EXPRESSION PROFILE OF TLR GENES IN BLACK BENGAL GOAT DURING DIFFERENT SEASONS

Thesis

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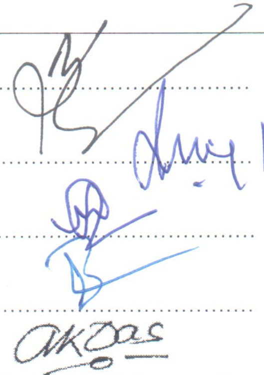

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ABBREVIATIONS

% : Percentage
< : Lesser than
> : Greater than
°C : Degree Celsius
ANOVA : Analysis of variance
GAPDH : Gleceraldehyde 3-Phosphate Dehydrogenase
bp : Base pairs
cDNA : Complementary DNA
Ct value : Cycle threshold values
DAHD : Department of animal husbandry and dairying
DEPC : Diethyl pyrocarbonate
DNA : Deoxy ribonucleic acid
dNTP's : Deoxynucleoside triphosphate
DW : Distilled Water
Fig. : Figure
h : Hour
HSP : Heat shock protein
HSF : Heat shock factors
IG : Immunoglobulin
ILs : Interleukins
KDa : Kilo Dalton
LN_2 : Liquid nitrogen
M : Molar
mg : Milligram
min : Minutes
$MgCl_2$: Magnesium Chloride

ml	: Millilitre
mm	: Millimeter
mM	: Millimolar
MM	: Master mix
Mol.Wt.	: Molecular weight
mRNA	: Messenger ribonucleic acid
NFW	: Nuclease free water
ng	: Nanogram
NTC	: No template control
OD	: Optical density
PAGE	: Polyacrylamide gel electrophoresis
PAMP	: Pathogen Associated Molecular Patterns
PBS	: Phosphate buffered saline
PBMC	: Peripheral blood mononuclear cells
PCR	: Polymerase chain reaction
PRR	: Pattern Recognition Receptor
pH	: -Log Hydrogen ion concentration
pmol.	: Pico mole
qRT-PCR	: Quantitative real-time PCR
RNA	: Ribonucleic acid
RT-PCR	: Reverse transcriptase polymerase chain reaction
SEM	: Standard Error of Mean
Sec	: Seconds
SDS	: Sodium dodecyl sulphate
TAE	: Tris Acetate EDTA buffer
Taq. Pol.	: Thermus aquaticus polymerase
TLR	: Toll Like Receptor
µg	: Microgram
µl	: Microliter

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Introduction

1 INTRODUCTION

Goats are an integral part of rural India's symbiotic system of crop and livestock production and make up a significant part of the livestock wealth of country. Goats are among the main meat-producing animals in India and goat meat (chevon) faces huge domestic demand, with no social, cultural, and religious restrictions, thus sharing the profit to the national GDP. India has a vast reserve of goat with total population of 140.54 million & average growth rate of 3.1% in the year 2007 which account for more than 25 percent of the country's total livestock and contribute more than Rs. 106 billion annually to the national economy, providing food and nutritional security to millions of marginal and small farmers and agricultural labourers (DAHD, 2010). About 90 percent of the world's goats are reared primarily for meat. In India about 42 percent of the total population of goats is slaughtered for meat every year. The total meat production from sheep & goat in the year 2009-10 is 830 tonnes. Goats provide not only livelihoods for much of the rural population, but also valuable animal protein to both rural and urban populations. They play an important role in income generation, capital storage, employment generation, and household nutrition.

Among the various meat-producing indigenous breeds of goats in India owned by small farmers, peasants, and landless labourers, the Black Bengal goat (*Capra hircus bengalensis*) is the most common. This breed of goat is mostly found in the Eastern & North-Eastern region of India. The state of West Bengal ranks first in India with 15.07 million goats out of total 123 million. (Annual Report, 2007-08, Govt. of West Bengal) and possesses a valuable genetic resource of dwarf Goats known as Black Bengal Goat (Syn. Bengal Goat). A number of factors make the rearing of black Bengal goats a preferred option among marginal and small farmers and even landless farmers, as this animal mainly depend on common grazing and forest lands for fodder (Biswas, 2010). Other factors for rearing black Bengal goat includes low capital intensity, prolific breeding, superior chevon quality, early sexual maturity, high-quality skin, low kidding intervals, good adaptability, no religious taboo against consumption, and steady returns (Dixit and Shukla, 1995). These factors have led to the realization that there is need to pay more attention to this goat breed, whose genetic material has been used since time immemorial. Thus there is need to have a study to understand how this breed of goat is adapting during different climatic season.

Black Bengal goats are generally reared under extensive farming system. While browsing during daytime, they are exposed to variable climatic conditions and thereby affected by both heat and cold stress. Since India is a tropical country the climatic variations are in broader range here. The lower critical temperature for goat is not specified but the limits of thermo neutrality for goats may be taken as a climatic environment having an air temperature of 13°C –27°C, relative humidity of 60-70 per cent and wind velocity of 5-8km/hr and a medium level of solar radiation (Mishra, 2009). In most domestic species including goat, hot as well as cold weather has deleterious effects on nutritional, physiological and reproductive functions. Exposure of goats to harsh environment causes depression of feed intake, reduction in production and increase susceptibility to wide range of microbes. This situation is often worsened by dry, poor quality forage. Adverse season is also detrimental to semen quality, embryo survival and foetal development. Goats are more susceptible to cold stress than hot weather. The grazing activity of domestic goats exposes them to a wide range of pathogenic organisms (cysts of helminths and nematodes) and pesticides (used by local farmers) as a part of environmental challenges. Moreover, incidents of infectious diseases such as enterotoxaemia, diarrhoea and various infections are more frequent during certain period of the year, i.e. rainy season. In tropical zone, rainy season provides high humidity and temperature for the growth of bacteria and several pathogens and facilitates their horizontal as well as vertical migration in domestic goat population. On the other hand, during rest of the year, the health of the domestic goats is optimum and produce maximum amount of milk and meat.

The immune system protects the livestock against different stressful situations. How this immune system precisely responds to stress, largely remains a mystery primarily due to the complexity of the immune and stress systems. Paradoxically the most common theory is that stress suppresses components of the immune system, thus enhancing disease susceptibility of an animal. Seasonal stress lowers the natural immunity of animals, making them more susceptible to disease. In general, animals will have less tolerance for parasitic and other opportunistic diseases. Therefore, it is important to understand the effects of seasonal stress on innate immunity when the animal becomes susceptible to various infections.

Animals live in a wide variety of microbe-rich environments and hence it is crucial to have a sensitive innate defence mechanism which relies in part by recognizing conserved molecules that are unique to some classes of potential pathogens. These molecules have been collectively called as pathogen-associated molecular patterns (PAMPs). Primary sensing of these PAMPs to alert the innate immune system is achieved by an array of germ line encoded receptors known as pattern recognition receptors (PRRs). One of the important PRRs that play a key role in innate immunity is the type I transmembrane proteins called Toll-Like Receptors (TLRs). TLRs recognize microbial markers namely protein, carbohydrate, lipid, nucleic acids and/or their combinations in an efficient, non-self-reactive means to initiate a complex signalling cascade to activate a wide variety of transcription factors and inflammatory cytokines (Takeda and Akira, 2004).

These cell surface molecules also activate complement, phagocytosis, inflammation and apoptosis in response to pathogen detection (West *et al.,* 2006) which finally culminate in the initiation of adaptive immunity through the induction of pro-inflammatory mediators (Janeway and Medzhitov, 2002). The expression of these TLRs is not restricted to cell types that are involved in antigen processing and presentation like macrophages, neutrophils and dendritic cells but also could be detected to variable levels in a wide range of tissues (Iqbal *et al.,* 2005). Basal TLR mRNA expression profiles in different cells and tissues are also suggestive of an individual's ability to respond to a challenge. The expression pattern and distribution of the TLRs have been shown to be characteristic of each species (Nalubamba *et al.,* 2008; Vahanan *et al.,* 2008; Zarember and Godowski, 2002). Over the last few decades there has been very good progress in identifying TLRs in different species of farm animals and also in implicating their role in diseases. Polymorphisms and mutations in the PRRs have been shown to be associated with disease susceptibility and resistance respectively indicating the importance of these receptors (Mucha *et al.,* 2009; Nalubamba *et al.,* 2008). Recently, the partial sequences for bovine i.e. Cattle, Buffalo, Sheep TLR1-10 were published (Menzies and Ingham, 2006). In goat all the TLRS 1-10 have been identified (Dhinakar Raj *et al.,* 2009).

Animals experience heat or cold stress when environmental variables such as ambient temperature, humidity, solar radiation and movement of air surpass the upper or lower limit of the thermoneutral zone, respectively. Goats adapted to a harsh environment perform better than other domesticated ruminants (Silanikove, 2000). These animals have developed adaptive mechanisms that allow their survival at very high (45 to 50 °C) as well as low temperatures (−20 to −40 °C). However, despite their extreme tolerance the productivity of these animals often declines due to thermal stress (Al-Tamimi, 2007). Thermotolerance in animals during prolonged heat or cold stress is characterized by the heat or cold shock response and adaptations associated with acclimation. The heat shock response confers transient thermal tolerance, in part due to the expression of heat shock proteins (HSPs). The induction of HSPs is remarkably rapid and intense as an emergency response. HSPs constitute a large group of chaperone proteins that are classified into several families on the basis of their molecular size and amino acid sequence similarity. FM Ritossa reported HSPs for the first time in 1960. HSPs are named according to their molecular weight. For example Hsp60, Hsp70 and Hsp90 refer to families of HSPs on the order of 60, 70 and 90 kilodaltons in size respectively (Li and Srivastava, 2004). HSPs are among the most well conserved proteins known. It account for 1-2% of total protein in unstressed cells which increases to 4-6% of cellular proteins when cells are heated (Crevel *et al.,* 2001).

Heat shock proteins are responsible for maintaining the balance between survival and an effective immune system in the organisms in order to acclimatize the stress (Morange, 2006). Recently it was observed that the mRNA expression of HSP60, HSP90 and ubiquitin were higher during peak summer season as compared with peak winter season in both tropical and temperate region goats whereas the expression of HSP70 was significantly higher during summer season as compared with winter season in tropical region goats which might play an important role in thermal stress tolerance against harsh environmental conditions (Dangi *et al.,* 2012). Studies also shows that HSP70 induces proinflammatory cytokine production via NF-kB signal transduction pathway and that HSP70 utilizes both TLR2 and TLR4 to transduce its proinflammatory signal (Asea *et al.*, 2002). However, the correlative expression of HSP family genes and Toll like receptor (TLR) genes during different seasons has not been studied in Black Bengal goats.

There is relative dearth of information on TLR gene sequences, expression profiles and their contribution to innate immunity in goats. Hence, this study was planned to demonstrate the expression profile of TLR genes during different season in peripheral blood mono nuclear cells (PBMCs) of Black Bengal goat.

Objectives:- Keeping in view of these facts, the present research work is formulated to carry out with the following objectives:-

1. **To study the expression profile of TLR genes in Black Bengal goat during different season.**
2. **To demonstrate the expression profile of HSPs (HSP60, HSP70, HSP90) in black bengal goat during different seasons**

Review of Literature

2 REVIEW OF LITERATURE

2.1 The Vertebrate Immune System

The vertebrate immune system is diffusely distributed throughout the body and consists of many interacting cells, tissues and soluble proteins (Janeway *et al.,* 2002). Collectively, these substances protect the body from infection, as well as rid or control infections once they have taken hold. Broadly, the immune system comprises two arms that differ in function and evolutionary history: the adaptive arm and the innate arm. The adaptive immune system is unique to the jawed vertebrates and consists of two branches termed humoral (B-cell) and cell mediated (T-cell). Humoral immunity is predominantly responsible for extracellular pathogen control through generation of soluble proteins (antibodies) specific to particular components (antigens) of invading cells or organisms. Cell-mediated immunity is generally responsible for intracellular pathogen control (cytotoxic T-cells) and/or managing B-cell and other immune responses (T-helper cells). Unlike other immune cells, B- and T-cells are capable of targeted defence against non-self substances via a diverse set of membrane receptors generated early in ontogeny. Although these defences can provide immunological memory of prior infections, they are developmentally expensive and slower to reach effectiveness in terms of pathogen control relative to the other main immune defence system, innate immunity.

The Innate immunity system also called nonspecific immune system serve as the first line of defense mechanism for host against different infectious microorganisms. This evolutionary mechanism of host defense which occurs very quickly upon the appearance of an antigen in the body is mediated by diverse array of cell types, such as macrophages, granulocytes, natural killer cells and a variety of substances secreted by these cells including antimicrobial peptides, destructive enzymes and complement, a protein complex responsible for rapid control of extracellular pathogens (Hoffmann 2003; Akira *et al.,* 2006). Innate immune defences are effective at controlling multiple parasite types, and they are much more quickly engaged than adaptive defences.

The innate immune system senses microbial infection with the help of special receptors that are predominantly expressed on sentinel cell called 'pattern recognition receptors (PPRs) that recognize the essential components of microbes (cell wall structures; nucleic acids), referred to as "Pathogen-associated molecular patterns" (PAMPs) (Janeway, 1989). Host sentinel cells (Macrophage, DCs) express various pattern recognition receptors (PRRs) like Toll Like Receptor that identifies diverse pathogen-associated molecular patterns (PAMPs), ranging from lipids, lipoproteins, proteins and nucleic acids, lipopolysaccharide (LPS), glycolipid etc. These PRRs are located on the cell surface & some located within the cytoplasm of sentinel cells. Binding of these PAMPs to these receptors activates intracellular signalling pathways which leads to the induction of inflammatory cytokines, chemokines, interferons (IFNs) by sentinel cells and upregulation of co-stimulatory molecules ultimately causing inflammation & other innate immune responses (Tizard, 2008).

Toll receptor was first identified in Drosophila as an essential receptor responsible for the establishment of the dorso-ventral polarity in developing embryos (Hashimoto *et al.,* 1988). In 2003, again Hoffmann & colleagues demonstration of susceptibility of Toll-mutant Drosophila flies to fungal infection aware that the innate immune system is particularly responsible for microorganism detection. A mammalian homologue of Toll receptor was identified in 1997 which was shown to induce the expression of responses by inflammatory genes (Medzhitov *et al.,* 1997). Thereafter, mammalian Toll receptors were identified one after another & designated as Toll Like Receptors (TLRs). Study shows that TLR4- deficient mice is unable to recognize LPS of bacteria (Hoshino *et al.,* 1999). A human homologue of the drosophila Toll protein was identified which found to induce production of inflammatory cytokines & expression of co-stimulatory molecule (Medzhitov *et al.,* 1997). So far, 10 mammalian members of TLR has been identified designated as [TLR1- TLR10] (Akira, 2004). Different TLRs & their associated ligands in mammals have been given below....

Table 1: TLR family and Their Ligands

TLR	Ligands (origin)
TLR1	Tri-acyl lipopeptides (bacteria, mycobacteria); Soluble factors (*Neisseria meningitides*)
TLR2	Lipoprotein/lipopeptides (a variety of pathogens); Peptidoglycan & Lipoteichoic acid (Gram + bacteria); Lipoarabinomannan (mycobacteria); A phenol-soluble modulin (*Staph. epidermidis*); Glycoinositolphospholipids (*Tryp. cruzi*); Glycolipids (*Trep. maltophilum*); Porins (*Neisseria*); Zymosan (fungi); Atypical LPS (*Leptospira interrogans, Porphyromonas gingivalis*) HSP70 (host)
TLR3	Double-stranded RNA (virus)
TLR4	LPS (Gram-negative bacteria); Fusion protein (RSV); Envelope proteins (MMTV); HSP60 (Chlamydia pneumoniae); Taxol (plant); HSP60; HSP70 (host); Type III repeat extra domain A of fibronectin; Oligosaccharides of hyaluronic acid; Polysaccharide fragments of heparan sulfate; Fibrinogen (host)
TLR5	Flagellin (bacteria)
TLR6	Di-acyl lipopeptides (mycoplasma)
TLR7	Imidazoquinoline; Loxoribine, Bropirimine (synthetic compounds)
TLR9	CpG DNA (bacteria)

(Jin and Lee, 2008)

In mammals, the family of Toll-like receptors (TLR) expressed on antigen presenting cells such as dendritic cells (DC) and macrophages serves as key PRRs which plays central role in induction of innate immune responses as well as the subsequent development of adaptive immune responses. TLRs are largely divided into two subgroups depending on their cellular localization and respective PAMP ligands. One group is composed of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10, which are expressed on cell surfaces and recognize mainly microbial membrane components such as lipids, lipoproteins and proteins. The other group consists of TLR3, TLR7, TLR8 and TLR9, which expressed exclusively in intracellular vesicles such as the endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes, where they recognize microbial nucleic acids (Kawai and Akira, 2010).

