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Mechanisms of acaricide resistance in the cattle tick *Rhipicephalus (Boophilus) microplus* in Sri Lanka

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ABSTRACT

High tolerance of ticks to acaricides is increasingly becoming a problem to cattle farmers. Resistance status of *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae)from two cattle farms of Sri Lanka were determined against different concentrations of pyrethroid permethrin, organophosphate malathion, organochlorine DDT and carbamate propoxur using Larval Packet Test (LPT) as recommended by Food and Agriculture Organization (FAO). Mechanisms of acaricide resistance were studied by conducting biochemical and PCR assays. Tick larvae were tested for the activity levels of acaricide metabolizing enzymes *i.e.*esterases, glutathione S-transferases (GSTs) and monooxygenases, and for altered target sites *i.e.*acetylcholinesterase (target site of organophosphates and carbamates) and sodium channel regulatory proteins (target site of pyrethroids and DDT). According to discriminating dosages specified by FAO for ticks both populations were 24–56% resistant to DDT. LC values showed that the both populations were susceptible to permethrin and resistant to malathion. Moderate insensitivity of AChEs and knock-down resistance (kdr) mutations were found as resistance mechanisms. GSTs and monooxygenases were not elevated. The kdr type mutation G72 V (G215 T in the gene) found in the sodium channel regulatory protein of *R. (B.) microplus* samples may be responsible for DDT resistance. Systematic and sophisticated insecticide resistance monitoring programmes and a better understanding on the mechanisms which govern resistance development are vital for future tick control programmes.

1. Introduction

Rhipicephalus (B.) microplus is one of the most abundant and widely distributed cattle tick species. It is an important external parasite on cattle and transmits tick-borne diseases like babesiosis [1]. Heavy infestations of *R. (B.) microplus* cause economic losses through affecting milk, meat and leather production of cattle. Acaricide resistance of ticks has become a major problem throughout the world and theresistance has been detected in *R. (B.) microplus* against almost all the chemicals that are registered for its control [2]. Indiscriminate use of acaricides by livestock farmers [3], the nature of the lifecycle of the tick and worldwide distribution of tick infestations have been shown to be the major contributing factors for the rapid development of acaricide resistance in ticks [4].Monitoring resistance and its underlying mechanisms to the recommended acaricides is urgent for an effective control of tick populations.

In general, the chemicals used as synthetic acaricides are pesticides which belong to organochlorines, organophosphates, carbamates and pyrethroids. Organophosphates (OPs) and pyrethroids are the most frequently used insecticide groups in tick control programmes although organochlorines and carbamates have also been used occasionally in the past [5]. Acetylcholinesterases, which breakdown the neurotransmitter acetylcholine, are the target site for both organophosphates and carbamates. Voltage gated Na^+ channel regulatory proteins of the nerve membrane are the target site for pyrethroids and DDT like organochlorines. In both insects and acarines, increased metabolism of insecticides (metabolic resistance) through elevated enzyme activity and/or decreased target site sensitivity are the two major mechanisms of insecticide resistance [6,7]. Metabolic resistance involves quantitative (through gene amplification, increased transcription and increased mRNA stability) and/or qualitative (through gene mutation) changes of insecticide metabolizing enzymes *i.e.*esterases, glutathione-S-transferases (GSTs) and monooxygenases. Increased activity of esterases is one of the most common type of metabolic resistance mechanism found among ticks, mites and insects as it can provide resistance to organophosphates which are esters of phosphoric acid. It is also effective on carbamates due to the existence of ester bonds in their chemical structures [8].

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Specific target site mutations, which do not affect the primary functions of the target but prevent its interaction with the acaricides, cause target site insensitivity. Reduction of the sensitivity of voltage-gated sodium channels to insecticides brings about the resistance phenotype 'kdr' (knock-down resistance) or `super kdr' (highly resistant type). Since this target site is shared by both DDT and pyrethroids, cross-resistance to pyrethroids had been shown by an DDT-resistant strain of *R. (B.) microplus* in Australia even before the commercial release of pyrethroids [9].We report here the status and the mechanisms of acaricide resistance of *R. (B.) microplus*, the most abundant and widely distributed cattle tick species in Sri Lanka.

2. Materials and methods

2.1. Collection and rearing of ticks

Tick samples were collected from two farms *i.e.* Koulwewa (7° 26′ N, 80° 11′ E) and Diyathure (7° 39′ N, 80° 31′ E), in Kurunegala district situated in the low country intermediate agro climatic zone of Sri Lanka from May 2014 to September 2015. The two farms were about 56 km away from each other.

