IMPACT OF HEAT STRESS ON DIFFERENT TOLL LIKE RECEPTORS EXPRESSION PATTERNS IN TWO DIFFERENT INDIGENOUS GOAT BREEDS

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CERTIFICATE

This is to certify that the thesis entitled ***"IMPACT OF HEAT STRESS ON DIFFERENT TOLL LIKE RECEPTORS EXPRESSION PATTERNS IN TWO DIFFERENT INDIGENOUS GOAT BREEDS''*** submitted by **Ms. SAVITHA S.T, I.D. No. DVHK 1605** in partial fulfillment of the requirements for the award of **DOCTOR OF PHILOSOPHY** in **VETERINARY BIOCHEMISTRY** of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar is a record of bonafide research work carried out by her during the period of her study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APC	Antigen presenting cells
bp	Basepair
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CD62L	Cluster of differentiation 62 Ligand
CPCSEA	Committee for the purpose of control and supervision of experiments on animals
CRH	Corticotropin releasing hormone
DAMPs	Damage associated molecular patterns
°C	Degree Celsius
Δ	Delta
DC	Dendritic cell
DNase	Deoxyribonuclease
dNTP	deoxy ribonucleotide triphosphate
E	East
ELU	Experimental livestock unit
ER	Endoplasmic reticulum
ET	Enterotoxaemia
et al	et alia
F	Forward
FAO	Food and agriculture organization
Fig	Figure
g	Relative centrifugal force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Gross domestic product

GI	Group I
GII	Group II
GIII	Group III
GIV	Group IV
GRP	Glucose regulated protein
h	Hour
HPA	Hypothalamo pituitary adrenal axis
HSF	Heat shock factor
HSP	Heat shock protein
5-HT	5-Hydroxy tryptamine
IACUC	Institutional animal care and use committee
IAEC	Institutional animal ethical committee
ICAR	Indian council of agricultural research
IgA	Immunoglobulin A
IFN	Interferon
IL	Interleukin
IPCC	Intergovernmental panel on climate change
Kg	Kilogram
LN_2	Liquid nitrogen
LXR	Liver X receptor
m	Metre
mg	Milligram
µL	Microlitre
µM	micromolar
µmol	Micromoles
min	Minute
ml	Millilitre

Mac1	Macrophage-1 antigen
MAPK	Mitogen activated protein kinases
MDA	Melanoma differentiation associated
MLN	Mesenteric lymph node
mm	Millimetre
mRNA	Messenger ribonucleic acid
MSU	Monosodium urate
N	North
NA	Nor adrenaline
NCBI	National centre for biotechnology information
NIANP	National institute of animal nutrition and physiology
ng	Nano gram
NOD	Nucleotide binding oligomerization domain
NK cells	Natural killer cells
NLR	NOD like receptor
OC	Osmanabadi Control
OD	Optical density
OHS	Osmanabadi heat stress
PAMPs	Pathogen-associated molecular pattern
PBMC	Polymorpho blood mononuclear cells
PCR	Polymerase chain reaction
%	Percentage
/	Per
PPARD	Peroxisome proliferator-activated receptor delta
PPR	Peste des petits ruminants
PRRs	Pattern recognition receptors
qPCR	Quantitative real-time PCR

R	Reverse
RH	Relative humidity
RIG1	Retinoic acid inducible gene protein 1
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Rectal temperature
RTqPCR	Real time quantitative polymerase chain reaction
RXR	Retenoid X receptor
s	Second
s	Swedberg unit
SAM	Sympathetic adrenal medullary axis
SATB1	Special AT-rich sequence binding protein 1
SBC	Salem black control
SBHS	Salem black heat stress
SE	Satandard error
SPSS	Statistical package for the social sciences
T_{db}	Dry bulb temperature
THI	Temperature humidity index
TLR	Toll–like receptors
TNF	Tumor necrosis factor
T_{wb}	Wet bulb temperature
U/μL	Units per micro litre
USA	United states of America
UV	Ultra violet

Introduction

I. INTRODUCTION

Climate change is the major environmental challenge being experienced by humankind throughout the globe. Climate change negatively affects almost all sectors currently. Intergovernmental panel on climate change reported that, the earth temperature may increase between 1.4 and 4.8^{0}C by the end of this century (IPCC, 2014). Among all climatic variables, heat stress seems to be the most crucial factor which negatively influence the livestock production, reproduction as well as the health status (Weindl *et al.,* 2015).

Among the livestock, small ruminants particularly goats are tipped to be the ideal climate animal model because of its superior thermo-tolerance, ability to survive on low pasture and water resources as well its high disease resistance capacity (Silanikove, 2000; Sophia *et al.,* 2016a; Pragna *et al.,* 2018a). Goats adapt better to the changing climate compared to other ruminant species. This is attributed to their better browsing capability with their anatomical advantage of upper lips and their ability to survive with less feedstuff in harsh climatic conditions in arid and semi-arid regions. So also, goats have the ability to thrive in different agro-ecological zones due to their water conservation ability owing to their speciality in browsing the feed. Therefore, goat species is expected to play a huge role for ensuring future food security. Further, research efforts are also needed to identify the most suitable indigenous breed with a potential to survive in different agro ecological zones. Propagating such animals among the poor and marginal farmers can help to ensure optimum economic return from livestock enterprises (Archana *et al.,* 2018). Among the various environmental stressors, heat stress is the major stressor that affects the immune response in the goats (Sophia *et al.*, 2016b).

The tropical environment in developing countries possess sound animal genetic resources to withstand the climatic extremes. However, over last few decades the indigenous germplasms are depleting particularly in the developing countries due to genetic erosion (Belew *et al.,* 2016). Indigenous breeds should be the primary focus to establish the impact of heat stress on livestock production as these animals are being refined over several generations to adapt to the existing environmental conditions (Aleena *et al.,* 2018; Sejian *et al.*, 2018). Indigenous breeds were established to have resistance capacity of various degrees to withstand high ambient temperature under tropical environmental conditions (Rashid *et al.,* 2013). Therefore, indigenous livestock can be projected as future animals to ensure food security under changing climate scenario (Katiyatiya *et al.,* 2017). Hence, research efforts are needed to identify suitable indigenous livestock breeds which has the ability to survive in different agro-ecological zones.

Heat waves are one of the extreme meteorological events in the changing climate scenario causing extreme heat stress which severely affects the growth of the animals (Bernabucci and Mele, 2014). Particularly, heat stress has negative influence on farm animal productivity and health because of the presence of close relationship between metabolic heat generation and production level (Bernabucci *et al.,* 2010). Heat stress may enhance or suppress the immune function of the animal. Generally, the thermal stress down regulates the innate immune functions like reduction in toll-like receptors

(*TLRs*), secretory IgA and intestinal intraepithelial lymphocytes production (Sophia *et al.*, 2016a). It has been established that glucocorticoids mediate this heat stress associated immune response in goats (Sophia *et al.*, 2016b).

Generally, heat stress negatively affects humoral and cell mediated immunity (Jin *et al.*, 2011). Toll-like receptors are one of the pattern recognition receptors (PRRs) which are found in serum, on the cell surface, in endosomes and in the cytoplasm (Medzhitov, 2001). These *TLRs* are expressed on variety of immune cells such as dendritic cells (DCs), natural killer (NK) cells, T cells, and B cells (Chen and Yu, 2016).

They recognize pathogen associated molecular pattern (PAMPs) which are expressed by the pathogens and danger associated molecular patterns (DAMPs) which are the components of damaged or apoptosed cells that act as endogenous stress signals (Vidya *et al.*, 2017). Moreover, presence of these *TLRs* in organs and their expression pattern in response to specific stimuli is one of the factor~~s~~ determining the disease resistance capability of an animal (Tirumurugaan *et al.*, 2010). Further, Paul *et al.* (2015) suggested that, goats under heat stress state showed significant up regulation of few *TLRs*, which play an important role in stimulating the innate immune response.

Although there are several reports available pertaining to establishing the impact of climate change associated heat stress on production and adaptation, still such efforts are very scanty in establishing its impact on immune status in livestock (Chauhan *et al.*, 2014; Sophia *et al.*, 2016a). In particular, research efforts of elucidating the molecular mechanisms related to heat stress influence on immune system are negligible. Therefore, sufficient research efforts are needed to establish the impact of heat stress on immune related functions to identify the intervening points to ameliorate its negative impact on immune functions. With these backgrounds, the current study was designed to comparatively assess the impact of summer season induced heat stress on the immune response related genes in two different indigenous Osmanabadi and Salem Black goat breeds. The primary objective of the study was to elucidate the impact of heat stress on the different intracellular *TLRs* expression pattern in both these goat breeds.

Currently sufficient research reports are available pertaining to establishing the impact of heat stress on both adaptation and production. However, such reports establishing heat stress impact on immune status particularly in the indigenous livestock population are very limited. Therefore, a study was designed in two different indigenous Osmanabadi and Salem Black goat breeds to comparatively assess their resilience capacity to heat stress based on their ability to maintain their immune status during exposure to adverse environmental conditions. A study was designed in two different indigenous goat breeds namely, Osmanabadi and Salem Black with the following objectives:

1. Assessing the expression patterns of different *TLRs* in heat stressed Osmanabadi goats.
2. To identify the expression patterns of different *TLRs* in heat stressed Salem Black goats.
3. Comparing the expression patterns of different *TLRs* between Osmanabadi goats and Salem Black goats to evaluate their immune status during heat stress.

Review of Literature

II. REVIEW OF LITERATURE

2.1 Introduction

There is a growing concern throughout the globe about the continuous increase in warming of earth's temperature since last three decades (IPCC, 2014). According to the latest study, the earth has experienced its third consecutive hottest year in 2016 (Greenfieldboyce, 2017). Further, IPCC projects the global earth temperature to increase to the tune of 2.7-4.8^{0}C by the end of this century (IPCC, 2014). The statistical significant variation in either the mean state of the climate or in its variability may persist for an extended period which may take typically decades or longer is considered as climate change. The natural internal processes or external forcing or persistent anthropogenic changes in the composition of the atmosphere or in land-use may lead to climate change. Climate change is of scientific curiosity that is no longer just one of many environmental and regulatory concerns (Vijaya *et al.*, 2012). The global average mean annual air temperature and variations in regional precipitation are due to climate change effect and these changes are expected to continue and may reinforce in future (Misra, 2014).

Heat stress is the sum of external forces acting on an animal that causes rise in body temperature and activates series of physiological responses (Polsky and von Keyserlingk, 2017). Heat stress has direct effect by impairment of productive and reproductive performance as well as animal health and welfare (St-Pierre *et al.,* 2003; Jin *et al.,* 2011; Weindl *et al.,* 2015). Generally, the animals respond to stress mainly through hypothalamo-pituitary-adrenal axis (HPA axis) of neuroendocrine system through the release of glucocorticoids which are the primary stress hormones (Sophia *et al.*, 2016a). Stress has effect on both innate and adaptive immune response in animals (Marketon and Glaser, 2008). Heat stress can down regulate the innate immune components like mucosal barrier, toll like receptors, secretory IgA, intestinal intraepithelial lymphocytes production (Deng *et al.*, 2012; Sophia *et al.*, 2016a), and expression of cytokines, responsible for humoral and cell mediated immune response in intestine.

A recent study reported that, around 20–30% of livestock are expected to be at risk of extinction (Al-Dawood, 2017) because of changes in weather and climate events. This may result in loss of specific gene pool which evolved from long term evolution thereby disturbing the ecological niche. Moreover, it has been proved that, exotic breeds and their crosses with indigenous breeds have lower upper critical temperature when compared with pure indigenous breeds (NAAS, 2016). According to Food and Agriculture Organization (FAO), there is a clear decline in the populations of indigenous breeds worldwide (FAO, 2015). Further, the livestock biodiversity conservation is strongly dependent on agricultural practices and management systems (Marsoner *et al.*, 2018). Hence the sustainable livestock productivity needs to be improved over the next 40 years in order to meet the world’s demand for agricultural products (FAO, 2011). Therefore, there is an urgent need to conserve the indigenous germplasms which possess the inherent ability to adapt to changing climate scenario.

Goats are multi-purpose animals because they can produce meat, milk, hide, fibre and also manure (Silanikove, 2000). Production per unit of investment is more in goats.

Goats can tolerate hot climatic condition and can thrive well in different agro-ecological zones compared to other farm animals and this could be attributed to their better thermo-tolerance, drought tolerance, ability to thrive on limited pastures as well as their high disease resistance capacity (Aleena *et al.,* 2018; Savitha *et al.,* 2019; Rashamol *et al.,* 2019). Although they are better disease resistant animals, still breed differences was reported on this particular aspect and heat stress was reported to impairs immune function to various degrees in different goat breeds (Al-Dawood, 2017; Vandana *et al.,* 2019; Rashamol *et al.,* 2019).

The pathogen associated molecular patterns (PAMPs) are the components of pathogens that are the key elements in initiation of innate immune response (Medzhitov and Janeway, 1997). The primary evolutionarily conserved mechanisms are brought through innate immunity that enable the differentiation between self and non-self-components through pattern recognition receptors (PRRs). Toll like receptors (*TLRs*) are one among the pattern recognition receptors (PRRs) and they have the ability to recognize the pathogens through pathogen associated molecular patterns (PAMPs) (Paul *et al.,* 2015). These receptors are present in important organs and their expression in response to particular stimuli is one of the factor~~s~~ determining the disease resistance capability of an animal (Sophia *et al.*, 2016a). Upon recognition, signalling cascade will trigger for transcription of proinflammatory and immunomodulatory factors which initiate both innate and adaptive immunity (Carmody and Chen., 2007).

The relevant information about heat stress impact on immune response and particularly on different *TLR* expression pattern~~s~~ of goats are very negligible. Heat stress has influence on expression of *TLR*'s in immune cells such that they modulate immune responsiveness to PAMPs to the full activation of innate and adaptive immune system to fight against pathogenic microorganisms in goats (Sophia *et al.*, 2016b; Vandana *et al.*, 2019). Stress stimuli had increased the expression of most of the *TLRs* in goats as well as in Tharparkar cattle and thereby altered their immune functions (Bharati *et al.*, 2017). It has been proved that, glucocorticoids will mediate the heat stress response in goats (Sophia *et al.*, 2016a) and cattle (Farooq *et al.*, 2010).

2.2 Climate change and Livestock production

Climate change and livestock production are considered to be very unique because of the dual role of livestock by contributing to the phenomenon through release of greenhouse gases (GHGs) and also getting affected by the already changing climate. Climate change is the long-term misbalance of temperature, wind and rainfall which are features of a specific region (Hidosa and Guyo, 2017). Global climate change is fundamentally caused by GHG emissions that result in warming of the atmosphere (IPCC, 2014; Rojas-Downing *et al.*, 2017). Global climate change is mainly attributed to the emission of GHGs which are carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). Among these GHGs, the potentiality of causing global warming by CH_4 and N_2O are approximately 23 and 300 times that of CO_2, respectively (Rust and Rust, 2013). Livestock sector contributes 40% to the agricultural Gross Domestic Product (GDP) in developing countries (FAO, 2009 and Sejian *et al.*, 2016). Generally, in developing countries, the demand by humans for animal products is increasing due to the continuous growth in the population and per capita consumption. These countries are facing a loss of about 25% of animal production through global warming (Seguin *et al.,* 2008; Nardone *et al.,* 2010).

The impact of climate change is expected to increase the vulnerability of livestock systems and reinforce existing factors that are affecting livestock production system~~s~~. Climate change has both direct and indirect effect on livestock. The direct effects are mediated through the alterations in air temperature, humidity, wind speed and other climate factors on the animal performance which includes growth, milk production, meat production as well as reproduction (Houghton, 2001; Rust and Rust, 2013). The indirect effects are through limited feed and water resources and sudden outbreak of diseases (Sejian *et al.,* 2018). By far the productive loses are incurred through the indirect pathways by altering the carrying capacity of rangelands, the buffering ability of ecosystems and their sustainability, and the distribution of livestock diseases and parasites (Thornton *et al*., 2007). The adverse impact of climate change on animals include decreased animal weight gain, lower reproduction rates and lower feed conversion rates in tropical regions. High temperature and humidity resulting from climate change increases the rate of development of lifecycle of parasites and pathogens (Mashaly *et al.,* 2004; Hidosa and Guyo, 2017). Climate change also mediates its impact through heat stress on both mortality and morbidity as well as on the incidence of climate-sensitive infectious diseases (Patz *et al.,* 2005; Rust and Rust, 2013).

2.3 Different factors that influence livestock production

Genetics, management and environmental factors affect the livestock production (Adediran *et al.,* 2010; Gaughan *et al.,* 2012; Sejian *et al.,* 2018). In addition, strain, nutrition, age, liveweight and sex have also been found to influence different productive responses in livestock (Abdullah *et al.*, 2010; Renaudeau *et al.*, 2012). Genetics is the major component for variation in livestock performance traits such as growth, milk production, meat production, reproduction and immune status (Berry *et al.,* 2014; Pragna *et al.,* 2018b; Archana *et al.,* 2018; Amitha *et al.,* 2019; Rashamol *et al.,* 2019; Friedrich *et al.,* 2015; Lalit *et al.,* 2016). Apart from sustaining the production, the animals need to be adapted to a particular environment to tag them as climate resilient animals/breeds. Several studies established breed differences for adaptive abilities of livestock (Rout *et al.,* 2016; Aleena *et al.,* 2018). Further, age and sex are also considered crucial other factors which influence livestock production and reproduction (Cameron *et al.,* 2001; Titi *et al.,* 2008; Do *et al.,* 2013). Similarly, these factors are also were found to influence the immune responses in different livestock species (Chowdhury *et al.,* 2015; Kihu *et al.,* 2015; Matos *et al.,* 2018). The components of climate includes temperature, humidity, rainfall, air movement, radiation, barometric pressure, and ionization (West, 2003). Climate change poses a major threat to the survival of many species, ecosystems and also for the sustenance of livestock production systems globally (Sejian *et al.,* 2018). Higher quantity and good nutritional quality pastures are available during rainy season whereas dry season’s pastures have poor nutritional quality with high fiber and low protein contents, which often results in decreasing the animal production (Butterworth, 1984; Fereja., 2016).

Nutrition is also one of the primary factors which influence all productive and adaptive responses of livestock (Sejian *et al.,* 2010; Sejian *et al.,* 2011; Shaji et al., 2017). The concentrate level of diet and nature of specific concentrate and forage feedstuffs~~,~~

impact level of milk production and characteristics of milk and milk products (Goetsch *et al.,* 2011; Idowu and Adewumi, 2017). Further, the energy component of the diet was found to influence milk production in high yielding dairy goats (Park and Haenlein, 2010; Idowu and Adewumi., 2017). Nutritional state is found to influence the ovulation rate, reproductive activity and other endocrine functions in many species including sheep (Naqvi *et al.,* 2013). Reproductive function at different levels of the hypothalamo-pituitary-gonadal axis is influenced by undernutrition in ruminants (Sejian., 2013).

2.4 Heat stress as the most crucial climatic factor affecting livestock

Heat stress has detrimental effects on livestock production. Usually, high producing animals are more sensitive to increased temperature and humidity (Rust and Rust., 2013). Moreover, more than 50% of the ruminant population are located in the tropics and it has been estimated that heat stress may cause an economic loss in 60% of the dairy farms around the world (Wolfenson *et al.,* 2000; Nardone *et al*., 2010). Heat stress can affect reproductive performance (Dahl *et al.,* 2017), mortality (Crescio *et al.,* 2010), water balance (Dahl *et al.,* 2017), as well as intake and utilization of nutrients (Bernabucci *et al.,* 2010). Heat stress also can lead to decreased gestation, reduced calving/lambing/kidding% and lower birth weight of calves/lambs/kids respectively (Tao and Dahl, 2013; Sejian *et al.,* 2012; Mabjeesh *et al.,* 2013; Karimi *et al.,* 2015). The indigenous animals can adapt to heat stress challenges but they do so by compromising their production as adaptation is biologically a very costly process and requires regular supply of energy for maintaining vital body functions (Sejian *et al.,* 2018).

2.5 Economic consequences of heat stress impact on livestock production

The world's agricultural Gross Domestic Product (GDP) about 40% is from the livestock sector and about 1.3 billion population of the world depends on animal husbandry for their livelihood (FAO, 2006). Wolfenson *et al.* (2000) reported that more than 50% of the bovine population is located in the tropics and about 60% of the dairy farms around the world are affected by heat stress causing severe economic losses. St. Pierre *et al.* (2003) reported that, increased temperature and humidity is inversely proportional to feed intake thus leading to decreased milk production. Heat stress causes significant economic burden to the dairy industry of about \$900 million per year (Baumgard and Rhoads, 2012). Heat stress caused increased mortality in dairy industry resulting in severe economic losses in Italy (Nardone *et al.*, 2010). Heat wave in 1995 caused severe economic losses to the extent of \$31 million in Iowa alone. The extreme heat stress has caused annual economic loss in dairy cattle of about \$800 million dollars to the US dairy industry (St-Pierre *et al.*, 2003). Further, St-Pierre *et al.* (2003) also established that the estimated cost of annual economic loss to be 897-1,500 million dollars to US dairy industry due to impact of heat stress on dairy cattle. Key *et al.* (2014) reported that, annual milk production in the average dairy was reduced due to heat wave and the loss was about \$39,000, totalling \$1.2 billion loss of production for the entire US dairy sector. It is estimated that there will be decrease of 0.85 kg in feed intake which causes ~36% reduction in milk production for every 1°C in air temperature above thermal neutral zone (West, 2003; Rhoads *et al.*, 2009). The annual economic loss is between 1.69 and 2.36 billion US dollars due to heat stress to the United States livestock industry, of which 50% occurs in the dairy industry alone (St-Pierre *et al.*, 2003).

