Ticks are obligate blood sucking ectoparasites recognised globally as vectors of various pathogens, next to mosquitoes. The present study is conducted on brown dog ticks collected from naturally infested dogs in and around Thrissur district. Adult immersion test was performed and the parameters such as engorged tick weight, egg mass weight as well as tick mortality were recorded to derive the reproductive indices, percentage inhibition of oviposition and percentage mortalities. Non-linear regression analysis of the dose response data yielded the LC50 values at 95% confidence intervals and, the slopes and regression coefficients of the different parameters were also derived. The Resistance factors (RF) were calculated to categorise resistance levels into I, II, III and IV. Resistance to amitraz was detected at level II in tick isolates collected from Choolissery and Mundur, at level III and level IV in isolates collected from Mannuthy and Paravattani, respectively. Two isolates collected from Mannuthy were found to be susceptible. One way ANOVA of resistance levels revealed that the resistance levels III and IV were significantly different from that of susceptible and resistance level II. This is the first report on detection of amitraz resistance levels in dog ticks from south India calling for proper implementation of tick control strategies to prevent spread of amitraz resistance.

Keywords: Adult immersion test, brown dog ticks, resistance levels, Kerala
Ticks rely completely on the host blood for development of each life cycle stage which is the reason for their notoriety as carriers of several pathogens (Ghosh and Nagar, 2014). *Rhipicephalus sanguineus*, the brown dog tick or the kennel tick is a three-host tick and is distributed across the world. The ticks are responsible for spreading a number of pathogens causing canine disease conditions such as babesiosis, ehrlichiosis, hepatoplasmosis and Q fever. Indiscriminate and frequent use of acaricides to control tick infestation has led to the development of acaricide resistance against majority of the drugs. A survey by FAO (2004) had revealed that tick population in India have become resistant to every acaricide available in the market. Inadequate managemental strategies, favourable climate and poor infrastructure facilities that offer favourable conditions for tick proliferation allowing ticks to survive throughout the year, have contributed to the development of acaricide resistance (Kumar et al., 2020). Deltamethrin resistance to brown dog ticks have been established in Kerala (Anand et al., 2021). Amitraz is a commonly used acaricide to control tick and mite infestations with its effects on mortality and reproduction. A meta-analysis of literature by Dzemo et al. (2022) had revealed that 254 out of 522 *Rhipicephalus (Boophilus) microplus* isolates collected and tested from ten different countries were resistance to amitraz. Only limited studies were conducted on evaluation of amitraz resistance in the dog ticks. Hence this study was carried out to explore the status of amitraz resistance in brown dog ticks.

**Materials and methods**

**Collection of ticks**

Engorged female ticks were collected from naturally infested dogs that were presented to the veterinary clinics, pet grooming centres, birth control centres, and from the dogs in kennels and animal shelters in and around Thrissur district. The ticks were collected in plastic vials covered with breathable cloth fabric to allow for ample air supply. Ticks collected from a group of animals were pooled and designated as an isolate, that were washed thoroughly and used for adult immersion test (AIT) within 24 hrs of collection as per Singh et al. (2014).

**Acaricide**

A total of 100 mg technical grade amitraz (Sigma-Aldrich, India) was diluted in 50 mL of 100% methanol for preparing the stock solution of 2000 ppm. Various dilutions of amitraz (10, 25 50, 100, 250, 500, 750, 1000 ppm) were prepared by diluting the stock solution in methanol for the experimental bioassay for each isolate.

**Adult Immersion Test (AIT)**

The engorged ticks were weighed and washed with distilled water to remove debris. Ticks in treatment groups were exposed to amitraz by immersing them into different working solutions for 2 min and the ticks in control group were immersed only in the diluent. Constant stirring of the ticks in the solution was carried out to ensure adequate exposure to the acaricide. After this, the ticks were dried with tissue papers and stored in glass vials covered with cloth fabric which were kept in the desiccator for 14 days to ensure favourable conditions for egg laying. Each concentration was replicated twice with 6-10 ticks per replication. Ticks that did not oviposit even after 14 days were considered dead. The parameters, viz. mortality, engorged tick weight and egg mass weight were recorded to calculate the resistance factors (RF), reproductive index (RI) and percentage inhibition of oviposition (%IO) using the formulae (Singh et al., 2014)

\[
\text{Reproductive Index (RI)} = \frac{\text{egg mass weight}}{\text{engorged tick weight}}
\]

\[
\text{Percentage inhibition of oviposition (%IO)} = \left(\frac{\text{RI control – RI treated}}{\text{RI control}}\right) \times 100
\]