Engorged and/or partially engorged female ticks (75–100) were collected from at least 15–20 individuals of cattle from each farm. The farm visits were done in early mornings *i.e.* 5.30 am – 7.00 am, so that engorged female ticks could be collected from the ground where the infested animals were tethered. Otherwise the ticks were removed carefully using a flat-headed forcep or by hand, avoiding any damage to the ticks. They were collected to small plastic bottles (5 individuals in each bottle) that were punctured with a fine needle to allow air circulation. A piece of cotton wool, moistened with water, was placed inside the container where the bottles were packed, to provide humidity. Ticks were transported to the laboratory within 24 h after collection and were transferred to the incubators where temperature and humidity were maintained at 27–28 °C and 85–95% RH respectively. Identifications were confirmed by examining under a stereo-microscope with a key introduced by Seneviratne [10]. Once the eggs were laid, they were allowed to hatch (3–4 weeks) and 14–21 day old tick larvae were used for the bioassay experiments.

2.2. Chemicals and equipment

Chemicals were purchased from Sigma chemicals U.K. unless otherwise stated. Technical grade acaricides (97–99% pure) were a gift from Liverpool School of Tropical Medicine, United Kingdom. UVmaxELx 800TM absorbance microtitre plate reader was from Molecular devices, Bio—Tek. USA. Protein assay kit was from BIO-RAD, U.K.

2.3. Acaricide bioassays

Acaricide bioassays were carried out using the Laval Packet Test method (LPT) as recommended by Food and Agricultural Organization [11]. Acaricide solutions with different concentrations were prepared using olive oil:acetone (1:2) as the solvent. Concentrations were determined by using published dose-response data for ticks and by conducting preliminary studies. An aliquot of 0.7 ml from each dilution was evenly distributed on a Whatman No.1 filter paper (7.5 cm × 9 cm) [12] and the control papers were prepared using the solvent alone. Acaricide impregnated papers were wrapped individually with aluminium foil and stored at 4 °C until use. They were used within one month after preparation and each paper was used up to a maximum of two trials.

An acaricide impregnated paper was folded along the middle line with the impregnated surface inside and two bulldog clips were fixed on two opening sides making a packet [11].Tick larvae (100) were inserted into the packet with the aid of a fine brush through the opening end and a third bulldog clip was used to seal the opening end. The packets were kept in separate humidity chambers (27–28 °C, 85–95 RH). Mortalities were recorded after a 24 h period. Larvae that showed no response to a needle touch under a hand lens were considered as dead. Data were considered only if the control mortalities were lesser than 20%, and the actual mortalities of valid experiments were adjusted with the control mortalities using Abott's formula [11].

2.4. Biochemical assays

All the biochemical experiments were carried out according to the procedures outlined by WHO [13] with slight modifications. Randomly selected 225 individuals of *R. (B.) microplus* larvae from each population were subjected to total protein, esterase, GST, monooxygenase and acetylcholinesterase assays in three replicates. Larvae (75) in each replicate were homogenized in 450 μl ice-cold distilled water. An aliquot of 100 μl was taken for AChE assay and the rest was centrifuged at 13,000 rpm for 2 min. The supernatant was used for esterase, GST, monooxygenase and protein assays [13]. For each population, the procedure was repeated with three separate homogenates and the average values were taken.

2.5. Detection of kdr mutations

DNA was extracted from six individuals of *R. (B.) microplus* larvaeusing DNeasy® Blood and Tissue Kit (QIAGEN) according to manufacturer's instructions. Each larva was homogenized in 50 μl distilled water and incubated at 56 °C for 6 h before applying to the column. PCR primers used were BmNaF5 5'TACGTGTGTTCAAGCTAGC3' and BmNaR5 5′ACTTTCTTCGTAGTTCTTGC 3′. These had been designed to amplify the exon region between domain II S4 loop and domain II S5 of the gene code for voltage gated sodium channel regulatory protein to detect resistance-conferring mutationL925I [14]. PCR reaction contained 2 μl of each primer, 14 μl of 2× GoTaq® Green Master Mix, 5 μl of DNA template and 4 μl of Nuclease-Free Water (Promega). The mixture was amplified in a thermocycler for 30 cycles (95C for 1 min followed by 30 cycles of 95 °C for 30s, 50 °C for 30s and 72 °C for 90s, then 72 °C for 7 min to complete extension). Products were viewed on an ethidium bromide stained 1% agarose gel. DNA sequencing was carried out using an automatic DNA sequencer (Applied Biosystems series 3500, USA) and the sequence was read for possible mutations.