The Toll like receptors (TLRs) are type-I transmembrane glycoproteins having N-terminal is outside the membrane & characterized by three major domains. The extracellular domain is composed of Leucine-rich repeats (LRR) that form a horse shoe structure with the ligand (PAMPS) binding to the concave surface.

There is a transmembrane domain and a intracellular domain that is homologous to that of interleukin (IL)-1 receptor and is known as Toll/IL1R (TIR) domain (Akira, and Takeda, 2004).

TIR poses Ig-like domain required for initiating downstream signalling & cascade of reaction inside the cytoplasm of sentinel cells. TIR domain contain proline residue which is common to all TLRs except TLR3 & substitution of proline by histidine cause dominant negative effect in TLR signalling (Hoshino *et al.,* 1999 and Underhill *et al.,* 1999). In course of TLR signalling downstream to the TIR domain, an adaptor protein called MyD88 was first characterized to play a crucial role. Later on during analysis of MyD88-independent pathway two more adaptor protein i.e, TIR domain containing adaptor protein (TIRAP/MyD88 –adaptor like (Mal) and TIR domain containing adaptor inducing IFN-β (TRIF) were identified (Horng, 2001, Fitzgerald *et al.,* 2001). Thus, TLR signalling pathway consists of mainly MyD88 dependent pathway (Common to all TLRs) & MyD88 independent pathway (mainly of TLR3 & TLR4) (Akira *et al.,* 2001). Individual TLRs trigger specific biological responses. For example, TLR3 and TLR4 generate both type I interferon and inflammatory cytokine responses, whereas cell surface TLR1-TLR2, TLR2-TLR6 and TLR5 induce mainly inflammatory cytokines. These differences are explained by the discovery of TIR domain–containing adaptor molecules, including MyD88, TIRAP (Mal), TRIF and TRAM, which are recruited by distinct TLRs and activate distinct signalling pathways (Kawai and Akira, 2006).

MyD88, the first identified member of this TIR family, is universally used by all TLRs except TLR3, and activates the transcription factor NF-kB and mitogen-activated protein kinases (MAPKs) to induce inflammatory cytokines. In contrast, TRIF is used by TLR3 and TLR4 and induces alternative pathways that lead to activation of the transcription factors IRF3 and NF- kβ and the consequent induction of type-I interferon and inflammatory cytokines. TRAM and TIRAP function as sorting adaptors that recruit TRIF to TLR4 and MyD88 to TLR2 and TLR4, respectively. Thus, TLR signalling pathways can be largely classified as either MyD88-dependent pathways, which drive the induction of inflammatory cytokines, or TRIF-dependent pathways, which are responsible for the induction of type I interferon as well as inflammatory cytokines (Kawai and Akira, 2010).

2.2 MyD88-dependent Pathway:

The MyD88-dependent response occurs on dimerization of the TLR receptor and is associated with the TIR domain of TLRs. Its primary effect is activation of NFκβ and Mitogen-activated protein kinase. When ligand bind to the extracellular domain of TLR than conformational change occurs in the receptor leading to recruitment of the adaptor protein MyD88. MyD88 then recruits IL-1 receptor associated kinase (IRAK). In the IRAK family IRAK-1 & IRAK-4 posses the kinase domain but IRAK-2 or IRAK-M (negative inhibitory role) is catalytically inactive. The importance of IRAK in TLR signalling is first identified by Janssens *et al.,* (2003). IRAK then got activated by phosphorylation and associate with the protein TRAF6. TRAF6 is a tumor necrosis factor receptor family member that mediates cytokine signalling pathway (Arch *et al.,* 1998). Upon stimulation by IRAK TRAF6 complex dissociates from the receptor and associates with TGF-β associated kinase-1 (TAK 1) & TAK1 binding protein TAB1 & TAB2. This complex of TRAF6, TAK1, TAB1 & TAB2 moves in the cytoplasm & then forms there a large complex with E2 ligases, Ub13 & uev1A (Li *et al.*, 2000). On binding, TAK1 phosphorylates IKKβ, which then phosphorylates IκB causing its degradation and allowing NFκB to diffuse into the cell nucleus and activate transcription and consequent induction of inflammatory cytokines (Kawai and Akira, 2006).

2.3 MyD88- independent pathway:

Both TLR3 and TLR4 utilize this MyD88-independent pathway also called TRIF-dependent pathway. The triggering agent for TLR3 & TLR4 is dsRNA and LPS respectively. For TLR4, LPS leads to activation of the receptor, recruiting the adaptor TRIF. TRIF activates the kinases TBK1 and RIP1, which creates a branch in the signaling pathway. The TRIF/TBK1 signaling complex phosphorylates IRF3 allowing its translocation into the nucleus and thereby induces IFN-β. IFN-β in turn activates Stat1, leading to the induction of several IFN inducible gene (Doyle *et al.,* 2002). Viral dsRNA acitivate the TLR3 receptor & ultimately induce IFN-β production. Hiscotts & Colleagues (2003) identified that IRF3 is associated with IkB kinases (Ikks), IKKε & IKKi. They also found that TBK1 & IKKε/IKKi induce IRF3 phosphorylation & leads to IFNβ production by TLR3 & TLR4.

Meanwhile, activation of RIP1 causes the polyubiquination and activation of TAK1 and NFκB transcription in the same manner as the MyD88-dependent pathway (Kawai and Akira, 2006). Thus TLR4 signalling pathway requires both MyD88-dependent as well as MyD88 independent pathway to induce inflammatory cytokines.

The TLR family is an important group of receptors helps in microbial elimination, such as recruitment of phagocytes to infected tissue & subsequent microbial killing by producing inflammatory cytokines like TNFα, IFN b and IL-1. These cytokines are critical for host defense like TNFα activates macrophage and PMN phagocytosis and killing; IFNαβ activates anti-viral mechanisms; IL-1 stimulates inflammation and fever.

The expression pattern of TLR mRNA have been reported in human (Zarember and Godowski, 2002), mice (Pruett *et al.,* 2004) and chicken (Iqbal *et al.,* 2005). Expression profile of toll like receptors in the tissues of water buffalo (*Bubalus bubalis*) and transcript profiling of pattern recognition receptors in a semi domesticated breed of buffalo (Toda) has also been reported from India (Vahanan *et al.,* 2008)**.** Subsequently differential expression of toll-like receptor and mRNA sequence analysis of selected tissues of goat (*Capra hircus*) has been studied by Dhinakar *et al.* (2009).

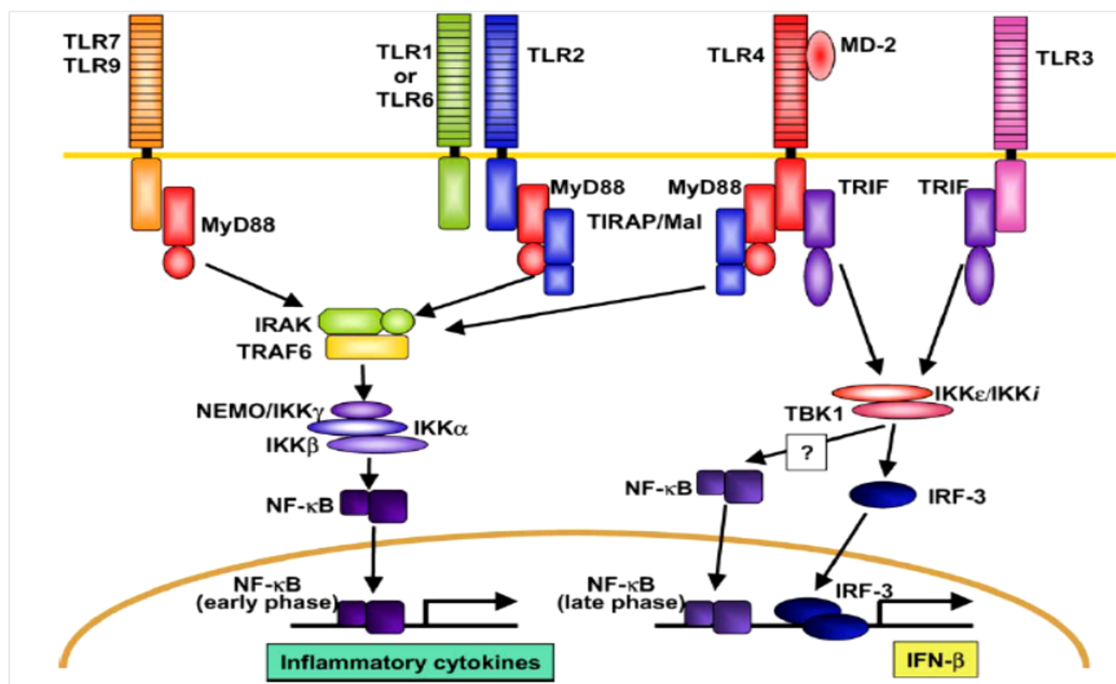


Fig 1: Overview of TLR signalling pathway (Anderson, 2000)

Although identification and basal expression level of Toll-like receptors 1–10 has been documented in certain bovine and ovine tissues (Menzies and Ingham, 2006), expression level of TLR genes in Black Bengal goat during different season remains to be elucidated.

2.4 Heat Shock Proteins:

Heat shock proteins (HSP) are highly conserved proteins found in all prokaryotes and eukaryotes. Under normal physiological conditions HSP are expressed at low levels . However, a wide variety of stressful stimuli including environmental (UV radiation, heat shock, heavy metals, and amino acids), pathological (viral, bacterial, parasitic infections or fever, inflammation, malignancy, autoimmunity), physiological stimuli (growth factors, cell differentiation, hormonal stimulation, or tissue development), induce a marked increase in intracellular HSP synthesis (Lindquist and Craig, 1988), known as the stress response. The primary function ascribed to HSP is as intracellular molecular chaperones of native, aberrantly folded or mutated proteins as well as in cytoprotection following the kinds of stressful stimuli mentioned above. HSPs were originally identified as proteins whose expression was markedly increased by heat shock (Lindquist, 1986).

2.5 Type of Heat Shock proteins:

2.5.1 Heat Shock Protein 60 (HSP 60): Mammalian HSP60 was first reported as a mitochondrial P1 protein (Gupta, 1995). HSP60 in eukaryotes is considered typically a mitochondrial chaperone (also called Cpn60) which also occurs in the cytosol, the cell surface, the extracellular space and in the peripheral blood under normal physiological conditions (Cappello *et al*., 2008). HSP60 has the capability of activating monocytes, macrophages and dendritic cells and also of inducing secretion of a wide range of cytokines (Hansen et al.,2003). HSP60 constitutes 15-30% of cellular proteins. Under normal physiological conditions, HSP60 is a 60 kD oligomer composed of monomers that form a complex arranged as two stacked heptameric rings (Cheng *et al.,*1990). It catalyze the folding of proteins destined for the matrix and maintains protein in an unfolded state for transport across the inner membrane of the mitochondrial (Koll et al., 1992). Cytoplasmic HSP also plays role in a “danger signal cascade” immune response (Itoh *et al.*, 2002).

2.5.2 Heat Shock Protein 70 (HSP 70): HSP70s are a family of ubiquitously expressed heat shock proteins. It is found in prokaryotes and eukaryotes (Tavaria *et al.*, 1996) and is mainly localized in the cytosol, mitochondria and endoplasmic reticulum and exhibit constitutive and inducible regulation. HSP 70 contains two distinct functional regions: a peptide binding domain (PBD) and the amino-terminal ATPase domain (ABD). Under normal conditions, HSP70 functions as ATP dependent molecular chapereon that assist the folding of newly synthesized polypetides, the assembly of multi protein complexes and the transport of proteins across cellular membranes. Under stressful conditions, elevated HSP70 levels allow cells to cope with increased concentrations of unfolded or denatured proteins (Panjwani *et al.,* 1999).

2.5.3 Heat Shock Protein 90 (HSP 90): HSP90 is a molecular chaperone and is one of the most abundant proteins expressed in cells (Csermely et al., 1998). It has been identified in the cytosol, nucleus and endoplasmic reticulum, and is reported to exist in many Tissues (Kunisawa *et al.*, 2006). There are two isoform of HSP90 in mammalian cells – HSP90α and HSP90β. It consists of four structural domains (Prodromou, 2003). A "charged linker" region that connects the N-terminus with the middle domain and middle domain is involved in client protein binding. It also increases the ATPase activity of HSP90. The C-terminal domain possesses an alternative ATP-binding site, which become accessible when N-terminal Bergerat pocket is occupied (Meyer *et al.,* 2003).In unstressed cells, HSP90 plays a number of important roles, which include assisting in folding (Buchner,1999), intracellular transport, maintenance and degradation of proteins as well as facilitating cell signalling.

2.6 Seasonal Variation in Immune Activity:

Intra-annual environmental conditions are in temporal flux over much of the planet. Winter and its associated low temperatures and food availability, however, make breeding and sometimes even survival difficult (Nelson *et al.,* 2002). Variation in immune defence over the year may occur for several reasons including (i) changes in disease threat over time, (ii) dynamism in the relative benefits of immune defence at certain times of the year versus others, or (iii) changes in environmental signals which portend impending disease threats (Nelson *et al.,* 2002).

Immune function is generally decreased during the winter in the wild but is enhanced in the laboratory during short-day conditions when all other factors are held constant (Demas and Nelson, 1996). Similarly changes in the abundance and distribution of immune cells on a seasonal basis are common with circulating cell densities tending to be higher in the winter months. In temperate regions of the world, disease threats fluctuate depending on the time of year. Thus, temporal changes in animals' immune defences may represent an effort to resist infection at those times of the year when certain diseases are most prevalent.

The annual cycle of changing day length (photoperiod) provides a reliable environmental cue to determine time of year (Goldman, 2001 ; Prendergast *et al.,* 2002). Photoperiod affects the immune system of several rodent species. Short days increase the number of circulating blood leukocytes, lymphocytes, T cells and NK cells, as well as spontaneous blastogenesis in whole blood and isolated lymphocytes and the cytolytic capacity of natural killer cells (Yellon, *et al.,* 1999). Moreover, short days suppress phagocytosis and oxidative burst activities of granulocytes and monocytes (Bilbo *et al.,* 2002). Short days also enhance lymphocyte proliferation in species ranging from mice to primates (Mann *et al.,* 2000 ; Nelson *et al.,* 2002). There are species differences in photoperiodic influences on immune function. Finally, the types of immune responses, such as enhanced primary defenses in the skin, lymph nodes and gastrointestinal tract, could vary because the types of infectious risks vary seasonally. However, the general pattern is that short day lengths are usually associated with enhanced immune function. Although behavioural adaptations are also important for overcoming invading pathogens, complex cascades of molecular and cellular responses have evolved to fight against invading pathogens.

Stressors vary throughout the day and across the seasons. Chronic exposure to stressors often compromises immunity and could have serious consequences for health and survival. Consequently, seasonal changes in immune responses have evolved as adaptive mechanisms to counter seasonal stress-induced immune suppression (Nelson *et al.,* 2002) these changes appear to be defined by seasonal fluctuations in energy availability.

Ideally, optimal immune status should be maintained throughout the year; however, this is not generally possible because immune function requires energy and energy availability and utilization varies seasonally.

Winter-enhancement of immune function requires curtailing other energetically demanding activities such as reproduction and growth (Bonneaud *et al.,* 2003 ; Lochmiller *et al.,* 2000). The cellular and molecular mechanism that mediates the effects of season and photoperiod on immune function is to be identified.

The initial response to an infectious agent often determines the outcome of infection and thus is a very important part of survival mechanisms. The extent to which photoperiod influences specific proinflammatory cytokines, toll-like receptors, natural killer (NK) cells and dendritic cells remains unspecified but important to pursue.

Gap of Knowledge:

To the best of our knowledge, no study on the expression profile of TLR genes and the cross-talk between TLR & HSP genes in domestic animals during different seasons has been carried out. So, once it has been determined this will help to elucidate their importance relating to the disease resistant ability of animals in various seasons.