2.6 Heat stress and immune system relationship in livestock

Stress is a constellation of events, comprises of stimulus (stressor), that precipitates a reaction in the brain (stress perception), that in turn activates biological stress response which is referred to as physiological fight-or-flight systems of the body (Dhabhar *et al.*, 2012). Selye, (1936) was the first person to introduce concept of stress and he putforth stress is a non-specific response of the body to any demand and/or change. The most accepted definitions of stress in humans and animals is “the biological response elicited when an individual recognizes a threat to its homeostasis” (Martínez-Miró *et al.,* 2016). Carroll and Burdick (2013) reported that prolonged stress causes deleterious effects on the immune system and health of livestock. The heat stressed birds showed alteration in behavioural, physiological and immunological responses which inflict unfavourable effect on their overall productivity, which is more pronounced during summer seasons (Mazzi *et al.*, 2003; Lara and Rostagno, 2013). Bird’s body has various physiological changes to maintain body temperature causing decreased immune response during summer stress condition (Pawar *et al.*, 2016). Chronic stress has deleterious effect. But response to stress has salubrious adaptive effects in the short run (Dhabhar *et al.*, 2012).

Central nervous system (CNS) takes a role in diversifying the immune response by complex bi-directional networks which involve nervous, endocrine and immune systems. The two pathways alter the immune response mainly through hypothalamic-pituitary-adrenal (HPA) and sympathetic-adrenal medullar (SAM) axes (Lara and Rostagno, 2013). The immune cells interact with each other through production of cytokines and immune cells also possess receptors for hormones, thereby hormones can alter immune function upon binding to their respective receptors. The alteration in the cytokine production has feedback regulation on brain leading to changes in the HPA axis, thus inducing sickness behaviour such as fever, loss of appetite, changes in sleep patterns and depression. Feedback mechanism is through the impact of interleukin-1 (IL-1) due to production of corticotropin releasing hormone (CRH) by hypothalamus. This CRH stimulate HPA axis to produce stress hormones. This networking is very complex to understand the body interactions (Glaser and Kiecolt-Glaser, 2005).

2.6.1 Sympathetic-adrenal-medullary axis response (SAM)

Generally, sympathetic nervous system releases catecholamines namely epinephrine and norepinephrine by chromaffin cells of the adrenal medulla upon activation and prepare the animal for a fight or flight response (Mifsud *et al.*, 2011). Further, stress also was shown to elevate the levels of neurotransmitters such as serotonin or 5-Hydroxy tryptamine (5-HT) and noradrenaline (NA) in notable brain regions (i.e. hypothalamus, hippocampus) (Mifsud *et al.*, 2011). Increases in heart rate and blood pressure are the result of this activation. Other changes include constriction of the intestine and skin vessels, dilatation of skeletal muscle vessels, dilatation of the pupils, or release of glucose and lipids from the liver are also observed (Martínez-Miró *et al.*, 2016). These changes are essential for animal to face the stressful stimulus or to escape from it (Martínez-Miró *et al.*, 2016).

2.6.2 Hypothalamic-pituitary-adrenal axis response (HPA)

NA and 5-HT act on the hypothalamus to activate the HPA axis resulting in the release of glucocorticoids from the adrenal cortex into the circulation (Heisler *et al.*, 2007). The increase in extracellular 5-HT in the hippocampus is based on the nature of the stressor it exposed to and its adaptation level (Barr and Forster, 2011). Mifsud *et al.* (2011) reported that, HPA pathway is activated by physical and psychological stressors. Apart from that, many studies have proved that the body responds to the different types of stress in distinct ways (Asres and Amha, 2014; Lees *et al.*, 2019).

Martínez-Miró *et al.* (2016) reported that, upon activation by the stressor, hypothalamus releases corticotropin-releasing hormone this in-turn acts on anterior pituitary to release adrenocorticotropic hormone (ACTH) by the anterior pituitary gland and ACTH acts on adrenal cortex to release glucocorticoids which in-turn acts on tissues to catabolise protein and fat to produce glucose. High level of cortisol have been associated to reduced rates of reproduction, suboptimal growth, decreased milk production and repression of immune function that could raise the condition for susceptibility to disease (Dobson and Smith., 2000; Carroll and Burdick Sanchez, 2013).

2.6.3 Immune system response

The immune response elicited depends on the mediator released in the process of stress: catecholamines or glucocorticoids (Dhabhar, 2009).

Martínez-Miró *et al.* (2016) reported that, at the beginning of stress catecholamines are released upon stressful stimulus thereby produce high level of leukocytes (mainly granulocytes and lymphocytes), which are released into the systemic circulation. On the other hand, when stressful stimuli are prolonged mainly cortisol will be produced and released in to the system thereby found decreased number of lymphocytes in blood and glucocorticoids can also decrease the production of cytokines and immunoglobulins (Ig).

Contact between the lymphoid and nervous systems as well as the presence of these receptors for endocrine hormones which disclose the existence of pathways of communication between the immune system, the nervous system and the endocrine system (Pedersen and Hoffman-Goetz, 2000). Heat stressed broilers and layers showed reduced relative weights of thymus and spleen which indicates immunosuppressive effect of heat stress while the laying hens showed decreased liver weights (Pawar *et al.*, 2016). Decreased levels of total circulating antibodies including specific IgM and IgG levels, both during primary and secondary humoral responses in broilers under heat stress condition has been reported by Pawar *et al.* (2016). Further, Aengwanich (2008) reported, heat stressed broilers showed decreased bursa weight along with decreased lymphocytes numbers in cortex and medulla of bursa. However, there are poor data on the understanding of immune response to heat stress in livestock with reference to genetic and cellular mechanisms and this warrants more research efforts in this novel researchable area.

2.7 Stress and innate immune response in livestock

Stress significantly affect immune cell distribution and function and this effect is primarily mediated by the release of glucocorticoids (Chrousos, 2010; Sophia *et al.*, 2016a). Stress hormones have role in influencing the adhesion cascade by the expression pattern of leukocyte CD62L (Dhabhar *et al.*, 2012).

The vital organ brain is one of the major site for immune cell traffic (Miller, 2010), and where immune cells play a role in facilitating homeostasis, neurogenesis, and function (Ziv *et al.*, 2006), may also be a target of stress-induced leukocyte redistribution. This particular enhancement of immune function has been observed in mice, rats, hamsters, and humans (Dhabhar *et al.*, 2012). Acute stress enhances the immune cell numbers in specific compartments and also the leukocyte function and therefore increases immunity (Viswanathan *et al.*, 2005). Further studies have been putforth that, expression of cell-surface protein CD62L mRNA by neutrophil was suppressed by glucocorticoid hormones action that is correlated with a glucocorticoid-induced neutrophilia (Weber *et al.*, 2001 and Dhabhar *et al.*, 2012) while inflammatory/classical (CD62L+) monocytes showed similar effect such as mobilization and trafficking during stress (Gordon and Taylor, 2005; Tacke and Randolph, 2006). In humans, PBMC chemotaxis and adhesion molecule (Mac-1) expressions were also increased by acute stress (Dhabhar *et al.*, 2012).

2.7.1 Heat stress and innate immunity

Generally, heat stress has negative influence that is reducing the weights of lymphoid organs like spleen, thymus and cloacal bursa (Aengwanich, 2008). Heat stressed poultry birds showed mild acute lymphocytic enteritis (Quinteiro-Filho *et al.*, 2010). The calves born to acute heat stress (HS) exposed cows for 1 wk at 3 wk before calving showed reduced expression of tumor necrosis factor-α and *TLR2* and higher expression levels of IL-1β, IL-1 receptor antagonist (IL-1RA), and *TLR4*, higher percentage of CD18(+) cells and neutrophils and lesser percentage of lymphocytes compared with the calves born to evaporative cooling (CT) exposed cows for 1 wk at 3 wk before calving/late gestational period (Strong *et al.*, 2015). The cows exposed to heat stress for 46 days of drying period before calving exhibited increased expression of mRNA of insulin growth factor1 (IGF1) and tumour necrosis factor (TNF) and upregulation of IL8 mRNA expression at 2 day relative to calving in peripheral blood mononuclear cells (PBMC) compared to cows exposed to cooling during 46 days of dry period (Tao *et al.,* 2013). Usage of cortisol or the synthetic glucocorticoids for eg. hydrocortisone, dexamethasone or prednisolone in cattle (Anderson *et al.,* 1999; Davis *et al.,* 2008), sows (Kranendonk *et al.,* 2005; Davis *et al.,* 2008), horses (Burguez *et al.,* 1983; Davis *et al.,* 2008), rats (Cox and Ford 1982; Davis *et al.,* 2008), mice (Van Dijk *et al.,* 1979; Davis *et al.,* 2008), guinea pigs (Fauci, 1975; Davis *et al.,* 2008) and humans (e.g. Fauci and Dale 1974; Dale *et al.,* 1975; Davis *et al.,* 2008) results in neutrophilia or lymphopenia, or both.

2.7.2 Heat stress and mucosal immunity

Under heat stress condition, the blood flow to intestine, integrity of intestinal epithelium, villi height and crypt depth decreases as well as causes desquamation of villi (Yu *et al.,* 2013). In intestine heat stress significantly reduces the innate immune components such as mucosal barrier, secretory IgA, intestinal intraepithelial lymphocytes production and down regulates the expression of *TLRs* and cytokines. Thus, ultimately heat stress was responsible for reducing both the humoral and cell mediated immune response (Deng *et al.,* 2012). Reduction of immune functions in intestine leads to translocation of bacterial infection to mesenteric lymph node (Liu *et al.,* 2012; Sophia *et al.,* 2016a). Additionally, heat stress directly alters jejunal tight junction proteins resulting in impaired intestinal barrier in bovine (Koch *et al.,* 2019).

2.7.3 Mechanism of inhibition of innate immunity

Glucocorticoids under stress conditions act to impede the pro-inflammatory cytokines, namely TNF-α, IL-6, IL-8, which are required to initiate an innate immune response through inhibition of p38 MAPK pathway which helps in maintaining their stability (Abraham *et al.*, 2006). GCs increase IL-10 which is an anti-inflammatory cytokine and is normally found at the end of immune response (Marchant *et al.*, 1994). Catecholamines acts through adrenergic receptors to increase cAMP levels that will in turn decrease the cytotoxic functions of NK cells (Whalen and Bankhurst, 1990). Suppression of NK cell activity due to hyperthermia is by the inhibition of perforin and granzymes (Koga *et al.*, 2005; Sophia *et al.*, 2016a).

2.8 Stress and adaptive immunity

The Th1 cells are concerned with cell mediated immune response and Th2 cells are involved in humoral immune response (Kaiko *et al.*, 2008). Alteration of immune function of Th1 to Th2 and vice versa is due to modulation of cytokine gene expression. Th1 cells secrete IFN-γ, IL 2, TNF β which contributes to cellular immunity. Th2 cells secrete IL-4, IL-10 and IL-13, which contribute to humoral immunity. IL-12 in association with IFN-gamma, converts naive T helper (Th0) cells to Th1 cells, whereas cytokines IL-4 and IL-10 induce Th2 cell production (Kidd, 2003; Roitt and Delves, 2001). Th1 and Th2 cell mediated together are inhibitory to each other (Elenkov and Chrousos, 1999). Potent and major antigen presenting cells (APCs) are the dendritic cells which are the bridge between innate and adaptive immune response as they are involved in phagocytosis and antigen presentation. Further, dendritic cells express more MHC II and Co-stimulatory molecules like CD80, CD83 and CD86 on their cell surface. The expression of these molecules on dendritic cells (DCs) were inhibited by glucocorticoids and thereby prevent its maturation (Girndt *et al.,* 1998).

2.9 Genes associated with immune functions during heat stress

Acute stress is beneficial in poultry whereas, chronic stress switches the T helper cell response to T regulatory cell and also TGF-β, regulatory cytokine production, thereby it suppresses immune response (Shini *et al.,* 2010). The expression of MHC class II and Co-stimulatory molecules like CD40, CD80 and CD86 by antigen presenting cells were reduced thereby delaying the DC maturation (Jin *et al.,* 2011). During heat stress condition the cytokine genes for both Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10) cell responses were down regulated. Further, acute heat stressed cattle showed up regulation of IL-17, which is a cytokine associated with innate immune response up to 48 hrs after heat stress (Sophia *et al.,* 2016a).

Under heat stress condition, humoral immune response (Cd83, HSPA1A & IL1A) showed increase in gene expression (Sophia *et al.,* 2016a). In pigs, enhanced expression of HSPs (HSP70, HSP90, and HSP27) and heat shock transcription factors (HSF1 and HSF2) were noticed under hot environment (Cui *et al.,* 2016). Heat stressed humans showed up regulation of *TLR2* and *TLR4* genes in monocytes (Zhou *et al.,* 2005). Under heat stress condition, the pro-inflammatory cytokines namely IL-6, IFN- β were down regulated which contributed to innate immune response (Jin *et al.,* 2011). The *TLR2* and *TLR4* mediates immune dysfunction in heat-stressed pigs (Ju *et al.,* 2014). The GRP94 is a glycoprotein in the endoplasmic reticulum (ER) which is involved in the processing and transport of secreted proteins and is essential for folding of *TLRs*. Therefore, GRP94 is vital for the initiation of the innate immune response (Cui *et al.*, 2016).

2.10 Significance of developing climate resilient livestock breeds

Currently, researchers have gained interest on the effect of non-infectious stressors on animal performance and used the term resilience to elaborate the wider aspects of an animal's response to different environmental challenges (Hermesch and Dominik, 2014). Resilience capacity is the ability of an animal to recover its normal biological functions after the exposure to the adverse climatic condition. This sustaining ability helps the animal to bounce back to the original state and perform better than expected. Resilience is a process and it is not a trait of an individual (Rashamol and Sejian, 2018). The cattle breeds Bos indicus or Bos taurus raised in hot climates for many generations are more superior with respect to body temperature control than the Bos taurus breeds in temperate climates (Carabaño, 2016). Naskar *et al.* (2012) stated that, exposure to climatic stress of hot and cold extremes has helped to develop the hardy breeds which are now resistant to the stressful environment. Awassi ewes showed better adaptation traits (lower thyroxine, rectal temperature (RT), and pulse rate levels) raised under extensive systems than those raised under intensive management systems (Naskar *et al.*, 2012).

The genes and pathways involved in resilient phenotypes in farm animals include: Neuropeptide Y gene; CRH receptor 1 gene and FK binding protein 5 gene (HPA axis); catechol-O-methyltransferase gene (noradrenergic and dopaminergic pathways); dopamine transported gene and dopamine receptor genes (dopaminergic pathway);

promoter region of serotonin transported gene and serotonin receptor genes (serotinergic pathway); brain-derived neurotrophic factor gene; and the fatty acid amide hydrolase gene (endocannabinoid pathway) need to be better characterised in order to understand the underlying mechanism of resilience capcacity of farm animals (Dincheva *et al.*, 2015).

Climate change has detrimental effects on agricultural productivity which threatens the future global food security. Moreover, agriculture sector is the main contributor to the global food security. Therefore, efforts should be made in such a way for the sustenance of agricultural productivity to ensure food security to meet the demands of growing human population. One of the best methods to achieve this goal is to improve the resilience capacity of livestock to gain the ability to withstand the adverse environmental condition as well as to maintain their productivity (Aleena *et al.*, 2018). Tremendous progress has been made in identifying advanced biotechnological tools which makes it possible to identify biological markers pertaining to diversified traits controlling production and adaptation. These efforts will improve not only the thermo-tolerance capacity but also in channelizing the available energy resources towards production pathway. These attempts will certainly help in sustenance of livestock production in the changing climate scenario thereby ensure both the livelihood security of farmers and also safeguard the food security of growing human population (Rashamol and Sejian, 2018). In addition to it, provision of comfortable, non-threatening environments as well as cognitive and emotional enrichment to the animal also enhance resilience capacity of livestock (Boissy and Lee, 2014).

2.11 Importance of utilizing indigenous livestock germplasms

Climate change poses a serious threat to livestock genetic resources (FAO, 2009). Therefore, there is an essentiality to develop appropriate agro-ecological zone breeds which possess the ability to survive and produce optimally under testing climatic condition. Indigenous animals which acquired the adaptive potential through several generations can survive in harsh climatic condition through their efficiency in tolerating high heat load, ability to conserve water during water scarcity, ability to survive on limited pastures, ability to walk long distances in search of limited pastures and high disease resistance capability (Alebachew *et al.,* 2017). Maintaining genetic diversity is of paramount importance to impart the indigenous animals ability to survive in different agro-ecological zone (Prentice and Anzar., 2011).

Aleena *et al.* (2018), established the significance of identifying the superior thermo-tolerant breed to survive in a specific locality. In addition, they also compared the adaptive potential of three different indigenous goat breeds (Osmanabadi, Malabari and Salem Black breeds) and established Salem Black breed to be the ideal breed to survive in Southern India through the changes associated with several phenotypic and genotypic traits (Aleena *et al.,* 2018). In a similar study on comparing the adaptive ability of Jamunapari, Barbari, Jakhrana and Sirohi goats, it was established that Sirohi breed exhibited higher potential to survive in harsh climatic condition based on its higher HSP70 expression pattern in vital organs of their body (Rout *et al.,* 2016).

2.12 Heat stress impact on immune response in goats

2.12.1 Impact on blood cells

Heat stress has effect on animal's haematological parameters particularly on the white blood cells, lymphocytes, neutrophil, eosinophil, monocyte, and granulocytes, which are considered immune cells (Alam *et al.*, 2011). There are few studies in sheep and goat which established the impact of heat stress on white blood cells (Sanusi *et al.*, 2010; Okoruwa, 2014; Al-Dawood, 2017).

Heat stress increases disease susceptibility due to impaired immune function (Kelley, 1985). Innate immune system is divided into two categories namely innate and adaptive immunity. Physical barrier of the skin and epithelia of mucus, leucocytes (macrophages, neutrophils and natural killer cells), non-immune cells (epithelial and endothelial cells), and certain soluble mediators (cytokines, eicosanoids and acute phase proteins) all fall under innate immunity. Inflammation is the primary immune response of the innate system to infection which is associated with heat, redness, pain, swelling and impaired function. It functions in two ways: one is by removing the injurious agents and initiate the tissue healing process (Al-Dawood, 2017).

Acute phase response starts developing at the site of infection that is at the local inflammation. Generally, trauma, infection, inflammation and stress or healing process activates the acute phase response and it is a complex systemic innate-defense system (Al-Dawood, 2017). Macrophages or blood monocytes at the site of inflammatory lesions or infections release inflammatory cytokines, especially interleukin-1, interleukin-6 and tumor necrosis factor-α leading to induction of acute phase response. Acute phase proteins are synthesised mainly in the liver. Therefore, cytokines are the mediators between the local site of injury and the hepatocytes of liver to produce and release the acute phase proteins (Jain *et al.*, 2011).

2.12.2 Haptoglobin and serum amyloid A concentrations:

Heat stressed goats showed increased concentrations of haptoglobin and serum amyloid A. Therefore, they are considered very useful sensitive markers in monitoring heat stress in goats. Goats subjected to heat stress showed increased haptoglobin concentration when they were metabolically challenged (Al-Dawood, 2017).

2.12.3 Gene expression regulation under heat stress

The Affymetrix Gene Chip Bovine Genome which is designed to monitor expression of approximately 23,000 transcripts, and identified 39 and 74 genes whose expression was up and down regulated respectively in heat stressed dairy goats blood cells (Salama *et al.,* 2012).

Many Ingenuity Pathways Analysis software was used to examine biological changes including identifying important pathways affected by heat stress. Heat stress also affect cell proliferation and death, free radical scavenging, inflammatory response and glycolysis/gluconeogenesis. The SATB1 (global chromatin organizer) and PPARD (peroxisome proliferator-activated receptor related to lipid metabolism) are the two transcription regulators of blood cells which are affected during heat stress. The blood immune cell functions are altered due to changes in their lipid metabolism during heat stress. Heat stress affects LXR/RXR signaling pathway and might play a role in the regulation of metabolism and inflammatory signaling (Salama *et al.,* 2014) there by the susceptibility of goats to infection increases due to altered functionality of immune cells during heat stress.

Immediate response to hyperthermia by the cell is through increased expression of heat shock proteins (HSP). These proteins helps in cytoprotective effects by folding of newly synthesized proteins and repairing and refolding damaged proteins under stress conditions (Kregel, 2002) further, avoiding cellular apoptosis. Incubation of bovine mammary epithelial cells *in vitro* at high temperatures had increased HSP70 mRNA expression (Collier *et al.,* 2008). Salama *et al.* (2014) observed that, milk cells of heat stressed dairy goats showed an increase in the expression of HSPA5, HSPA9, and HSP90B1 genes. The concept widely accepted is that changes in gene expression are an integral part of the cellular and molecular response to thermal stress. Changes in gene expression include activation of heat shock transcription factor 1 (HSF1), increased expression of HSPs and decreased expression and synthesis of other proteins (Sharma *et al.,* 2013).

