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IDENTIFICATION OF MUTATIONS IN ACETYLCHOLINESTERASE 2 GENE OF ACARICIDE RESISTANT ISOLATES OF *RHIPICEPHALUS* (*BOOPHILUS) MICROPLUS*

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Abstract: Monitoring of acaricide resistance and identification of resistance mechanisms are two important facets of sustainable tick control. To identify the role of mutations in esterase genes in the development of acaricide resistance, carboxylesterase (CES) and acetylcholinesterase 2 (AChE2) genes were PCR amplified and sequenced. PCR-RFLP assay using G1120A substitution site in carboxylesterase gene was performed. Four amino acid substitutions viz, V297I, S364T, H412Y and R468K, were found in acetylcholinesterase 2 gene of resistant field isolates and in reference resistant lines. The PCR-RFLP assay failed to distinguish between susceptible and resistant isolates indicating absence of G1120A site in Indian tick populations. The findings of this study highlighted the possible association of multiple substitutions in acetylcholinesterase 2 gene in conferring resistance in *R. (B.) microplus* isolates resistant to organophosphate (OP) compounds.

Keywords: acaricide resistance, acetylcholinesterase 2, carboxylesterase, *Rhipicephalus (Boophilus) microplus*.

Introduction

Ticks are obligate hematophagus organisms and the most important ectoparasites of domestic animals. In India, *Rhipicephalus (Boophilus) microplus* is the most common tick species infesting milk and meat producing animals, accountable for the control cost to the tune of more than 498.2 million USD annually (Minnjauw and Mcleod, 2003). Extensive use of acaricides has led to resistance development against almost all chemicals registered for the control of the this important tick species (He *et al.,*1999, chen *et al.,* 2009). In India, reports of acaricide resistance are pouring from many parts during the last few years (Kumar *et al.,* 2011, Kumar *et al.,* 2014, Sharma *et al.,* 2012, Singh *et al.,* 2014, Ghosh *et al.,* 2015).

Mutations in the target site of insecticides have been implicated in resistance development. In *R. (B.) microplus* a point mutation (G1120A) has been identified in

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carboxylesterase gene of resistant and moderately resistant strains from Mexico (Hernandez *et al.,* 2000). Although a direct correlation between this mutation and pyrethroid resistance could not be established, this mutation was thought to occur simultaneously with other events that contribute to resistance development (Baffi *et al.,* 2007). This mutation creates an EcoRI restriction site in the mutant individuals and thus its use as a monitoring tool was explored in the form of a PCR-RFLP assay (Baffi *et al.,* 2007, Hernandez *et al.,* 2002). Recently, four novel amino acid substitutions were identified in AChE2 gene of resistant field isolates collected from the state of Bihar, India (Ghosh *et al.,* 2015). Bendele et al. (2015) reported OP resistance associated allelic variants in AChE1 gene of laboratory strains of *R.(B.) microplus* while Temeyer et al. (2013) identified substitutions and sequence polymorphism in *R.(B.) microplus* AChE1, AChE2 and AChE3 transcripts.

However, in India the role of AChE2 gene mutations in acaricide resistance development has not been suitably addressed. Thus, the present experiments were aimed to identify the possible role of target site modification in acetylcholinesterase 2 gene in conferring organophosphate resistance in ticks collected from the wide geographical regions of India. The applicability of PCR-RFLP assay utilizing G1120A mutation in carboxylesterase gene as monitoring tool in Indian context is also assessed.

Materials and methods

Sampling

The tick isolates used for the study were collected from different agro-climatic regions of India. The latitude and longitude of collection centers are : in trans-gangetic plain region 30.4750º–30.9100ºN and 74.5150º–75.8500ºE, western dry region of 27.6200ºN and 75.1500ºE, western Himalayan region of 29.4722ºN and 79.6479ºE, middle gangetic plain region having the latitude of 25.4200º – 26.6333ºN and longitude of 82.0000º – 85.9000ºE, central plateau and hill region located at the latitude of 24.58000º – 25.3500ºN and longitude of 73.6800ºE and lower gangetic plain region of 22.5300ºN and 88.3300ºE. The information regarding commonly used acaricides at surveyed farms was compiled. An isolate comprised of pooled ticks collected from an area and after generation of sufficient adults it was tested at different grades of DC using adult immersion test (AIT) and categorized on the basis of resistant factor (RF).