**Statistical analysis**

The data analysis was carried out in the GraphPad Prism 5 software. Non-linear regression analysis of the log dose concentrations and mortality percentages was applied to calculate the \(\text{LC}_{50}\) at 95% confidence intervals (CI). The slopes and regression coefficients of mortality, RI and IO (%) were also derived. One way ANOVA of the obtained data on resistance levels and \(\text{LC}_{50}\) was carried out.
out to ascertain significance levels. The RFs were calculated as the quotient between LC$_{50}$ of field tick isolate and LC$_{50}$ of field susceptible strain of *R. sanguineus* s. l. obtained in the study. Depending on the RF, the resistance levels were characterised at level I (RF= 1.5-5), level II (RF= 5.1-25), level III (25.1-40), level IV (RF > 40) and susceptible (RF<1.4) (Sharma et al., 2012).

**Results and discussion**

A total of eight isolates comprising of approximately 800 ticks were screened for amitraz resistance. Methanol was used as the diluent as amitraz was insoluble in water and organic solvent such as methanol facilitates the penetration and adsorption of acaricide across the exoskeleton of the tick (Sharma et al., 2012). The LC$_{50}$ (95% CI), R$^2$ (goodness of fit) values and slopes of mortality, along with the resistance factors and resistance levels were calculated for field isolates of *R. sanguineus* s. l. (Table 1). Non-linear regression graphs of mean mortality of ticks were plotted against the log concentrations of amitraz and the regression graphs of highly resistant ticks are provided in Fig.1 and Fig.2 (RL: III and IV). In the eight isolates screened, resistance to amitraz was detected in six isolates at levels II, III and IV whereas two isolates were susceptible.

In an isolate collected from Mannuthy from a dog reportedly unexposed to amitraz, 100 per cent mortality was observed at the lowest concentration of 10 ppm and hence regression analysis could not be performed. In another tick isolate collected from Mannuthy which was collected from a dog reportedly unexposed to amitraz, the LC$_{50}$ calculated was 2.353 ppm which is the lowest LC$_{50}$ recorded in the study, hence this isolate was used as the susceptible isolate to calculate the RFs. The LC$_{50}$ value obtained in the study was nearer to the LC$_{50}$ value of susceptible *R. (B). microplus* isolate derived by Dutta et al. (2017). The LC$_{50}$ values of the remaining six isolates were in the range of 11.67-274.8 ppm which were in agreement with the LC$_{50}$ ranges (27.5-77.4 ppm) of Dutta et al. (2017).

**Table 1. Categorisation of resistance levels based on AIT**

<table>
<thead>
<tr>
<th>Locality of collection</th>
<th>LC$_{50}$ (in ppm)</th>
<th>LC$_{50}$ at 95% C. I.</th>
<th>R$^2$</th>
<th>Slope</th>
<th>Resistance factor (R.F)</th>
<th>Resistance level (R.L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choolissery</td>
<td>33.94</td>
<td>22.79 to 50.56</td>
<td>0.9274</td>
<td>35.7103</td>
<td>14.42</td>
<td>Level II</td>
</tr>
<tr>
<td>Choolissery</td>
<td>19.76</td>
<td>12.27 to 31.80</td>
<td>0.883</td>
<td>34.66898</td>
<td>8.39</td>
<td>Level II</td>
</tr>
<tr>
<td>Mundur</td>
<td>20.44</td>
<td>14.11 to 29.62</td>
<td>0.9736</td>
<td>35.69864</td>
<td>8.68</td>
<td>Level II</td>
</tr>
<tr>
<td>Mundur</td>
<td>11.67</td>
<td>4.699 to 29.01</td>
<td>0.8512</td>
<td>34.56759</td>
<td>5.38</td>
<td>Level II</td>
</tr>
<tr>
<td>Paravattani</td>
<td>274.8</td>
<td>156.8 to 481.5</td>
<td>0.9087</td>
<td>33.54259</td>
<td>116.7</td>
<td>Level IV</td>
</tr>
<tr>
<td>Mannuthy</td>
<td>68.29</td>
<td>30.21 to 154.3</td>
<td>0.8683</td>
<td>42.78728</td>
<td>29.02</td>
<td>Level III</td>
</tr>
<tr>
<td>Mannuthy</td>
<td>Not calculated since cent per cent mortality was observed at the lowest concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig.1. Regression plot of Paravattani isolate