The HSPs prime role is folding of newly synthesized proteins, transport of proteins into cell compartments, disaggregation of protein complexes and other functions (Zeng *et al.,* 2004). Among the various HSPs, HSP70 is one of the most temperature sensitive and is positively correlated with thermotolerance in livestock. Stress induces expression pattern of HSP70 gene in bovine lymphocytes, in kidneys of goats, in myocardium, in lung cells and in hepatocytes and liver (Sharma *et al.,* 2013) thereby indicating that heat shock proteins provide protection from toxic effects of thermal stress.

2.12.4 Immunological responses of goats to heat stress

Studies in rats explained that stress stimulates HPA axis further it stimulates the release of catecholamines and glucocorticoids which modulate immune cells and thus cytokine production. In addition to that, cold stress results in higher expression of proinflammatory cytokines like IL2 and IL6 (Sparke *et al.,* 2001). Acute heat stress in broiler chickens resulted in significant increased secretion of IL-2 by splenic lymphocytes. They change, behaviour, metabolism and neuro-endocrine secretion by systemic action. The primary mediator of metabolic response to inflammation by inducing production of a broad array of acute phase proteins is by IL-6. Muscle protein degradation is linked with IL-1 and IL-6 and production of hepatic acute phase proteins as a response to inflammatory stimuli (Sarangi, 2018). The relative mRNA expression of IL2 and IL6 were found to increase significantly in winter as compared to summer in tropical as well as temperate region goats (Marai *et al.,* 2001; Sarangi, 2018). In a recent study Rashamol *et al.* (2019) established the impact of heat stress on different cytokine gene expression in Malabari goats. These authors established significantly lower expression levels of IL-18, TNF-α, IFN-β, and IFN-γ genes during heat stress exposure in this breed and attributed this to the compromised immune status of Malabari goats during heat stress exposure.

Heat stress in the humid tropical regions showed decreased health status of calves by increasing body temperature, inducing rapid dehydration, reducing feed intake and weakens the immune response. The circulating immunoglobulins were found to be reduced during heat stress exposure in these calves (Yun *et al.,* 2014).

2.13 Pathogen recognition receptors, toll like receptors and its significance

Historically, immunity is classified into two ways to fight against pathogens or allergens: innate and adaptive immunity. Innate immune system is the chief defence of body against pathogens (Saeidi *et al.,* 2014).

2.13.1 Pathogen recognition receptors

The pattern-recognition receptors (PRRs) are the germline-encoded receptors of the innate immune system component which are capable of identifying pathogen associated molecular patterns (PAMPs). The PAMPs are expressed by the pathogens (Akira *et al.*, 2001), cancer, or necrotic cells of the host (Abdelsadik and Trad, 2011) and are capable of differentiating between self and nonself cells.

The PRRs are found in both the cell membranes and in the cytosol. The toll like receptors are the most studied PRRs which are localized either to the cell surface or within endosomes. RNA helicase family (retinoic acid inducible gene protein 1, RIG-1 and melanoma differentiation-associated gene-5, MDA5) and the nucleotide binding and oligomerization domain (NOD)-like receptor (NLR) family (Fukata *et al.*, 2009) fall under the cytoplasmic PRRs. The PRRs recognize endogenous cellular products associated with tissue injury and self-danger signals such as HSPs and defective nucleic acids (Shi *et al*, 2003). Nucleotide binding oligomerization domain (Nod)-like receptor (NLR) are one of the special type of PRRs, and play an important role in identification of cytosolic microbial and danger components (Jin *et al.*, 2014).

2.13.2 Toll like receptors

Toll like receptors (*TLRs*) are transmembrane proteins (Zhao *et al.,* 2014). They belong to members of the interleukin-1 (IL-1R) superfamily and they are structurally related to Drosophila Toll. The *TLRs* are evolutionarily conserved molecules and they were first traced in vertebrates on the basis of their homology with Toll, a molecule that stimulates the production of antimicrobial proteins in *Drosophila melanogaster* (Trinchieri and Sher, 2007). Till date, around 10 *TLRs* in humans and 12 in mice have been identified (*TLR1* to *TLR9* and *TLR11* to *TLR13*) have been identified (Jin *et al.*, 2014). For all *TLRs*, ligands have been identified except for human *TLR* 10, mouse *TLR* 12 and mouse *TLR* 13 (Lee *et al.,* 2012).

The *TLRs* are found not only on antigen processing and presentation cells like macrophages, neutrophils and dendritic cells but also detected to variable levels in a wide range of tissues (Iqbal *et al.*, 2005). The *TLRs* are categorized into two types based on their pathogen-sensing and subcellular localization. The *TLR1*, *TLR2*, *TLR4*, *TLR5*, and *TLR6* belong to cell surface and they sense structural protein components of pathogens. The *TLR3*, *TLR7*, *TLR8* and *TLR9* are located intracellularly in the endosome compartments and sense foreign nucleic acids (Kawai and Akira, 2011).

The membrane components of bacterial origin are identified by surface *TLRs* (*TLR1-TLR6*), molecular components on fungus and parasites are detected by *TLR2*, *TLR4*, *TLR6* (verify the whole sentence) and in some cases structural components of viruses were identified by *TLR2* and *TLR4* (Koupenova *et al.*, 2015). *TLR7* and *TLR8* sense single stranded RNA, *TLR3* identifies double stranded RNA, *TLR9* detects double stranded DNA. Mainly endosomal *TLRs* takes the control against viral infection, as well they also fight against bacterial, fungal and parasite RNA/DNA (Kawai and Akira, 2011). The polymorphism of *TLR* genes in livestock might affect immune related traits (Jann et al., 2009) and this explains the observed variation in disease resistance. The *TLR* mRNAs expression pattern have been revealed in mice (Pruett *et al.,* 2004), humans (Bharati *et al.,* 2017), cattle and sheep (Menzies and Ingham, 2006), goat (Tirumurugaan *et al.,* 2010) and buffalo (Vahanan *et al.,* 2008).

2.13.3 Significance of *TLRs* in ischemia and reperfusion injury

TLRs mediate recruitment of leukocyte to infected tissues and the uptake of microorganisms by phagocytic cells. Triggering antigen-presenting cells (APCs) such as dendritic cells (DCs) and activation of both T- and B-cell-mediated immune responses are due to ligation of *TLRs* (Ioannou and Voulgarelis, 2010). In another study by Chao (2009) showed down regulation of *TLR2*, *TLR4*, or MyD88 in ischemic injury reduce myocardial inflammation. In burn injuries, *TLR4* is found to be responsible for the increased T cell response and graft inflammation, sterile injury and alloimmune responses in tissue transplantation. Similar pattern of upregulation of *TLR2* and *TLR4* on immune cells and non-immune cells during sepsis condition is associated with tissue injury in organs. Recent studies have shown that *TLR2* act as pro-atherogenic and *TLR3* is involved in the maintenance of the integrity of the blood vessel wall (Vidya *et al.,* 2017).

2.13.4 Significance of *TLRs* in tissue repair and regeneration

Especially, in liver and intestinal epithelium, *TLR* response is very important in tissue injuries and subsequent tissue repair and regeneration (Rakoff-Nahoum and

Medzhitov, 2008). Majorly, *TLR2* signaling contributes to wound healing process (Yu *et al.,* 2010).

2.13.5 Significance of *TLRs* in allergy and infection

According to Nadeem *et al.* (2016) when *TLR7* agonist is given, it activates the *TLR7* which has a function of enhancing antioxidant network in the lung. Therefore, *TLR7* provides protection against ROS-mediated airway reactivity and inflammation. Further, *TLR4* expression deficiency is associated with enhanced susceptibility to Candida infection. The *TLR2* has important role in the production of proinflammatory cytokines that recruits neutrophils to the site of infection and both *TLR2* and *TLR4* are found to be associated with the pathogenesis of Candidiasis (Netea *et al.,* 2002).

2.13.6 Significance of *TLRs* in auto-immune disorders

Mainly the endogenous *TLR* ligand-mediated signalling pathways are involved in auto-immune disorders. The damaged cells release uric acid which forms endogenous monosodium-urate monohydrate (MSU) crystals. This acts as Damage-associated molecular pattern molecules (DAMP) and triggers the *TLR2* activation ultimately leading to cartilage degeneration (Akahoshi *et al.,* 2007).

2.13.7 Significance of *TLRs* in tumorigenesis and tumor progression

The *TLRs* have both positive and negative impact on tumorigenesis. Study has shown that, *TLR* agonists promote the survival chance of malignant cells as well as increase their resistance to chemotherapy (Shchebliakov *et al.,* 2010). The DAMPs released by apoptosed tumor cells themselves are associated with tumor progression (Li *et al.,* 2009). In contrast, activation of *TLR3* and *TLR5* signalling were reported to induce antitumor T-cell response (Kaczanowska *et al.,* 2013). Also, the *TLRs* plays a significant role in total body irradiation (TBI) cancer immunotherapy (Paulos *et al.,* 2007).

2.13.8 Significance of *TLRs* in vaccines

The *TLRs* play a very significant role in control of adaptive immune response through maturation of DC's, induction of cytokines and co-stimulatory proteins expressions, and reversal of tolerance and they also act as natural adjuvants to vaccines which contain attenuated live or heat-killed viruses or bacteria. The *TLRs* as natural adjuvants in vaccines, they help DCs in better antigen presentation, subsequently leading to a better immune response (Van Duin *et al.,* 2006).

2.14 Heat stress impact on different *TLR* expression patterns in livestock

Expression pattern of basal *TLR* mRNAs in different cells and tissues indicates an individual's ability to respond to a challenge. Therefore, the expression pattern and dispersion of the *TLRs* have been shown to be characteristic of each species (Tirumurugaan *et al.,* 2010).

There is a vast progress in identifying *TLRs* in divergent species of farm animals and also shown their role in diseases. The immune profile in PBMC of 21 days heat stressed local Bama miniature pigs showed up regulation of the Toll Like Receptors (*TLR*) 2, 4 which are actively involved in identification of conserved molecules of microbes such as lipoprotein and lipopolysaccharides respectively (Ju *et al.,* 2014).

The *TLR2* and *TLR4* genes were upregulated in human monocytes under heat stress condition (Sophia *et al.,* 2016b). The expression pattern of *TLR* 1-10 mRNA in Black Bengal goats had seasonal influence which revealed that significant increases in *TLR* genes were observed during summer season in breed (Paul *et al.,* 2015).

It is also a well-established fact that, *TLRs* play a significant role in activation of innate immune response during heat stress in mice (Dehbi *et al.,* 2012) and goat (Bharati *et al.,* 2017).

Expression dynamics of pro-inflammatory cytokines such as IL-2 mRNA expression were modulated in chicken lymphocytes (Han *et al.,* 2010), pig (Ju *et al.,* 2014) and goat (Maurya *et al.,* 2013) PBMCs and IL 6 mRNA expression in livestock (Ju *et al.,* 2014) under heat stress condition.

The heat stressed PBMC in Tharparkar cattle showed significant higher expression pattern of *TLR2* and *TLR4* genes (Bharati *et al.,* 2017). Further, in the same study it has been established that, *TLR2, TLR4* and IL6 could possibly play an important role to elicit immune response in Tharparkar cattle to ameliorate the thermal insults during long term heat stress acclimation. Further, Bovine skin showed higher expression of *TLR2* while ovine gut associated tissues showed lower levels of expression (Menzies and Ingham, 2006). PBMC of heat stressed water buffalo (*Bubalus Bubalis*) showed higher expression pattern of *TLR3* mRNA (Vahanan *et al.,* 2008). Immune cells *TLRs* expressions were found to be altered during heat stress condition further *TLRs* modulate immune responsiveness to PAMPs for complete activation of innate and adaptive immune system to fight against pathogenic microorganisms in goats (Paul *et al.,* 2015).

In a study on Osmanabadi goats, Sophia *et al.* (2017) established the significant influence of heat stress, nutritional stress and combined stress (heat and nutritional) different *TLRs* expression and opined that immune functions were altered by the stress stimuli. The increased *TLR* expression during heat stress in this breed was the reflection of active immune system of this breed under harsh conditions establishing firmly their adaptive capability.

Further, Paul *et al.*, 2015 reported that, the PBMCs of Black Bengal goats showed significant higher expression patterns of *TLR2* and *TLR4* genes during heat stress exposure. In the same study it was established that *TLR1-10* mRNA expressions were significantly higher except the *TLR5* in the PBMC of Black Bengal goats in hot condition (Paul *et al.*, 2015).

In addition, Sophia *et al.*, (2017) reported that the multifold expression of *TLR3* in spleen of heat stressed indigenous Osmanabadi goats showed that the splenic *TLR3* can act as an immunological marker in goats under environmental stress conditions. In an another study, Vandana *et al.* (2019) reported that the heat stressed Malabari goats showed decreased expression pattern of *TLR1* gene compared to control Malabari goats (Vandana *et al.*, 2019). Tirumurugaan *et al.* (2010) reported that heat stressed Kanni breed of goats showed increased expression of *TLR1* genes in organs such as uterus, skin, lymph node, PBMC and lungs. Vandana *et al.* (2019) also established that heat stressed Malabari goats showed increased expression pattern of *TLR2* gene compared to control Malabari goats. Both Vandana *et al.* (2019) and Sophia *et al.* (2017) established significantly lower expression pattern of *TLR4* mRNA in mesenteric lymph node of Malabari and spleen of Osmanabadi goat breeds respectively.

The above review clearly indicates that *TLRs* are important traits whose expression pattern gets altered during heat stress exposure in different livestock species. Further, the above review of literature also discloses that the *TLRs* are important component of innate immune responses and the expression patterns of these different receptors during heat stress could potentially be correlated with thermo-tolerance in livestock.

Materials and Methods

III. MATERIALS AND METHODS

3.1 Study site

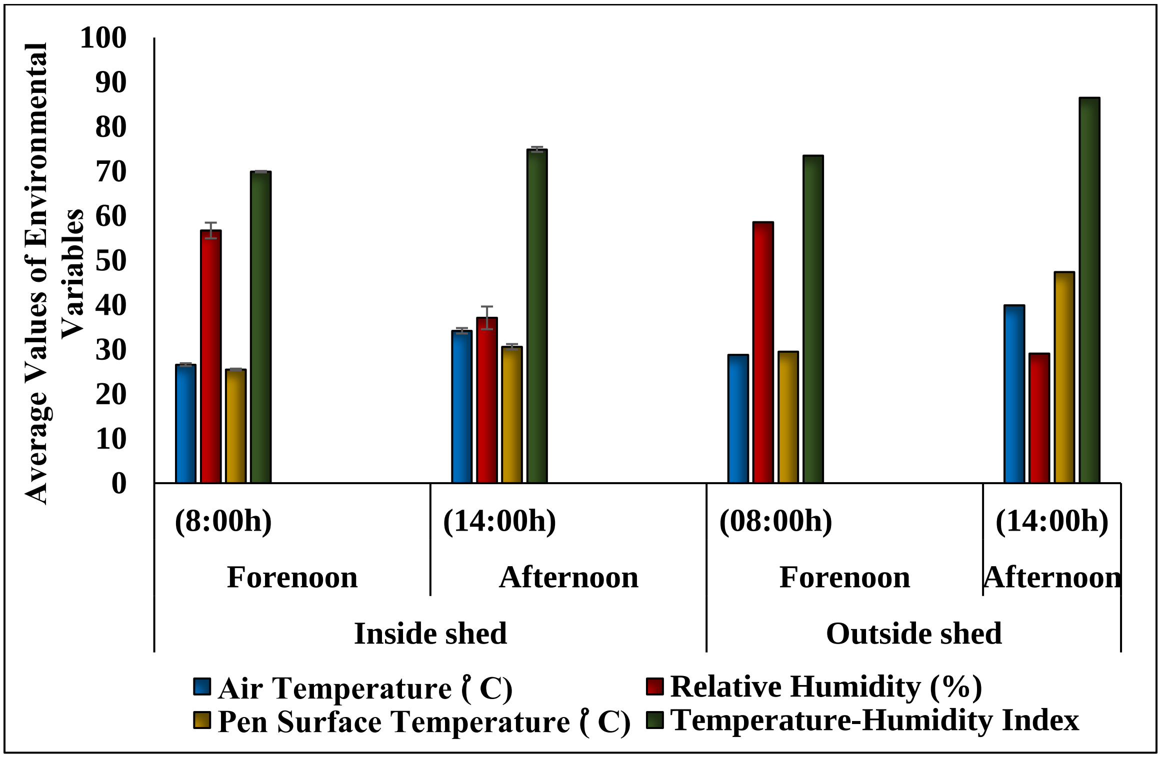
The live animal experiment was carried out in the experimental livestock unit (ELU) of the ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru, India which is located on latitude 77°36'25.3"E, longitude 12°57'04.3"N and altitude of 920 m above mean sea level in southern Deccan plateau of the country. The mean annual minimum and maximum ambient temperature of this region ranged between 15ºC and 36ºC respectively. The mean annual relative humidity ranged from 20% to 85%. The average annual precipitation in this region varied between 200 mm and 970 mm with erratic distribution. The experimental study was conducted during the month of April and May 2017 (peak summer period). The maximum-minimum temperatures, relative humidity, dry and wet bulb temperature, pen surface temperature and temperature-humidity index (THI) during the study period (45 days) are listed in Table-1 and Fig 1. THI was calculated by the formula described by McDowell (1972). Accordingly, the formula used was THI = 0.72 (T_{db} + T_{wb}) + 40.6 where, T_{db} = Dry bulb temperature in ^{0}C; T_{wb} = Wet bulb temperature in ^{0}C. The THI values of 72 and less were considered as comfortable, while the THI values between 75 and 78 were considered stressful and THI above 78 was considered extreme distress to the animals.

Ambient temperature and relative humidity describes the diurnal variations of temperature-humidity index (THI) both inside and outside the shed. The THI index falls within the comfort zone both during forenoon and afternoon inside the shed and during morning outside the shed. However, the THI was significantly higher during afternoon outside the shed inducing extremely severe heat stress to both the heat stress groups of Osmanabadi and Salem Black goat breeds.

Table 1: Description of microclimate (environmental variables) during the study

Environmental Variables	**Mean**			
	Inside Shed		**Outside Shed**	
	Forenoon (8.00 h)	**Afternoon** (14.00 h)	**Forenoon** (8.00 h)	**Afternoon** (14.00 h)
Air Temperature (ºC)	26.6±0 .31	34.2±0.22	28.8±0.61	39.9±0.63
Relative Humidity (%)	56.7± 1.76	37.1± 1.62	58.6± 2.54	29.1± 1.75
Pen Surface Temperature (ºC)	25.5±0 .20	30.6±0.46	29.5±0.61	47.4±0.76
Temperature-Humidity-Index	69.9±0.16	74.9±0.14	73.5±0.56	86.5±0.39

Fig. 1: Average microclimate (environmental variables) of the study location during the experimental period



3.2. Animals

Two indigenous female goat breeds of India were used in the present study, ie., Osmanabadi (OS) and Salem Black (SB). All the goats procured were under the age group of 10 months to 1 year. The animals were maintained as per institute standard management practices (Plate 2A and Plate 2B).

3.2.1 Osmanabadi

Osmanabadi (Plate 1A) is a dual-purpose goat breed reared for meat and milk purpose. It is a hardy breed. This breed originated in the semi-arid areas of central tropical India. The Osmanabadi breed derives its name from its habitat and distributed in Ahmedanagar, Solapur and Osmanabad districts in Maharashtra (Motghare *et al.*, 2005; Deokar *et al.,* 2006). It has spread over a wide range of agro-climatic conditions in Maharashtra and adjoining parts of Karnataka and Andhra Pradesh. The goats are large in size. Majority of them are black in colour (73 per cent), while white, brown or spotted color is rarely found. The average body weights of adult male and female animals are 34 kg and 30 kg, respectively. The average daily yield of milk ranges from 0.5 to 1.5 kg for a lactation length of about 4 months. In favourable conditions the does will breed regularly twice a year and twinning is common in this breed.

3.2.2 Salem Black

The Salem Black goat breed (Plate 1B) is one of the important Indian goat breed originated from north-western part of Tamil Nadu, India. The breed owes its name from its place of origin (Salem) and colour (black).

Plate 1A: Osmanabadi breed

Plate 1B: Salem Black breed

The Salem Black breeds are completely black in colour and found to be distributed in the Salem, Dharmapuri, Krishnagiri, Erode, Karur and Namakkal districts of Tamil Nadu. Generally, these goats are tall with a lean body. The characteristic features of ears are found to be medium-long, leaf like and semi-pendulous. In adults the body weight ranges from 35 to 39 kg and from 25 to 30 kg for males and females respectively (Thiruvenkadan *et al.,* 2014). THI of native track of Salem Black breed is 82.

3.3 Technical program

Twelve animals of Osmanabadi and twelve Salem Black breeds of goats weighing between 15 and 20 kg were used in the present experimental study. In addition, the study was conducted after obtaining approval from the Committee for the purpose of control and supervision of experiments on Animals (CPCSEA), Ministry of Environment, Forest and Climate Change, Government of India for subjecting the animal to heat stress (NIANP/IAEC/2/2017). In addition, all handling and management procedures performed in the study were in accordance with the ethical standards of the Institutional Animal Care and Use Committee (IACUC, ICAR-NIANP).

The experimental study duration was for a period of 45 days between April-May 2017. The animals were housed in well ventilated sheds made up of galvalume roofing at a height of 2.4m and open from side and maintained under proper hygienic conditions. Prophylactic measures, ie., deworming against endo and ectoparasitic infestations and vaccination against goat diseases like goat pox, peste des petits ruminants (PPR), enterotoxaemia (ET), were carried out as prescribed by the health calendar of the institute to ensure that the animals were in healthy condition throughout the study period.

