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Expression profilingof Ecto-NOX Disulfide-Thiol Exchanger 2 (*ENOX2*) gene during heat stress in Attappady black and Malabari goats[#]

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Abstract

Thermal stress is one of the major factors negatively influencing livestock production. Goats are known for their ability to adapt to adverse environmental conditions as they possess unique adaptive traits due to behavioural, morphological, physiological, and largely genetic bases. There is a high need to select goats that are suited to a wide range of geographical and harsh environments. The present study was carried out to analyse the expression profile of ENOX2 gene in heat stress susceptible (HSS) and heat stress tolerant (HST) individuals in Attappady black and Malabari goat breeds. The temperature humidity index (THI) was calculated during the period of the experiment. Total RNA was isolated from the whole blood of HST and HSS groups of both breeds. The relative expression pattern of ENOX2 gene showed 1.74 and 5.27 fold higher expression when compared to the control (HST) in Attappady black and Malabari goats, respectively.

Keywords: *ENOX2, HST, HSS, fold change*

Heat tolerance (HT) is a complex trait that is regulated by multiple quantitative trait loci (QTLs). There are several candidate genes that play major roles in the adaptation mechanisms of goats to heat stress (Dikmen *et al.,* 2012). Among these candidate genes, *ENOX2* gene plays the role of improving the long-term heat stress resistance in goats (Kaushik *et al.,* 2016).

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The *ENOX2* gene encodes the protein ecto-nox disulfide-thiol exchanger 2, a tumour specific member of ECTO NOX family of cell surface NADH oxidases. The protein is a tumour specific member of the Ecto-Nox family and it has two enzymatic activities, *viz.* protein disulfide interchange and hydroquinone catalysis of oxidation of NADH (Tang *et al.,* 2014). The NOX proteins regulate a wide range of physiological functions such as cell growth, apoptosis, cytoskeleton remodelling and play a significant role in host defence (Morre *et al.,* 2000; James and Morre, 2003; Cave *et al.,* 2006; Rada *et al.,* 2008). The *ENOX2* gene in goat is 82543bp in length (accession no. NW 017189517) and is located on the X chromosome with 13 exons. The NOX produced ROS carry regulatory function in cytoskeletal remodelling, gene expression, proliferation, differentiation, migration, and cell death (Brown and Griendling, 2009; Morre and Morre, 2012). The present study was planned to analyse the expression profile of *ENOX2* gene in heat stress tolerant (HST) and heat stress susceptible (HSS) animals of Attappady black and Malabari goats in response to heat stress.

Materials and methods

The experiment was carried out on 12 Attappady black and 12 Malabari goats maintained at the University Goat and Sheep Farm, Mannuthy, during the months of March and April.

Recording of physiological and environmental parameters

The physiological parameters such as heart rate, respiratory rate and rectal temperature were recorded before and after grazing (at 7 AM and 3 PM). The ambient temperature and relative humidity were recorded using a digital temperature and a humidity meter. The temperature-humidity index (THI) was calculated using the formula $THI = T_{air}\,T_{air} - (0.55 - [0.55 \times RH/100]) \times (T_{air}\,T_{air} - 58)$, where THI = temperature humidity index, $T_{air}\,T_{air}$ = average air temperature in Fahrenheit, RH = average relative humidity in percentage (LPHSI, 1990).

Classification of goats into heat stress tolerant (HST) and heat stress susceptible groups (HSS)

Goats were classified into heat stress tolerant (HST – Rectal Temperature(RT)<38.5 °C, Respiration Rate(RR)<40, Heart Rate(HR)<80) and heat stress susceptible groups (HSS– RT>39 °C, RR>80, HR >95) based on the recorded physiological parameters (Kaushik *et al.,* 2016). The blood samples were collected from 12 adult Attappady black and 12 adult Malabari goats, six samples from HST and 6 six from HSS group.

Quantitative PCR (qPCR)

Three millilitres of blood were collected from the jugular vein of 24 goats in vacutainer tubes coated with an anticoagulant EDTA. Ribonucleic acid (RNA) was extracted using Origin total RNA isolation kit. Isolated RNA was checked for concentration and purity by Nanodrop spectrophotometer. Quality was assessed by one percent agarose gel electrophoresis. Complementary DNA was synthesised from isolated RNA using iScrip t™t™cDNA Synthesis Kit (Bio-Rad, USA). The qPCR was used to find out the relative expression of *ENOX2* gene in blood samples of heat stress tolerant and heat stress susceptible goats from Attappady black and Malabari breeds. The relative quantification of gene expression was carried out using Illumina Eco® Q- RT PCR system using SYBR green chemistry.

Primers were custom synthesised using primer3 V.0.4.0 software (Untergasser *et.al.,* 2012) and a gradient PCR was carried out to fix the optimum annealing temperature and specific amplification was detected by running samples on 2.5 percent agarose gel using a molecular weight marker of 100 bp size. The primer sequences used for qRT-PCR for *ENOX2* and *GAPDH* are listed in Table 1.