Materials and Methods

3 MATERIALS AND METHODS

3.1 Materials:

The proposed study was accomplished by using a number of materials and techniques which are described in this section. The first part deals with the chemicals used, followed by the methodologies applied to achieve the target. Standard protocols have been followed everywhere with slight modifications.

3.1.1 ANIMALS

Eighteen healthy Black Bengal goats divided into three age groups (n=6) : Group: I, below two years; Group: II, between 2-5 years; Group:III, above 5 years maintained at animal farm under IVRI, ERS centre were used for the present study. The animals were maintained under well ventilated and proper hygienic conditions. Prophylactic measures against goat diseases were carried out as prescribed by the health calendar of the institute to ensure that the animals were in a healthy condition throughout the study. Blood samples were collected at peak winter, moderate season and peak summer through jugular puncture using heparin as anticoagulant.

3.1.2 CHEMICALS/REAGENTS

Chemicals for molecular biology were obtained from different companies like Promega, Sigma, Bio-Rad, Invitrogen, New England Biolabs, as per requirement. 100 bp and 50 bp DNA ladder (New England Biolabs), Loading dye (MBI, Fermentas) were used. The chemicals like Tris, One Step RNA Reagent (G-Biosciences, Canada) Ethanol, Chloroform, Isopropyl alcohol, Agarose (Himedia), Ethidium Bromide, DEPC (AMRESCO) were used.

3.1.3 COMMERCIAL KITS

iScript™ cDNA synthesis Kit (Bio-Rad Laboratories, CA), Block PCR kit (New England Biolabs Inc), Ssofast™Eva Green® qPCR kit (Solis Biodyne) were used.

3.1.4 BUFFERS AND REAGENTS

The details of the media, buffers and other solutions used in this study, are given in the appendix or at respective places. The molecular biology grade reagents/chemicals were used for preparation of various buffers etc. using autoclaved distilled/MilliQ/nuclease free water; whereas the buffers and reagents used for RNA work were prepared in diethylpyrocarbonate (DEPC) treated distilled water using nuclease free water.

3.1.5 PRIMER SEQUENCES

To amplify the genes, a set of gene specific primers were designed from the published sequence. These primers were designed by the IDT (Integrated DNA Technologies) using Beacon software and the details have been given in Table 2.

3.1.6 GLASSWARES AND PLASTIC WARES

For RNA work, RNase-free plastic wares and glassware were used, and they were thoroughly treated with 0.1% DEPC overnight at 37°C. It was further autoclaved to make it DNase and RNase free before use. For PCR and other DNA related work plastic wares were autoclaved (121°C for 15 minutes at 101.3 kpa or 1 atmospheric pressure) and then used.

3.1.7 EQUIPMENTS

Major equipments used were as follows:

1. Agarose gel electrophoresis apparatus (Biorad)
2. Air displacement pipettes viz. P10, P100, P1000 (Finnpipette, Finland)
3. Gel documentation analysis system (AlphaImagerTM1220, Alpha Innotech Corporation, USA)
4. Hot air oven (Yorco instrument, Bombay)
5. Ice flaking machine (Harrison Scientific Instrument Co. Delhi)
6. Microcentrifuge (Heathrow Scientifc, USA)

Table 2: Gene Transcripts, Primer sequence and resulting fragment size

Target	Sequence of nucleotide	Fragment size (bp)	EMBL/Reference
TLR1	For: 5'-ACTTGGAATTCCTTCATTACGA-3' Rev:5'-GGAAGACTGAACACATCATGGA-3'	176	**HQ263209.1**
TLR2	For: 5'-TTCCGTCTCTTTGATGAG-3' Rev: 5'-CTTGGTGTTCATGATCTTC-3'	114	**JQ911706.1**
TLR3	For: 5'-GATGTATCGCCGTGCAAAGACA-3' Rev: 5'-TGCATATTCAAACTGCTCTGCT-3'	195	**HQ263210.1**
TLR4	For: 5'-CTTGCGTCCAGGTTGTTCCTAA-3' Rev: 5'-CTGGGAACCTGGAGAAGTTATG-3'	153	**JF825527.1**
TLR5	For: 5'-CCTCCTGCTCAGCTTCAACTAT-3' Rev: 5'-TATCTGACTTCCACCCAGGTC-3'	172	**FJ659852.1**
TLR6	For: 5'-CCTTGTCTTTCACCCAAATAGC-3' Rev: 5'- GTTGGTCTTCCAGTGAGT-3'	150	**HQ263211.1**
TLR7	For: 5'-TCTTGAAGGAAAGGACTGGTTA-3' Rev: 5'-AAGGGGCTTCTCAAGGAATATC-3'	205	**HQ263216.1**
TLR8	For: 5-'CGCACCGTCTAGGATTTATT 3' Rev: 5'-AAGCCGGGTCAGATTGGT 3'	209	**JF825528.1**
TLR9	For: 5'-CTGACACCTTCAGCCACCTGAG-3' Rev: 5'-TGGTGGTCTTGGTGATGTAGTC-3'	156	**HQ263217.1**
TLR10	For: 5'-ATGGTGCCATTATGAACCCTAC-3' Rev: 5'-CACATGTCCCTGTGGTGTCTAA-3'	248	**HQ263213.1**
HSP70	For: 5'-GACGACGGCATCTTCAAG -3' Rev: 5'-GTTCTGGCTGATGTCCTTC -3'	132	**FJ975769.1**
HSP60	For: 5'-ACTGGCTCCTCATCTCACTC -3' Rev: 5'-CTGTTCAATAATCACTGTCCTTCC -3'	148	**NM_001166609.1**
HSP90	For: 5'-GCATTCTCAGTTCATTGGCTATCC- 3' Rev: 5'-GTCCTTCTTCTCTTCCTCCTCTTC- 3'	190	**NM_001012670.1**
GAPDH	For: GCGATACTCACTCTTCTACTTTCGA Rev: TCGTACCAGGAAATGAGCTTGAC	82	**U85042.1**

EMBL – accession number or reference of published sequence

7. Non refrigerated Centrifuge (Remi, India)
8. Refrigerator, BPL India Ltd.
9. -20 ^{0}C Deep freezer (Vestfrost)
10. -80 ^{0}C Sanyo Biomedical freezer
11. Spinix vortex machine
12. Nanodrop spectrophotometer (Thermo Scientific, USA)
13. Real time PCR (Stratagene Mx 3005P QPCR System, Agilent Technologies, USA)
14. Thermal cycler (Eppendorf, Germany).
15. Sanyo, microwave oven
16. Scientronic Double distillation apparatus
17. Weighing balance (Sartorius, Germany)
18. Dry bath (Bangalore Genei)
19. Laminar Flow (ESCO)
20. Spectrophotometer (Electronics Corporation of India Limited)

3.2 METHODS

3.2.1 COLLECTION OF BLOOD

Blood samples were collected using heparin (10U/ml) as anticoagulant once at peak winter, moderate cold and peak summer by jugular vein puncture. Sterile gloves were worn during RNA isolation and precautions were taken to minimise ribonuclease activity. The environmental conditions at the time of blood collection are given in Table 3.

Table 3. The environmental conditions at time of blood collection

Season	**Tropical region** Temperature	**Mean Temperature**
Winter	2 to 13°C	8.1°C
Moderate	18 to 25°C	22.21°C
Summer	26 to 44°C	35.38°C

3.2.2 PROCEDURE FOR PBMCs ISOLATION

1. 5 ml Histopaque-1077 (Sigma) was pipetted into each of 15 ml conical centrifuge tube. Sufficient time was allowed for histopaqaue to reach at room temperature.
2. 5 ml blood was slowly layered on the top of each histopaque layer by keeping the centrifuge tubes at 45 degree angle without mixing the two layers.
3. Centrifuge at 400 × g for exactly 30 minutes at room temperature (25°C).
4. Tubes were carefully removed from centrifuge without disturbing gradient. Using a pipette, the upper plasma layer was slowly aspirated off from the opaque interface containing mononuclear cells.
5. With a sterile transfer pipette, the opaque interface was transferred into a fresh 15 ml conical centrifuge tube, avoiding carryover of the lower clear layer (Histopaque).
6. The tubes were filled to 10 ml with room temperature 1X PBS (pH 7.4) and gently mixed by inversion.
7. The tubes were centrifuged at 250 × g for 10 minutes.
8. Supernatant was removed and discarded.
9. The PBMCs pellet was resuspended in 5 ml cold PBS (pH 7.4) and centrifuged at 250 × g for 10 minutes.
10. Supernatant was poured off and centrifuged at 250 × g for 10 minutes after resuspending PBMCs in cold PBS.
11. Pour off the supernatant.

3.2.3 ISOLATION OF TOTAL RNA USING TRIZOL REAGENT

1. The PBMCs pellet was resuspended in 500 µl of DEPC-PBS (PH 7.4) and transferred to 2 ml nuclease free (DEPC treated) microcentifuge tube.
2. 1 ml one step RNA reagent was added into each tube and incubated at room temperature for 15 minutes.
3. Tubes were shaken vigorously by hand and incubated at room temperature for 10-15 min.
4. Tubes were centrifuged at 12,000 x g for 15 mins at 4°C.
5. Following centrifugation, the mixture was separated into a lower red phenol-chloroform phase an interphase and a colorless upper aqueous phase.

6. Fresh set of 1.5 ml eppendorf tubes were labeled.
7. The upper aqueous layer was removed carefully into the labeled tube without disturbing the interphase.
8. 500 µl of isopropanol was added to each of these tubes.
9. The samples were Incubate at room temperature for 10 min.
10. Tubes were centrifuge at 12,000 x g for 10 mins at 4°C.
11. A pellet was visible at this stage. The supernatant was removed.
12. The pellet was washed with 1ml of 75% ethanol and mixed by vortexing.
13. Centrifuged at 7500 x g for 5 mins at 4°C.
14. Supernatant was poured off to waste and tubes were inverted on clean tissue towel for 10 minutes to let the ethanol evaporate.
15. Depending on the size, the pellet was resuspend in 15-200 µl of DEPC-treated water.
16. RNA samples were stored at –80°C.

3.2.4 QUANTIFICATION OF RNA

The total RNA was quantified and purity was checked using the nanodrop spectrophotometer reading. 1 µl of resuspended total RNA was used and absorbance at 260 nm and 280 nm wavelengths were recorded against nuclease free water as blank. RNA samples showing the OD 260: OD 280 value more than 1.8 was considered to contain no protein and taken for further use.

3.2.5 CONFIRMATION OF RNA BY GEL ELECTROPHORESIS

The quality and integrity of the total RNA was checked using denaturing agarose gel (1%) electrophoresis and visualization under UV light. Two intact bands of 28s and 18s with smearing indicated good quality and intactness of RNA.

3.2.6 SYNTHESIS OF FIRST STRAND cDNA

The first strand cDNA was synthesized from the isolated total RNA. Reverse transcription was carried out in 20 µl reaction mixtures. Calculation was done by using the concentration of total RNA from nanodrop reading (ng/µl) to take one µg of total RNA for each reaction and dissolved in nuclease free water to make final volume 15µl.

Constant amount(1 µg) of isolated total RNA is reverse transcribed using iScript^{TM} cDNA Synthesis Kit (BIO-RAD laboratories, CA) with master mix- 15µl RNA + nuclease free water, 4 µl 5x iScript reaction mix, 1µl iScript reverse transcriptase as per manufacturer's instruction. One microgram of random hexamer primer was added and then incubated at 65°C for 5 minutes. Snap cooled in ice and following mixture was added:

The resulting complimentary DNAs (cDNAs) were used in quantitative RT-PCR (qRT-PCR) reactions.

Components	Volume per Reaction
5x iScript reaction mix	4 µl
iScript reverse transcriptase	1 µl
Nuclease-free water	variable
RNA template (1pg for 1µg total RNA)	x µl
Total volume	20 µl

Reaction mixture was mixed to RNA-primer complex and spinned, followed by incubation at 25°C for 5 minutes and 42°C for 60 minutes. Reaction was stopped by incubating for 5 min at 70°C and finally at 4°C forever. The cDNA is stored at -20°C for long term use.

3.2.7 CONFIRMATION OF cDNA WITH GAPDH PRIMERS

The integrity of the cDNA was checked by PCR with GAPDH primers. The amplification of 54 bp GAPDH gene fragment from the cDNA indicated that the cDNA was made from the RNA extracted from harvested PBM cells and was of good quality.

3.2.8 OPTIMIZATION OF END POINT PCR

End point PCR conditions were optimized to amplify goat HSP60, HSP70, HSP90 and Ubiquitin gene sequences in gradient thermo cycler. Factor specific primers were used for the amplification of genes. The annealing temperature was standardized using cDNA prepared from mRNA of goat PBMCs by PCR. The reaction was carried out at different annealing temperatures, primer concentrations, $MgCl_2$ concentration, template DNA and Taq polymerase.

The optimum temperature of 55°C for Ubiquitin, 57°C for HSP60, 58°C for HSP70 and HSP90 and 60°C for HSP110 were found to be most suitable for annealing for respective primers and was used in subsequent polymerase chain reaction. The concentration of different component which were found suitable for the optimum amplification are as follows.

Components	Quantity
cDNA template	1.0 µl
PCR-H_2O	18.30 µl
10x Buffer	2.50 µl
$MgCl_2$	1.50 µl
dNTP Mix (10 mM)	0.50 µl
Primer For (10 µM)	0.50 µl
Primer Rev (10 µM)	0.50 µl
Roche Taq Polymerase	0.20 µl
Total	**25.0 µl**

The above reactants were added to a nuclease free thin walled 0.2 ml microcentrifuge tube prechilled on ice. The contents were gently vortexed and then spun down to collect at the bottom of tube by brief centrifugation.

The reaction was carried out in a thermal cycler using the following cycling parameters that have been found optimum for amplification of gene fragments.

Step I : 95° C for 5 min for initial denaturation

Step II : 95°C for 30 sec for denaturation

Step III : 60^0C for TLR 1, 4, 5, 7, 10, GAPDH ; 55^0C for TLR2, 8; 50^0C for TLR3 ; 61^0C for TLR6, 9, HSP70; 57^0C for HSP60 & for 58^0C for HSP90 for 30 sec for annealing

Step IV : 72°C for 30 sec extension

Step V : II to IV repeated for 40 cycles for all the genes.

Step VI : 72°C for 10 min for final extension.

3.2.9 AGAROSE GEL ELECTROPHORESIS

The confirmation of amplification of specific RT-PCR amplicon during optimisation and expression of each determined factors was done by agarose gel electrophoresis (annexure). 2% agarose was mixed with 35 ml 1X TAE buffer and heated in a microwave oven for 40-60 sec until gel mixture becomes crystal clear. Then the gel mixture was cast into the gel casting tray fitted with the comb. The gel was allowed to solidify and after complete solidification, the comb was removed. The PCR product (around 5 µl) were mixed with 0.5 µl of 10X Saf Runner C-MeTM gel loading dye and loaded into the wells. For the comparison, a 50 bp molecular weight marker was gel electrophoresed in parallel to the RT-PCR amplicons. The gel was run at a voltage of 5 V/cm till the running dye crossed at least two third of the gel. The bands were visualized under UV light and re-corded on a gel documentation system (GELDOC, USA).

3.2.10 REAL TIME PCR

Quantitative Real-time PCR was performed with Solis Biodyne Eva Green ® qPCR kit and Stratagene Mx3000P (Agilent technology USA) spectroflouremetric thermal cylcer operated by MxPro™ QPCR software. Reaction setup was performed in area separate from nucleic acid preparation or PCR product analysis.

Pipetting was done with sterile filter tips. Proper care was taken to minimize the light expose to qPCR master mix. Careful pipetting was done without creating bubbles to avoid interference in reading of fluorescence by the instrument. No template control (NTC) was put for gene quantification for checking the contamination in the reaction components other than the cDNA. To ensure the cDNA samples were not contaminated with genomic DNA, reactions were set up using 10 ng of non-reverse transcribed RNA in place of cDNA. Failure to generate a detectable signal signified the samples as DNA free. In negative control, only the real time master mix and primers were added. For reaction set up optically clear caps were used. 1 µl of cDNA was taken. Following master mix was prepared:

MASTER MIX

Components	Quantity
Nuclease free water	4.0 µl
Primer forward	0.25 µl
Primer reverse	0.25 µl
Eva green mix	5.0 µl
Total volume	**9.5 µl**

Touching of the optical surface of the caps without gloves was avoided. Strips were centrifuged before starting the cycling programme to spin down the solution to the bottom of the tubes and to remove any possible bubbles. GAPDH was taken as housekeeping gene. Three segmented qPCR amplification programme was used as given in Table 3.