The animals were randomly allocated into two groups of six animals each from each breed, OC (n=6; Osmanabadi control) (GI), OHS (n=6; Osmanabadi heat stress) (GII), SBC (n=6; Salem Black control) (GIII) and SBHS (n=6; Salem Black heat stress) (GIV). All the animals were stall fed with a diet consisting of 60% roughage (Hybrid Napier) and 40% concentrate (Maize 36kg, wheat bran 37kg, soybean meal 25kg, mineral mixture 1.5kg, common salt 0.5kg/100kg) and the chemical composition, fibre fraction and their nutritive value details are enlisted in Table-2. The GI and GIII animals were maintained in the shed (Plate 2C) in thermo-neutral condition while GII and GIV animals were exposed outside to summer heat stress (Plate 2D) between 10:00h to 16:00h to expose to heat stress during experimental period. The GI and GIII animals were fed and watered ad-libitum inside the shed while GII and GIV animals were fed and watered ad-libitum while they are exposed to summer heat stress in the outside environment. All the cardinal weather parameters were recorded twice daily both inside and outside the shed throughout the study period. The animals were slaughtered at the end of the study for sample collection for conducting gene expression study.

3.4 Expression of different *Toll like receptors* in mesenteric lymph nodes

3.4.1 Principle

Samples were lysed and homogenized in lysis buffer, which contains guanidine thiocyanate, a chaotropic salt capable of protecting RNA from endogenous RNases. The lysate is then mixed with ethanol and loaded on a purification column. The chaotropic salt and ethanol cause RNA to bind to the silica membrane while the lysate was spun through the column.

Table 2: Ingredients and chemical composition of concentrate mixture and hybrid Napier hay fed to goats

Attribute	**Concentrate mixture (kg/100 kg)**	**Napier hay** (*Pennisetum purpureum*)
Ingredients		
Maize	36	-
Wheat bran	37	-
Soybean meal	25	-
Mineral mixture	1.5	-
Salt	0.5	-
Chemical composition (%)		
Dry matter	92.9±0.079	94.0±0.289
Organic matter	95.9±0.190	95.4±0.298
Crude protein	19.6±0.176	6.21±0.098
Ether extract	1.82±0.183	1.49±0.026
Total ash	4.10±0.190	4.64±0.298
Fibre fractions (%)		
Neutral detergent fibre	40.4±1.400	82. 9±0.881
Acid detergent fibre	11.1±0.239	64.6±1.950
Acid detergent lignin	2.14±0.029	12.3±0.651
Nutritive value		
Total digestible nutrients %*	72.2	55.0
Digestible energy (kJ/kg) *	13.3	10.1
Metabolizable energy (kJ/kg) *	10.9	8.28

*Calculated values

Plate 2A: Feeding and Managemental practices for Osmanabadi and Salem Black goats

Plate 2B: Feeding practices for Osmanabadi and Salem Black goats

Plate 2C: Control group kept inside the shed

Plate 2D: Heat stress group animals exposed to sun for six hours a day

Subsequently, impurities were effectively removed from the membrane by washing the column with wash buffers. Pure RNA was then eluted under low ionic strength conditions with nuclease-free water.

3.4.2 Sample collection and storage

The mesenteric lymph node (MLN) samples were collected from all the animals of control and heat stress groups immediately after slaughtering. The lymph node samples were cut into small pieces, washed in phosphate buffered saline and immersed in RNA shield (Zymo Research, USA) and snap freezed in liquid nitrogen. Then samples were shifted and stored at –80 °C in deep freezer till further use.

3.4.3 Sample preparation for RNA isolation

The tissues were removed from the RNA shield (Zymo Research, USA) after thawing and immediately processed for RNA isolation. The total RNA was isolated from tissues using the GeneJET RNA Purification Kit (#K0731 and #K0732; Thermo Scientific, Lithuania) and the procedure was done as per manufacturer's protocol which is as follows:

About 20-25 mg of tissue sample was weighed and transferred into 20 ml centrifuge tube. Further, liquid nitrogen (LN_2) (–196 °C) was added immediately. Thereafter, 300 μL of lysis buffer supplemented with β-mercaptoethanol (10 μL/ml) was added and homogenized using hand held homogenizer (WT 130 Dyna-ken Selangor, Malaysia) for 3 minutes at 12000 rpm. After homogenization, the content was transferred to 1.5 ml microcentrifuge tube and vortexed for 10 s. To the vortexed lysate, 10 µL of proteinase K (200µg/mL) in 590 µL of Tris-EDTA buffer (pH-8.0) was added and again vortexed and then incubated at 25 °C for 10 min. Later, it was centrifuged for 8 min at 12,000 g and the supernatant was transferred into a new RNase-free microcentrifuge tube. To the supernatant, 450 µL of ethanol was added and mixed well.

Then 700 µL of lysate was transferred to a spin column with a 2 ml collection tube and centrifuged for 1 min at 12,000 g. After discarding the flow through (eluent), 700 µL of wash Buffer - 1 was added and centrifuged for 1 min at 12,000 g, this was followed by two time washing with 600 µL and 250 µL of wash Buffer - 2 followed by centrifugation at 12,000 g for 1 and 2 min, respectively.

About 40µL of warm nuclease-free water was added to the membrane, and centrifuged at 10,000 g for 1 min to elute RNA. The purified RNA samples were stored at -80 °C until cDNA synthesis.

3.4.4 DNase treatment

Total RNA isolated from different samples were treated with 1 µL DNase (TURBO DNA-free, Ambion, USA) to eliminate the genomic DNA contamination in total RNA. Subsequently to DNase treatment, 1 µL of RNase inhibitor (20 U/µL, Invitrogen, USA) was added. After DNase treatment quality and quantity of the isolated RNA was analysed using UV-Vis spectrophotometer (ND-1000, Thermo Scientific, USA).

3.4.5 RNA quantity and quality

The purity and quantity of isolated RNA was analysed using the spectrophotometer (Nano Drop-1000, Thermo Scientific, USA). One microlitre of RNA sample was directly measured at 260 nm. The 260/230 ratio were used to assess the contamination of salts and other solvents and the 260/280 ratio was used to assess the contamination of proteins. The samples with 260/230 ratio (1.5-2.0) and 260/280 ratio (1.8-2.0) were used for gene expression study by RT-PCR. The quality of RNA was checked by A260/A280 ratio and quantified as 1OD = 40μg/ml using Nanodrop spectrophotometer. The quantity of RNA was expressed as ng/μL. Integrity of the total RNA was checked using denaturing agarose gel (1%) electrophoresis and visualized under UV light. Two intact bands of 28s and 18s with smearing indicated good quality and intactness of RNA.

3.5 cDNA synthesis

The total RNA was reverse transcribed into cDNA using Thermo scientific revertaid first strand cDNA synthesis kit (#K1622) for Real Time quantitative polymerase chain reaction (RTqPCR) (Thermo Scientific, Lithuania). The procedure was performed as per manufacturer's protocol as follows: 4 μL of 5X Reaction Mix, 2 μL Maxima Enzyme Mix, 1 μg of Template RNA and 20 μL of nuclease-free water were added into a sterile RNAase-free tube. Then the contents were mixed gently and centrifuged and subjected to reverse transcribing PCR (10 min at 25°C, followed by 20 min at 50°C and the reaction was terminated by heating at 85°C for 5 min). The reverse transcription reaction product was used directly for qPCR (real time PCR) analyses or stored at -20^{0}C.

The product of the first strand cDNA synthesis was diluted to a final concentration of 25 ng/μL with nuclease-free water and 2 μL of diluted cDNA was used for each reaction in qPCR.

3.6 Primer design and synthesis for *TLR* 1-10

TLR 1-10 primer sequences used for amplifying the target regions of the *TLR* genes have been published by Paul *et al.* (2015). These primer sequences are as described in Table-3.

3.7 Quantitative RT-PCR analysis

The relative expression of selected genes were studied using SYBR green chemistry (#K0221; Maxima SYBR green qPCR master mix, Fermentas, USA). The 20μL reaction was carried out in duplicates using 50 ng of template (cDNA) and 0.5 μM primer concentrations. The qPCR reaction conditions were: enzyme activation at 95 °C for 10 min and amplification cycle (40 cycles; initial denaturation at 95°C for 15 s, annealing at 60°C for 30s and extension at 72°C for 30 s).

The melt curve analysis was performed to check the non-specific amplification. The Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; Fig.2), Hypoxanthine phosphoribosyl transferase 1 (*HPRT1*; Fig.3), and Succinate dehydrogenase complex flavoprotein subunit A (*SDHA*; Fig.4) genes were used as reference genes for internal control. The melt curve and amplification plot (Fig.5) of no template control (NTC) indicates non contamination with other templates.

Table 3: Primer sequences for different *TLR* genes (*TLR* 1 – 10), *GAPDH, SDHA* and *HPART1* reference genes

Gene ID	Primers	Primer Sequence (5’-------------3’)	Annealing Temp	Product Size (bp)	Accession No.
TLR-1	F	5-ACTTGGAATTCCTTCATTACGA-3	60	176	HQ263209.1
	R	5-GAAGACTGAACACATCATGGA-3			
TLR-2	F	5-TTCCGTCTCTTTGATGAG-3	60	114	JQ911706.1
	R	5-CTTGGTGTTCATGATCTTC-3			
TLR-3	F	5-GATGTATCGCCGTGCAAAGACA-3	60	195	HQ263210.1
	R	5-TGCATATTCAAACTGCTCTGCT-3			
TLR-4	F	5-CTTGCGTCCAGGTTGTTCCTAA-3	60	153	JF825527.1
	R	5-CTGGGAACCTGGAGAAGTTATG-3			
TLR-5	F	5-CCTCCTGCTCAGCTTCAACTAT-3	60	172	FJ659852.1
	R	5-TATCTGACTTCCACCCAGGTC-3			
TLR-6	F	5-CCTTGTCTTTCACCCAAATAGC-3	60	150	HQ263211.1
	R	5-GTTGGTCTTCCAGTGAGT-3			
TLR-7	F	5-TCTTGAAGGAAAGGACTGGTTA-3	60	205	HQ263216.1
	R	5-AAGGGGCTTCTCAAGGAATATC-3			
TLR-8	F	5-CGCACCGTCTAGGATTTATT-3	60	209	JF825528.1
	R	5-AAGCCGGGTCAGATTGGT-3			
TLR-9	F	5-CTGACACCTTCAGCCACCTGAG-3	60	156	HQ263217.1
	R	5-TGGTGGTCTTGGTGATGTAGTC-3			
TLR-10	F	5-ATGGTGCCATTATGAACCCTAC-3	60	248	HQ263213.1
	R	5-CACATGTCCCTGTGGTGTCTAA-3			
GAPDH	F	5-GGTGATGCTGGTGCTGAGTA-3	60	265	AF030943.1
	R	5-TCATAAGTCCCTCCACGATG-3			
HPRT1	F	5- GCCCCAGCGTGGTGATTAG-3	60	145	XM_018044253.1
	R	5-ACATCTCGAGCCAGTCGTTC-3			
SDHA	F	5- GCTAAAGTTTCAGATGCGATTTCTG-3	60	128	XM_018065656.1
	R	5- CGT GTT GAA ACC TGC TTC GG-3			

The single distinct peak of melting curve for each gene indicates that a distinct single PCR product is very characteristic of a given double-stranded DNA sequence which has been amplified without the formation of unwanted products like primer dimer, non-specific amplification. However, in the NTC there was no distinct peak indicating the components of PCR were free from any template contamination. Amplification plot showed variation of log (An amplification plot shows the variation of log (ΔRn) with PCR cycle number. The relative gene expression was analysed using the formula, $2^{-\Delta\Delta C}$T (Livak and Schmittgen, 2001).

The C_T cycle values provided by qPCR instrumentation were imported into a spreadsheet program such as Microsoft Excel. To perform the analysis, data were reported from a quantitative gene expression experiment and a sample spread sheet was described. The mean C_T values of all target genes are normalized using *GAPDH*, *HPRT1* and *SDHA* as reference genes gives ΔC_T values. By assuming any one sample as control the $\Delta\Delta C_T$ values were calculated. The fold change difference were then analysed using $2^{-\Delta\Delta C_T}$ method. The results were expressed in fold change as compared to untreated control (control = 1fold).

3.8 Statistical analysis

The changes in relative expression of different genes in relation to the reference gene were analysed using SPSS (16.0) software using one-way analysis of variance (ANOVA). The changes in relative expression patterns of toll like receptor mRNAs in relation to *GAPDH*, *HPRT1* and *SDHA* as the house keeping genes were analysed by ANOVA to compare the means between the groups. The significance level was set at $P<0.01$.

Fig. 2: Melt curve and amplification plot of *GAPDH gene* expression pattern

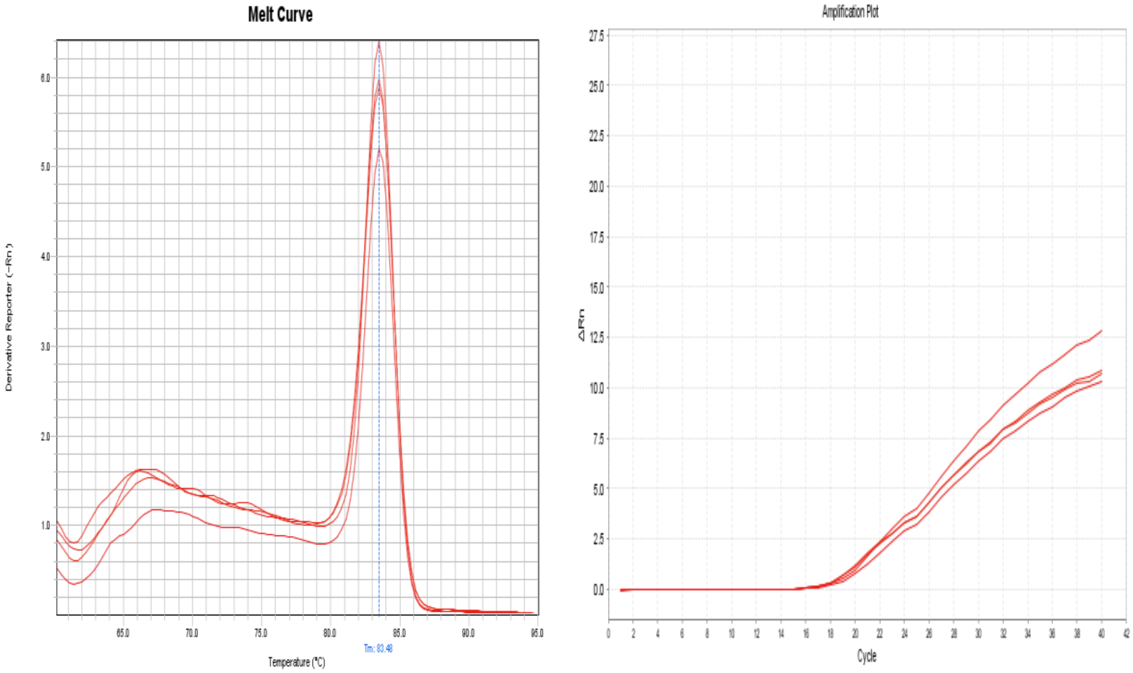


Fig. 3: Melt curve and amplification plot of *HPRT1 gene* expression pattern

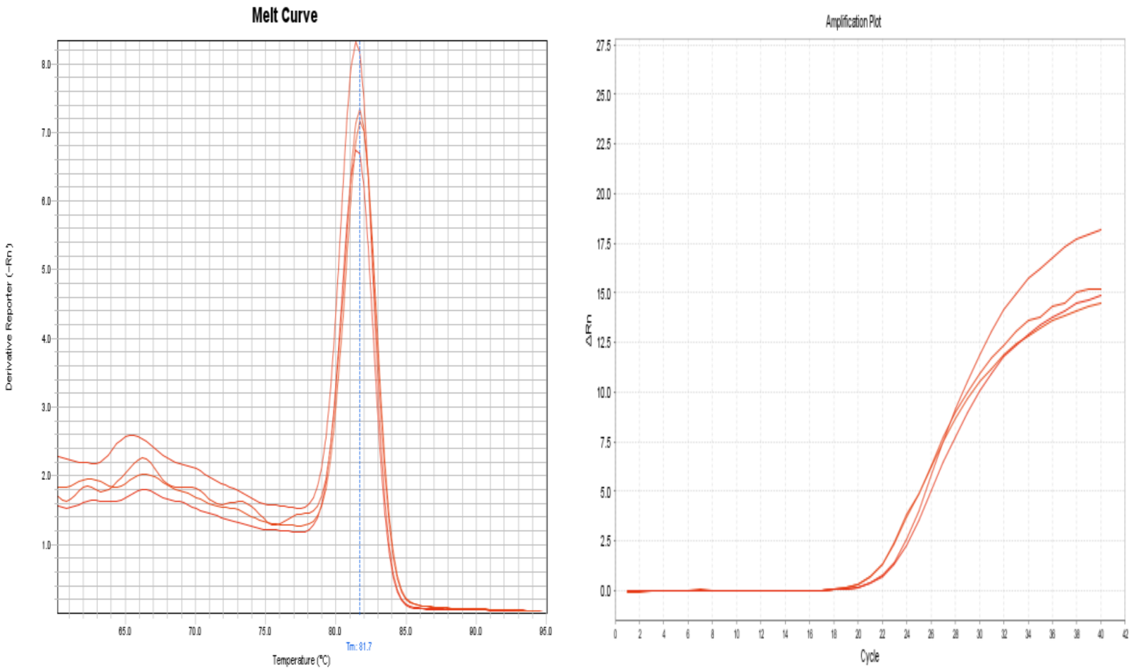


Fig. 4: Melt curve and amplification plot of *SDHA gene* expression pattern

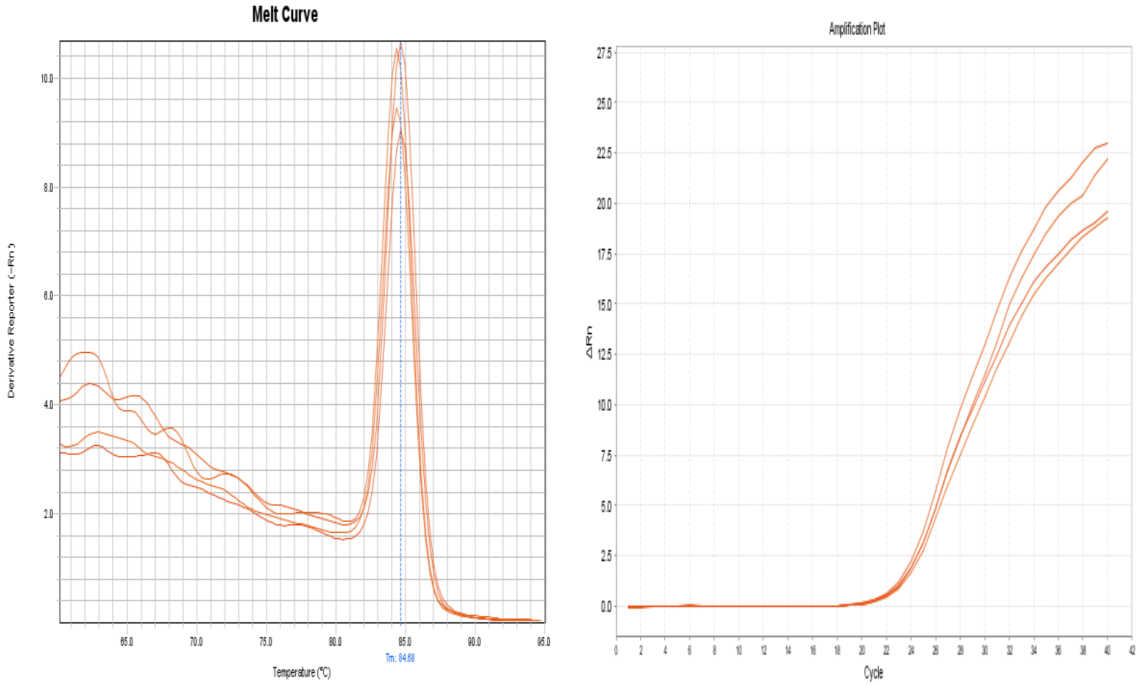
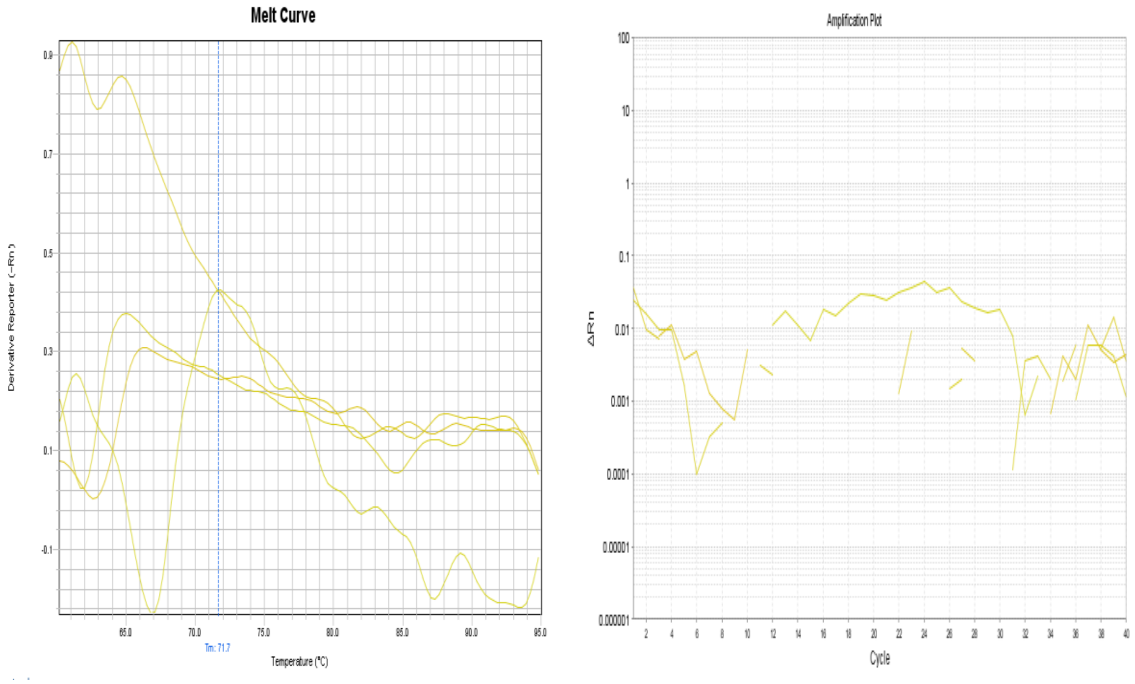


Fig. 5: Melt curve and amplification plot in No Template Control



Results

IV. RESULTS

4.1 Temperature-Humidity Index

The differences in the THI values between inside and outside the shed for the entire study duration are depicted in Fig. 6. The THI values both inside and outside the shed in the morning were 69.9±0.16 and 73.5±0.14, respectively while in the afternoon the values were 74.9±0.56 and 86.5±0.39, respectively. The THI values for the entire study duration during the morning were not stressful to the animals kept both inside and outside the shed. However, the obtained THI values ($P<0.01$) during the afternoon indicated that the animals inside the shed were not stressed while the animals kept outside the shed were under extreme distress. This difference in THI between inside and outside the shed was highly significant ($P<0.01$).