PCR amplification, cloning and sequencing of targeted site of carboxylesterase (CES) and acetylcholinesterase 2 (AChE2) genes

The cDNA synthesized from RNA extracted from the adults of reference IVRI-I, IVRI-III, IVRI-IV, IVRI-V lines and field isolates, were used as a template for amplification of the 372 bp fragment of CES gene encompassing G1120A mutation site and the 1692 bp AChE2 gene. Amplification of CES gene was carried out in a 25µl reaction using primers described previously (Hernandez *et al.,* 2000). Each reaction contained 10X buffer, 100ng cDNA, 0.5 µM of each primer, 0.2 mM dNTP and 1.5 units of Dream Taq DNA polymerase (Thermo Scientific, USA). The reaction was incubated initially at 95ºC for 3 min followed by 35 cycles of 95ºC for 30s, 58ºC for 30s, 72ºC for 30s. The AChE2 gene was amplified as per the protocol adopted by Ghosh et al. (2015). The PCR products were purified using Gene JET gel extraction Kit (Thermo Scientific, USA). For PCR-RFLP, the 372 bp PCR products of CES gene were digested with the *EcoRI* restriction enzyme using 1 µg of the PCR product per sample. The purified PCR products of CES (372bp) and AChE2 (1692bp) were ligated with T/A cloning vector, pTZ57R/T (InsTAClone, Thermo Scientific, USA) and subsequently transformed into *E. coli* DH5α cells. Positive clones were verified by restriction enzyme digestion and outsourced to DNA sequencing facility at University of Delhi, South Campus for double stranded sequencing. The forward and reverse sequence data of 5 clones from each isolate were analyzed by multiple sequence alignment using Clustal W algorithm in Lasergene software (DNAStar Inc., Madison, USA) and BTI software (Gene Tool Lite, USA).

Results

Resistant status of field isolates

Eight isolates were collected from unorganized and organized farms. Amongst the frequently used acaricides in the surveyed farms, a maximum usage of 88% was reported for deltamethrin while a minimum usage of 6% was reported for amitraz. The use of diazinon was comparatively less at present due to reduction of efficacy. Although ivermectin was initially introduced for the control of endo-parasites, its use has increased considerably (59% of the surveyed farms) for the control of ecto-parasites.

All the collected tick isolates were found to be resistant to diazinon in the range of level I-IV with RF from 4.15 to 57.85 (Table 1).

PCR-RFLP analysis of carboxylesterase and sequence analysis of AChE2 genes

The PCR product of 372 bp was amplified in all the samples (Fig.1). Following EcoRI digestion, no change in product size was seen. All the PCR products showed the original band of 372 bp which indicated non digestion of the PCR products. The analysis of

forward and reverse sequences also did not show any point mutation at position 1120 in any of the Indian isolates (Accession nos. GU830960, GU830963, GU830964, JN979988).

The alignment of nucleotide and deduced amino acid sequences of AChE2 amplified from field isolates and reference resistant lines with IVRI-I sequence led to the identification of five nucleotide substitutions in reference resistant lines as well as in all resistant isolates except LDH (Accession nos HQ184942, HQ184944, HQ184946, HQ184947, JN624773, KC493618, KC493619, KT215343, KC493616, KC493617). One of the substitutions was a silent mutation (G138A) while the other 4 mutations, i.e., G889A, T1090A, C1234T and G1403A lead to V297I, S364T, H412Y and R468K amino acid substitutions in AChE2 gene (Table 1).

Discussion

Acaricide resistance is a global problem associated with use of chemicals for tick control. In India, sporadic information regarding reduced efficacy of commonly used acaricides was provided by livestock owners, but scientific evidence was generated only recently, with the adoption of standardized resistance monitoring tools. Resistance to diazinon, deltamethrin, cypermethrin and amitraz has been established in several regions of the country despite early optimism that because of their rapid toxicological action targeted organism will not be able to develop resistance against these groups of insecticides (Kumar *et al.,* 2011, Kumar *et al.,* 2014, Sharma *et al.,* 2012, Singh *et al.,* 2014).

It was hypothesized that both point mutation and over-expression of the enzymes are involved in development of resistance to SP and OP compounds. But there was a general lack of information regarding the acaricide resistance linked mutations in CES and AChE2 gene in Indian strains of *R. (B.) microplus*. In the present study, no mutation was detected at 1120 position in the 372 bp CES gene in reference resistant lines and in any of the field isolates. This finding was further verified by the nucleotide sequencing of this particular region. None of the Indian isolates despite of their varying resistance status had the G1120A mutation. The findings of this study were in contrast to the observations of Hernandez et al. (2002), Baffi et al. (2007) and Chen et al. (2009) where the authors utilized the G1120A point mutation to develop molecular tools for rapid detection of pyrethroid resistance in ticks. It is inferred that, in Indian situation, the PCR-RFLP with 372bp segment of CES gene is not a suitable tool for resistance monitoring.