Table 3. Thermal cycler protocol of real time PCR

Segment	Thermal profile	Time	No. of cycles	Comments
Segment 1	95° C	15 min	1 cycle	Hot start PCR
Segment 2	95°C	10 sec	35cycles	Denaturation
	60^0C for TLR1,4,5,7 10, GAPDH			
	61^0C for TLR6,9, HSP70			
	53^0C for TLR 3	30 sec	40 cycles	Annealing
	55^0c for TLR 2,8			
	57^0C for HSP60			
	58^0C for HSP90			
	72°C	30 sec		Extension
Segment 3	95°C	1 min	1 cycle	Dissociation curve analysis
	65°C	30 sec		
	65-95 °C	2 degree per min		
	95 ^{0}C	30 Sec		

The amplification and denaturation data was acquired. After the run has ended, cycle threshold (Ct) values and amplification plot for all determined factors were acquired by using the "EvaGreen® (with Dissociation Curve)" method of the real time machine (Stratagene MxPro3005 (Agilent technologies, USA).

3.2.10.1 DETERMINATION OF EFFICIENCY OF PRIMERS

Efficiencies were determined by running a standard curve for each assay prior to processing experimental samples. A standard curve was obtained by serial dilution of the cDNA containing the template and a regression line equation in relation to the threshold values (Ct) was formulated.

To obtain best accuracy level 6 serial dilutions of cDNA were taken in triplicate. Slopes were calculated using Mxpro QPCR software (Stratagene Mx3005P, Agilent Technologies, USA).

3.2.10.2 CALCULATION OF RELATIVE EXPRESSION

Optical data were collected at end of each extension step, and relative expression of PCR product was determined by the equation (Pfaffl, 2001) given below:

$$\text{Ratio} = \frac{(E_{target})^{\Delta Ct\ target\ (control-sample)}}{(E_{ref})^{\Delta Ct\ ref\ (control-sample)}}$$

Where, **ratio** is the relative expression, $\mathbf{E_{target}}$ is the real time efficiency of target gene transcript, and $\mathbf{E_{ref}}$ is the real time efficiency of housekeeping gene transcript

3.2.11 Statistical Analyses

The statistical significance of differences in mRNA expressions of the examined factors was assessed by Two way ANOVA using Graph Pad-4.0 software. Differences were considered significant if $p<0.05$.

Results

EXPRESSION OF mRNA FOR HSP60

The relative expression of mRNA for HSP60 in PBMCs of Black Bengal goats is presented in Fig. 28. The moderate or thermo neutral season values were used as calibrator.

The mRNA expression of HSP60 during winter season was found 1.28. 0.8 and 1.02 times higher or lower as compared to calibrator in three age groups (0-2 yrs, 2-5 yrs and > 5yrs respectively) whereas it was 2.90, 4.6 and 6.6 times, respectively more as compared to calibrator during summer season (Fig.28). During summer season, the mRNA expression of older age group was found significantly higher ($p<0.05$) in comparison to I and II age group. However, during winter season mRNA expression level among the three age groups was found non-significant ($p>0.05$). The mRNA expression of all age groups during summer season was statistically significant ($p <0.05$) and level increased with the age of animals. The mRNA expression values in all age groups during summer season was found to be significantly higher than the winter season.

EXPRESSION OF mRNA FOR HSP70

The relative expressions of mRNA for HSP70 in PBMCs of Black Bengal goats during peak winter and peak summer are presented in Fig. 29. The moderate or thermo neutral season values were used as calibrator.

The mRNA expression during winter season was found to be 1.5, 0.27 and 0.35 times as compared to calibrator in three age groups (0-2 yrs, 2-5 yrs and >5yrs, respectively) whereas it was 2.6, 1.41 and 1.38 times more as compared to calibrator during summer season. The mRNA expression of I age group was found to be significantly higher ($P<0.05$) in comparison to II and III age groups during both winter and summer seasons. However, during summer season the mRNA expressions in all age groups were significantly higher ($P <0.05$) than the corresponding values during winter season.

EXPRESSION OF mRNA FOR HSP90

The relative expressions of mRNA for HSP90 in PBMCs of Black Bengal goats during peak winter and peak summer are presented in Fig. .30 The moderate or thermo neutral season values were used as calibrator.

During winter the mRNA expression was found to be 1.4, 0.9 and 0.98 times as compared to calibrator in three age groups (0-2 yrs, 2-5 yrs and >5yrs, respectively) whereas it was 7.0, 2.37 and 6.61 times higher as compared to calibrator during summer season. During winter season mRNA expression level among the three age groups was found non-significant ($p>0.05$). However during summer season mRNA expression was found significantly higher ($p<0.05$) in group I and III as compared to group II.

EXPRESSION OF mRNA FOR TLR1

The relative expression of mRNA for TLR-1 of Black Bengal goats during peak winter & peak summer are presented in Fig. 18. The moderate or thermo-neutral season values were used as calibrator.

During winter mRNA expression was found to be 0.33, 0.2 and 0.17 times as compared to calibrator in three age groups (0-2 yrs, 2-5 yrs and >5 yrs), respectively, whereas it was 0.27, 1.3 and 0.43 times as compare to calibrator during summer season. During winter season mRNA expression level among the three age groups was found non -significant. However, during summer season mRNA expression was found significantly higher ($P<0.05$) in group II as compared to group I and III.

EXPRESSION OF mRNA FOR TLR2

The relative expression of mRNA for TLR-2 of Black Bengal goats during peak winter & peak summer are presented in Fig. 19. The moderate or thermo-neutral season values were used as calibrator.

During winter mRNA expression was found to be 0.24, 0.23 and 0.35 times as compared to calibrator in three age groups (0-2 yrs, 2-5 yrs and >5 yrs) respectively whereas it was 4.05, 2.66 and 0.42 times as compare to calibrator during summer season. During winter season mRNA expression level among the three age groups was found non -significant. However, during summer season mRNA expression was found significantly higher ($p<0.05$) in group I and II as compare to group III.

EXPRESSION OF mRNA FOR TLR-3

The relative expression of mRNA for TLR-3 of Black Bengal goats during peak winter & peak summer are presented in Fig. 20. The moderate or thermo-neutral season values were used as calibrator.

During winter mRNA expression was found to be 5.75, 0.37 and 5.47 times as compared to calibrator in three age groups (0-2 yrs, 2-5 yrs and >5 yrs), respectively, whereas it was 0.59, 5.5 and 4.2 times as compare to calibrator during summer season. During winter season mRNA expression level was found to be significantly higher (p<0.01) in group I and III as compare to group II. However, during summer season mRNA expression was found significantly (p<0.05) higher in group II and III as compared to group I.

EXPRESSION OF mRNA FOR TLR4

The relative expression of mRNA for TLR-4 of Black Bengal goats during peak winter & peak summer are presented in Fig. 21. The moderate or thermo-neutral season values were used as calibrator.

During winter mRNA expression was found to be 0.41, 0.47 and 0.32 times as compared to calibrator in three age groups (0-2 yrs, 2-5 yrs and >5 yrs), respectively, whereas it was 3.19, 3.5 and 0.75 times as compare to calibrator during summer season. During winter season mRNA expression level among the three age groups was found non-significant. However, during summer season mRNA expression was found to be significantly (p<0.05) higher in group I and II as compared to group III.

EXPRESSION OF mRNA FOR TLR5

The relative expression of mRNA for TLR-5 of Black Bengal goats during peak winter & peak summer are presented in Fig. 22. The moderate or thermo-neutral season values were used as calibrator.

During winter mRNA expression was found to be 0.25, 0.11 and 0.26 times as compared to calibrator in three age groups (0-2 yrs, 2-5 yrs and >5 yrs), respectively, whereas it was 0.25, 0.14 and 0.38 times as compared to calibrator during summer season. During both in winter and summer season mRNA expression level among the three age groups was found non-significant (p>0.05).

EXPRESSION OF mRNA FOR TLR6

The relative expression of mRNA for TLR-6 of Black Bengal goats during peak winter & peak summer are presented in Fig. 23. The moderate or thermo-neutral season values were used as calibrator.

During winter mRNA expression was found to be 0.48, 0.38 and 0.08 times as compared to calibrator in three age groups (0-2 yrs, 2-5 yrs and >5 yrs), respectively, whereas it was 1.2, 1.9 and 2.0 times as compared to calibrator during summer season. During winter season mRNA expression level among the three age groups was found non -significant. However, during summer season mRNA expression was found to be significantly higher (p<0.05) in group II and III as compared to group I.

EXPRESSION OF mRNA FOR TLR7

The relative expression of mRNA for TLR-7 of Black Bengal goats during peak winter & peak summer are presented in Fig. 24. The moderate or thermo-neutral season values were used as calibrator.

During winter mRNA expression was found to be 0.28, 0.24 and 0.39 times as compared to calibrator in three age groups (0-2 yrs, 2-5 yrs and >5 yrs), respectively, whereas it was 0.33, 0.60 and 0.29 times as compared to calibrator during summer season. During winter season mRNA expression level among the three age groups was found to be non-significant. However, during summer season mRNA expression was found to be significantly higher (p<0.05) in older age group as compared to group I and group II.

EXPRESSION OF mRNA FOR TLR8

The relative expression of mRNA for TLR-8 of Black Bengal goats during peak winter & peak summer are presented in Fig. 25. The moderate or thermo-neutral season values were used as calibrator.

During winter mRNA expression was found to be 1.96, 2.48 and 0.62 times as compared to calibrator in three age groups (0-2 yrs, 2-5 yrs and >5 yrs) respectively whereas it was 0.80, 3.77 and 2.79 times as compared to calibrator during summer season.

During winter season mRNA expression level was found to be significantly higher ($p<0.05$) in group I and II as compared to group III. However, during summer season mRNA expression was found to be significantly ($p<0.05$) higher in group II and III as compare to group I.

EXPRESSION OF mRNA FOR TLR9

The relative expression of mRNA for TLR-9 of Black Bengal goats during peak winter & peak summer are presented in Fig. 26. The moderate or thermo-neutral season values were used as calibrator.

During winter mRNA expression was found to be 0.19, 0.13 and 0.26 times as compared to calibrator in three age groups (0-2 yrs, 2-5 yrs and >5 yrs), respectively, whereas it was 1.36, 0.27 and 0.49 times as compared to calibrator during summer season. During winter season mRNA expression level among the three age groups was found non -significant. However, during summer season mRNA expression was found to be significantly higher ($P<0.05$) in group I as compared to group II and group III.

EXPRESSION OF mRNA FOR TLR10

The relative expression of mRNA for TLR-10 of Black Bengal goats during peak winter & peak summer are presented in Fig. 27. The moderate or thermo-neutral season values were used as calibrator.

During winter mRNA expression was found to be 0.08, 0.84 and 0.32 times as compared to calibrator in three age groups (0-2 yrs, 2-5 yrs and >5 yrs, respectively, whereas it was 0.35, 2.12 and 1.45 times as compared to calibrator during summer season. During winter season mRNA expression level among the three age groups was found non -significant. However, during summer season mRNA expression was found significantly ($P<0.05$) higher in group II and III as compare to group I.