4.2 Expression pattern of different *TLRs* in Osmanabadi goats

The differences in the expression pattern of different *TLRs* between OC and OHS groups in the Osmanabadi breed were described in Table-4. The difference in the expression pattern of *TLR1* between the OC and OHS groups was not statistically significant. But the *TLR2* expression pattern showed a significant difference between the groups. The level of *TLR2* expression was significantly ($P<0.01$) higher in the OHS group as compared to the OC group. Similarly, the *TLR3* expression pattern also showed a significant ($P<0.01$) difference between the groups with higher expression patterns recorded in the OHS group. However, the expression pattern of *TLR4* did not differ between the groups in this breed. Likewise, the expression pattern of both *TLR5* and *TLR6* also did not vary between the groups. However, the expression pattern of *TLR7* differed between the groups with significantly (P<0.01) higher value recorded in the OHS group. But again, the *TLR8*, *TLR9* and *TLR 10* expression patterns also did not differ between the groups.

4.3 Expression pattern of different *TLRs* in Salem Black goats

The differences in the expression pattern of different *TLRs* between SBC and SBHS groups in the Salem Black breed were described in Table-5. The difference in the expression pattern of *TLR1* between the SBC and SBHS groups was statistically significant (P<0.01) with higher expression patterns reported in the SBHS group. However, the *TLR2* did not express in both SBC and SBHS groups in this study. The level of *TLR3* expression was significantly (P<0.01) higher in the SBHS group as compared to the SBC group. Similarly, the *TLR4* expression pattern also showed a significant difference between the groups with higher (P<0.01) expression patterns recorded in the SBHS group. Likewise, the expression pattern of both *TLR5* and *TLR6* also were significantly (P<0.01) higher in the SBHS group as compared to the SBC group. Similarly, the expression pattern of *TLR7*, *TLR8*, *TLR9* and *TLR10* also were significantly (P<0.01) higher in the SBHS group as compared to the SBC group.

4.4 Comparative assessment of different *TLR* expression patterns between Osmanabadi and Salem Black goat breeds

4.4.1 Expression pattern of different cell surface Toll-Like Receptor genes

4.4.1.1 Differential expression pattern of *TLR1* between Osmanabadi and Salem Black goats

The melt curve and amplification plot of *TLR1* expression pattern between control and heat stress groups of female Osmanabadi and Salem Black goat breeds were described in Fig. 8 and Fig.9, respectively. The differences in the expression pattern of *the TLR1* gene between Osmanabadi and Salem Black breed goats is depicted in Fig. 10. The fold changes of expression patterns of *TLR1* gene between control and heat stress groups of Osmanabadi and Salem Black breeds were 1.00 ± 0.02, 1.11 ± 0.16 and 1.00 ± 0.04, 2.20 ± 0.04, respectively. The expression patterns of *the TLR1* gene did not differ in Osmanabadi breed while significantly ($P<0.01$) up regulated in the SBHS group as compared to rest all groups (OC, OHS and SBC).

4.4.1.2 Differential expression pattern of *TLR2* between Osmanabadi and Salem Black goats

The melt curve and amplification plot of *TLR2* expression pattern between control and heat stress groups of female Osmanabadi and Salem Black goat breeds were described in Fig. 11 and Fig.12, respectively. The differences in the expression pattern of *the TLR2* gene between Osmanabadi and Salem Black breed goats is shown in Fig. 13. The fold changes of expression patterns of *the TLR2* gene between control and heat stress groups of Osmanabadi breed were 1.00 ± 0.1 and 5.18 ± 0.32, respectively. However, *TLR2* did not get expressed in the Salem Black bread while significantly higher expression was recorded in the OHS group as compared to the OC group.

4.4.1.3 Differential expression pattern of *TLR4* between Osmanabadi and Salem Black goats

The melt curve and amplification plot of *TLR4* expression pattern between control and heat stress groups of female Osmanabadi and Salem Black goat breeds were described in Fig. 14 and Fig.15, respectively. The differences in the expression pattern of *the TLR4* gene between Osmanabadi and Salem Black breed goats is illustrated in Fig. 16. The fold changes of expression patterns of *TLR4* gene between control and heat stress groups of Osmanabadi and Salem Black breeds were 1.00 ± 0.07, 0.89 ± 0.05 and 1.00 ± 0.06, 4.50 ± 0.02, respectively. The expression patterns of *the TLR4* gene significantly ($P<0.01$) up-regulated in the SBHS group as compared to rest all groups (OC, OHS and SBC).

4.4.1.4 Differential expression pattern of *TLR5* between Osmanabadi and Salem Black goats

The melt curve and amplification plot of *TLR5* expression pattern between control and heat stress groups of female Osmanabadi and Salem Black goat breeds were described in Fig. 17 and Fig.18, respectively. The differences in the expression pattern of *the TLR5* gene between Osmanabadi and Salem Black breed goats is shown in Fig. 19. The expression pattern of *the TLR5* gene also significantly ($P<0.01$) up-regulated in the SBHS group as compared to SBC group animals. The fold changes of expression patterns of *TLR5* gene between control and heat stress groups of Osmanabadi and Salem Black breeds were 1.00 ± 0.04, 0.82 ± 0.04 and 1.00 ± 0.05, 3.91 ± 0.19, respectively.

4.4.1.5 Differential expression pattern of *TLR6* between Osmanabadi and Salem Black goats

The melt curve and amplification plot of *the TLR6* expression pattern between control and heat stress groups of female Osmanabadi and Salem Black goat breeds is depicted in Fig. 20 and Fig. 21, respectively. The differences in the expression pattern of *the TLR6* gene between Osmanabadi and Salem Black breed goats is depicted in Fig. 22. The expression pattern of *the TLR6* gene also was significantly ($P<0.01$) up-regulated in the SBHS group as compared to the rest of all groups (OC, OHS and SBC). The fold changes of expression patterns of *TLR6* gene between control and heat stress groups of Osmanabadi and Salem Black breeds were 1.00 ± 0.04, 0.90 ± 0.1 and 1.00 ± 0.02, 2.30 ± 0.01, respectively.

4.4.1.6 Differential expression pattern of *TLR10* between Osmanabadi and Salem Black goats

The melt curve and amplification plot of *TLR10* expression pattern between control and heat stress groups of female Osmanabadi and Salem Black goat breeds were described in Fig. 23 and Fig.24, respectively. The differences in the expression pattern of *the TLR10* gene between Osmanabadi and Salem Black breed goats is illustrated in Fig. 25. The *TLR10* expression pattern also showed a similar trend of up-regulation ($P<0.01$) as that of another cell surface *TLRs* studied in the SBHS group as compared to rest all groups (OC, OHS and SBC). The fold changes of expression patterns of *TLR10* gene between control and heat stress groups of Osmanabadi and Salem Black breeds were 1.00 ± 0.06, 0.93 ± 0.07 and 1.00 ± 0.03, 3.54 ± 0.04, respectively.

4.4.2 Expression patterns of different intracellular Toll-Like Receptor genes

4.4.2.1 Differential expression pattern of *TLR3* between Osmanabadi and Salem Black goats

The melt curve and amplification plot of *TLR3* expression pattern between control and heat stress groups of female Osmanabadi and Salem Black goat breeds were described in Fig.26 and Fig.27, respectively. The differences in the expression pattern of *the TLR3* gene between Osmanabadi and Salem Black breed goats is shown in Fig. 28. The fold changes of expression patterns of *TLR3* gene between control and heat stress groups of Osmanabadi and Salem Black breeds were 1.00 ± 0.26, 6.18 ± 2.56 and 1.00 ± 0.03, 10.62 ± 0.58, respectively. The expression pattern of *TLR3* was similar in both Osmanabadi and Salem Black breeds. The expression patterns of *the TLR3* gene were significantly higher in (P<0.01) both OHS and SBHS groups as compared to their respective control groups, OC and SBHS, respectively. Further, on a comparison between the heat stress groups, the expression pattern of *TLR3* was significantly higher (P<0.01) in the SBHS group as compared to the OHS group.

4.4.2.2 Differential expression pattern of *TLR7* between Osmanabadi and Salem Black goats

The melt curve and amplification plot of *the TLR7* expression pattern between control and heat stress groups of female Osmanabadi and Salem Black goat breeds were described in Fig. 29 and Fig.30, respectively. The differences in the expression pattern of *the TLR7* gene between Osmanabadi and Salem Black breed goats is illustrated in Fig. 31. The fold changes of expression patterns of *TLR7* gene between control and heat stress groups of Osmanabadi and Salem Black breeds were 1.00 ± 0.08, 1.84 ± 0.29 and 1.00 ± 0.01, 2.11 ± 0.04, respectively. Like *TLR3*, *TLR7* expression pattern also showed a similar trend in both OHS and SBHS group with significantly higher (P<0.01) expression in these groups as compared to their respective control groups (OC and SBC groups). However, the expression pattern between the OHS and SBHS was comparable.

4.4.2.3 Differential expression pattern of *TLR8* between Osmanabadi and Salem Black goats

The melt curve and amplification plot of *TLR8* expression pattern between control and heat stress groups of female Osmanabadi and Salem Black goat breeds were described in Fig. 32 and Fig.33, respectively. The differences in the expression pattern of *the TLR8* gene between Osmanabadi and Salem Black breed goats is shown in Fig. 34. As compared to *TLR3* and *TLR7*, the expression pattern of *TLR8* was different between the breeds. The fold changes of expression patterns of *TLR8* gene between control and heat stress groups of Osmanabadi and Salem Black breeds were 1.00 ± 0.01, 1.16 ± 0.07 and 1.00 ± 0.03, 1.83 ± 0.01, respectively. The expression pattern of *TLR8* between the control and heat stress group of the Osmanabadi breed was comparable while in Salem Black breed the expression pattern differed (P<0.01) between the control and heat stress group. The expression pattern of *TLR8* in the SBHS group was significantly higher (P<0.01) as compared to the SBC group.

4.4.2.4 Differential expression pattern of *TLR9* between Osmanabadi and Salem Black goats

The melt curve and amplification plot of *TLR9* expression pattern between control and heat stress groups of female Osmanabadi and Salem Black goat breeds were described in Fig. 35 and Fig.36, respectively. The differences in the expression pattern of the *TLR9* gene between Osmanabadi and Salem Black breed goats is illustrated in Fig. 37. The fold changes of expression patterns of *TLR9* gene between control and heat stress groups of Osmanabadi and Salem Black breeds were 1.00 ± 0.02, 0.83 ± 0.01 and 1.00 ± 0.04, 2.78 ± 0.01, respectively. The expression patterns of *the TLR9* gene significantly ($P<0.01$) up-regulated in the SBHS group as compared to rest all groups (OC, OHS and SBC). However, the expression pattern of *the TLR9* gene was comparable between OC and OHS groups.

Table 4: Differences in the expression patterns of different *TLRs* between the control and heat stress groups of Osmanabadi goat breeds

Different *TLR* Expression Pattern	Osmanabadi Breed		Level of Significance
	Control (OC)	Heat Stress (OHS)	
TLR1	1.00 ± 0.02	1.11 ± 0.16	--
TLR2	1.00 ± 0.1	5.18 ± 0.32	**
TLR3	1.00 ± 0.26	6.18 ± 2.56	**
TLR4	1.00 ± 0.07	0.89 ± 0.05	--
TLR5	1.00 ± 0.04	0.82 ± 0.04	--
TLR6	1.00 ± 0.04	0.90 ± 0.1	--
TLR7	1.00 ± 0.08	1.84 ± 0.29	**
TLR8	1.00 ± 0.01	1.16 ± 0.07	--
TLR9	1.00 ± 0.02	0.83 ± 0.01	--
TLR10	1.00 ± 0.06	0.93 ± 0.07	--

TLR-Toll-Like Receptor; OC- Osmanabadi Control; OHS-Osmanabadi Heat Stress

**Indicates statistical significance between control and heat stress values within a row at $p < 0.01$.

Table 5: Differences in the expression patterns of different *TLRs* between the control and heat stress groups of Salem Black goat breeds

Different *TLR* Expression Pattern	Salem Black Breed		Level of Significance
	Control (SBC)	Heat Stress (SBHS)	
TLR1	1.00 ± 0.04	2.20 ± 0.04	**
TLR2	0.00 ± 0.00	0.00 ± 0.00	--
TLR3	1.00 ± 0.03	10.62 ± 0.58	**
TLR4	1.00 ± 0.06	4.50 ± 0.02	**
TLR5	1.00 ± 0.05	3.91 ± 0.19	**
TLR6	1.00 ± 0.02	2.30 ± 0.01	**
TLR7	1.00 ± 0.01	2.11 ± 0.04	**
TLR8	1.00 ± 0.03	1.83 ± 0.01	**
TLR9	1.00 ± 0.04	2.78 ± 0.01	**
TLR10	1.00 ± 0.03	3.54 ± 0.04	**

TLR-Toll-Like Receptor; OC- Osmanabadi Control; OHS-Osmanabadi Heat Stress

**Indicates statistical significance between control and heat stress values within a row at $p < 0.01$.

Fig. 6: Differences in the THI values between inside and outside the shed for the entire study duration

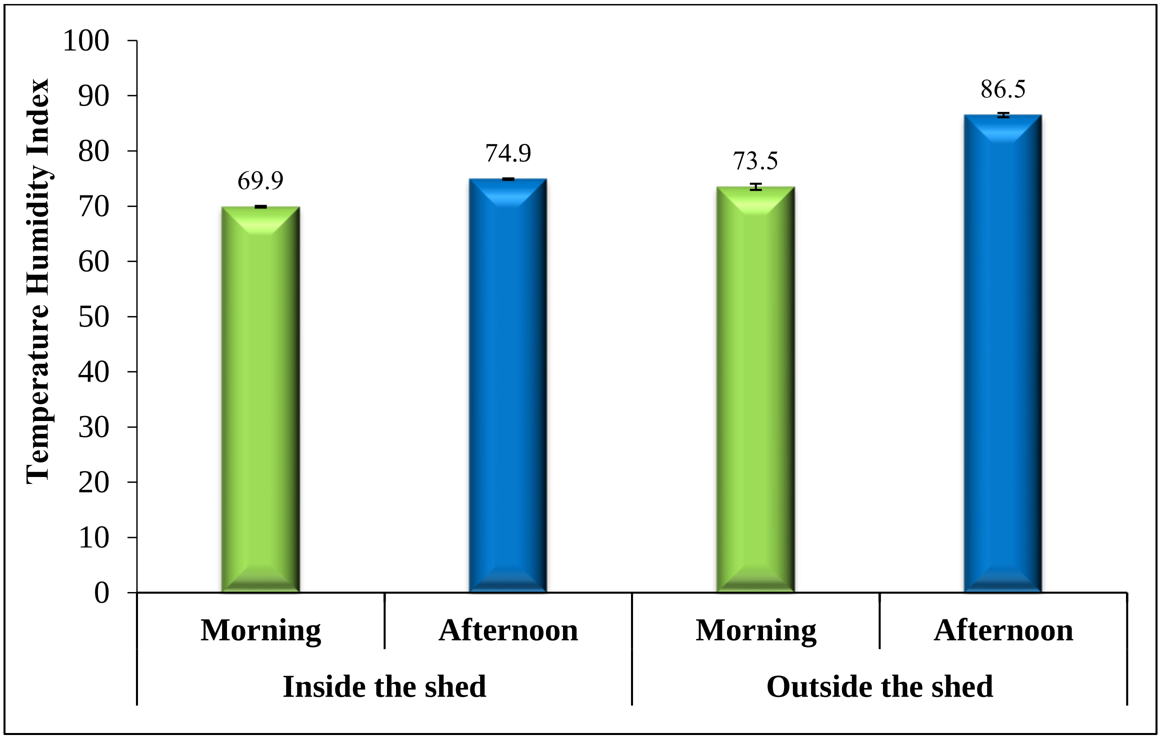


Fig. 7: Agarose gel (1%) electrophoresis indicating RNA integrity

Fig. 8: Melt curve and amplification plot of *TLR1* expression pattern in female Osmanabadi (Control and Heat stress) goats

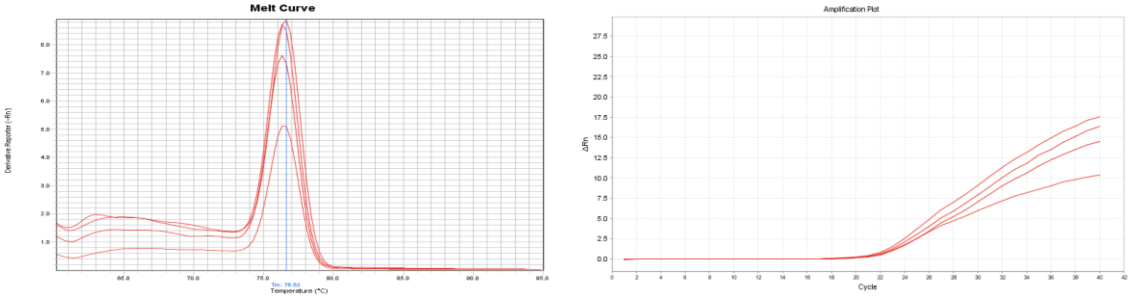


Fig. 9: Melt curve and amplification plot of *TLR1* expression pattern in female Salem Black (Control and Heat stress) goats

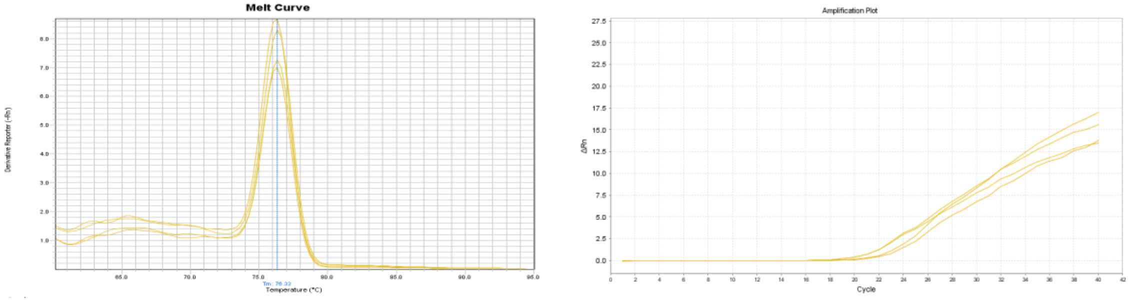
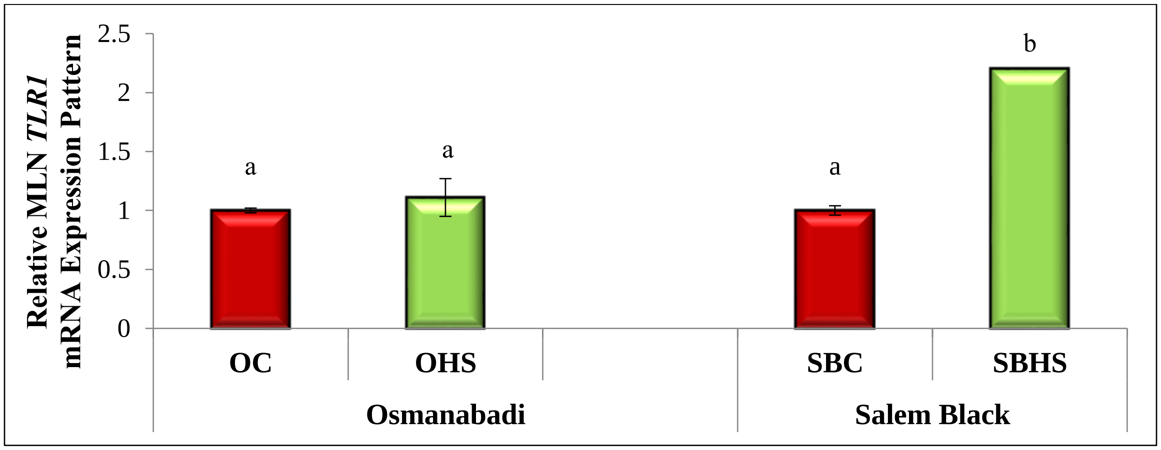


Fig. 10: Relative mesenteric lymph node *TLR1* mRNA expression pattern in female Osmanabadi (Control and Heat stress) and Salem Black (Control and Heat stress) goats



The values bearing different superscripts both within and between breeds differ significantly at P < 0.01. The values bearing same superscript both within and between breeds do not differ with each other.