2.6. Statistical analysis

Probit analysis of the concentration – response data was carried out using Sigma Plot (version 10). Mortality data of the ticks were probit transformed and plotted against the log values of the acaricide concentrations. LC_{50} values for each acaricide, for each population were determined by applying regression equation analysis, considering 95% confidence interval levels. Analysis of Variance (ANOVA) was applied using Minitab (version 15) to test for significant variation between the LC50 values of the two populations.

3. Results

Log-probit mortality curves for permethrin, malathion, DDT and propoxur were prepared by plotting percentage mortalities against acaricide concentrations (Figs. 1–4). LC50 values between the two populations were significantly different only for DDT (Table 1). Diyathure population showed relatively higher LC50 values indicating a higher resistance than that of the Koulwewa population for all acaricides except for propoxur where the higher LC50 value was for the Kowulwewa population.

According to the DDT discriminating dosage (2%) recommended by the FAO for *R. (B.) microplus*, both Diyathure and Koulwewa populations are resistant to DDT and the calculated resistance percentages are 56.04% and 24.11% respectively. Discriminating dosages for permethrin and malathion are not available for *R. (B.) microplus*. However, the LC_{50}

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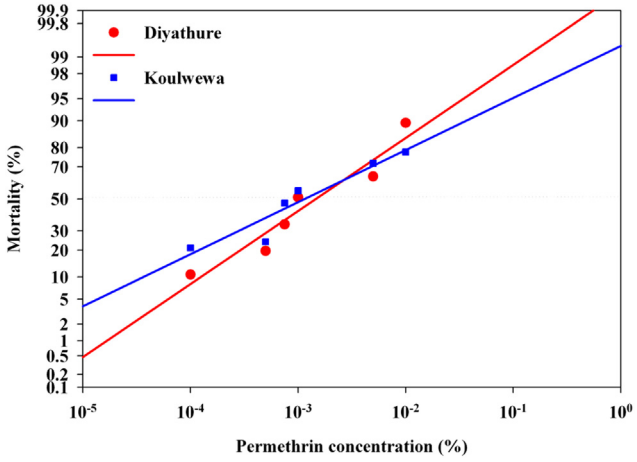

Fig. 1. Log-probit mortality curves for Diyathure and Koulwewa populations of *R. (B.) microplus* larvae for Permethrin as determined by Larval Packet Test.

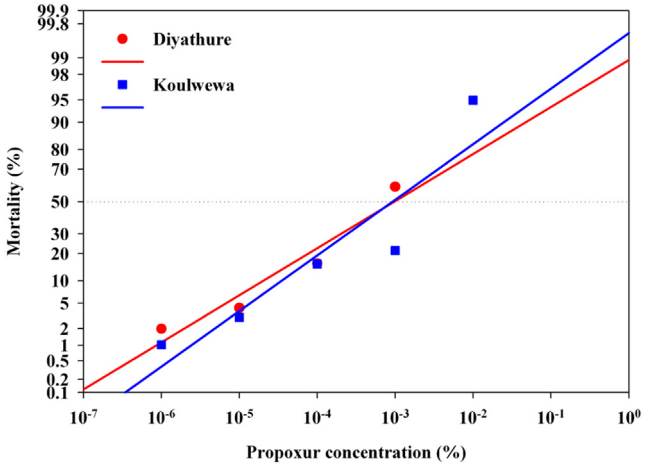

Fig. 3. Log-probit mortality curve for Diyathure and Koulwewa populations of *R. (B.) microplus* larvae for propoxur as determined by Larval Packet Test (LPT) acaricide bioassays [11].

values obtained for malathion in both populations (0.035% and 0.026% for Diyathure and Koulwewa respectively) were higher than that of a malathion susceptible *R. (B.) microplus* strain IVRI-I (0.00144%) [15]. Therefore the resistance ratios for malathion at LC50 are 24.3 and 18.06 respectively for Diyathure and Koulwewa populations. The LC50 value for permethrin susceptible *R. (B.) microplus* tick population of Gonzalez, Mexico has been reported as 0.02% [12] which is approximately ten times higher than the LC50 value obtained for the Diyathure and Koulwewa populations of *R.(B.) microplus*. The LC90 values for permethrin (0.017% and 0.037% for Diyathure and Koulwewa respectively) were lower than that of a susceptible *R. (B.) microplus* strain (Munoz strain) (0.068%) [16]. No discriminating dosage or LC values for propoxur are available for *R. (B.) microplus* for comparison.