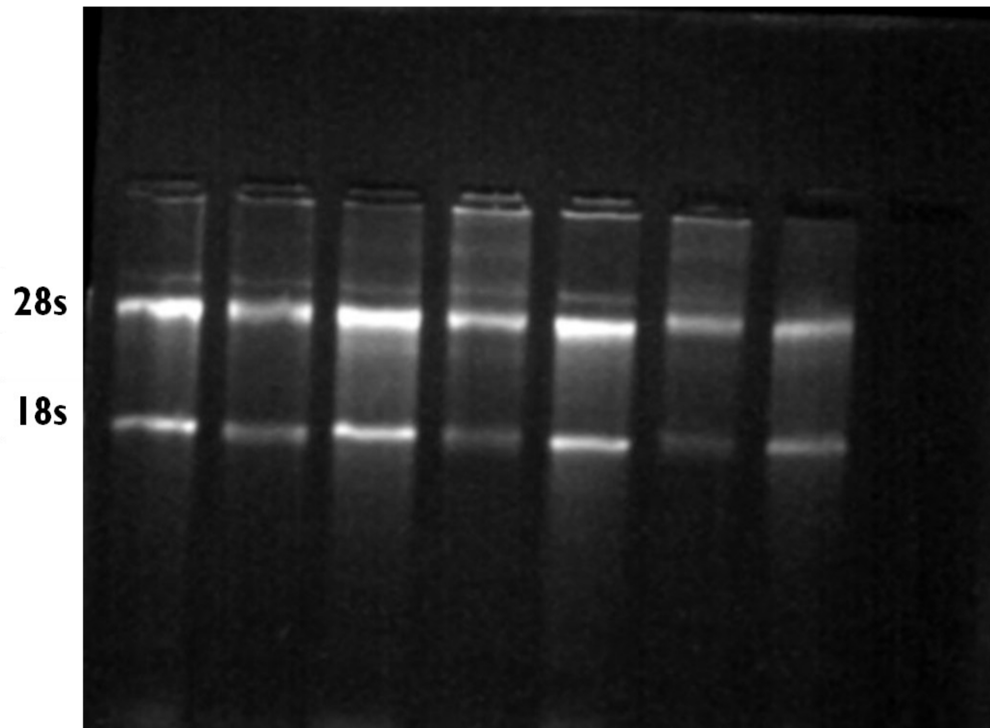


Fig. 2: Gel Picture showing integrity of total RNA Sample

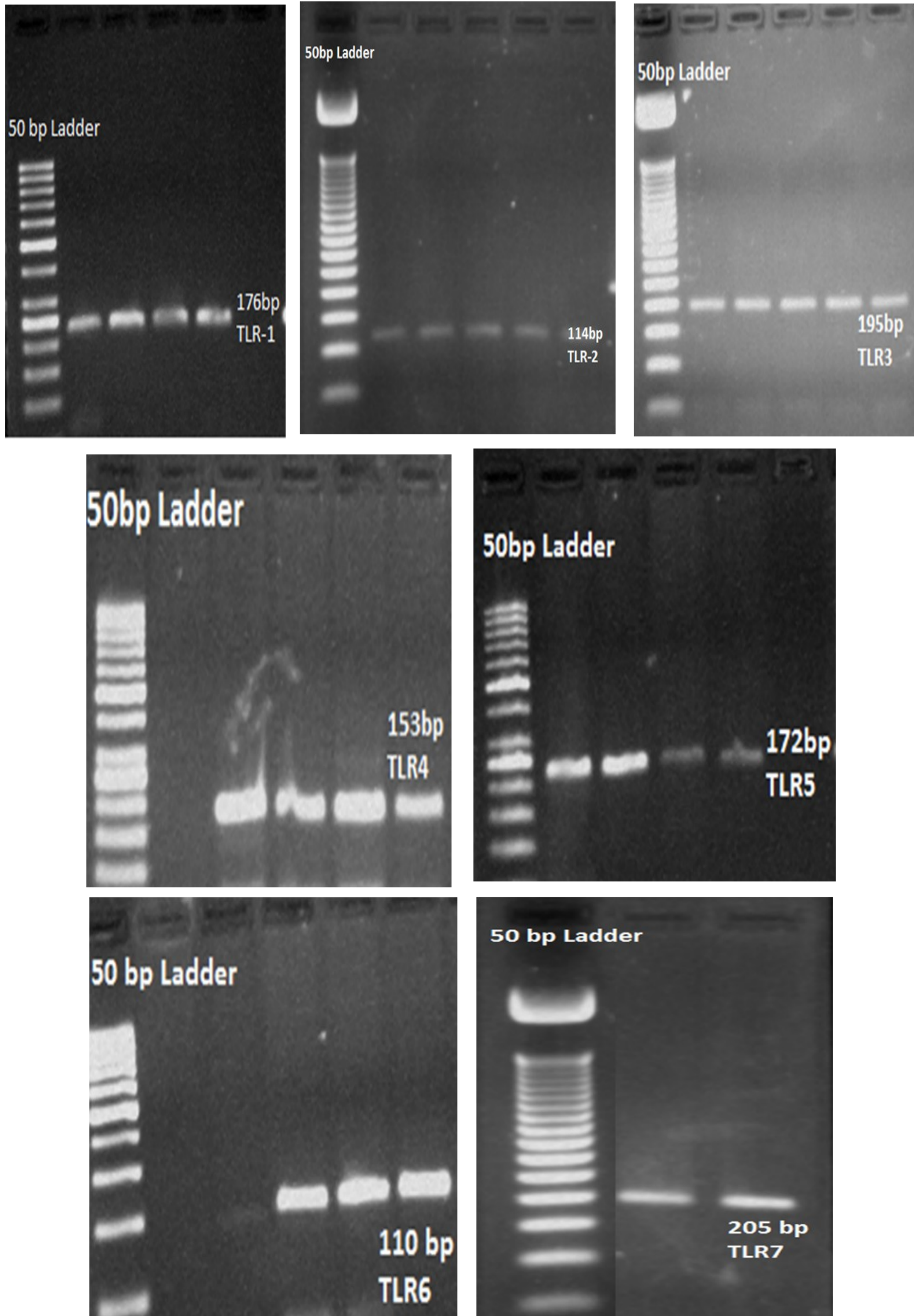
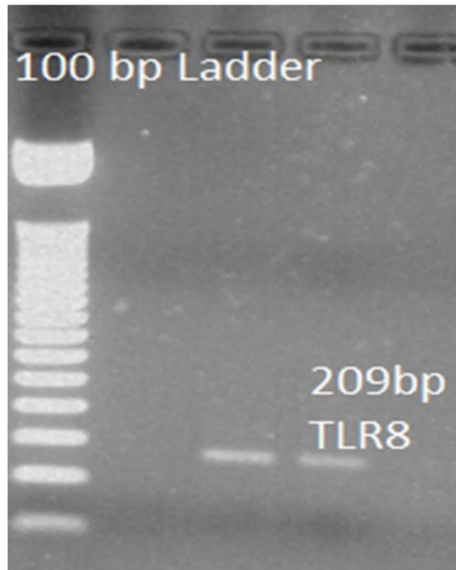
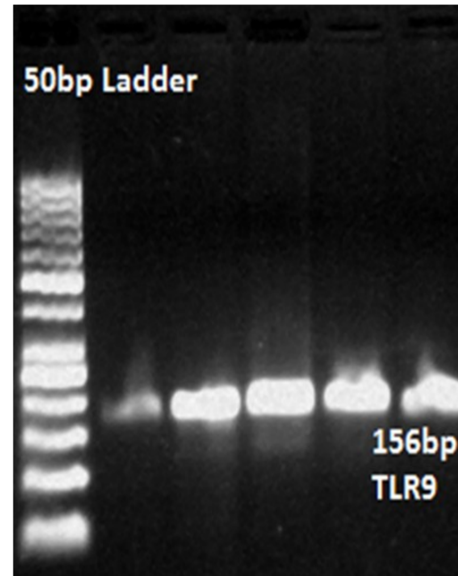
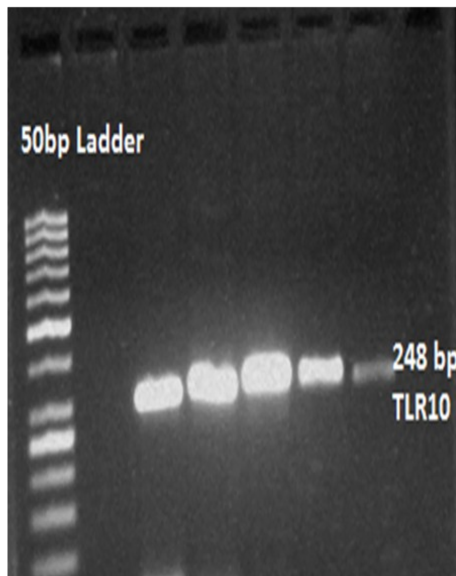
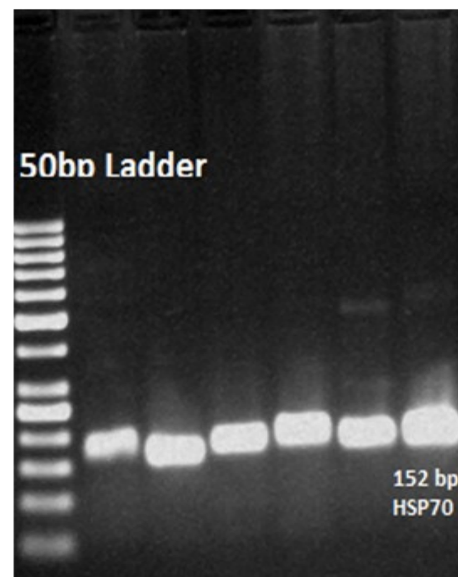


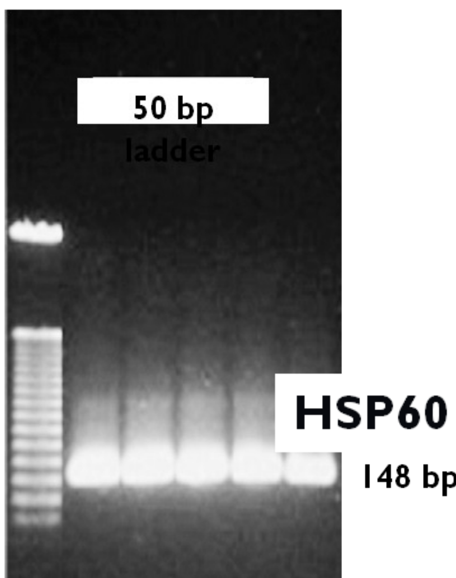
Fig.3 : Gel Picture showing PCR amplification product of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6 and TLR7











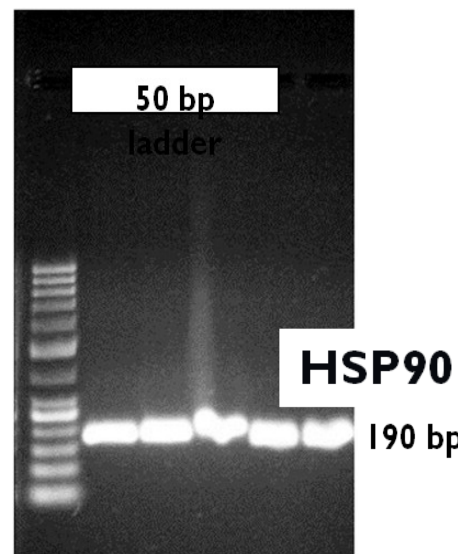
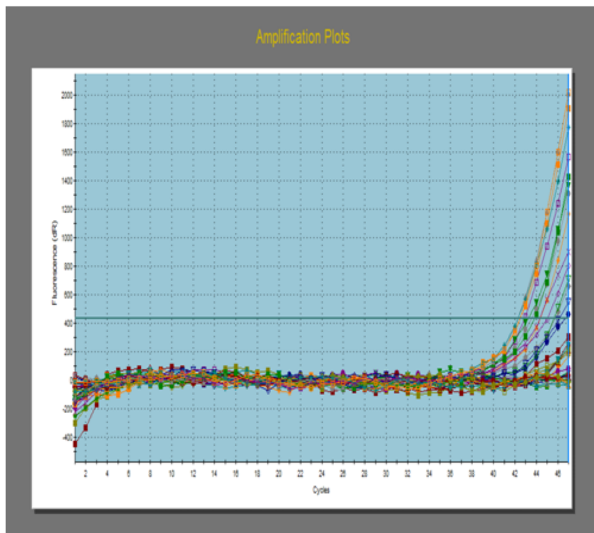


Fig.4 : Gel Picture showing PCR amplification product of TLR8, TLR9, TLR10, HSP60, HSP70 & HSP90



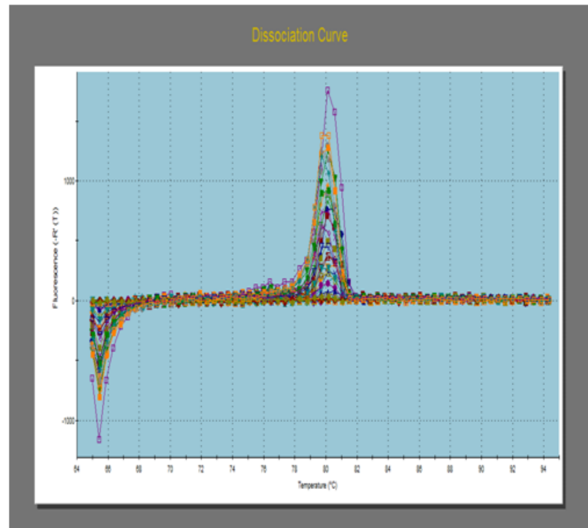
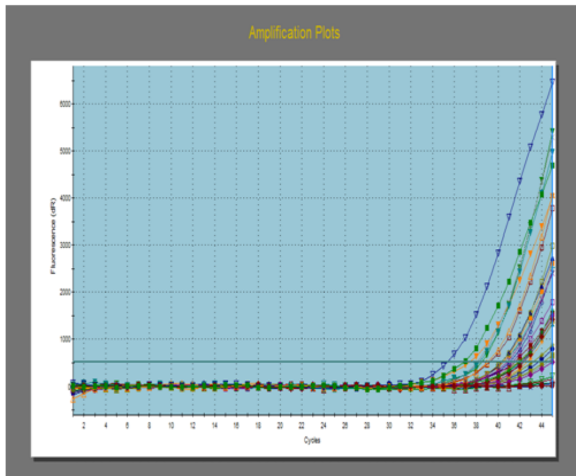


Fig.5 : Amplification & Dissociation plot for TLR1



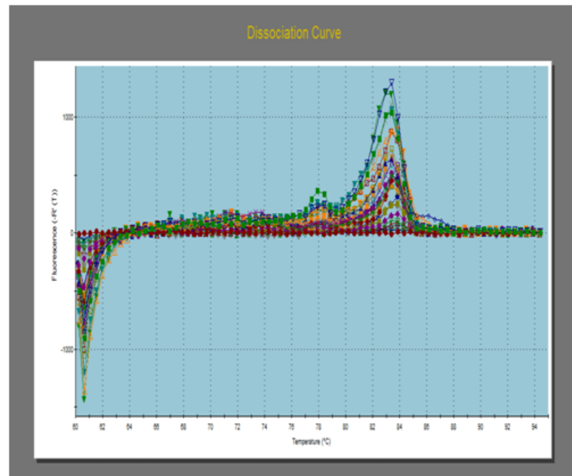
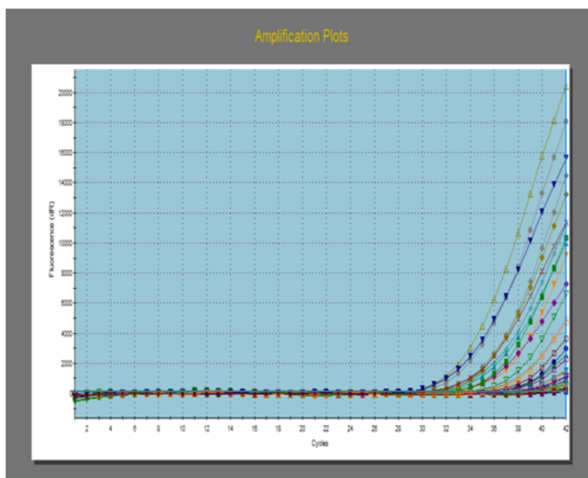


Fig.6 : Amplification & Dissociation plot for TLR2



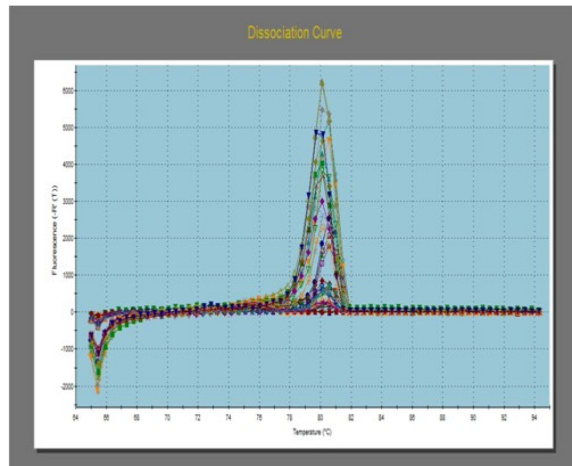
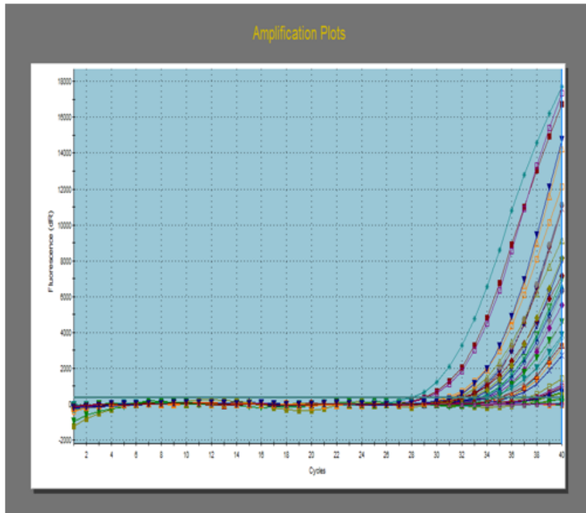


Fig.7 : Amplification & Dissociation plot for TLR3



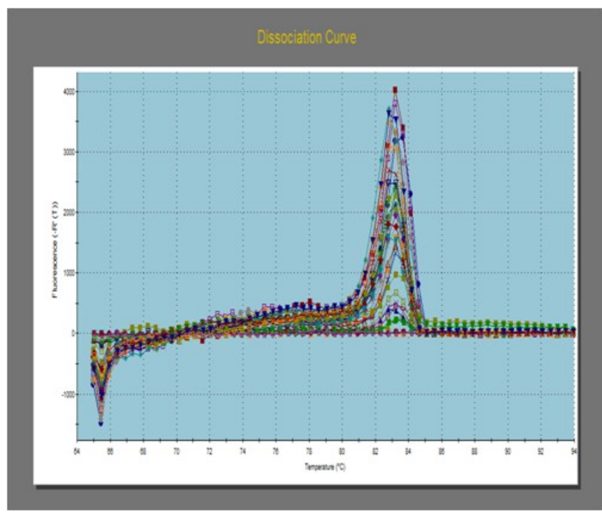
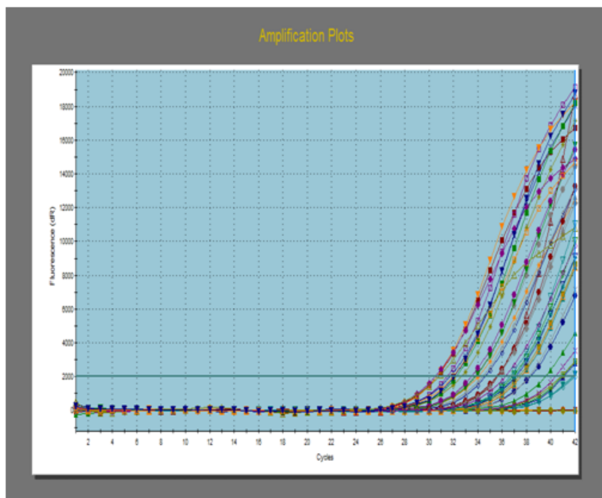


Fig.8 : Amplification & Dissociation plot for TLR4



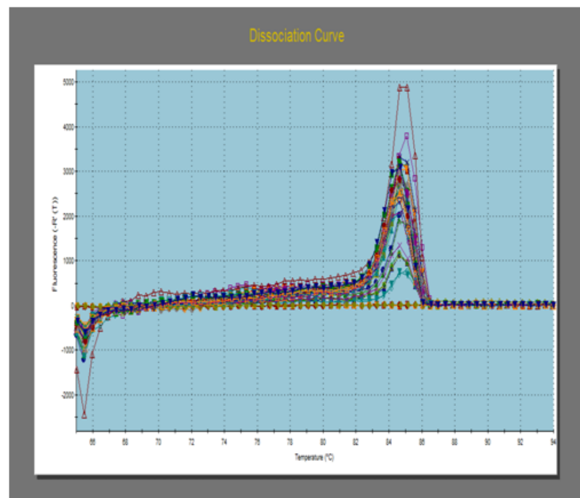
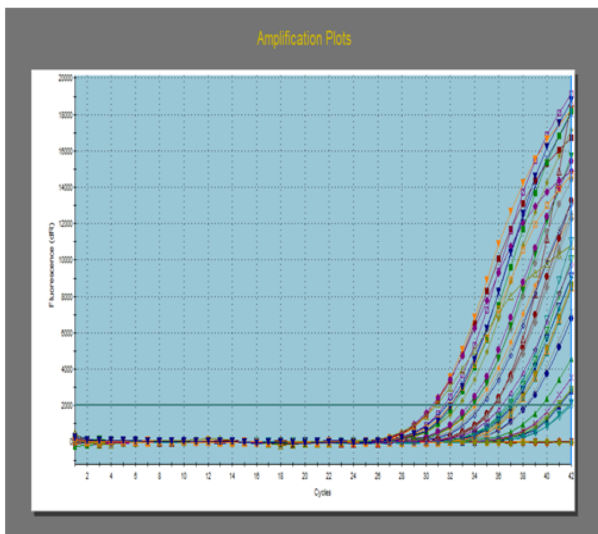


