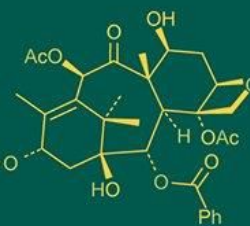
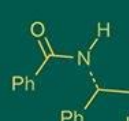


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Molecular characterization of deltamethrin resistance in *Rhipicephalus microplus* ticks infesting cattle in Nagpur district of Maharashtra using allele-specific PCR

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Abstract

Rhipicephalus microplus is the most predominant tick species infesting cattle in the Nagpur district of Maharashtra state. In recent years, several reports have indicated that deltamethrin is becoming less effective in controlling tick infestations, highlights the importance of assessing the resistance status of this acaricide in field populations of cattle ticks. In the present study, resistance to deltamethrin was investigated using allele-specific polymerase chain reaction (AS-PCR), a molecular technique that helps detect specific point mutations associated with acaricide resistance. The DNA extraction was carried out from larvae procured from *R. microplus* ticks from five talukas of the Nagpur district and were subjected to allele specific PCR. The AS-PCR results showed that ticks from all five talukas were homozygous resistant to deltamethrin. Further sequence analysis of the PCR amplicons confirmed a point mutation in the sodium channel gene, where cytosine (C) was replaced by adenine (A) in the domain II S4-S5 linker region, indicating the genetic basis of resistance in these ticks.

Keywords: Cattle, ticks, deltamethrin, resistance, molecular, characterization

Introduction

Rhipicephalus microplus is one of the major tick species infesting livestock and poses serious problems in livestock management and disease control. It is a common ectoparasite of cattle and other farm animals, causing considerable economic losses due to reduced milk and meat production, irritation, and damage to the skin. It acts as a vector for tick-borne diseases such as Babesiosis and Anaplasmosis which may cause high morbidity and sometimes mortality in affected animals resulting in reduced health and productivity. (Gosh and Nagar, 2014) [6]. Ticks cause harm to livestock through multiple effects, including toxicity, blood loss due to feeding, irritation, damage to hides, and subsequent weight loss (Furman and Loomis, 1984) [3]. Tick control using of chemicals is the most commonly used method, however frequent and indiscriminate use and lower dosing of acaricides is responsible for development of acaricidal resistance (Sagar *et al.* 2020) [18]. Amongst the synthetic pyrethroids, deltamethrin is the most widely used acaricide for tick control in India. (Kumar *et al.* 2020) [9]. Several studies across India have reported the development of deltamethrin resistance in *Rhipicephalus microplus*, with varying resistance levels observed in Indian states such as in Haryana, Gujarat, Rajasthan, Jammu & Kashmir, Kerala and Telangana. Synthetic pyrethroid acts by targeting the voltage-gated sodium channels of nerve cells in ticks, leading to paralysis and death. Allele-specific polymerase chain reaction (AS-PCR) is used to detect resistance to synthetic pyrethroids by identifying specific point mutations in target genes, thereby confirming resistance in *Rhipicephalus microplus*. (Morgan, 2009) [14]. The information regarding the resistance status of deltamethrin in cattle ticks from the Nagpur region of Maharashtra would be valuable for developing appropriate alternative strategies to manage acaricidal resistance in ticks.

Materials and Methods

Fully engorged adult female ticks were collected manually from naturally infested cattle and

were sterilized using 1% sodium hypochlorite solution. The identification of ticks was done based on standard morphological keys as per Walker *et al.*, (2003) [22]; Soulsby, (1982) [19] and Geevarghese and Mishra, (2011) [4]. After identification the engorged female ticks were treated with 25 ppm deltamethrin for 2 minutes and allowed for egg laying in a BOD incubator at $27 \pm 1^\circ\text{C}$ and 80-85% relative humidity. About 14 days-old larvae after hatching were then stored in 70% alcohol at room temperature for genomic DNA extraction.

Genomic DNA extraction

The preserved larvae of *R. microplus* ticks were washed in normal saline, dried and weighed to get a weight of 25mg. The weighed larvae were transferred in sterile zip lock pouches and crushed in mortar and pestle to make it homogenized. Properly crushed larvae were then transferred

to 2 ml sterile Eppendorf tube by rinsing the larval side of the pouch with normal saline. Then genomic DNA extraction of crushed larvae was carried out using HIMEDIA HiPurA™ Multi-Sample DNA Purification Kit as per manufacturer protocol with some modification. The quality and quantity of obtained DNA was assessed by agarose gel electrophoresis as well as by Nanodrop 2000C spectrophotometer and then stored at -20°C for further processing.

Amplification of genomic DNA template by Allele-Specific PCR

Allele-specific PCR was performed by using template DNA obtained from larvae of *R. microplus* ticks by using primers and PCR conditions as described by Rineshkumar (2010) [16] and Amrutha *et al.* (2021) [1]. The primer details are mentioned in Table 1

Table 1: Primer details

Sr. No.	Name of primer	Nucleotide sequence
1	ASS-Forward Allele-specific susceptible	F-5'GGAAAACCATCGGTGCTC3'
2	ASR-Forward Allele-specific resistant	F-5'GGAAAACCATCGGTGCTA3'
3	ASC-Reverse Allele specific common	R-5'CTTCGTAGTTCTTGCCAAAG3'
4	R1-Forward Nonspecific control	F-5'TACGTGTGTTCAAGCTAGC3'

Susceptible reaction

For susceptible reaction, ASS-Forward Allele-specific susceptible primer was used as forward primer and ASC-Reverse Allele-specific common primer was used as reverse primer.

Resistant reaction

ASR-Forward Allele-specific resistant primer was used as forward primer and ASC-Reverse Allele-specific common primer was used as reverse primer in resistant reaction.