Fig. 11: Melt curve and amplification plot of *TLR2* expression pattern in female Osmanabadi (Control and Heat stress) goats

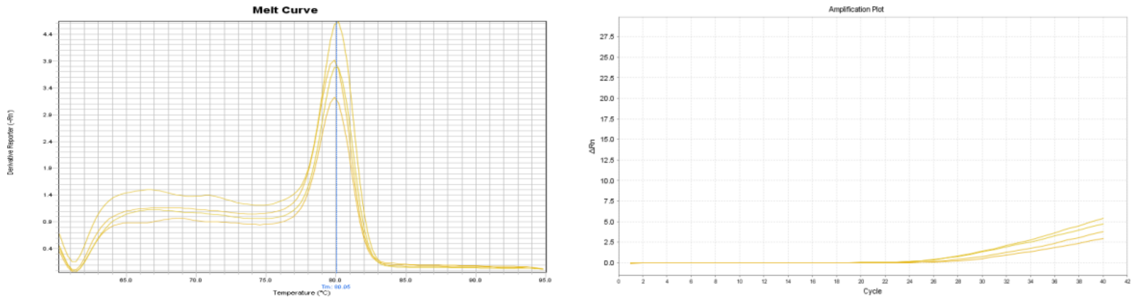


Fig. 12: Melt curve and amplification plot of *TLR2* expression pattern in female Salem Black (Control and Heat stress) goats

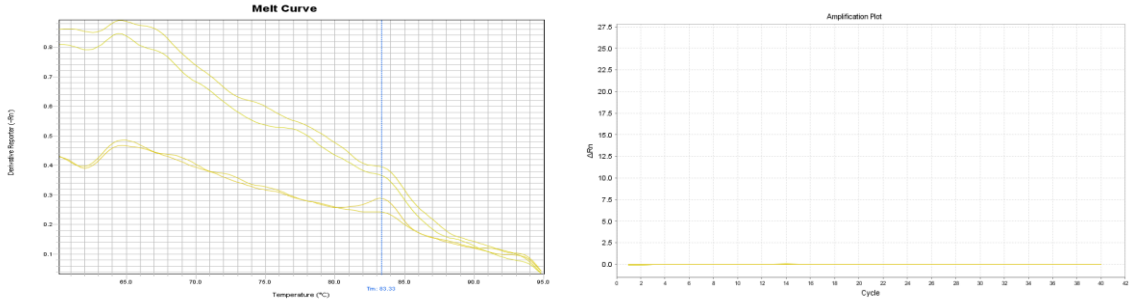
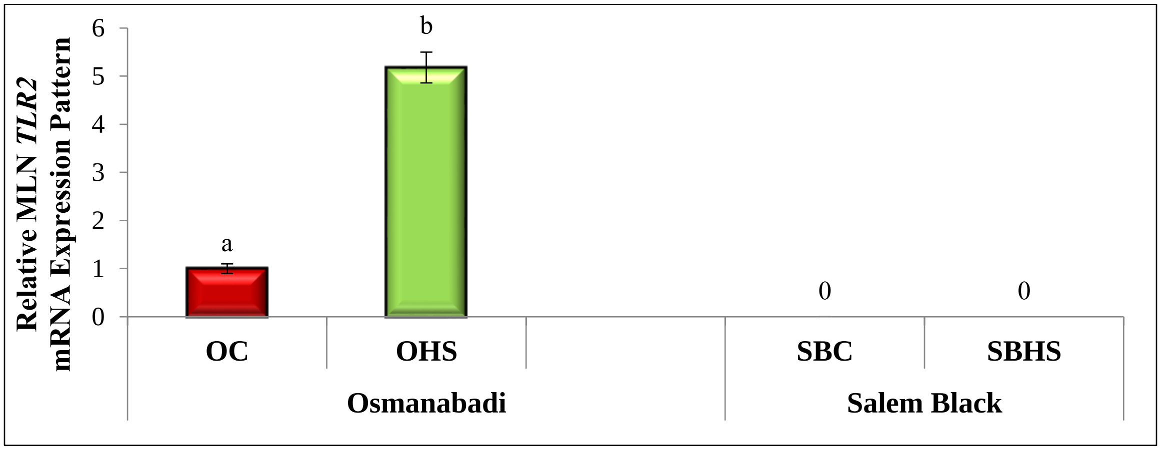


Fig. 13: Relative mesenteric lymph node *TLR2* mRNA expression in female Osmanabadi (Control and Heat stress) and Salem Black (Control and Heat stress) goats



The values bearing different superscripts both within and between breeds differ significantly at P < 0.01. The values bearing same superscript both within and between breeds do not differ with each other.

Fig. 14: Melt curve and amplification plot of *TLR4* expression pattern in female Osmanabadi (Control and Heat stress) goats

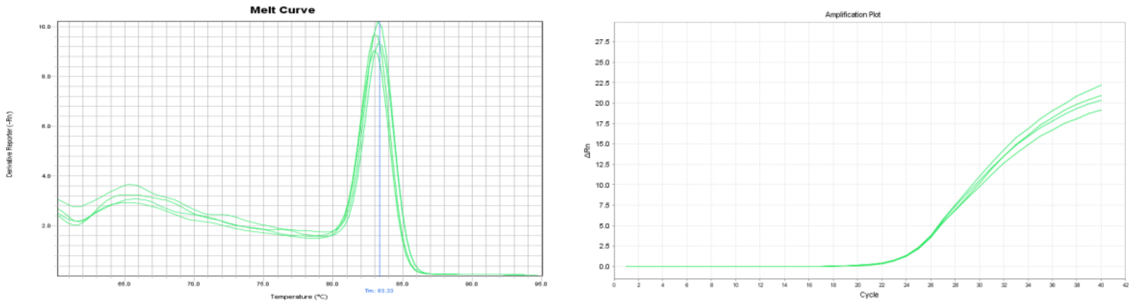


Fig. 15: Melt curve and amplification plot of *TLR4* expression pattern in female Osmanabadi (Control and Heat stress) goats

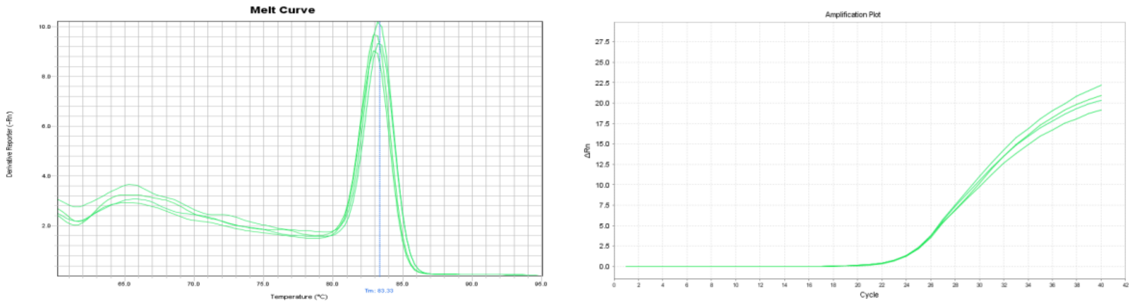
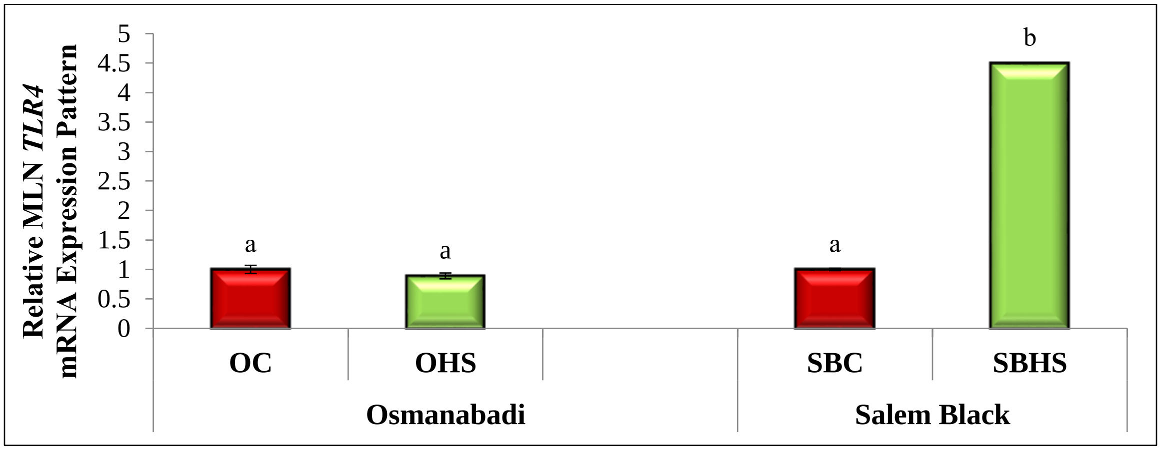


Fig. 16: Relative mesenteric lymph node *TLR4* mRNA expression in female Osmanabadi (Control and Heat stress) and Salem Black (Control and Heat stress) goats



The values bearing different superscripts both within and between breeds differ significantly at $P < 0.01$. The values bearing same superscript both within and between breeds do not differ with each other.

Fig. 17: Melt curve and amplification plot of *TLR5* expression pattern in female Osmanabadi (Control and Heat stress) goats

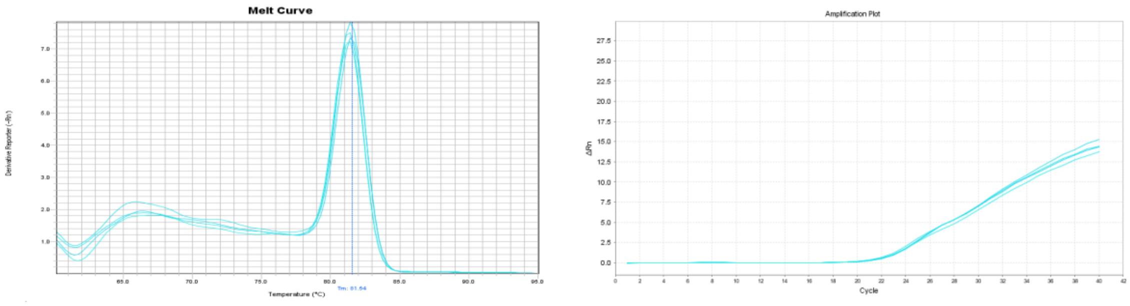


Fig. 18: Melt curve and amplification plot of *TLR5* expression pattern in female Salem Black (Control and Heat stress) goats

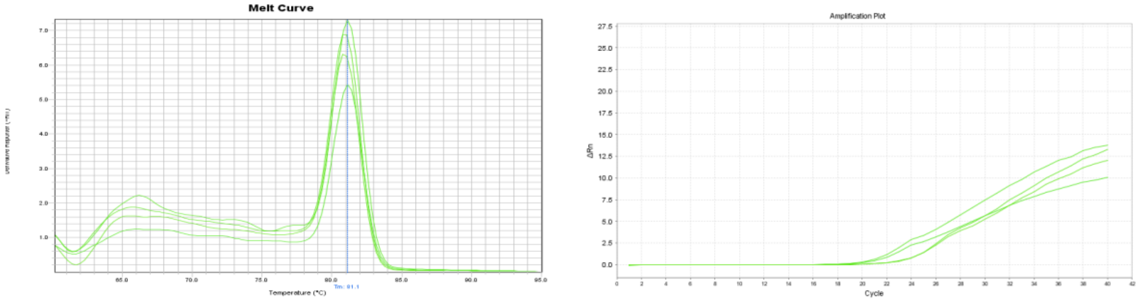
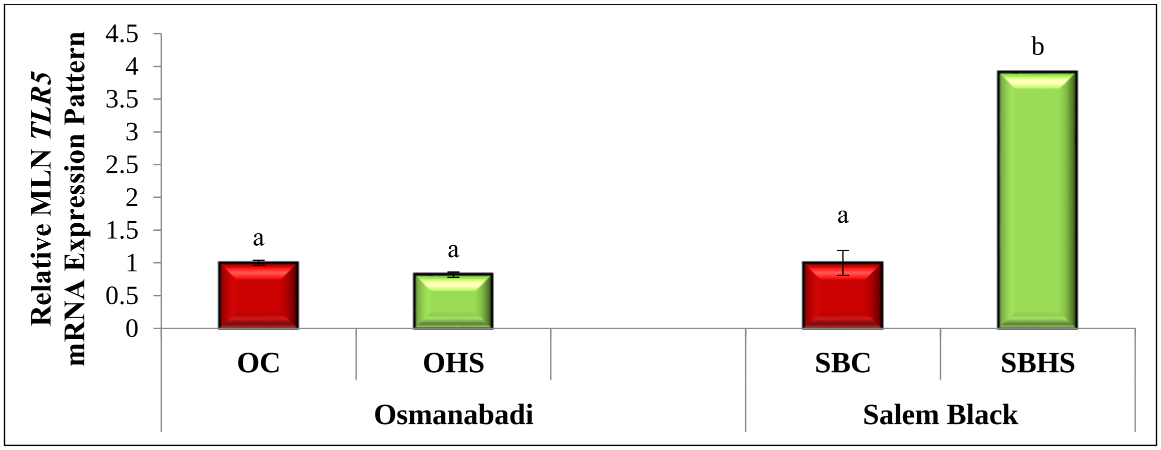


Fig. 19: Relative mesenteric lymph node *TLR5* mRNA expression in female Osmanabadi (Control and Heat stress) and Salem Black (Control and Heat stress) goats



The values bearing different superscripts both within and between breeds differ significantly at $P < 0.01$. The values bearing same superscript both within and between breeds do not differ with each other.

Fig. 20: Melt curve and amplification plot of *TLR6* expression pattern in female Osmanabadi (Control and Heat stress) goats

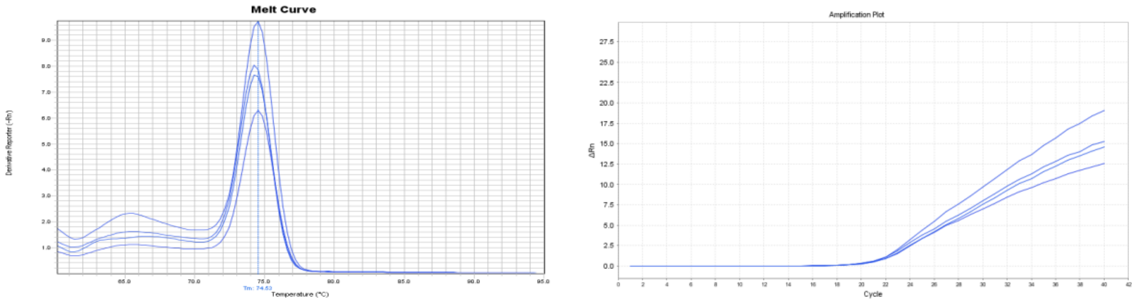


Fig. 21: Melt curve and amplification plot of *TLR6* expression pattern in female Salem Black (Control and Heat stress) goats

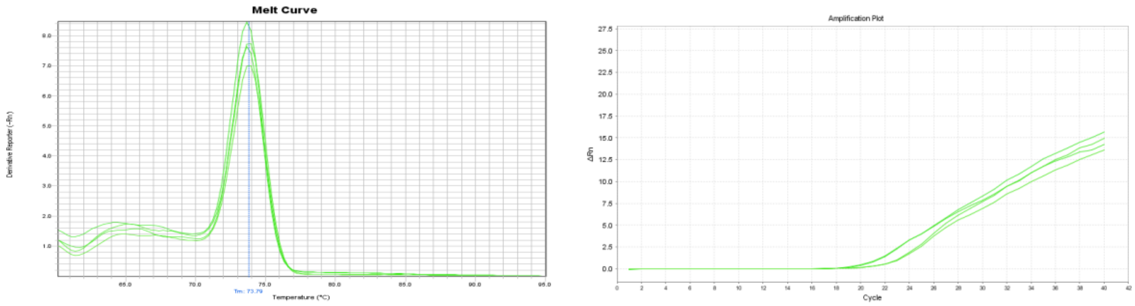
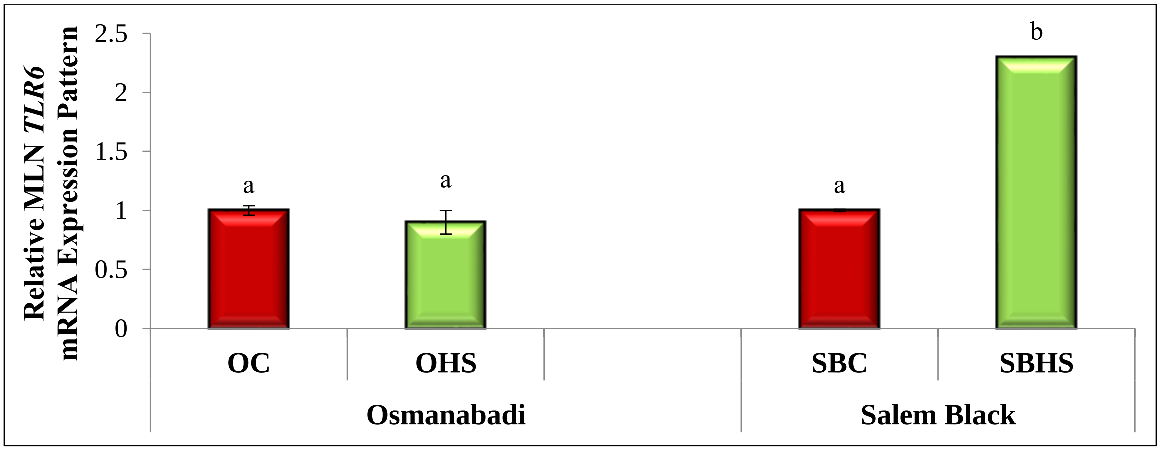


Fig. 22: Relative mesenteric lymph node *TLR6* mRNA expression in female Osmanabadi (Control and Heat stress) and Salem Black (Control and Heat stress) goats



The values bearing different superscripts both within and between breeds differ significantly at $P < 0.01$. The values bearing same superscript both within and between breeds do not differ with each other.

Fig. 23: **Melt curve and amplification plot of *TLR10* expression pattern in female Osmanabadi (Control and Heat stress) goats**

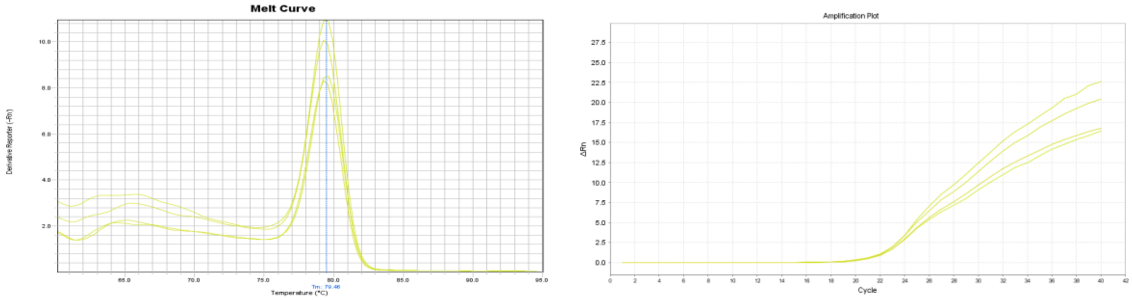


Fig. 24: **Melt curve and amplification plot of *TLR10* expression pattern in female Salem Black (Control and Heat stress) goats**

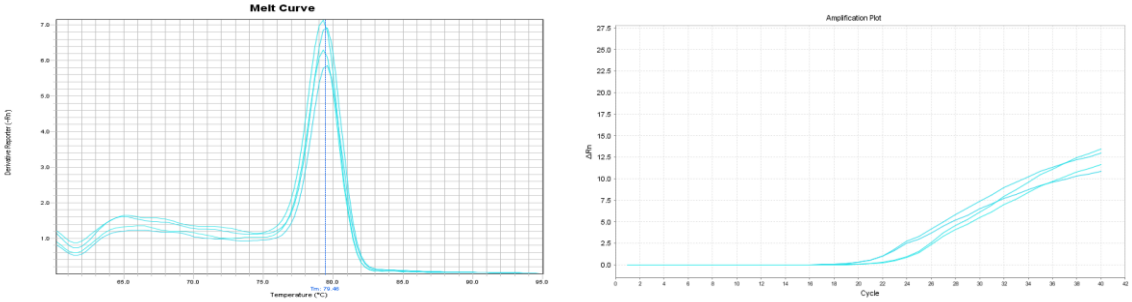
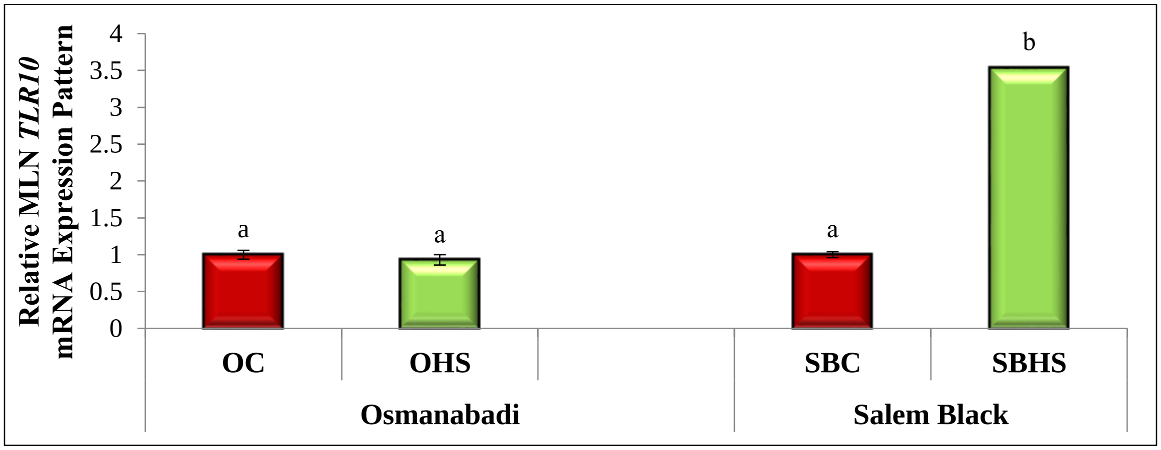


Fig. 25: **Relative mesenteric lymph node *TLR10* mRNA expression in female Osmanabadi (Control and Heat stress) and Salem Black (Control and Heat stress) goats**



The values bearing different superscripts both within and between breeds differ significantly at $P < 0.01$. The values bearing same superscript both within and between breeds do not differ with each other.