Mean specific activities of esterases and GSTs, monooxygenase amounts and the percentage remaining activities of propoxur-inhibited AChEs are shown in Table 2. AChE assay can be used to distinguish susceptible homozygous (< 30% remaining activity) heterozygous (30% - 70% remaining activity) and resistant homozygous (>70% remaining activity) percentages of a population for insensitive AChE mechanism [13]. Accordingly the both populations are heterozygous for insensitive AChE mechanism.

The sequence data of the partial sodium channel regulatory gene were edited using the software Bioedit (version7) and compared with the sequence data obtained from NCBI database (Accession number: AF134216) for the relevant portion of the gene of *R. (B.) microplus*. A non-silent mutation was found from one individual (out of six larvae) at position 215, substituting guanine with thymine which cause an amino acid conversion Glycine to Valine at the 72nd position of the amino acid sequence (Fig. 5).

4. Discussion

In vitro bioassays are the most suitable method to detect resistance in arthropod populations mainly due to its simplicity and low cost [2]. Although several bioassay techniques including adult immersion test (AIT), larval packet test (LPT), and larval immersion test (LIT) have been recommended to detect acaricide resistance in *R. (B.) microplus* [11], LPT has been identified as the most definitive and most frequently used method [17]. Present study used LPT bioassays to investigate the resistance spectrum of the tick populations where malathion, propoxur, DDT and permethrin were used to represent the major groups of acaricides OPs, carbamates, OCs and pyrethroids respectively.

Organochlorine DDT wasthe first synthetic insecticide introduced to Sri Lanka in early 1950s and was heavily used in both health sector (mainly to control malaria vectors) and agricultural sector. Use of DDT was discontinued in 1975 mainly due to development of resistance in malaria vectors and its persistence in the environment [18]. OPs malathion and temephos replaced DDT in the health sector mainly in malaria vector control programmes in the country. Other OPs such as

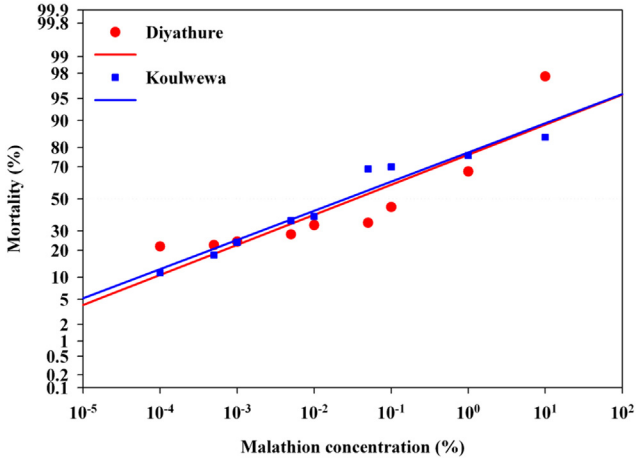

Fig. 2. Log-probit mortality curves for Diyathure and Koulwewa populations of *R. (B.) microplus* larvae for Malathion as determined by Larval Packet Test (LPT) acaricide bioassays [11].

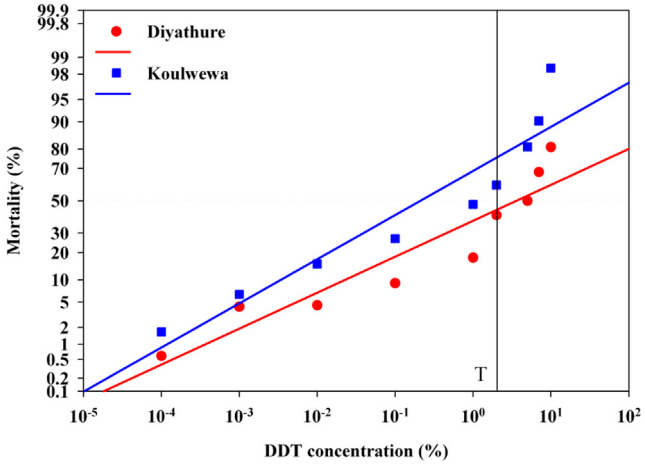

Fig. 4. Log-probit mortality curve for Diyathure population of *R. (B.) microplus* larvae for DDT as determined by Larval Packet Test (LPT) acaricide bioassays [11].