The qRT-PCR was carried out using Maxima SYBR Green qPCR Master Mix, forward and reverse primers and cDNA. Thermal profile of reactions for both *ENOX2* and *GAPDH* was 95°C for 8min, 95°C for 10s, 56°C for 15s, 72°C for 20s. Dissociation (melt) curve analysis was

Table 1. Sequences and properties of primers designed for Quantitative Real Time PCR

Sl. No.	Name	Accession No.	Sequence (5'- 3')	Product size (bp)
1	*ENOX2* F	XM_018044266.1	CTCCGGCCTTCTGACAAAGT	263
	ENOX2 R		GCAGGACTTCTGACTGGTCC	
2	GAPDH F	XM_005680968	TGGAGAAACCTGCCAAGTATG	127
	GAPDH R		TGAGTGTCGCTGTTGAAGTC	

done after each PCR. The protocol for melt curve analysis was 95°C for 15s, 55°C for 15s followed by 95°C for 15s. Data acquisition was performed during the final denaturation step.

Relative quantification was performed by $2^{-\Delta\Delta C_T}$ $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Cycle threshold (C_T) values was the number of PCR cycles required for the fluorescence signal to cross the threshold line, gene expression levels were quantified with C_t values as raw data for real-time PCR. The RT-PCR was normalized to a reference gene (*GAPDH*). The heat tolerant group was selected as the control for expression analysis. Statistical comparison between relative quantification (RQ) values between different samples was done using the Analysis of variance (ANOVA) and independent sample t-test (SPSS V.21). Duncan's multiple range test was used to test the significance of differences between sub classes at 95 per cent ($p<0.05$) confidence interval.

Results and discussion

The maximum ambient temperature (°C), relative humidity (%) and temperature humidity index recorded were 30.185±0.14, 92.44±1.52 and 85.44, respectively. Minimum ambient temperature, relative humidity and temperature humidity index recorded were 28.363±0.27, 85.68±3.99 and 79.43, respectively. The mean ambient temperature was 29.24±0.25 (°C). Relative humidity ranged from 71.58 to 97.03% and the peak THI (85.44) was recorded during the 6th week (2nd week of April) of the experiment.

Relative expression of caprine ENOX2 gene in different heat stress groups

The mean C_T values and calculated ΔC_T, $\Delta\Delta C_T$ with fold change ($2^{-\Delta\Delta C_T}$) values of *ENOX2, GAPDH* genes for HST and HSS groups are mentioned in Table 2. Heat tolerant group was

Table 2. Relative expression of caprine *ENOX2* gene in heat stress tolerant and heat stress susceptible groups of Attappady black and Malabari goats

Breed	Group	Mean C_T ±SE		ΔC_T±SE	$\Delta\Delta C_T$±SE	Fold change from control ($2^{-\Delta\Delta C_T}$)
		ENOX2	*GAPDH*			
Attappady black (n-12)	Heat stress tolerant (control)	35.12±0.37	27.84±0.54	7.30±0.39	0±0.39	1[a]
	Heat stress susceptible	33.52±0.54	27.02±0.27	6.50±0.58	-0.8±0.58	1.74[b]
Malabari (n-12)	Heat stress tolerant (control)	36.37±0.78	28.95±0.92	7.42±0.61	0±0.92	1[a]
	Heat stress Susceptible	34.49±0.92	29.47±0.61	5.02±0.31	-2.4±0.61	5.27[b]
Overall (n-24)	Heat stress tolerant (control)	35.39±1.38	28.79±0.75	6.6±1.11	0±1.11	1[a]
	Heat stress susceptible	33.91±0.59	27.91±0.76	6±0.63	-0.6±0.63	1.52[b]

Values with different superscripts differs significantly ($p<0.01$)

taken as the control. Level of expression of *ENOX2* was 1.74 fold and 5.27 fold higher in the HSS group of Attappady black and Malabari goats, respectively. Relative expression was significantly ($p<0.01$) high in the heat stress susceptible (HSS) group when compared to the heat stress tolerant (HST) group. Expression of *ENOX2* was found to be significantly low in Attappady black goats when compared to Malabari goats ($p<0.01$).

Kaushik *et al.* (2016) studied the expression profile of *ENOX2* gene in different tissues of HST and HSS goats of native Barbari, Jamunapari, Jakhrana and Sirohi breeds. They reported 12.45 and 5.41-fold higher *ENOX2* gene expressions in Heat stress-susceptible (HSS) goats when compared to heat stress-tolerant (HST) goats during thermal stress. The significantly ($p<0.01$) high expression of *ENOX2* gene observed in heat stress susceptible (HSS) group in Attappaddy and Malabari breeds **are** in accordance with the above results reported in other Indian breeds. The level of expression of *ENOX2* gene in the HST group is significantly ($p<0.01$) low in Attappady black goats when compared to Malabari goats. The results indicate the significant down regulation of *ENOX2* gene in HST animals. Among breeds, Attappady black goats were found to be more heat tolerant as these animals showed a down regulation of 2.99 fold *ENOX2* gene when compared to Malabari goats (Table.3).

Conclusion

From the present study, we can conclude that Attappady black goats are relatively more heat tolerant than Malabari goats. The result indicated the potential role of *ENOX2* gene on heat resilience. Hence, it may be used as a candidate gene for heat tolerance in goats.

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Conflict of interest

The authors report no conflict of interest.

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Table 3. Relative expression of caprine *ENOX2* gene in heat stress tolerant groups of Attappady black and Malabari goats

Breed	Mean C_T ±SE		ΔC_T±SE	$\Delta\Delta C_T$±SE	$2^{-\Delta\Delta C}{}_T$
	ENOX2	*GAPDH*			
Attappady black	35.09±2.21	27.70±1.01	7.39±1.86	0±1.86	1 [a]
Malabari	35.69±1.87	29.88±0.99	5.81±1.28	-1.58±1.28	2.99 [b]

Values with different superscripts differs significantly ($p<0.01$)

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