Fig. 26: **Melt curve and amplification plot of *TLR3* expression pattern in female Osmanabadi (Control and Heat stress) goats**

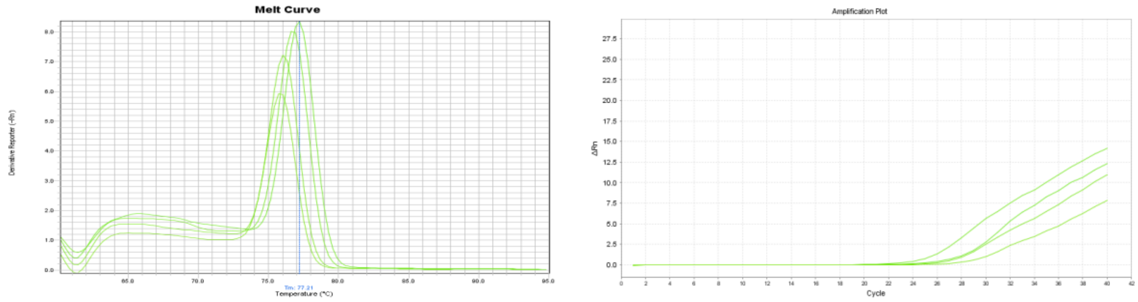


Fig. 27: **Melt curve and amplification plot of *TLR3* expression pattern in female Salem Black (Control and Heat stress) goats**

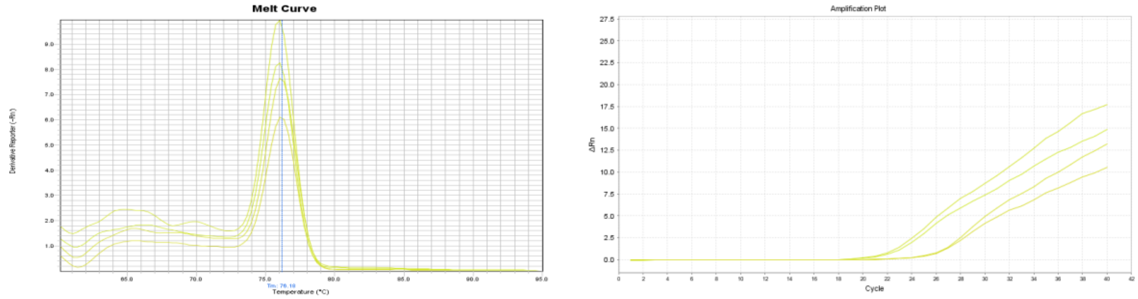
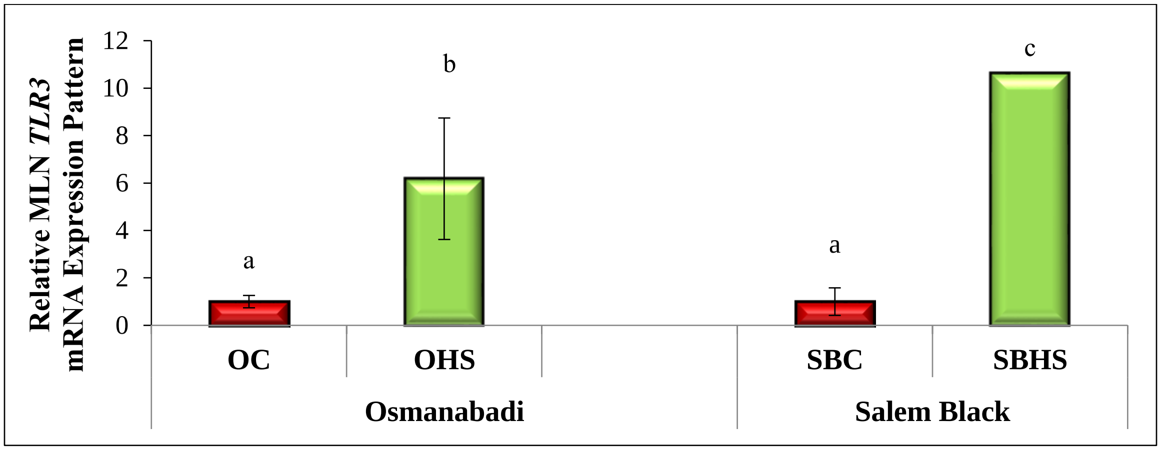


Fig. 28: **Relative mesenteric lymph node *TLR3* mRNA expression pattern in female Osmanabadi (Control and Heat stress) and Salem Black (Control and Heat stress) goats**



The values bearing different superscripts both within and between breeds differ significantly at $P < 0.01$. The values bearing same superscript both within and between breeds do not differ with each other.

Fig. 29: **Melt curve and amplification plot of *TLR7* expression pattern in female Osmanabadi (Control and Heat stress) goats**

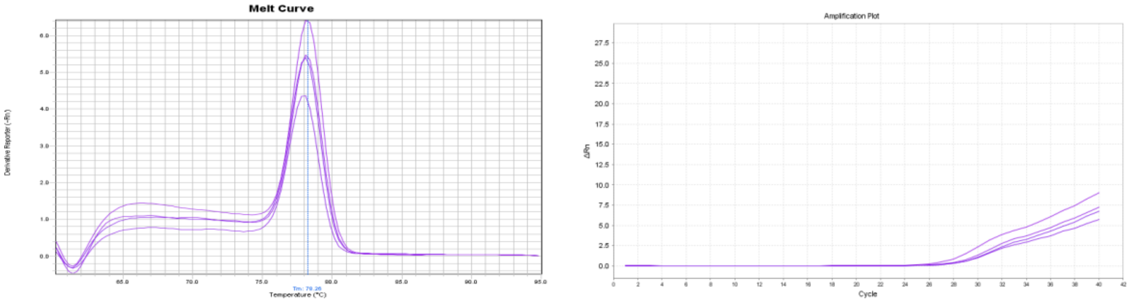


Fig. 30: **Melt curve and amplification plot of *TLR7* expression pattern in female Salem Black (Control and Heat stress) goats**

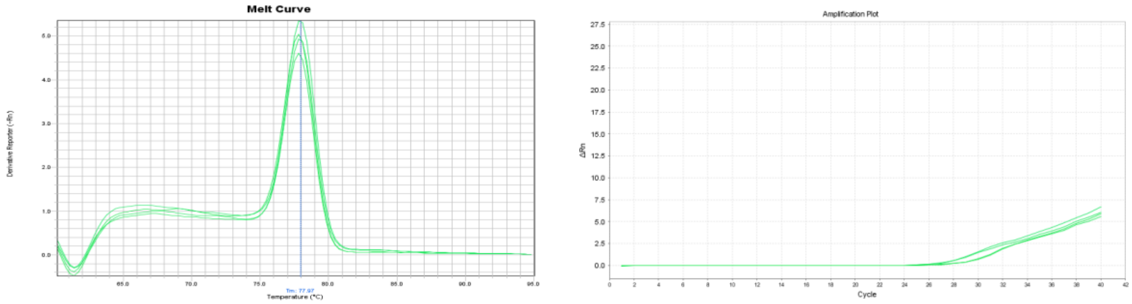
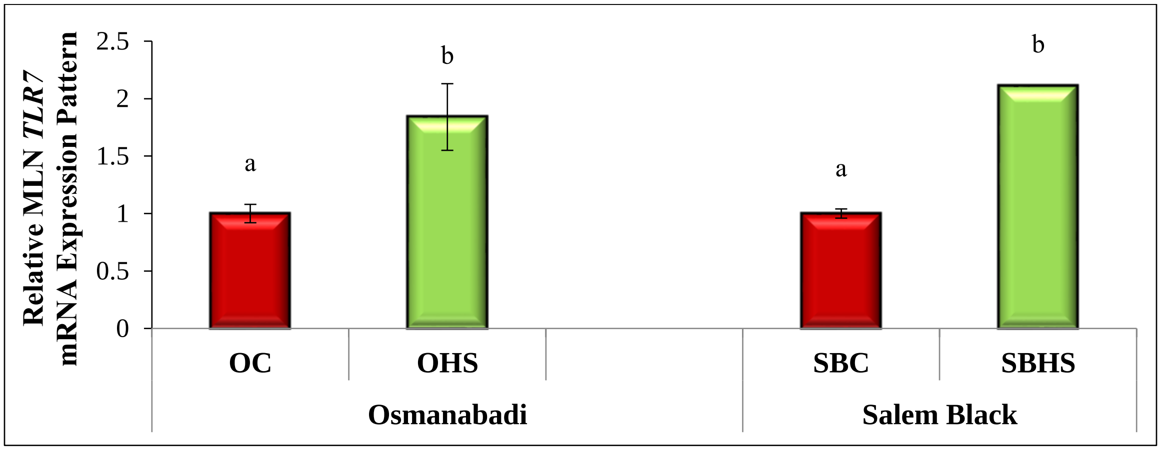


Fig. 31: **Relative mesenteric lymph node *TLR7* mRNA expression pattern in female Osmanabadi (Control and Heat stress) and Salem Black (Control and Heat stress) goats**



The values bearing different superscripts both within and between breeds differ significantly at $P < 0.01$. The values bearing same superscript both within and between breeds do not differ with each other.

Fig. 32: Melt curve and amplification plot of *TLR8* expression pattern in female Osmanabadi (Control and Heat stress) goats

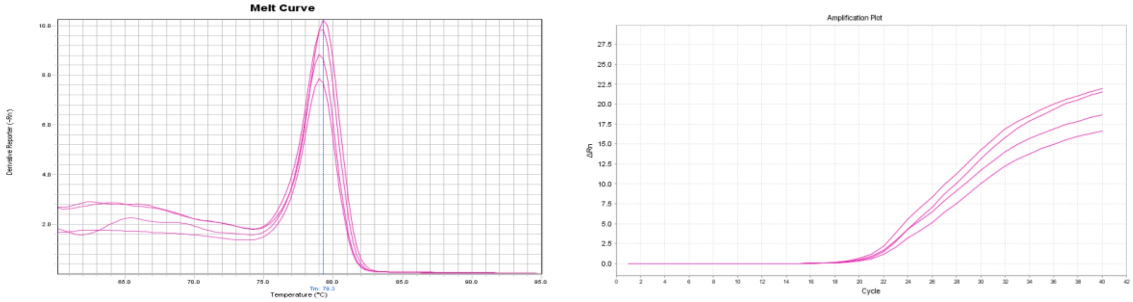


Fig. 33: Melt curve and amplification plot of *TLR8* expression pattern in female Salem Black (Control and Heat stress) goats

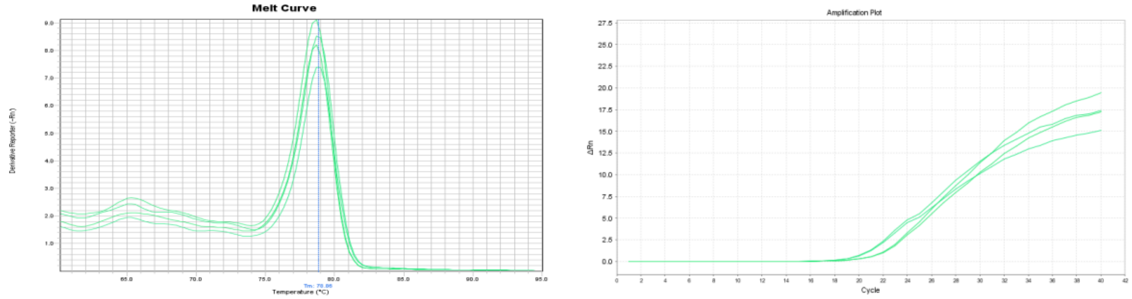
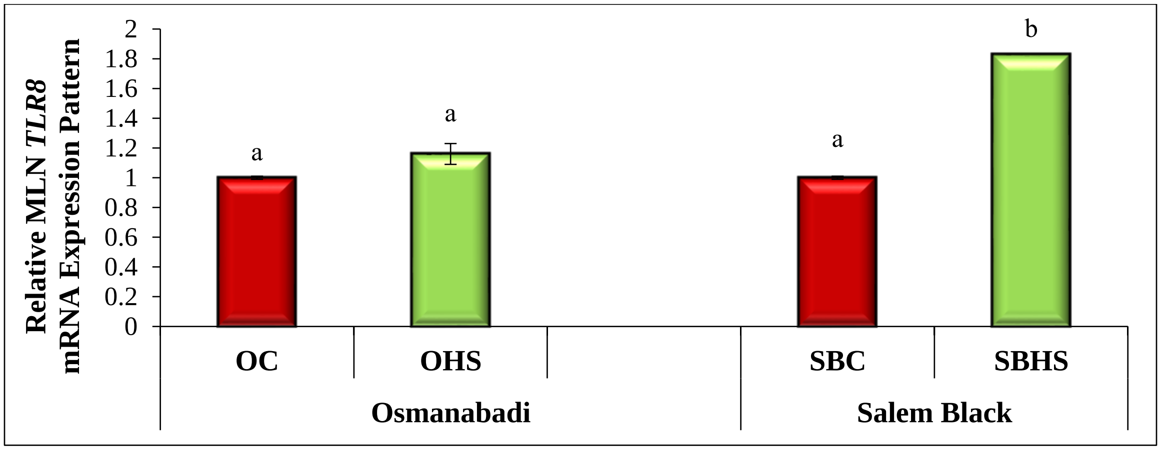


Fig. 34: Relative mesenteric lymph node *TLR8* mRNA expression pattern in female Osmanabadi (Control and Heat stress) and Salem Black (Control and Heat stress) goats



The values bearing different superscripts both within and between breeds differ significantly at $P < 0.01$. The values bearing same superscript both within and between breeds do not differ with each other.

Fig. 35: Melt curve and amplification plot of *TLR9* expression pattern in female Osmanabadi (Control and Heat stress) goats

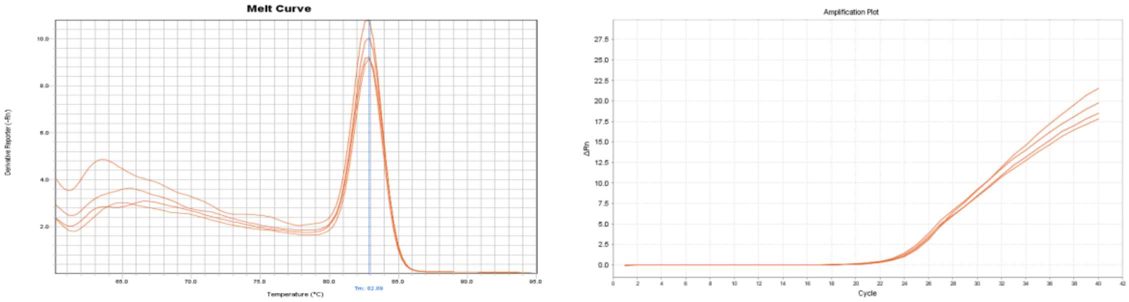


Fig. 36: Melt curve and amplification plot of *TLR9* expression pattern in female Salem Black (Control and Heat stress) goats

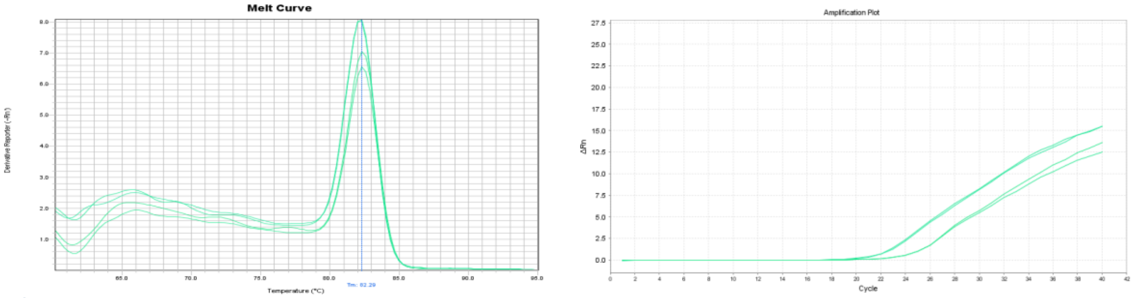
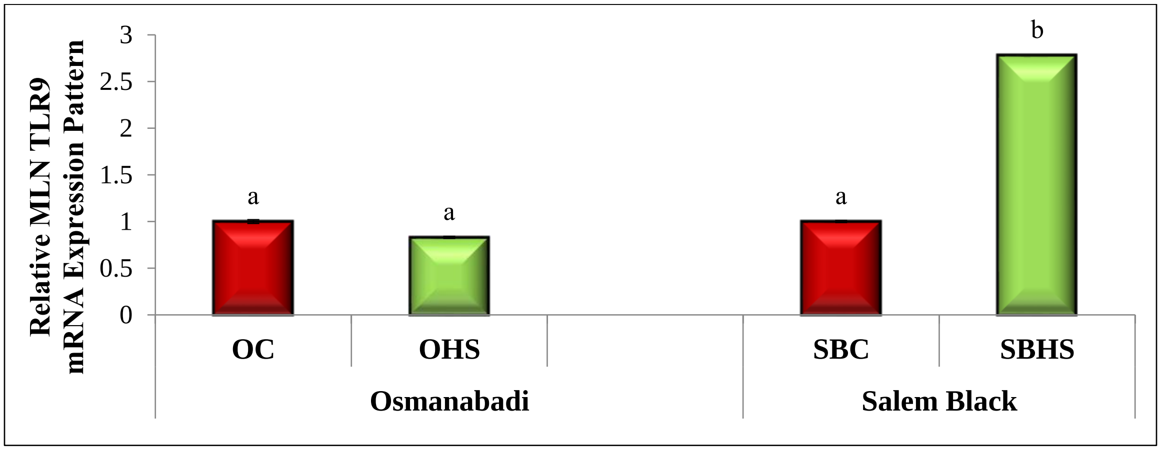


Fig. 37: Relative mesenteric lymph node *TLR9* mRNA expression pattern in female Osmanabadi (Control and Heat stress) and Salem Black (Control and Heat stress) goats



The values bearing different superscripts both within and between breeds differ significantly at $P < 0.01$. The values bearing same superscript both within and between breeds do not differ with each other.

Discussion

V. DISCUSSION

In the changing climate scenario, research efforts are needed to identify the most suitable livestock breed which can withstand the adverse environmental conditions to sustain production. To claim an animal to be thermo-tolerant, it should possess sound disease resistance capability with potent immune competency. In this line, the present study is first of its kind to comparatively assess the better immune competency of two indigenous goat breeds Osmanabadi and Salem Black breed during exposure to summer heat stress. The study was of practical relevance as the global community are working towards improving the resilience capacity of indigenous breeds to climate change and sustaining their production to ensure food security by 2050. It is also very essential to identify a breed with better immune competency which may impart disease resistance capacity to cope with the sudden outbreak of diseases emerging as a result of climate change. In this line, the results of this study may give valuable clue about choosing the best breed which has the ability to survive in different agro-ecological zone.