Fig.9 : Amplification & Dissociation plot for TLR5



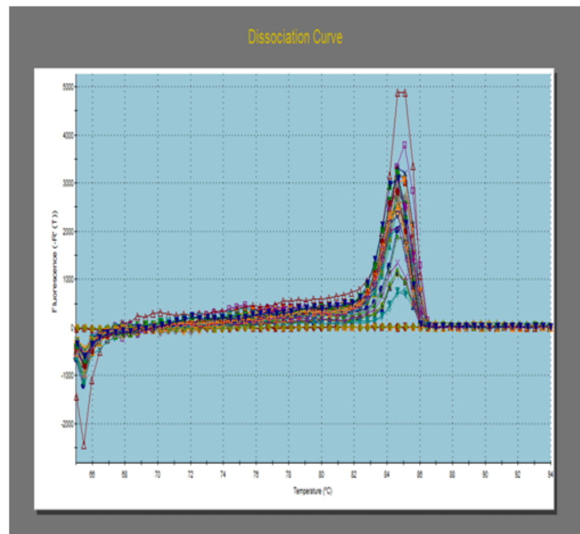
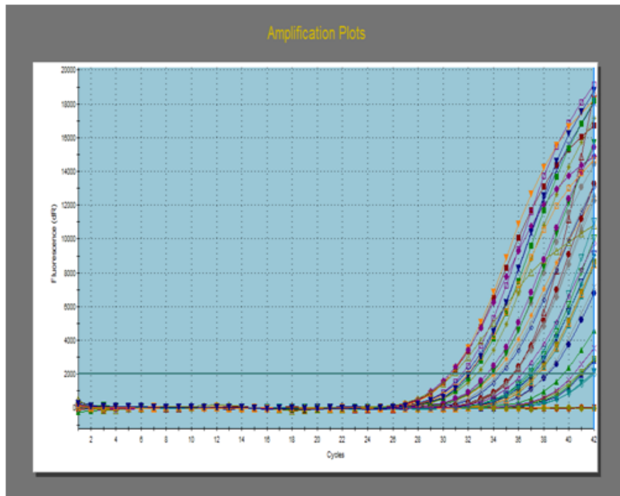


Fig.10 : Amplification & Dissociation plot for TLR6



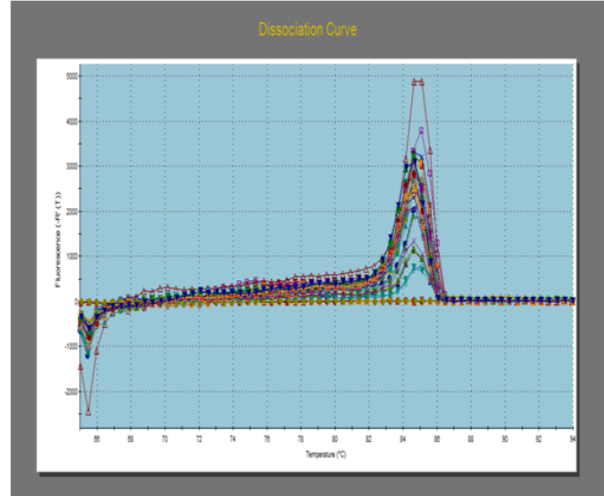
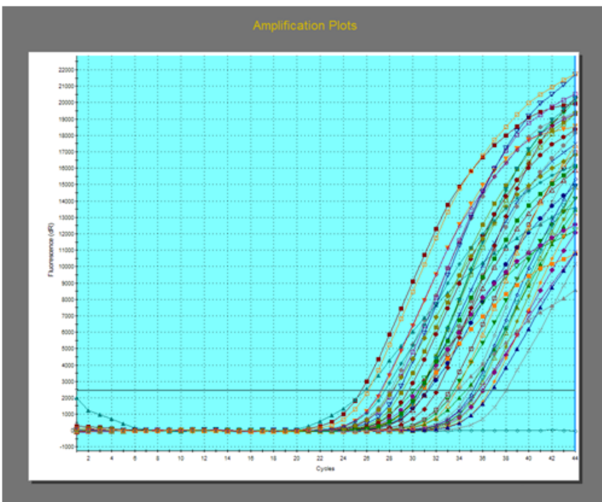


Fig.11 : Amplification & Dissociation plot for TLR7



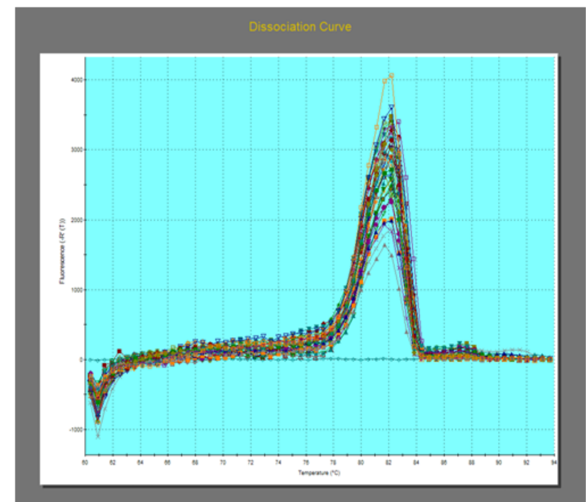
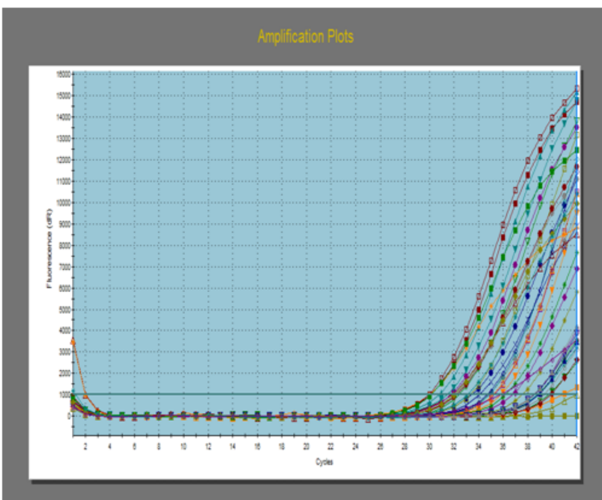


Fig.12 : Amplification & Dissociation plot for TLR8



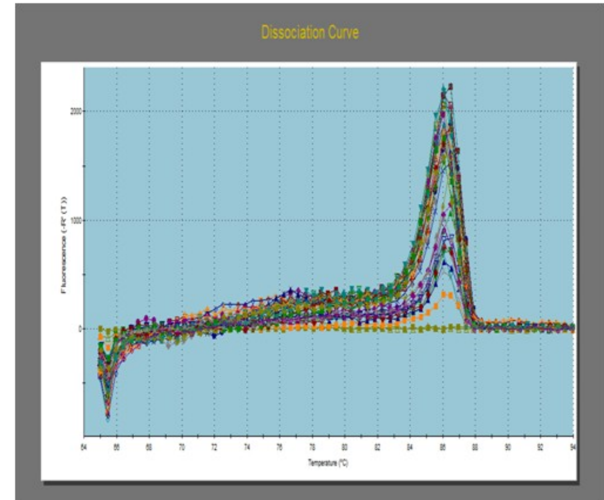
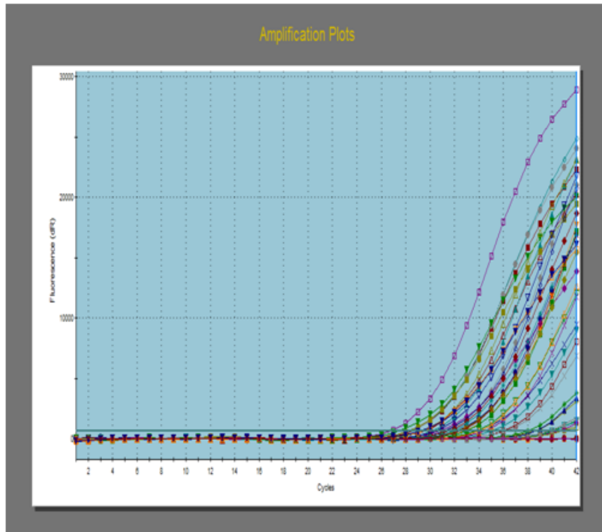


Fig.13 : Amplification & Dissociation plot for TLR9



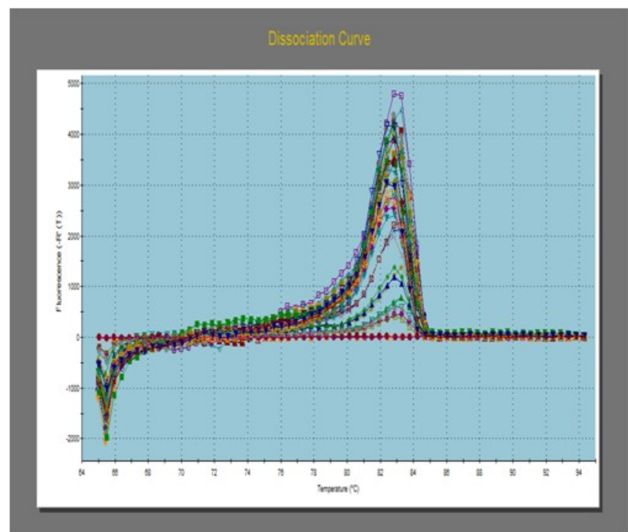
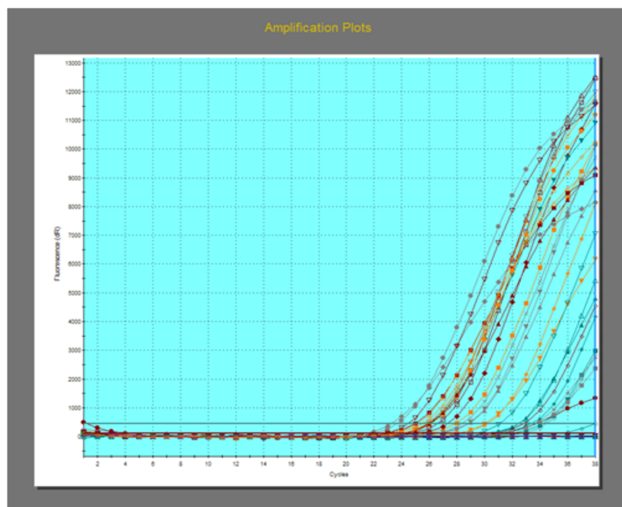


Fig.14 : Amplification & Dissociation plot for TLR10



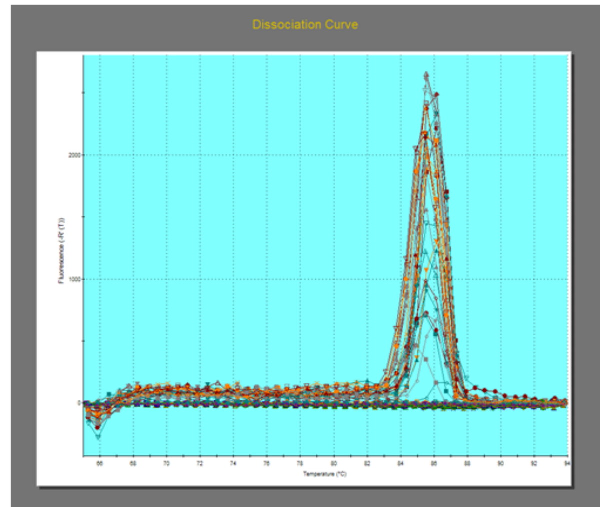
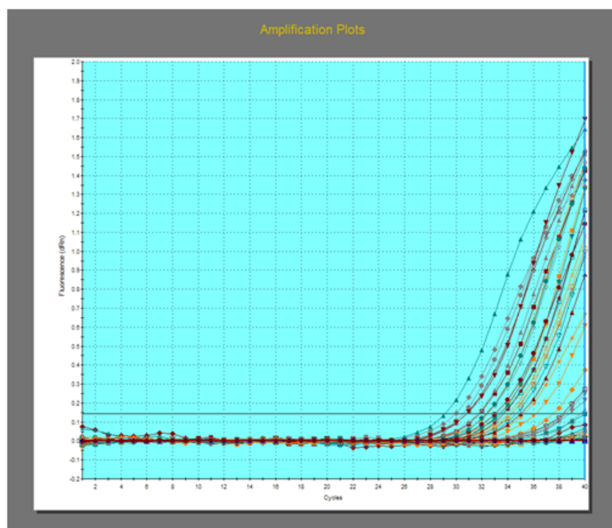


