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Differential mRNA expression profiling of *Heparan sulphate 3-O-sulphotransferase 1(HS3ST1)* gene in Vechur (*Bos indicus*) and crossbred (*Bos indicus* X *Bos taurus*) cattle of Kerala in response to *Rhipicephalus annulatus* infestation

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Abstract: *Heparan sulphate 3-O-suphotransferase* 1 (*HS3ST1*) gene encodes a suphotransferase which transfers a sulfuryl group (SO_3) to glucosamine residue to form the 3-*O*-sulfo glucosamine in the antithrombin binding motif of heparan sulphate. Antithrombin processes both anticoagulant and anti-inflammatory activities. This study was undertaken to assess the differential cutaneous expression profiles of *HS3ST1* gene elicited in response to *R. annulatus* infestation in Vechur (*Bos indicus*) and crossbred Holstein Friesian (CBHF) (*Bos Taurus* x *Bos indicus*) calves after 24 hours of infestation. Both genetic groups exhibited significant up-regulation of the gene after 24 hours. The up-regulation of *HS3ST1*gene might trigger the anti- inflammatory effects of antithrombin whereby there is controlled progression of the inflammation preventing shock and organ failure.

Keywords: *HS3ST1* expression, Antithrombin, 3- *O*- sulfation, cell- signalling.

Introduction

Rhipicephalus species are the predominant obligate, blood sucking ectoparasites in cattle causing severe economic strain on cattle production in India. The tick is endemic to the Indian sub- continent and is continuing to spread due to the breakdown of control measures and development of acaricide resistant strains (Ghosh and Nagar, 2014). The direct effects in infested cattle are marked loss of production parameters (weight gain and milk production), anaemia, skin lesions (reduced leather value) and weakening of the host immune system

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while the indirect effects are brought about by the multitude of bacterial, viral, rickettsial and protozoan diseases transmitted to the host during the blood feeds (Hurtado and Giraldo-Ríos, 2018). Alternative safe and sustainable methods of tick control were explored by many investigators to avoid excessive use of chemical acaricides. Most prominent was the effort to identify naturally tick resistant cattle breed or to develop a herd which could effectively counter the effects of a tick infestation through selective breeding. *Bos indicus* animals were reported to be more resilient to tick infestation than *Bos taurus* cattle (Wambura et al.,1998; da Silva et al., 2007; Piper et al., 2008). Several attempts were made to identify specific genes or group of genes which contributed to tick resistance and incorporate these markers as selection criteria in cattle breeding in endemic areas (Martinez et al., 2006; Carvalho et al., 2008).

Heparan sulphate is a linearglycosaminoglycan, present close to the cell surface or in the extracellular matrix of all animal tissues, complexed with other such chains and a protein core to form a proteoglycan. The binding of the molecule to different protein ligands enables it to perform a wide spectrum of biological functions spanning over cell- matrix interactions, cell signalling, cell growth and division, tissue morphogenesis (Häcker et al., 2005; Bülow and Hobert, 2006). Heparan sulphate has been indicated in wound healing (Olczyk et al., 2014; Uijtdewilligen et al., 2018), initiation of immune response (Kodaira et al., 2000; Johnson et al., 2002), modulation of inflammatory reactions (Taylor and Gallo, 2006). The binding of heparan sulphate with different proteins is dependent on the series of structural modifications it acquires during its polymerisation with EXT1 and EXT2 polymerases including a series of sulphation of glucosamine units and uronic acid epimerization at specific locations (Esko and Selleck, 2002; Multhaupt and Couchman, 2012; Thacker et al., 2014). Since these modifications do not occur uniformly there are regions of high, intermediate and low sulphation resulting in generation of structural variations and ligand- binding sites in heparan sulphate aiding its diverse functions. The last step in these sequential modifications is the 3-*O* sulphation, brought about by *HS3ST* gene family, involving only a finite number of chains (Thacker et al., 2014).

HS3ST1 enzyme transfers the sulphuryl group (SO_3) from 3′-phosphoadenosine 5′-phosphosulphate to the 3-OH position of a glucosamine residue to form the 3-*O*-sulfo glucosamine, a pentasaccharide epitope, which is the specific structural motif for binding of heparan sulphate to antithrombin (Edavettal et al., 2004). Unlike other suphotransferases that

have signal-anchor domains and are type II membrane integral proteins in Golgi apparatus, HS3ST1 lacks a transmembrane domain and is likely to be an intraluminal enzyme.