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Table 1
LC_{50} values of permethrin, malathion, DDT and propoxur as determined by Larval Packet test (LPT) acaricide bioassays (FAO, 2004) for Diyathure and Koulwewa populations of *R. (B.) microplus*.

Acaricide	P	N	Slope* ± SE	LC_{50}(%)* (95% CI)	χ^2 (d.f)	*p* values†
Permethrin	Diy	1469	14.36 ± 1.84	0.0017 (0.0008–0.0029)	7.36 (5)	0.195
	Koul	1363	12.23 ± 1.48	0.0012 (0.0006–0.0026)	6.99 (5)	0.220
Malathion	Diy	1763	6.353 ± 0.97	0.0353(0.0061–0.2672)	15.28 (8)	0.054
	Koul	1799	7.169 ± 0.74	0.0260(0.0125–0.0550)	5.43 (8)	0.711
DDT	Diy	1175	6.064 ± 1.46	3.684 (0.8778–29.758)	15.07 (8)	0.058
	Koul	1688	8.257 ± 1.16	0.2111 (0.0919–0.8150)	15.74 (8)	0.050
Propoxur	Diy	1028	10.19 ± 1.62	0.00094 (0.0001–0.018)	5.31 (4)	0.150
	Koul	1179	9.63 ± 2.22	0.0010 (0.0001–0.092)	8.37 (4)	0.079

P = Population, Diy = Diyathure, Koul = Koulwewa, N = Number of larvae, SE = Standard error, CI = Confidence Intervals, χ^2 = Chi squared value evaluating the goodness of fit of the linear regression to log-probit transformed data, R^2 = Regression Coefficient.
* No significant difference between the populations were observed for each acaricide ($p > 0.05$).
† $p \geq 0.05$.

Table 2
Mean activity levels of insecticide detoxifying enzymes, and % remaining activity of the target site enzyme acetylcholinesterase, in Diyathure and Koulwewa *R. (B.) microplus* populations as determined by the biochemical assays ($n > 225$ for each value).

	Tick metabolic enzyme activity**			Target site activity
Population	Est. activity[a]	GSTactivity[b]	MO amount[c]	AChE[d]e*
Koulwewa	0.09 ± 0.02	0.38 ± 0.06	0.14 ± 0.05	36.50 ± 10.10*
Diyathure	0.08 ± 0.04	0.13 ± 0.08	0.34 ± 0.18	37.62 ± 07.86*

[a] Esterase activity (μmol min^{-1} mg^{-1}).
[b] Glutathione S-transferase activity (μmol min^{-1} mg^{-1}).
[c] Monooxygenase amounts (equivalent units of cytochrome P450).
[d] Activity of propoxur inhibited acetylcholinesterasesas a percentage of uninhibited activity.
[e] Heterozygous for altered AChE mechanism as determined by WHO guidelines [13].

coumaphos, ethion, diaxathion and dursban were also introduced for tick control [19]. Although the carbamates were introduced parallel to OPs in Sri Lanka, its use was restricted to agricultural sector. In late 1990s pyrethroids were introduced to both health and agricultural sectors as a new alternative. The most popular acaricides currently used in Sri Lanka for cattle tick control are flumethrin (Bayticol) (a pyrethroid), coumaphos (Asuntol) (an OP) and amitraz (a formamidine).

DDT bioassay results show that Sri Lankan tick populations are highly resistant to DDT even after four decades of DDT cessation. Previously published work shows that high DDT resistance prevails in Sri Lankan insect populations as well [20,21]. This may be due to the selection of the same molecular mechanisms, which were originally developed to provide DDT resistance, by the subsequently used insecticides. Alternatively, the resistance mechanisms which were subsequently developed in populations may be capable of providing DDT resistance as well. As shown in Fig. 3, the mortality curves for both Diyathure and Koulwewa populations did not fit into regression lines indicating that the populations had heterogeneous responses for all the insecticides tested. Characteristic sigmoid shape mortality curves were prominent for DDT where the susceptible homozygous, heterozygous and resistant homozygous percentages are clearly visible. Presence of similar susceptibility/resistance levels found in cattle from two farms 56 km away from each other indicates that the same acaricide resistance spectrum of cattle ticks is distributed across a wide geographical area.

