatives. Makwarela *et al.* (2025) [18] critically examined current control strategies, noting that while chemical acaricides remain effective, they are increasingly challenged by resistance development and environmental concerns. Recent reports have identified emerging innovations, including nanotechnology-enhanced acaricides and next-generation vaccines, as promising avenues for improved tick control (Barrero *et al.*, 2017; Nganso *et al.*, 2022; Barbieri *et al.*, 2023; Lew-Tabor and Rodrigues, 2023; Lempereur *et al.*, 2025) [19-22, 5].

4.4 Comparison with Existing Knowledge and Mechanistic Hypotheses

The present study contributes to the sparse literature on *Hyalomma* acaricide resistance in India. Kadam *et al.* (2025) [14] reported that all *H. anatolicum* isolates from Maharashtra remained susceptible to coumaphos, with resistance factors <1.6. Our observation of partial survival at 200 ppm in *H. marginatum* from Tamil Nadu may reflect species-specific differences, regional variation in acaricide selection pressure, or simply the earlier stage of resistance evolution in Maharashtra populations. Multi-centric studies employing standardised protocols are urgently needed to

establish the national resistance landscape for *Hyalomma* species.

The observed patterns of this study show identical tolerance across three OPs favours a mechanism involving enhanced metabolic detoxification (e.g., elevated esterase or glutathione S-transferase activity) rather than a target-site mutation specific to a single compound. Metabolic resistance often confers cross-resistance to multiple OPs, whereas target-site insensitivity may be more compound-specific. Kadam *et al.* (2025) [14] reported elevated α - and β -esterase activity in resistant *R. microplus* isolates, although no significant correlation was found with resistance ratios, suggesting that multiple mechanisms may operate simultaneously. Further biochemical or molecular assays (e.g., esterase activity assays, sequencing of *ace* genes) would be required to confirm the underlying mechanism in our *H. marginatum* population.

5. Conclusion

This study provides preliminary evidence that adult *Hyalomma marginatum* ticks in the northeastern agroclimatic zone of Tamil Nadu, exhibit a dose-dependent response to three organophosphate acaricides, with 50% survival at 200 ppm, a concentration that would be expected to cause complete mortality in fully susceptible populations. This pattern is consistent with emerging acaricide resistance. In contrast, larvae remain fully susceptible to the discriminating concentrations of Chlorpyrifos, Malathion, and Diazinon. The identical response profile across all three OPCs suggests potential cross-resistance within the organophosphate class.

The findings underscore the critical need for systematic, large-scale acaricide resistance surveillance in *Hyalomma* populations across Tamil Nadu and India more broadly. Such surveillance should employ standardised FAO protocols with adequate replication, concurrent susceptible reference strains, and formal dose-response analysis to enable calculation of resistance ratios and lethal concentrations. Adoption of high-throughput bioassays such as the Larval Tarsal Test could substantially accelerate this effort.

A precautionary approach to tick control is warranted until comprehensive resistance data are available. Integrated Tick Management strategies that minimise chemical selection pressure, rotate acaricide classes with different modes of action, and incorporate non-chemical interventions, including pasture spelling, breed resistance, botanical acaricides, and biological control, should be promoted among livestock keepers in the region.

Future research should aim to: (i) confirm the observed resistance phenotype using larger sample sizes and full dose-response assays; (ii) establish zone-specific discriminating doses for *Hyalomma marginatum* through multi-centric studies; (iii) elucidate the underlying resistance mechanism(s) through integrated biochemical (esterase, GST, monooxygenase assays) and molecular (AChE gene sequencing, transcriptomic analysis) approaches; (iv) investigate the relationship between on-farm acaricide use history and resistance status; and (v) evaluate the field efficacy of alternative acaricide classes and non-chemical control methods against OP-tolerant populations.

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