Investigations by Smits et al., (2017) revealed that an SNP rs16881446G/G genotype was independently associated with the severity of coronary artery disease and atherosclerotic cardiovascular events in mice. The same was associated with reduced *HS3ST1* expression in human primary endothelial cells. They could conclude that effect of HS3ST1is not limited to helping the association of heparin sulphate with antithrombin but also for promoting the protective anti-inflammatory effects of antithrom bin. Levy et al., (2016) in their review of the clinical applications of anti-inflammatory properties of antithrombin in humans, suggested two different mechanisms- coagulation-dependent effects and coagulation-independent effects- through which antithrombin exerted its anti-inflammatory action.

This study investigated the expression profiles of *HS3ST1* gene in pre-sensitized Vechur (*Bos indicus*) and crossbred Holstein Friesian (CBHF) (*Bos taurus x Bos indicus*) calves 24 hours after artificial tick challenge with *R. annulatus* larvae.

Materials and methods

The design and nature of this experiment was approved by the CPCSEA, India (letter no. F.No.25/21/2017-CPCSEA dated 20-10-2017) and the Institutional Animal Ethics Committee (IAEC), Kerala Veterinary and Animal Sciences University.

Relative quantification of expression of HS3ST1 gene

Vechur and CBHF male calves (2 each) of six months were randomly selected from Instructional Livestock Farm Complex, College of Veterinary and Animal Sciences, Pookode and pre-sensitised to *R. annulatus* prior to the start of the experiment (da Silva et al., 2007). Punch biopsies of 8 mm diameter were collected from each calf just before the tick challenge with 10,000 larva and after 24 hours of tick challenge from the area where the ticks were released. The collected samples were transported to the laboratory in RNA later and stored in 80°C freezer until RNA isolation. RNeasy Fibrous Tissue Mini kit (Qiagen) was used for isolating RNA from skin biopsies. The integrity of the extracted RNA was checked using agarose gel electrophoresis. The concentration and quality of RNA was checked by NanoDrop spectrophotometer (Thermo Scientific, USA). Complementary DNA (cDNA) was synthesised from 1000 ng of isolated RNA in 20 µl reaction volume using Revert Aid first strand cDNA synthesis kit (Thermo Scientific) as per manufacturer's protocol and stored at -40°C until used.

The primers for qPCR of *HS3ST1* were designed and custom synthesized (Sigma Aldrich) from published bovine mRNA sequence available in GenBank (Accession no. NM_001076122.1) using Primer3 software while primers for the internal control gene, *GAPDH* were adopted from published research article (Naicy *et al*., 2016). Details of primers used for the amplification of cDNA of the genes are listed in the Table 1. The qPCR reactions were carried out in triplicate. Each reaction was of10 µl volume containing 5µl iTaq universal SYBR green supermix (BioRad), 1µl template cDNA, 0.3 µl each of forward and reverse primers (10 pM/µl) and 3.4µl nuclease free water. The non-template control (NTC) for each gene and reverse transcription minus (RT minus) control for each cDNA sample (RNA as template), a positive control with cDNA synthesized from control RNA (provided with RevertAid first strand cDNA synthesis kit) and a negative control (with nuclease free water) were set up along with the sample reactions.

PCR reaction consisted of an initial denaturation at 95°C for three minutes followed by 40 cycles of denaturation (95°C, 20 seconds), annealing (variable °C,15 seconds) and elongation (72°C, 15 seconds). Dissociation (melt) curve analysis was done after the PCR reaction. The protocol for melt curve analysis was 95°C for 15 S, 55°C for 15 S followed by 95°C for 15 S. The data obtained was analysed for relative quantification by $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Statistical comparison between samples was performed using Analysis of variance (ANOVA) and independent sample t-test (SPSS V.21) and subsequent Duncan's multiple range test (p-value of ≤ 0.05 was considered statistically significant).

Results

The amplification plot and melt curve *HS3ST1* are given in Figures 1 and 2 respectively. The relative quantification of *HS3ST1* expression in Vechur (*Bos indicus)* and CBHF (*Bos taurus)* are given in Table 2. There is significant upregulation of the gene expression at 24 hours following the tick challenge in both Vechur and CBHF calves. The fold change in expression of the gene exhibited after the challenge compared to the initial level in CBHF was 25.63 while in Vechurit it was 1.952 (Figure 3).