LC50 of a susceptible *Rhipicephalus sanguineus* population in Panama was 0.039% [22] indicating that the Sri Lankan populations studied are highly susceptible for permethrin. This may be due to the late introduction of pyrethroids for cattle tick control programs in the country. Although the carbamates have never been used as tick controlling agents in Sri Lanka, exposure to carbamates used for agriculture must have resulteda moderate propoxur resistance in cattle ticks. OP resistance, which has been developed due to the usage of OPs in tick control programmes for the last two decades in Sri Lanka, is shown by malathion resistance of the populations.

Elevation of esterases is a well-known mechanism for organophosphate resistance and to a lesser extent for carbamate resistance in insects and acarines [6]. Carboxylesterase activity obtained from both populations of *R. (B.) microplus* of the Kurunegala district was higher than that of a susceptible strain (0.02 ± 0.007 μmol/min/mg) of the mosquito *Culex quinquefasciatus* [23]. The 30% insensitivity of acetylcholinesterases shown by the tick populations, the target site of both organophosphates and carbamates, to the propoxur inhibition also indicates that altered acetylcholinesterases are partly responsible for organophosphate and carbamate resistance shown by the tick populations. Insensitive AChE mechanism has also been reported from Sri Lankan *Aedes* mosquitoes as well [24].Higher GST levels have been linked to high DDT resistance in mosquitoes [6]. However, DDT susceptible populations of *Anopheles gambiae* and *Culex quinquefasciatus* have shown activity levels of GSTs(*i.e.* 0.42 and 0.34 μmol/min/mg respectively) similar to that of present tick populations [25,26], indicating that the DDT resistance observed in *R. (B.) microplus* is not due to GSTs.For Sri Lankan mosquitoes *An. culicifacies*, *An. subpictus* and *Cx tritaeniorynchus,* the mean values were 0.24 ± 0.14, 0.30 ± 0.24 and 0.35 ± 0.33 μmol/min/mg respectively [23,27]. Monooxygenases are an important and diverse family of heme-containing enzymes that elevates as a resistance mechanism mainly against pyrethroids. Monooxygenase amounts of both Diyathure and Koulwewa cattle tick populations were similar to

Fig. 5. Sequence of *R. (B.) microplus* (GenBank accession number AF134216) (A), aligned and compared with Sequence analysis of a portion of S 4–5 linker gene, domain II of voltage gated sodium channel in *R. (B.) microplus* of Koulwewa population (B) indicating the G215 T mutation and translated amino acid sequence of *R. (B.) microplus*(C), aligned and compared with translated amino acid sequence analysis of a portion of S4–5 linker gene, domain II of voltage gated sodium channel in *R. (B.) microplus* of Koulwewa population (D) indicating the G72 V mutation.

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the amounts previously reported for permethrin susceptible *An. culicifacies* and *An. Subpictus* populations *i.e.* 0.35 equivalent units of cytochrome P450 [21,28].

To date, more than thirty unique resistance associated `kdr'-type mutations or combination of mutations have been detected in pyrethroid and DDT resistant insect populations [29]. `kdr' associated leucine to phenylalanine L1014F point mutation has been previously reported from Sri Lankan *Cx quinquefasciatus* [30] and *An. Subpictus* [20]. In *R. (B.) microplus*G72 V (G215Tin the gene) in the domain II S4–5 linker, which is the mutation found in the present study, has been reported previously from Mexico [31] and Australia [32,33]. In addition, two other pyrethroid associated kdr type mutations have also been reported from *R. (B.) microplus*,L64I (gene mutation C190A) in the same domain II S4–5 linker from Australia, Africa and South America [33], and the mutation F1550I (gene mutation T2134A) in domain III S6 fromMexico and Texas [14,16,33,34].

In mosquitoes, several candidate genes involved in insecticide resistance have been identified using microarray studies and transcriptome analysis. It has been suggested that resistance involves multiple genes and multiple complex mechanisms [35]. Not many studies have taken place to understand insecticide resistance and underlying molecular mechanisms in ticks. Systematic and sophisticated insecticide resistance monitoring in the field populations of ticks and a better understanding of the mechanisms governing resistance development is vital for future tick control programmes.

5. Conclusions

Acaricide resistance of *R. (B.) microplus* in Sri Lanka is at an emerging state. Resistance to DDT and moderate level resistance to organophosphates and carbamates were evident. Insensitive acetylcholinesterases and kdr type mutations were detected as developing molecular mechanisms of resistance. This is the first study on acaricide resistance of *R.(B.) microplus* in Sri Lanka andisland-wide studies are needed to make predictions on its prevalence to adopt management strategies in acaricide usage preventing or delaying possible resistance development.

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