Fig.15 : Amplification & Dissociation plot for HSP 60



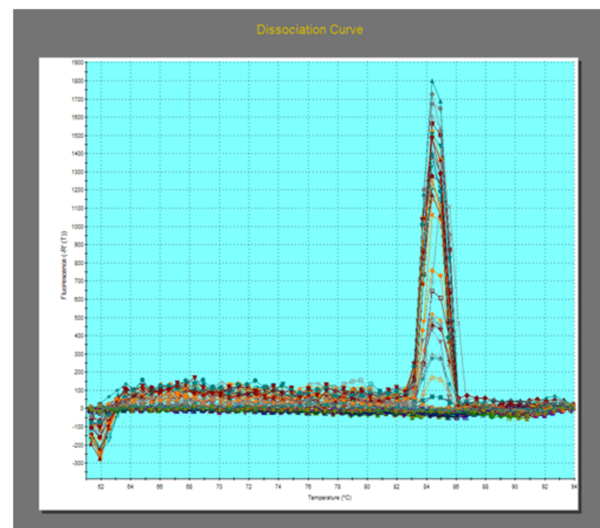


Fig.16 : Amplification & Dissociation plot for HSP 70

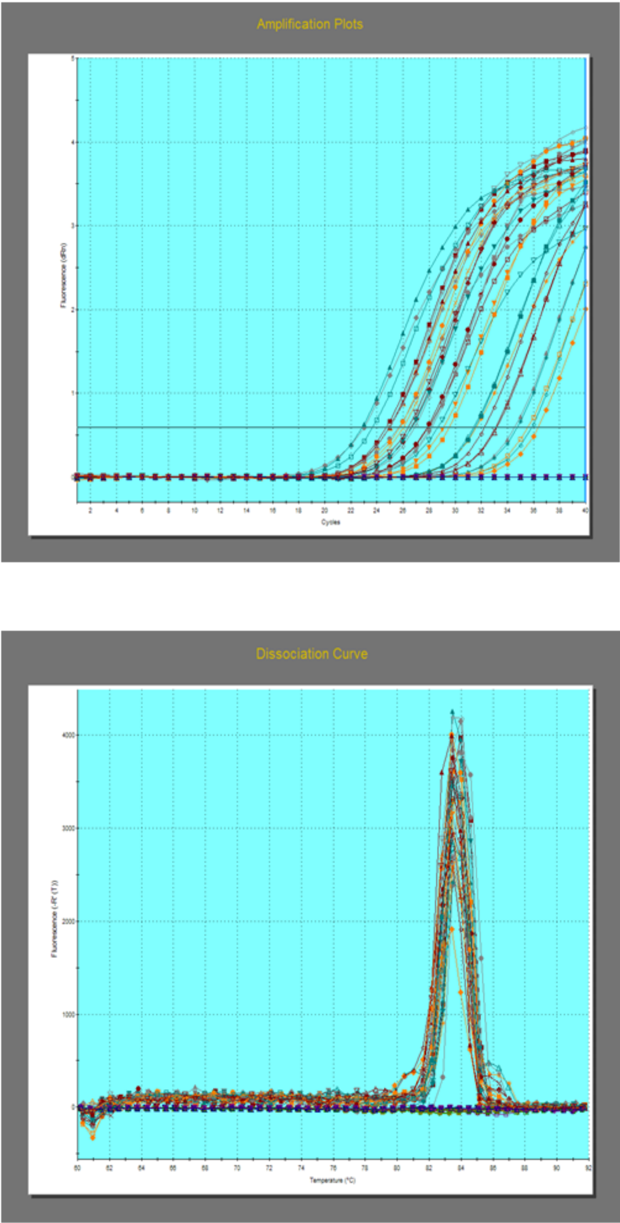


Fig.17 : Amplification & Dissociation plot for HSP 90

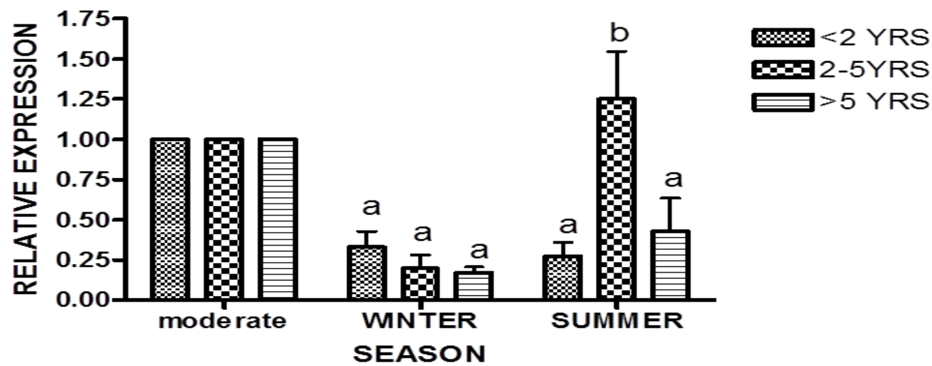


Fig.18 : Expression Profile of TLR1 in Black Bengal Goat

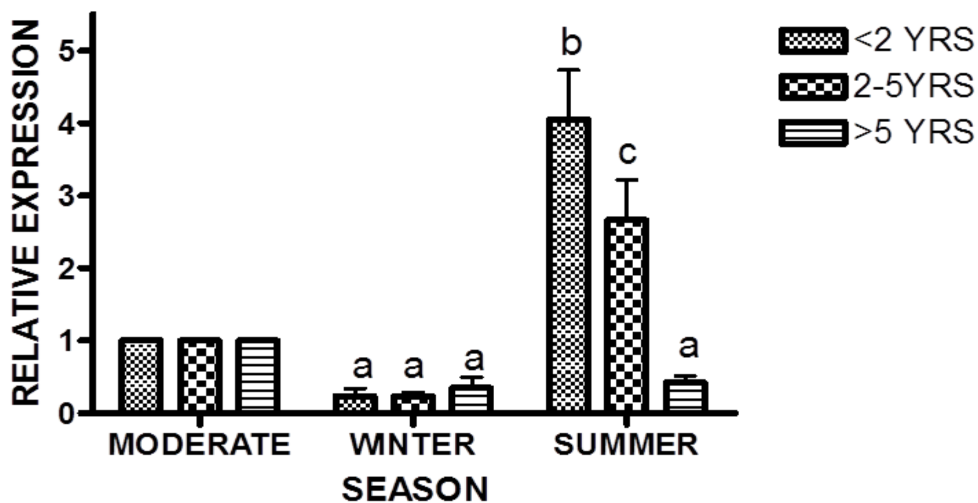


Fig.19 : Expression Profile of TLR 2 in Black Bengal Goat

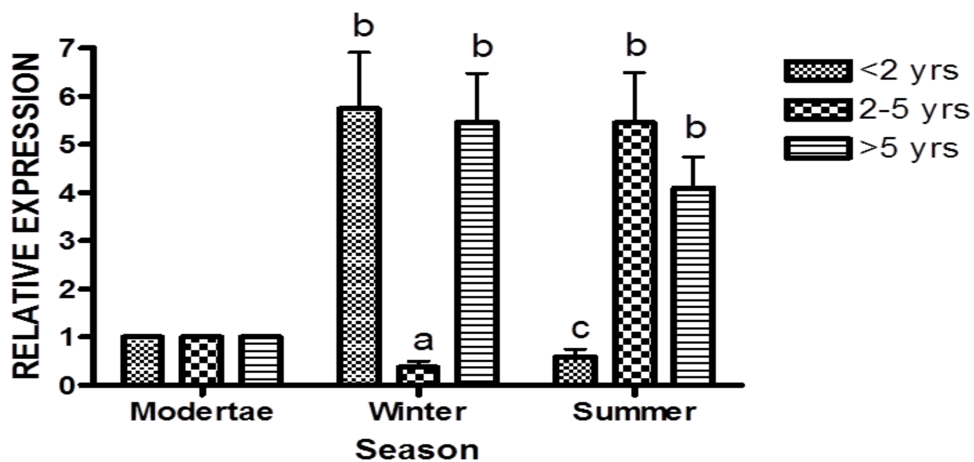


Fig.20 : Expression Profile of TLR 3 in Black Bengal Goat

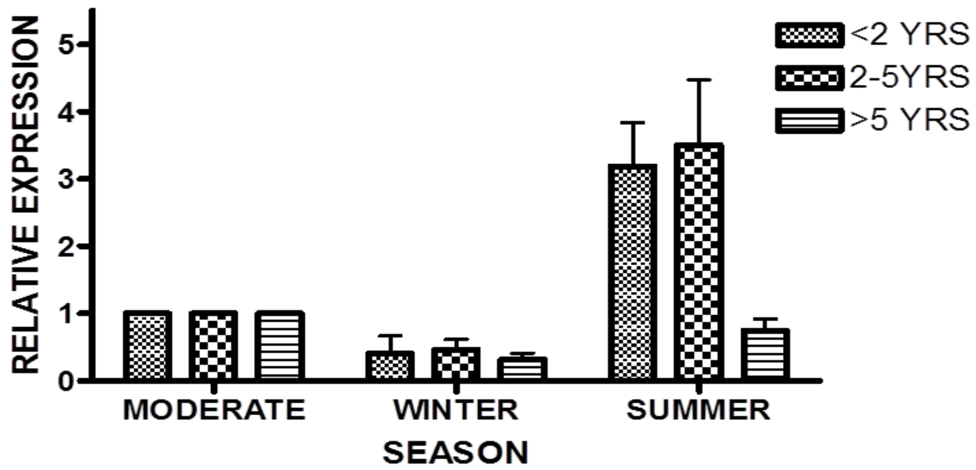


Fig.21 : Expression Profile of TLR 4 in Black Bengal Goat

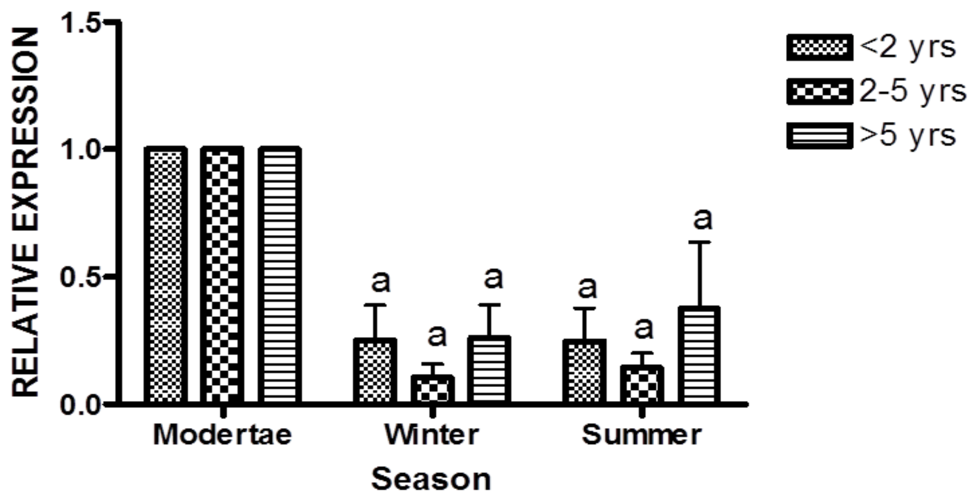


Fig.22 : Expression Profile of TLR 5 in Black Bengal Goat

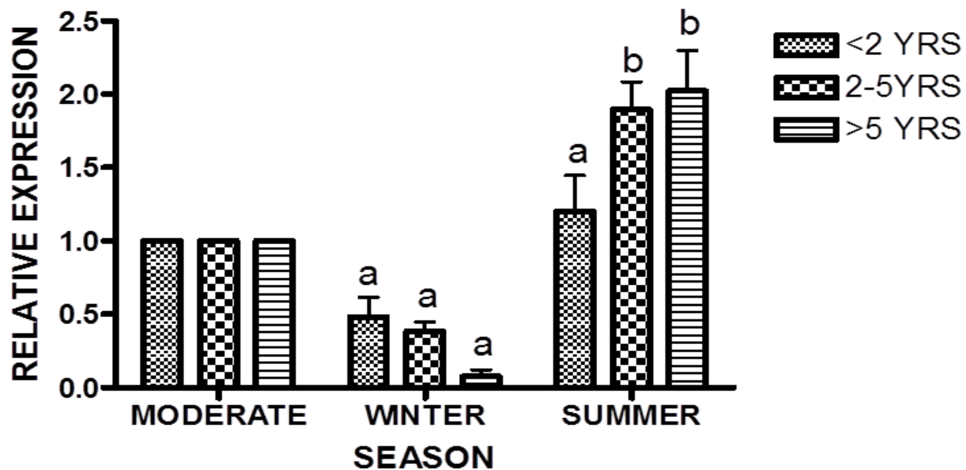


Fig.23 : Expression Profile of TLR 6 in Black Bengal Goat

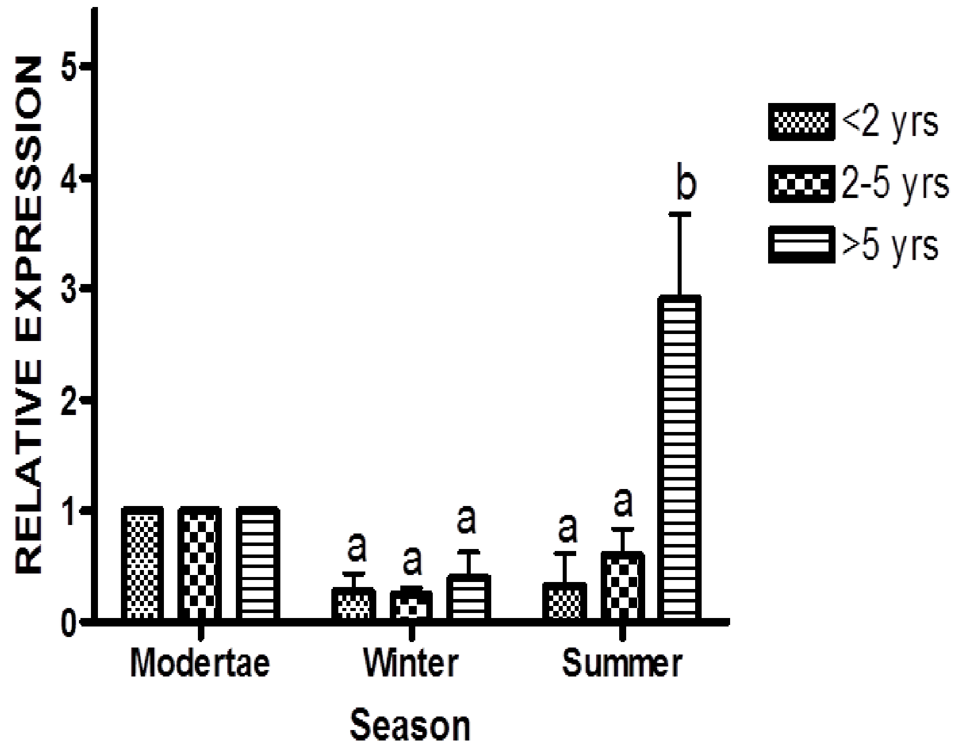


Fig.24 : Expression Profile of TLR 7 in Black Bengal Goat

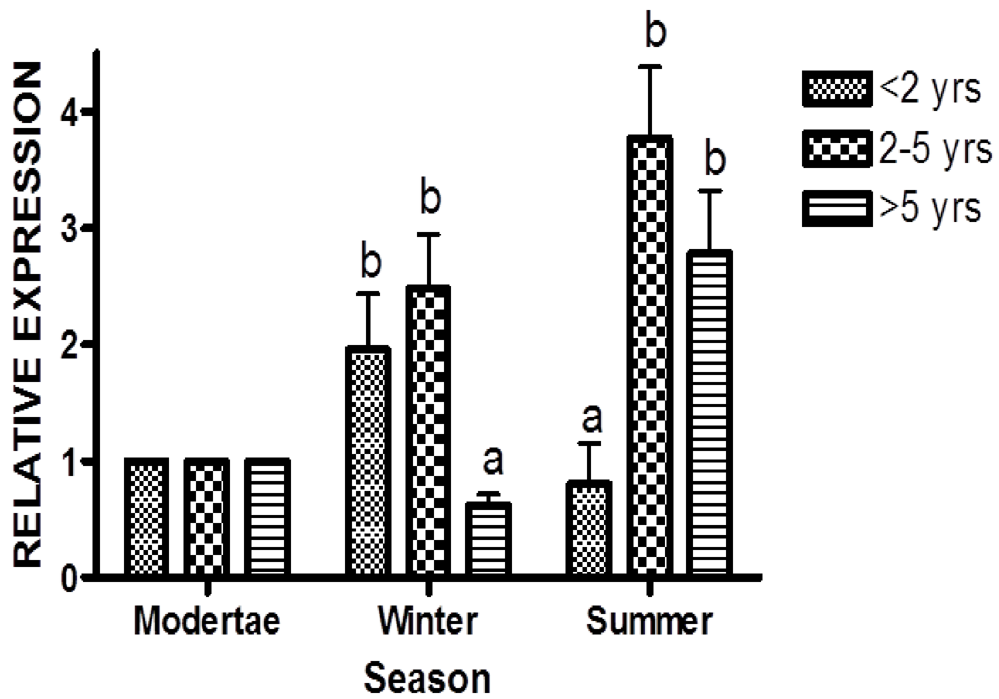


Fig.25 : Expression Profile of TLR 8 in Black Bengal Goat

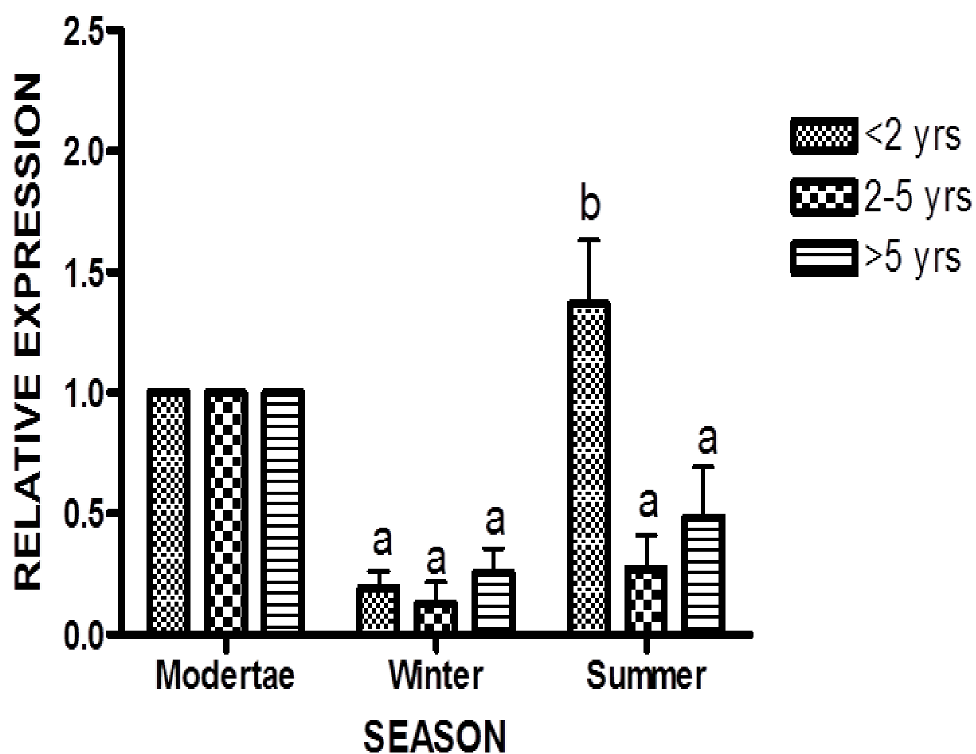


Fig.26 : Expression Profile of TLR 9 in Black Bengal Goat

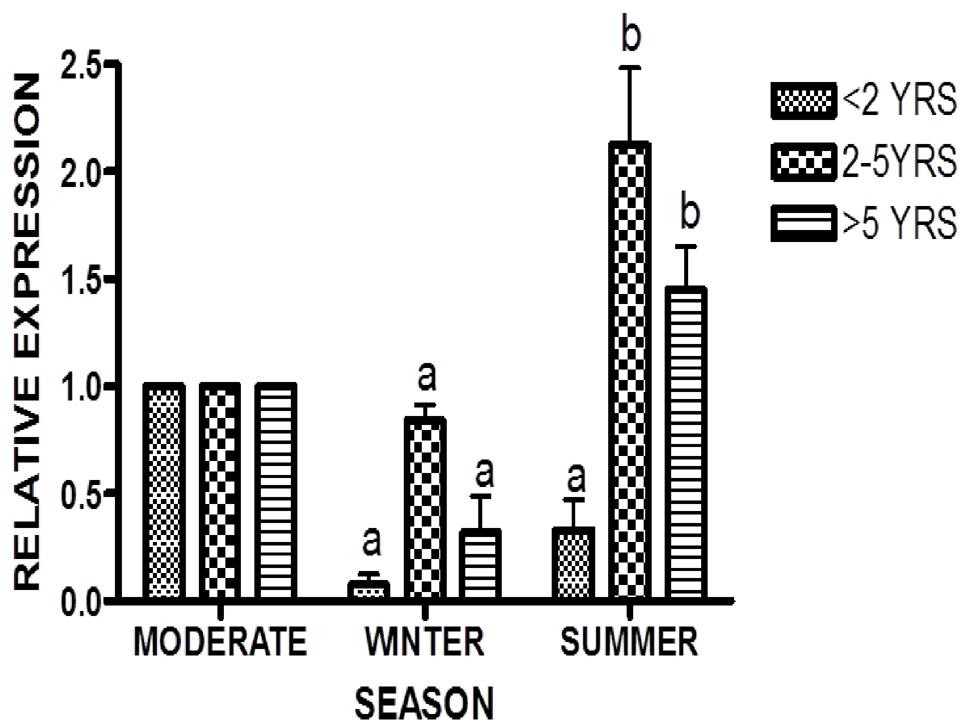


Fig.27 : Expression Profile of TLR 10 in Black Bengal Goat

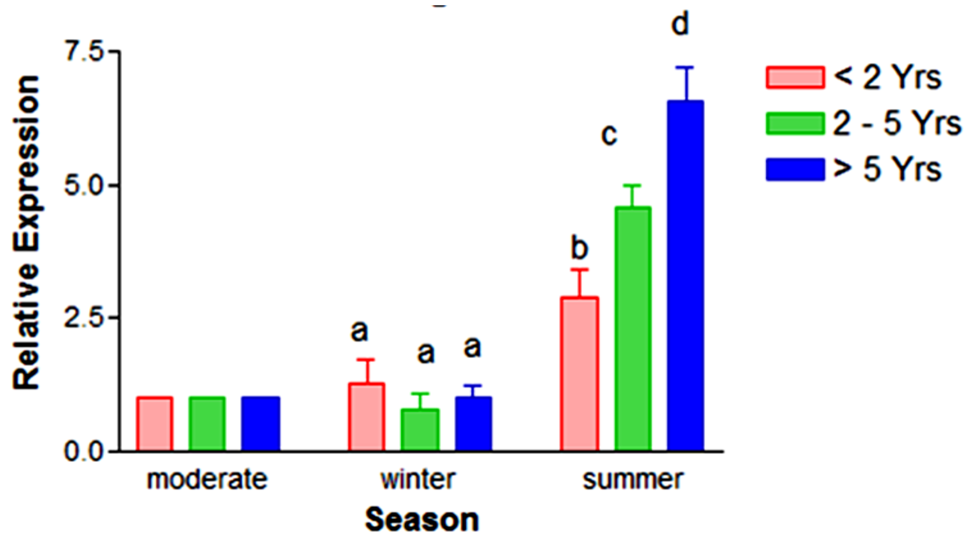


Fig.28 : Expression Profile of HSP60 in Black Bengal Goat

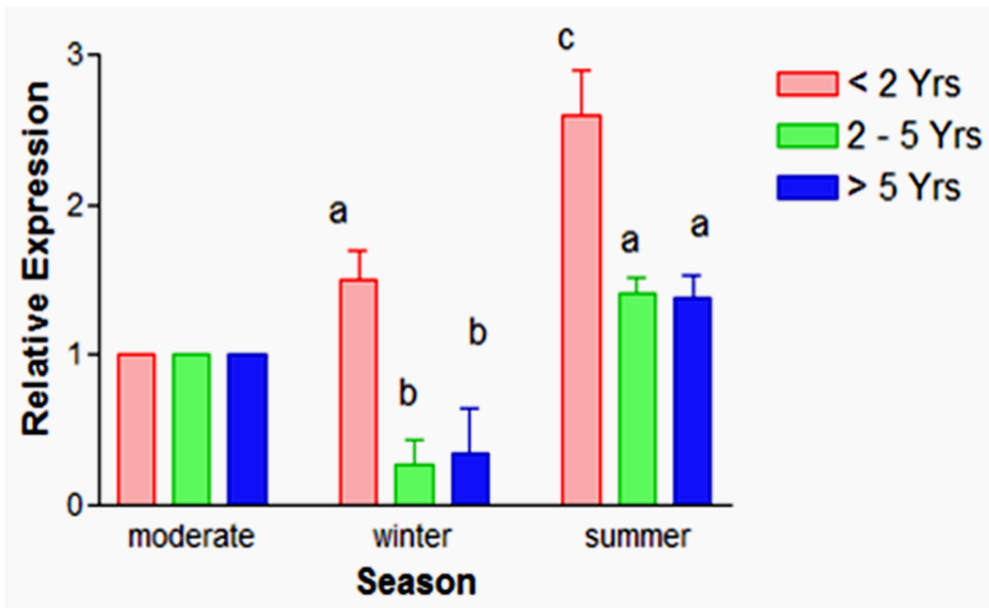


Fig.29 : Expression Profile of HSP70 in Black Bengal Goat

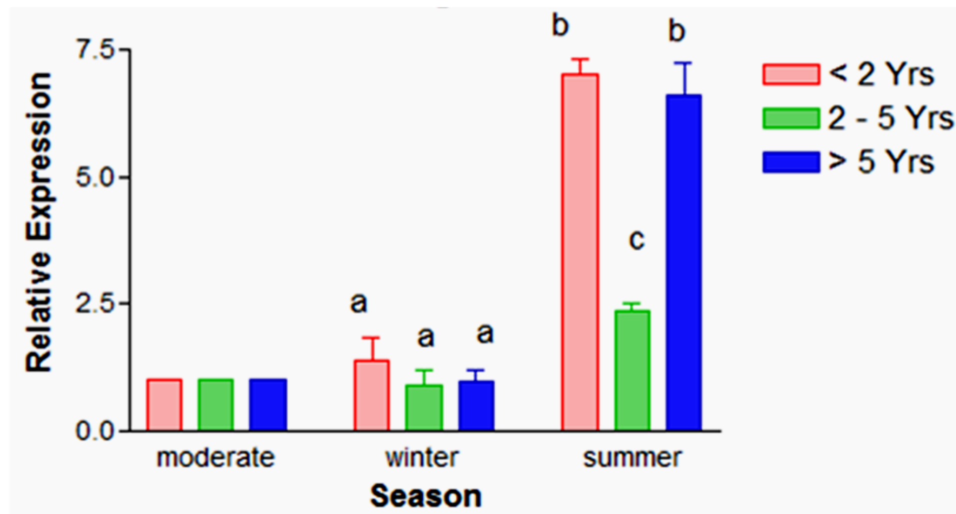


Fig.30 : Expression Profile of HSP90 in Black Bengal Goat

5 DISCUSSION

Thermal stress triggers a complex program of gene expression and biochemical adaptive responses (Lindquist *et al.,* 1986). Biologically, the ability to survive and adaptation to thermal stress appears to be a fundamental requirement of cellular life, as cellular responses to stress are ubiquitous among both eukaryotes and prokaryotes. It is widely accepted that changes in gene expression are an integral part of the cellular responses to thermal stress. Goats are well adapted to different environmental & geographical conditions and are found in extreme hot areas as well as extreme cold condition. Goat must have developed some signalling pathways that allow their survival in very high as well as cold temperature. The expression profiling may contribute to the effort of identifying elements not previously known to involve in the cellular responses to heat and cold stress. A detailed understanding of the genes involves in heat and cold tolerance may help in integrating the understanding of function and interaction of the involved elements.

Toll-like receptors (TLRs) are a family of at least 10 proteins that function as central mediators of the innate immune response to diverse pathogens as well as to endogenous molecules released by injured or dying cells (Kawai and Akira, 2010). It is accepted that heat stress can alter innate immunity response by inducing the expression of stress protein such as HSPs (Zhou *et al.,* 2005). Heat stress might affect TLRs expression of immune cells and then modulate immune responsiveness to PAMPs to the full activation of innate & adaptive immune system to fight against pathogenic microorganisms. To the best of our knowledge, no study has been done so far regarding the expression of TLR genes in either domestic animal or goat during different seasons.

In the present study, we have examined the effects of thermal stress on the expression profile of TLRs & HSP in PBMCs of Black Bengal goats during different seasons. During summer and winter, the animals were exposed to an ambient temperature beyond their comfort zone so they were under thermal stress in both the seasons.

It has been seen from our studies that almost all the TLRs showing significantly ($P<0.005$) higher expression during hot summer season in either of the age group of animal as compared to the moderate season. And a reverse i.e. lower level of expression of almost all the TLR genes has been found during winter season in all the age groups as compare to both moderate as well as summer season. Among the ten TLR familes found in goat, the TLR2 & TLR4 are regarded as the major Pattern Recognization Receptor (PRR) for both Bacteria (Gm +ve and Gm -ve) and other endogenous ligands (HSPs). In the present investigation, the mRNA expression levels of both TLR 2 & TLR4 among the three age groups remain unchanged during winter season. However during summer season, mRNA expression of TLR2 was found to be significantly ($P<0.05$) higher in group I and III as compared to group II and in case of TLR4, higher expression was found in group I and II as compared to group III. Our findings are in conformity with the other findings reporting that heat shock up regulates the expression of TLR2 & TLR4 in human monocyte (Zhou *et al.,* 2005). Further, fever range temperature is also reported to promote TLR4 expression in dendritic cells, leading to increase production of various cytokines (Yan *et al.,* 2007). Our findings are also in accordance with the previous studies of Zhou *et al.* (2007) that hyperthermia differentially regulates TLR 2 & TLR4 mediated immune response. Thus the higher expression of TLR2 & TLR 4 during thermal stress, supports that summer stress could enhance the innate immunity responses to PAMPs by promoting TLR expression and signalling in immune cells.

Cross talk between TLR & HSP: HSP70 family of proteins are most temperature sensitive and highly conserved among heat shock proteins. In the present investigation, the mRNA expression of HSP70 was found to be significant higher in animals below two years compared to the other age groups during both winter and summer season with the expression being higher in all age groups during summer season.