Discussion

R. annulatus ticks are pool feeders which need to remain attached to their host for many days from larval to adult stage. The ability of the host to react to the skin injury at the bite site and mount an immune response against the tick salivary proteins determines the efficiency at which it can limit the blood feed, cause tick rejection and overcome the infestation. Wound

healing process includes the formation of haemostatic plug, vasoconstriction, inflammation and tissue remodelling. Gracia et al., (2017) suggested a strong genetic basis for the variation in host response among individuals.

Glucosaminoglycan, Heparan sulphate, is modified during its biosynthesis by a variety of deacetylases and suphotransferases, forms proteoglycans (HSPG) with a number of protein molecules which determines its function. These HSPGs are found either tethered to the cell membranes through various transmembrane domains, found extracellular or is intraluminal. On event of tissue injury or presence of immunogenic agents (like tick salivary gland extracts), inflammation sets in and there is elevated activity of enzymes like metalloproteases and heparanases. This results in shedding of soluble haparan sulphate fragments from the cellular membrane and extracellular matrix. These are sensed by the toll like receptor factors (especially TLR 4) on the immature dendritic cells leading to rapid maturation and migration of dendritic cells to the lymphoid tissue and subsequent stimulation of cellular immune response through the T lymphocytes (Kodaira et al., 2000; Johnson et al.,2002). Heparan sulphate also favours phagocytosis and produce B cell stimulation.

The cascading immune responseis controlled by the release of cytokines interleukin4 (IL 4) and IL13. IL 4 has dual action initially it stimulates the B cells to produce IgE but it is a negative regulator of Th1 inflammation and will produce the upregulation of associated genes including *HS3ST1*. *HS3ST1* is a rate limiting enzyme in the formation of heparan sulphate-antithrombin proteoglycan. This complex is in turn responsible for moderating the pace of inflammatory changes through the anti-inflammatory action of antithrombin (Collins and Troeberg, 2018). The up regulation of *HS3ST1* observed in calves used in the present study indicates a robust stimulation of the cellular immune system on tick challenge.

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during infestations according to the genetic composition of the bovine hosts, *Bos taurus* and *Bos indicus*. Exp Parasitol., 118: 587- 591

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Table 1: Sequence and properties of primers designed for qPCR

Gene	Primer	Primer sequence (5'-3')	Annealing temperature	Product size(bp)
GAPDH	GAPDH-F	TGGAGAAACCTGCCAAGTATG	60°C	127
	GAPDH-R	TGAGTGTCGCTGTTGAAGTC		
HS3ST1	HS3ST1- F	TTCCCCGAGATCCAGAAGGT	63°C	97
	HS3ST1- R	GGCAGTAGAAGCCCTTGGTT		

Table 2: Relative expression profile of *HS3ST1* in Vechur and CBHF cattle

		Mean C_T ±SE		$^{\Delta C}T$ ±SE	$^{\Delta\Delta C}T$ ±SE	Fold change	P value
		HS3ST1	*GAPDH*				
Vechur	Before	21.705± 0.40	15.04 ± 0.53	6.665± 0.66	0.0 ± 0.66	1	0.036*
	After	21.12 ± 0.13	15.5 ± 0.05	5.7 ± 0.14	- 0.96±0.14	1.952	
CBHF	Before	25.475± 0.39	15.475 ± 0.06	10.44 ± 0.39	0.338 ±0.39	1	0.050*
	After	21.26 ± 0.08	15.37± 0.05	5.75 ±0.09	-4.68± 0.09	25.634	