Being the target of OP insecticides, acetylcholinesterases are the enzymes of interest to understand the molecular basis of OP resistance. Both, the altered enzymatic activity and

target site insensitivity in AChE genes have been investigated by various authors in different organisms (Pan *et al.,* 2010, Chang *et al.,* 2014). In *R.(B.) microplus,* three AChE genes were characterized as AChE1, AChE2 and AChE3 (Baxter *et al.,* 2002, Hernandez *et al.,* 1999, Temeyer *et al.,* 2004), however, none of these genes were conclusively linked with resistance. Recently, amino acid substitutions in AChE1, AChE2 and AChE3 genes of different field strains were identified but due to the presence of multiple copies and transcript polymorphism in each gene, these substitutions were not directly correlated with OP resistance (Temeyer *et al.,* 2013). In India, the information regarding mutations in any of the AChE genes was scanty, until recently when four amino acid substitutions in AChE2 gene of resistant isolates of *R. (B.) microplus* collected from the state of Bihar were identified (Ghosh *et al.,* 2015) and that observation is further validated in the present study. All four substitutions were detected in the reference resistant lines and in field isolates except in LDH isolate. Although it remains to be seen if these mutations are correlated with OP resistance, their presence in only moderate to high OP resistant isolates provides some insight into the possible OP resistance mechanisms operating in Indian isolates of *R.(B.) microplus*.

Conclusion

In India, the *R. (B.) microplus* populations have developed resistance to organophosphate acaricides. Identification of mutations in AChE2 gene of resistance isolates provides some information about possible mechanism of resistance development. However, further work is required to confirm the role of these mutations in OP resistance. The PCR-RFLP assay proposed as a diagnostic tool in Mexican strains was found not feasible for Indian strains of *R. (B.) microplus*.

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Fig.1: Amplification of AChE2 (1692bp) and CES(372bp) genes (M: 100bp plus DNA ladder).

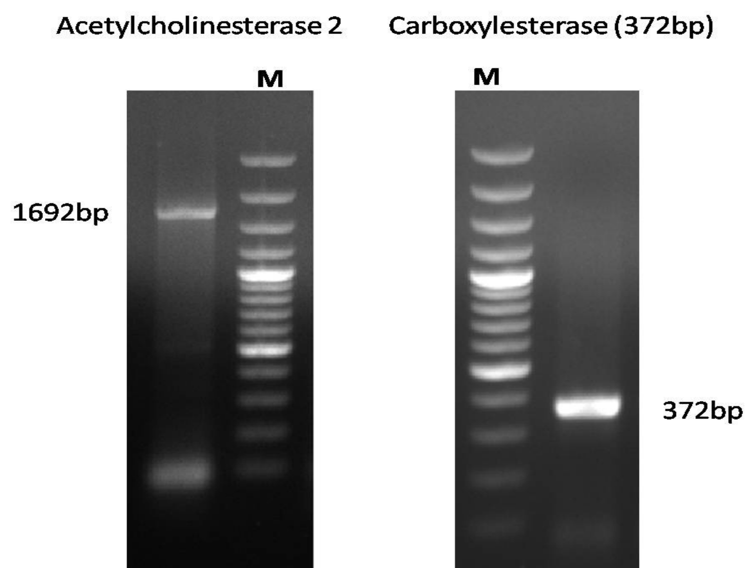

Table 1: Resistance Factor (RF), Resistance level of field isolates and amino acid substitutions detected in the AChE2 gene

Isolate (Accession No.)	RF (Diazinon)	Resistance Level	Amino Acid substitution site			
			297	**364**	**412**	**468**
IVRI-I	1	S	V	S	H	R
LDH	4.15	I	V	S	H	R
BLW	22.78	II	I	T	Y	K
DRB	14.48	II	I	T	Y	K
UDP	38.92	III	I	T	Y	K
DNP	37.79	III	I	T	Y	K
SUL	57.85	IV	I	T	Y	K
SKR	16.3	II	I	T	Y	K
MKT	14.01	II	I	T	Y	K
IVRI-III	28.4	III	I	T	Y	K
IVRI-V	25.5	III	I	T	Y	K