Control reaction

R1-Forward Non-specific control primer was used as forward primer and ASC-Reverse Allele-specific common primer was used as reverse primer in control reaction.

The PCR product of 5 μl was loaded in each well along with the DNA ladder (100 bp) in 1.5% agarose gel and samples were run at 90 volts for 45 min. PCR product was sent for sequencing. After sequencing of PCR product, the data obtained from sequencing was analysed in megalign software for detection absence/presence of mutation in gene.

Results and Discussion

The detection of deltamethrin resistance in *R. microplus* ticks at molecular level and to detect absence/presence of mutation in sodium channel gene AS-PCR was performed from Nagpur, Kamptee, Ramtek, Kalmeshwar and Umred taluks of Nagpur district of Maharashtra. In the present research *R. microplus* ticks from all five taluks of Nagpur district were found homozygous resistant for deltamethrin showing a single band of 101 bp size in resistant reaction with the corresponding region of 100 bp DNA ladder. The presence of band in only resistant reaction showed that the larvae were homozygous resistant. The presence of a band in the control reaction at 161 bp size was used to verify the successful PCR reaction. The PCR product was commercially sequenced. The sequence was blasted in NCBI and was compared with related sequences in GenBank. The mutation in para sodium channel gene in *R.*

microplus against deltamethrin was detected in the present study by the replacement of cytosine (C) with adenine (A) in domain II S4-5 linker region. For this the query sequence was aligned with the published sequences of *R. microplus* isolate Izatnagar sodium channel gene, partial cds (Sequence ID: HM579820.1), *R. microplus* strain IVRI-IV sodium channel gene, partial cds (Sequence ID: JQ693154.1), *R. microplus* isolate IVRI-V para-sodium channel gene, partial cds (Sequence ID: KX670384.1), *R. microplus* isolate Rampur para-sodium channel gene, partial cds (Sequence ID: KY873587.1), *R. microplus* isolate Moradabad para-sodium channel gene, partial cds (Sequence ID: KY873589.1) and *R. microplus* isolate Mathura para-sodium channel gene, partial cds (Sequence ID: KY873592.1) in megalign software (Upadhaya *et al.* 2020; Nagar *et al.* 2018) [20, 15]. The sequence is submitted to BankIT NCBI and published with Accession no.-PP357617.

Discussion

The detection of resistance-associated mutations in the voltage-gated sodium channel (VGSC) gene clearly indicates that target-site insensitivity plays a major role in the development of synthetic pyrethroid resistance in these tick populations. A higher amount of resistance occurs in ticks as ticks have higher reproductive potential leading to the inheritance of resistant factors resulting in a greater number of resistant ticks. By knowing the mechanism of resistance development rapid and accurate diagnostic tools for early detection of resistance can be developed (FAO, 2004) [2]. AS-PCR enables the detection of specific point mutations in genes associated with resistance, thereby confirming synthetic pyrethroid resistance in *Rhipicephalus microplus*. (Morgan, 2009) [14]. The findings of the present study are consistent with earlier studies conducted in different parts of India. Deltamethrin resistance in six agro climatic zones of India in Indian tick populations and associated it with target-site insensitivity, including mutations in the sodium channel gene was reported by Vatsya *et al.* (2011) [21]. Amrutha *et al.* (2021) [1] reported

multiple mutations (e.g., C190A, A271G) in the voltage-gated sodium channel gene using AS-PCR, confirming their association with resistance. Rineshkumar, (2010) ^[16] revealed the deltamethrin resistance in Sultanpur and Bareilly isolates of *R. microplus* ticks with allele frequencies of 88% and 20% respectively. Similarly, Kumar *et al.* (2013) ^[8] Identified C190A mutation in domain II of the sodium channel gene, confirming its role in deltamethrin resistance., Gosh *et al.* (2015) ^[5] from Bihar, Kumar *et al.* (2016) from Rajasthan, Lenka *et al.*, (2016) from south India, Nagar *et al.* (2018) ^[15] from Uttar Pradesh, Upadhy *et al.* (2020) ^[20] from Assam, Kumar *et al.* (2021) from Haryana detected the presence of mutation in S4-5 linker region of sodium channel gene in *R. microplus* ticks by performing PCR. The studies from abroad, Guerrero *et al.* (2001) ^[7] from Mexico; Rodriguez-Vivas *et al.* (2012) ^[17] from Mexico; Lovis *et al.* (2012) ^[13] from America, Australia and Africa also reported the presence of a mutation in sodium channel gene in synthetic pyrethroid-resistant *R. (Boophilus) microplus* ticks. The detection of resistance in the Nagpur region has significant practical implications. Since *R. microplus* is a major vector of diseases like babesiosis and anaplasmosis, ineffective tick control can lead to increased disease incidence, reduced animal productivity, and economic losses for farmers. Therefore, regular monitoring of resistance is crucial for effective disease and tick management.

Conclusion

The present study clearly indicated that *Rhipicephalus microplus* ticks from all selected talukas of Nagpur district of Maharashtra found to develop homozygous resistance to deltamethrin confirmed by molecular analysis. Mutation in the sodium channel gene, where cytosine was replaced by adenine, was also identified and is likely responsible for this resistance. The findings revealed that this drug will not be further effective for tick control in this region. Therefore, it is needed to restrict the indiscriminate use of deltamethrin and further monitoring for acaricidal resistance status in ticks is necessary. The adaptation of an alternative and integrated tick control approaches are important to deal the problem and to prevent further spread of resistance. Overall, the study provides the baseline information on the status of deltamethrin resistance in the Nagpur region, which can be implemented in planning effective and sustainable tick control strategies such as integrated tick management and the use of biological or ethnoveterinary methods for sustainable tick control.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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