*P≤0.05 is significant

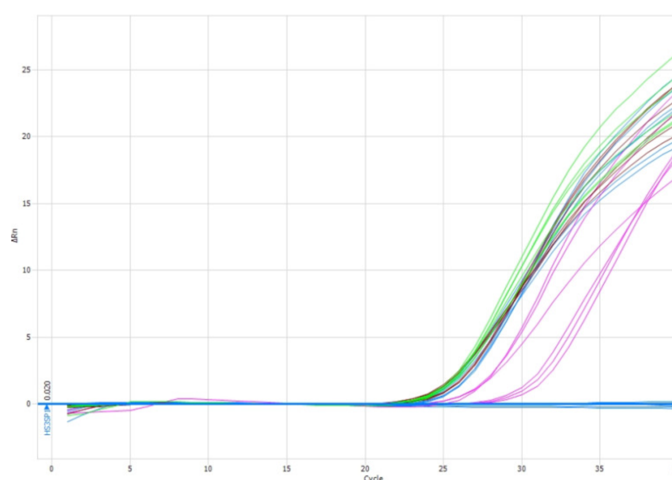

Figure 1: *HS3ST1* Amplification Plot

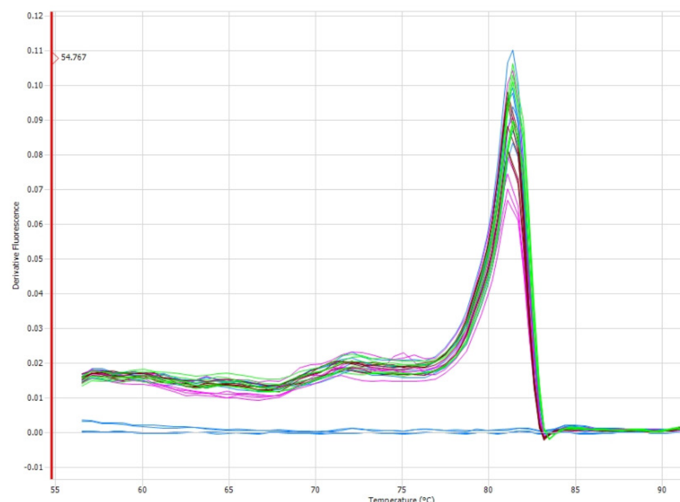

Figure 2: *HS3ST1* Derivative melt curve

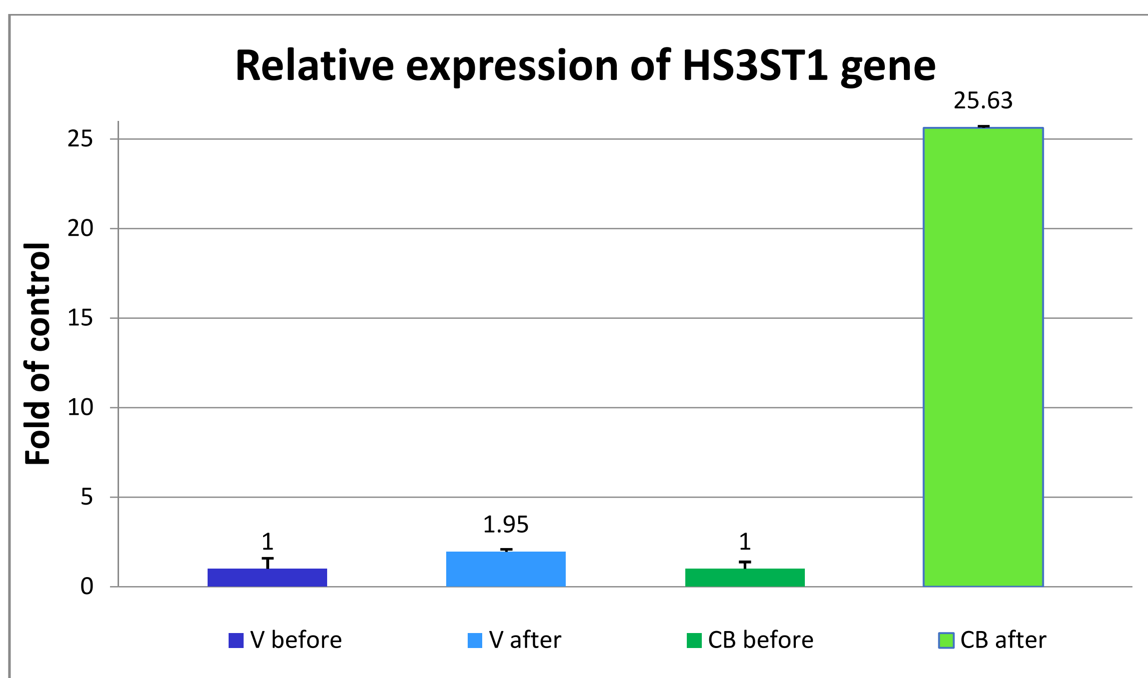

Figure 3: Relative expression of *HS3ST1* following *R. annulatus* challenge