Fig.2. Regression plot of Mannuthy isolate

---


Adult immersion test for assessment of amitraz resistance in *Rhipicephalus sanguineus* ticks
The highest LC\textsubscript{50} observed was 274.8 ppm in an isolate collected from Paravattani that showed a resistance level IV. The LC\textsubscript{50} value for Mannuthy isolate was 68.29 ppm which was categorised at resistance level III. Isolates collected from Choolissery and Mundur had LC\textsubscript{50} values at a range of 11.67-33.94 that were categorised as level II resistant. Similar studies employing AIT in cattle ticks were conducted by Dutta \textit{et al.} (2017) in Jammu and Kashmir reporting level I resistance, Singh \textit{et al.} (2013) in Gujarat who reported level II resistance, Singh \textit{et al.} (2014) in Punjab who reported level II resistance and Kumar \textit{et al.} (2014) who reported resistance levels I, II and III from northern and eastern states of India.

Adult Immersion Test with Discriminating Dose (DD) was not conducted, as Jonsson \textit{et al.} (2007) showed that DD concept is not effective for amitraz, by comparing both the classical AIT and AIT-DD in two series of experiments. One way ANOVA (Tukey’s multiple comparison test) with the resistance levels and the LC\textsubscript{50} values of the ticks from all the isolates revealed that no significant difference exists between susceptible vs resistance level II. While the difference between susceptible and resistance levels III and IV were highly significant with a p-value of <0.05 (Table 2).

Effects of amitraz on egg laying were studied by comparing the egg masses of different isolates exposed to different concentrations of amitraz to that of the controls, which was achieved by calculating the slopes of RI and IO (%). The slopes obtained for RI were in the range of -0.228 to -0.1197 which were nearer to the slopes of RI obtained by Dutta \textit{et al.} (2017) and Kumar \textit{et al.} (2014). The negative slopes of RI in all the isolates indicated that reproduction (egg laying) was adversely affected by amitraz. Positive slopes were obtained with the IO (%) ranging between 23.22 to 37.58 which also suggested that as concentration of amitraz increases the egg laying capacity was inhibited implying that the efficiency of converting live weight into egg mass was affected among the surviving ticks which was in agreement with the range of slopes of IO (%) arrived by Dutta \textit{et al.} (2017).

Studies employing the AIT to detect amitraz resistance were scarce, but a study employing AIT-DD in Karnataka by Rani \textit{et al.} (2018) for evaluating the efficacy of three different acaricides in different species of ticks had shown that ticks were susceptible to amitraz. A study by Rodriguez-Vivas \textit{et al.} (2016) in Mexico employing the larval immersion test to detect amitraz resistance revealed that low levels of resistance (RR= 1.68-4.58) were prevalent in dog ticks. Another study employing larval packet test (LPT) by Bandara \textit{et al.} (2016) from Sri Lanka had also shown that low levels (RR= 1.52) of amitraz resistance is prevalent. There are no published records on amitraz resistance in brown dog ticks in India.

**Conclusion**

The present study detected the presence of amitraz resistance at levels II, III and IV in tick isolates collected from Thrissur district, Kerala. There are no available reports on amitraz resistance levels detection in brown dog ticks of India. The ticks attaining resistance levels III and IV is alarming, and if left undetected will lead to massive failure of the drug. The spread of amitraz resistance should be viewed seriously in the light of increasing ticks and tick-borne diseases and the indiscriminate use of this acaricide should be checked in the light of present study’s findings of high levels of amitraz resistance. Alternative tick control strategies such as the employment of semiochemicals,

<table>
<thead>
<tr>
<th>Tukey’s Multiple Comparison Test</th>
<th>Mean Difference</th>
<th>Q value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible vs Level II</td>
<td>-19.13</td>
<td>4.785</td>
<td>NS</td>
</tr>
<tr>
<td>Susceptible vs Level III</td>
<td>-65.97</td>
<td>14.29</td>
<td>S</td>
</tr>
<tr>
<td>Susceptible vs Level IV</td>
<td>-272.5</td>
<td>59.03</td>
<td>S</td>
</tr>
<tr>
<td>Level II vs Level III</td>
<td>-46.84</td>
<td>11.72</td>
<td>S</td>
</tr>
<tr>
<td>Level II vs Level IV</td>
<td>-253.3</td>
<td>63.37</td>
<td>S</td>
</tr>
<tr>
<td>Level III vs Level IV</td>
<td>-206.5</td>
<td>44.74</td>
<td>S</td>
</tr>
</tbody>
</table>

NS – Non-significant  S- Significant
phytoacaricides and vaccine development which are still in their infancy should be robustly studied. Further investigations on acaricidal resistance should be carried out involving different species of ticks to understand the true nature of acaricidal resistance in the state.

References