To claim the breed to be climate resilient, the animals should possess thermo-tolerance and disease resistance capacity. The upregulation of TLRs in animals generally reflects the immunocompetency of the breed. In this study most of the TLRs are overexpressed only in Salem Black breed whereas in Osmanabadi breed most of the TLRs expression did not differed between the groups. Therefore, on comparative basis it was concluded that Salem Black breed possessed better immunocompetency than Osmanabadi breed. Although both the breeds are indigenous in nature the study attempted to identify the better indigenous breed with superior immunocompetency for the study location. As the primary hypothesis of the study was to identify the best climate resilient breed in a specific agroecological zone, it is very vital to compare a breed which is native to the study location to prove the hypothesis of other indigenous breed of different agroecological zone origin may be better to survive in the target location and provide a better output for the farmers. This is the reason why Osmanabadi breed was chosen for the study which is native to the study location and another breed that is Salem Black was brought from different agro-ecological zone to comparatively assess their climate resilient capacity in terms of maintaining their immunocompetency. The results from the study proved that it is better for the farmers of Karnataka to rear Salem Black breed than Osmanabadi because of their superior immunocompetency as reflected by the over expression of almost all the TLRs.

The THI was considered the best indices to quantify the heat stress response in livestock (Marai *et al.,* 2007). It provides a reliable methodology to assess the stress status of the animals and provide important clue to identify the intervening points for implementing amelioration strategies. The significantly higher THI value during afternoon in the outside environment clearly indicates that, both OHS and SBHS group animals are subjected to extremely severe heat stress as described by McDowell (1972). With the THI value of 86.5 recorded during outside exposure in all stress groups clearly indicated that these animals were subjected to extremely severe heat stress. This ensures that the technical design of subjecting two groups one from each breed to heat stress was justified. The previous study in our laboratory comparatively assessed the heat stress resilience capacity of Salem Black and Osmanabadi breeds in terms of changes associated with behavioural, physiological and endocrine mechanisms (Aleena *et al.*, 2018). This study further established the superior thermo-tolerance ability of the Salem Black breed over Osmanabadi breed (Aleena *et al.,* 2018). In addition, the study also established that respiration rate, rectal temperature, plasma cortisol and HSP70 to be the ideal biological markers for assessing the heat stress resilience capacity of indigenous goat breeds. Since these results were already established in our previous study such variables were not repeated in the current study.

During heat stress exposure there will be tissue injuries, cell necrosis and tissue damage which releases endogenous DAMPs to induce sterile inflammatory responses (West *et al.,* 2010). The TLR expression is a prerequisite step for inducing the inflammatory response as the DAMPs which are produced during tissue injury are recognized by these TLRs. The increased TLR expression in the affected immune cells on heat stress exposure helps in the synthesis various pro inflammatory cytokines and chemokines which in turn induces adaptive immune response to combat against the DAMPs produced in heat stress affected immune cell. Thus, TLRs mediated immune responses are also a prerequisite for the generation of adaptive immune responses in heat stressed animals. Hence, TLRs represents the first line of host defence mechanism against DAMPs associated with heat stress induced tissue injury and therefore play a key role in keeping intact the adaptive immunity (Sophia *et al*., 2016).

Similar to our finding, Paul *et al.* (2015) also reported significantly higher *TLR1* expression during exposure to summer season induced heat stress in Black Bengal goats. Further, Tirumurugaan *et al.* (2010) also reported increased expression of *TLR1* in different organs such as uterus, skin, lymph node, PBMC and lungs of heat stressed

Kanni breed goats. These breeds are also indigenous in nature with good disease resistance capacity in their respective region of origin. Therefore, increased expression pattern of *TLR1* in Salem Black goats could be due to the sustained efforts of these animals to maintain the immune status. Tirumurugaan *et al.* (2010) explained that the increased expression of *TLR1* in lymph nodes of the gastro-intestinal tract could be due to the constant activation by antigens. The non-significant effect of heat stress on *TLR1* expression in Osmanabadi breed could be attributed to the fact that the study location was the native track of this breed. Among the cell surface *TLRs* only *TLR2* was up regulated in Osmanabadi breed. This indicates the functional significance of this *TLR* in imparting immune competency to Osmanabadi breed. However, *TLR2* did not express at all in Salem Black breed. This indicates the breed difference in expression pattern of *TLR2* in goats.

Among the *TLRs*, *TLR4* was established to be associated with the damage-associated molecular patterns (DAMPs) to induce immune response during heat stress by producing pro-inflammatory cytokines (Bharati *et al.*, 2017). Like *TLR1*, *TLR4* also showed similar expression pattern between the breeds with no difference between the groups in Osmanabadi breed and significantly higher expression of *TLR4* recorded in SBHS group of Salem Black breed. However, contrasting report of significantly lower *TLR4* expression pattern were recorded in other indigenous goat breeds such as Malabari (Vandana *et al.,* 2019) and Osmanabadi goats (male) (Sophia *et. al.,* 2017). These findings suggest the superiority of Salem Black breed to maintain immune status as compared to even other indigenous goat breeds. Similar to our findings there are also reports of higher expression of *TLR4* in response to heat stress in different species (Ju *et* *al.,* 2014; Paul *et al.,* 2015; Bharati *et al.,* 2017). This difference between these studies could be attributed to the difference in the magnitude of heat stress as well as the difference in the adaptive capacity of different breeds.

Similar to the trends of *TLR1* and *TLR4*, *TLR5* also showed the same expression pattern between the breeds with no difference between the groups in Osmanabadi breed and significantly higher expression of *TLR5* recorded in heat stress group of Salem Black breed.

In a similar study in our laboratory, Vandana *et al.* (2019) reported significantly lower expression of *TLR5* and they attributed this to the lower thermo-tolerance ability of the Malabari goats. The non-significant influence of heat stress on *TLR5* expression in Osmanabadi breed suggests the ability to withstand heat stress but the significantly higher expression of *TLR5* in Salem Black breed indicates the ability of this breed to mount appropriate immune response even during exposure to adverse environmental condition. Similar to the no effect of heat stress in female Osmanabadi goats in the current study was similar to the findings reported by Sophia *et al.,* (2016b) in male Osmanabadi breed.

The expression pattern of *TLR6* also was similar like that of *TLR1*, *TLR4,* and *TLR5* in the current study. Similar results of significantly higher *TLR6* expression pattern were also reported in other species (Plain *et al.,* 2010; Paul *et al.,* 2015; Srikanth *et al.,* 2017). These authors attributed this to the superior thermo-tolerance of these animals. Hence the similar finding in Salem Black breed could be attributed to the climate resilient capacity of this breed. This argument was supported by the findings of Aleena *et al.* (2018) who established superior thermo-tolerance of Salem Black breed over other two indigenous Osmanabadi and Malabari breeds when exposed to heat stress. The non-significant influence of heat stress on *TLR6* expression pattern in Osmanabadi breed could be attributed to the origin of this particular breed to the study location. Quantitative expression pattern of *TLR10* also followed the same pattern as that of *TLR1*, *TLR4*, *TLR5* and *TLR6*. Similar findings of no effect of heat stress on *TLR10* expression pattern also was established by Vandana *et al.* (2019) and Sophia *et al.* (2016b) in Malabari and Osmanabadi breeds respectively. In contrast to these findings, significantly higher *TLR10* expression was reported in the heat stress group of Salem Black breed. Similar to this finding on *TLR10* expression in Salem Black breed, Paul *et al.* (2015) also established significant up regulation of *TLR10* in Black Bengal goats during exposure to heat stress. This finding again supports the argument of superior thermo-tolerance of Salem Black breed over Osmanabadi breed in maintaining the immune status during exposure to hot and humid tropical environmental conditions.

The results indicated that Salem Black breed possessed superior ability to maintain the immune response even during exposure to extreme climatic conditions. This is evident from the significant up regulation of *TLR1*, *TLR4*, *TLR5*, *TLR6* and *TLR10* in heat stress groups of this breed as compared to control group reflecting that the animals were able to mount appropriate immune response during exposure to extremely severe heat stress. The result points towards the excellent resilience capacity of Salem Black breed to heat stress challenges. This was evident from their ability to maintain superior immune status by up regulating all cell surface *TLRs* during exposure to adverse environmental condition.

The expression pattern of *TLR3* was significantly higher in both OHS and SBHS groups indicating the similar expression pattern of this particular gene between the two breeds. This was in contrast to the finding reported by Vandana *et al.* (2019) in Malabari goat breed that no significance in expression pattern of *TLR3* gene between control and heat stress group and attributed this to the thermo-tolerance capacity. The significantly higher *TLR3* gene expression in both Osmanabadi and Salem Black breed in the current study could be attributed to the better immune competency of these two breeds over Malabari. Similarly, there are also reports suggesting significantly higher *TLR3* expression in heat stressed indigenous goats (Paul *et al.,* 2015; Sophia *et al.,* 2017). These findings indicate that *TLR3* could be a good indicator of thermo-tolerance in indigenous goat breeds.

The *TLR7* expression pattern also followed the similar pattern as that of *TLR3* with significantly higher expression pattern recorded in both OHS and SBHS groups indicating the similar expression pattern of this particular gene between the two breeds. Likewise, most of the studies in farm animals also reported significantly higher level of expression pattern of *TLR7* in heat stressed animals as compared to the control group (Paul *et al.,* 2015; Sophia *et al.,* 2016b, 2017). Hence, *TLR7* could serve as reliable indicator for assessing the thermo-tolerance in farm animals.

Contrasting expression pattern was recorded for *TLR8* as compared to the expression pattern for *TLR3* and *TLR7* between Osmanabadi and Salem Black breeds. Among these breeds, only in Salem Black breed the *TLR8* expression was significantly higher in heat stress group. However, the *TLR8* expression pattern was similar between the control and heat stress group in Osmanabadi breed. This was similar to the findings reported by Vandana *et al.* (2019) who established no difference in expression pattern of *TLR8* between the heat stress and control groups in Malabari goat breed. This suggests the better ability of Salem Black breed to withstand heat stress in terms of maintaining the immune status as compared to both Osmanabadi and Malabari breeds. This finding was further supported by the findings of Aleena *et al.* (2018) who established better thermo-tolerance of Salem Black goat breed over Osmanabadi and Malabari breed based on differences in phenotypic and genotypic traits.

Similarly, the expression pattern of *TLR9* also followed the same pattern like that of *TLR8* gene between these two breeds with significantly higher *TLR9* gene expression recorded only in the heat stress group of Salem Black breed while in Osmanabadi, its expression level was comparable between the control and heat stress group. Like our findings in Salem Black breed, there are also reports which established higher expression pattern of *TLR9* in heat stressed animals (Takeshita *et al.,* 2001; Paul *et al.,* 2015). This gain supports the argument of better heat tolerance ability of Salem Black breed and point towards the better immune competency of this particular breed. The non-significant influence of heat stress on the expression pattern of *TLR9* in female Osmanabadi goats in this study was similar to the findings reported in male Osmanabadi goats by Sophia *et al.* (2016b, 2017). This indicates that irrespective of sex, the expression pattern of *TLR9* during heat stress exposure followed the same pattern in Osmanabadi breed. Likewise, Vandana *et al.* (2019) also reported no difference in expression pattern of *TLR9* gene between the control and heat stress group of Malabari breed. This again indicates that *TLR9* could be other immunological variable to reflect the immune status during heat stress exposures in different breeds of goats. The significantly higher *TLR9* gene expression in Salem Black breed again establishes the supremacy of this breed to withstand heat stress without compromising the immune status.

A study was designed in two different indigenous goat breeds namely, Osmanabadi and Salem Black, to comparatively assess the thermo-tolerance during heat stress exposure. Based on this preliminary findings in the current study we can conclude that Salem Black breed possess a better thermo-tolerance capacity as compared to Osmanabadi breed which is native to the study location. This was evident from the significantly higher expression of majority of cell surface *TLRs* such as *TLR1*, *TLR4*, *TLR5*, *TLR6* and *TLR10* in SBHS group as compared to OHS group. Further, the study also established significantly higher expression of all four intracellular *TLRs* in the heat stress group of Salem Black breed as compared with Osmanabadi breed. The higher expression of most of cell surface and intracellular *TLRs* in the SBHS group indicates the superior resilient capacity of Salem Black goats as compared with Osmanabadi breed to maintain immune status even during exposure to adverse environmental condition. Although, the Salem Black breed was brought from different agro-ecological zone to the current study location, still it was able to mount better immune response as compared to its native counterpart Osmanabadi breed. Further, the non-significant influence of heat stress on majority of *TLRs* studied in Osmanabadi breed indicates the indigenous nature of this breed to cope to existing climatic condition of its origin. In addition, the significantly higher expression of *TLR1*, *TLR3*, *TLR4*, *TLR5*, *TLR6*, *TLR7*, *TLR8*, *TLR9* and *TLR10* in the heat stress group indicates the reliability of these genes to act as biological markers for assessing the immune status of Salem Black breed during heat stress exposure.

Summary

VI. SUMMARY

The study is the first of its kind to assess the thermo-tolerance of two different indigenous goat breeds based on immune system related gene expression patterns. The study was of practical relevance as the global community are working towards improving the resilience capacity of indigenous breeds to climate change and sustaining their production to ensure food security by 2050. It is also very essential to identify a breed with better immune competency which may impart disease resistance capacity to cope with the sudden outbreak of diseases emerging as a result of climate change. In this line, the results of the present study may give valuable clue about choosing the best breed which has the ability to survive in different agro-ecological zone.

Among the domesticated ruminant species goat has been projected as the ideal animal that can withstand the harmful effects of climate change. Among the various environmental stressors, heat stress is the major stressor that affects the immune response in the goats. However, there are very limited reports in establishing the impact of heat stress on immune response in goats. Currently sufficient research reports are available pertaining to establishing the impact of heat stress on both adaptation and production. However, such reports establishing heat stress impact on immune status particularly in the indigenous livestock population are very limited. Therefore, a study was designed in two different indigenous breeds of goat, namely, Osmanabadi and Salem Black to comparatively assess their resilience capacity to heat stress based on their ability to maintain their immune status during exposure to adverse environmental conditions. Therefore, a study was conducted to establish the differences in expression pattern of different *toll-like-receptors (TLRs)* in Osmanabadi and Salem Black goats and to compare the expression patterns of these *TLRs* between the breeds subjected to summer season induced heat stress.

The study was conducted for a period of 45 days in twenty-four number of 10 months to one-year old female Osmanabadi and Salem Black breed goats. The animals were randomly allocated into four groups of six in each, OC (n=6; Osmanabadi control); OHS (n=6; Osmanabadi heat stress); SBC (n=6; Salem Black control) and SBHS (n=6; Salem Black heat stress). The OC and SBC animals were maintained in the shed in comfort condition while OHS and SBHS animals were exposed outside to summer heat stress between 10:00h to 16:00h during experimental period. The THI values for the present study were calculated as per the method described by McDowell (1972). The animals were slaughtered at the end of the study and their mesenteric lymph node samples were collected for assessing the different toll-like receptor genes. The targeted genes were *TLR1*, *TLR2*, *TLR3*, *TLR4*, *TLR5*, *TLR6*, *TLR7*, *TLR8*, *TLR9* and *TLR10* genes. The relative expressions of selected genes were studied using SYBR green chemistry after designing appropriate primers for the target genes. The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), Hypoxanthine phosphoribosyl transferase 1 (*HPRT1*), and Succinate dehydrogenase complex flavoprotein subunit A (*SDHA*), genes were used as reference genes and the relative expression of all target genes were analysed using the formula $2^{-\Delta\Delta C}$T.

The THI values for the entire study duration during morning were not stressful to the animals kept both inside and outside the shed. However, the obtained THI values

(P<0.01) during afternoon indicated that the animals inside the shed were not stressed while the animals kept outside the shed were under extreme distress. The significantly higher THI value during afternoon in the outside environment clearly indicates that both OHS and SBHS group animals are subjected to extremely severe heat stress as described by McDowell (1972). This ensures that the technical design of subjecting two groups one from each breed to heat stress was justified.

The difference in expression pattern of *TLR1* between the OC and OHS groups was not statistically significant. But the *TLR2* expression pattern showed significant difference between the groups. The level of *TLR2* expression was significantly (P<0.01) higher in OHS group as compared to OC group. Similarly, the *TLR3* expression pattern also showed significant difference between the groups with higher (P<0.01) expression pattern recorded in OHS group. However, the expression pattern of *TLR4* did not differ between the groups in this breed. Likewise, the expression pattern of both *TLR5* and *TLR6* also did not vary between the groups. However, the expression pattern of *TLR7* differed between the groups with significantly (P<0.01) higher value recorded in OHS group. But, again the *TLR8*, *TLR9* and *TLR10* expression pattern also did not differ between the groups.

The difference in expression pattern of *TLR1* between the SBC and SBHS groups was statistically significant (P<0.01) with higher expression pattern reported in SBHS group. However, the *TLR2* did not express in both SBC and SBHS groups in this study. The level of *TLR3* expression was significantly (P<0.01) higher in SBHS group as compared to SBC group. Similarly, the *TLR4* expression pattern also showed significant difference between the groups with higher (P<0.01) expression pattern recorded in SBHS group. Likewise, the expression pattern of both *TLR5* and *TLR6* also was significantly (P<0.01) higher in SBHS group as compared to SBC group. Similarly, the expression pattern of *TLR7*, *TLR8*, *TLR9* and *TLR10* were also significantly (P<0.01) higher in SBHS group as compared to SBC group.

The results on the comparative analysis of all *TLRs* expression between the two breeds indicated that the expression of *TLR1*, *TLR4*, *TLR5*, *TLR6* and *TLR10* genes in both the breeds showed different expression pattern. The expression pattern of all these genes did not differ between the control (OC) and heat stress (OHS) group in Osmanabadi breed but significantly higher (P<0.01) expression of these genes were reported in heat stress group (SBHS) as compared to control group (SBC) in Salem Black breed. Further, the results indicated that the expression of *TLR3* and *TLR7* followed the same pattern between both Osmanabadi and Salem Black breeds with significantly higher (P<0.01) expression in heat stress group of both these breeds. However, *TLR8* and *TLR9* genes showed different expression patterns between the breeds with significantly higher (P<0.01) expression of these two genes recorded in Salem Black breed. The *TLR2* expression was significantly higher (P<0.01) in heat stress group of Osmanabadi breed, but *TLR2* did not expressed in Salem Black breed. The higher expression of most of *TLRs* in the SBHS group as compared to OHS group indicates the superior resilient capacity of Salem Black goats to maintain immune status even during exposure to adverse environmental condition.

The study is the first of its kind to comparatively assess the thermo-tolerance between Osmanabadi and Salem Black, two different goat breeds, during heat stress exposure. Based on this preliminary findings in the current study we can conclude that Salem Black breed possess a better thermo-tolerance capacity compared to Osmanabadi breed which is native to the study location. This was evident from the significantly higher expression of majority of cell surface *TLRs* such as *TLR1*, *TLR4*, *TLR5*, *TLR6* and *TLR10* in SBHS group compared to OHS group. Further, the study also established significantly higher expression of all four intracellular *TLRs* in the heat stress group of Salem Black breed compared to Osmanabadi breed. The higher expression of most of cell surface and intracellular *TLRs* in the SBHS group indicates the superior resilient capacity of Salem Black goats compared to Osmanabadi breed to maintain immune status even during exposure to adverse environmental condition. Although, the Salem Black breed was brought from different agro-ecological zone to the current study location, still it was able to mount better immune response as compared to its native counterpart Osmanabadi breed. Further, the non-significant influence of heat stress on majority of *TLRs* studied in Osmanabadi breed indicates the indigenous nature of this breed to cope to existing climatic condition of its origin. In addition, the significantly higher expression of *TLR1*, *TLR3*, *TLR4*, *TLR5*, *TLR6*, *TLR7*, *TLR8*, *TLR9* and *TLR10* in the heat stress group indicates the reliability of these genes to act as biological marker for assessing the immune status of Salem Black breed during heat stress exposure.

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Abstract

VIII. ABSTRACT

The study was conducted to establish the differences in expression pattern of different *toll-like-receptors (TLRs)* in Osmanabadi and Salem Black goats and to compare the expression patterns of these *TLRs* between these breeds subjected to summer season induced heat stress. The animals were randomly allocated into four groups of six animals each, OC (n=6; Osmanabadi control); OHS (n=6; Osmanabadi heat stress); SC (n=6; Salem Black control) and SBHS (n=6; Salem Black heat stress). The OC and SC animals were maintained in the shed in comfort condition while OHS and SBHS animals were exposed outside to summer heat stress between 10:00h to 16:00h during experimental period. The results indicated that the expression of *TLR1, TLR4, TLR5, TLR6* and *TLR10* genes in both the breeds showed different expression pattern. The expression pattern of all these genes did not differ between the control (OC) and heat stress (OHS) group in Osmanabadi breed but significantly higher ($P<0.05$) expression of these genes were reported in heat stress group (SBHS) as compared to control group (SBC) in Salem Black breed. Further, the results indicated that the expression of *TLR3* and *TLR7* followed the same pattern between both Osmanabadi and Salem Black breeds with significantly higher ($P<0.01$) expression in heat stress group of both these breeds. However, *TLR8* and *TLR9* genes showed different expression patterns between the breeds with significantly higher ($P<0.05$) expression of these two genes recorded in Salem Black breed. The *TLR2* expression was significantly higher ($P<0.01$) in heat stress group of Osmanabadi breed. But *TLR2* did not expressed in Salem Black breed. The higher expression of most of *TLRs* in the SBHS group as compared to OHS group indicates the superior resilient capacity of Salem Black goats to maintain immune status even during exposure to adverse environmental condition.

Keywords: Heat stress; Goat; Immune response; *TLR*; THI; Thermo-tolerance