Increased expression of HSP70 mRNA upon exposure to heat stress has been reported in caprine PBMCs (Dangi *et al*., 2012), bovine lymphocytes (Patir and Upadhyay, 2007; Mishra *et al*. 2010). TLR also senses the endogenous ligands such as HSP 60 (Ohashi *et al.,* 2000), HSP70 (Vabulus *et al.,* 2002) that are released during heat stress and other oxidative stresses. It has been reported that HSPs could activate the TLR2 & TLR4 (Chen *et al.,* 1999 ; Beg *et al.,* 2002). Through binding to TLR2 & TLR4, recombinant HSP70, HSP60 can stimulate sentinel cells to release cytokines and increase their antigen-presenting capacity (Gobert *et al.,* 2004 ; Vabulus *et al.,* 2002). Therefore it might be reasonable to suspect that during summer season, the up-regulation of TLR2 & TLR4 were caused by the induction of HSPs. And the exposure of HSPs to those TLRs may result in the activation of dendritic cells & macrophages and production of immune enhancing cytokines that are essential in survival of host infection. Hence, all those findings support the concept that the thermal microenvironment initiates physiological responses that amplify immune protection during compromised state in animals.

The other Toll like Receptors i.e. TLR1, TLR6, TLR7 and TLR10 also follows the same pattern of higher expression during summer season compared to winter season in either of the age group and lower expression in winter season in all the age groups than that of summer as well as moderate season.

The diacelyated and triacelyated lipopeptides found on the surface of all the bacteria are recognize by the heterodimer formed between TLR2-TLR6 and TLR2-TLR1 respectively (Takeda et al., 2001). As TLR1 and TLR6 works in dimerization, thus there may be the possibility of synergistic up-regulation of those TLRs like that of TLR2 & TLR4. Again TLR3 & TLR8 in our studies shows higher level of expression in different age groups both in winter and summer season compared to moderate season. Both TLR3 & TLR8 are expressed in the endosomal vesicles of cell and recognize the double stranded RNA and single stranded RNA of viruses (Alexopoulou *et al*., 2001). Thus, upregulation of TLR3 & TLR8 genes during winter and summer season may provide the means to fight against the viral infection.

However, in all of the age groups of goats studied, a non-significant difference of TLR5 expression during both summer and winter season was noticed. Interestingly, in all the age groups, the expression of TLR5 was lower in both summer and winter as compared to moderate season. The possible reason for lower expression of TLR5 may be due to the low level of exposure to bacterial flagellin (bindling ligands for TLR5) during this period.

TLR9 has been shown to be necessary in the recognition of CpG motifs and plays a critical role in CpG ODN-mediated activation of immune responses (Takeshita *et al.,* 2000). Our studies shows that during summer season, mRNA expression of TLR9 was significantly (P<0.05) higher in group I as compare to group II and group III. The Chen *et al.,* (2005) finding regarding upregulation of TLR9 expression in human B cells supports our observations. Possible role of up-regulation of TLR9 in heat stress could render immune cells more sensitive to microbes and helps in host defence.

As winter season or short day length causes depression of immunity due to reduced food availability and increased thermoregulatory demands, thus it may be a reason of lower expression of almost all the TLR genes during winter season as compared to moderate and summer. Moreover, most aspects of innate arm of the immune system including cytokine responses to pathogens are inhibited by winter like day length (Bilbo *et al*., 2002).

In the present study, mRNA expression of HSP60 in all age groups during summer season was significantly (P <0.05) higher and level increased with the age of animals. However, during winter season mRNA expression level among the three age groups was found non-significant (P>0.05). Our findings are attuned with the findings of S. Dangi *et al*., 2012 who reported similar findings of mRNA expression in PBMCs of tropical region goats. Again, during summer season mRNA expression of HSP90 was found significantly higher (P<0.05) in group I and III as compared to group II. Previous studies also showed increased HSP90 expression due to heat stress in caprine PBMCs (Dangi *et al*., 2012), in heart, liver and kidney of broilers (Lei *et al*., 2009).

Summary and Conclusions

6 SUMMARY AND CONCLUSION

Environmental factors such as, ambient temperature, solar radiation, and humidity have direct and indirect effects on animals and impact worldwide livestock production. Since goats are usually reared under extensive farming condition in village condition thus they become more susceptible to heat stress where ambient temperature may increase or decrease as per seasonal variation. The grazing activity also exposes them to a wide range of pathogenic organisms (cysts of helminths and nematodes) and pesticides (used by local farmers) as a part of environmental challenges. Stressors vary throughout the day and across the seasons. Chronic exposure to stressors often compromises immunity and could have serious consequences for health and survival. Measures to deal with stress at the cellular level include temporary modifications in gene expression to survive changing environments, as well as altering cellular structure and function to deal with more permanent adverse conditions. A detailed understanding of the genes involves in heat and cold tolerance may help in integrating the understanding of function and interaction of the involved elements. Toll like receptors (TLR) are a family of germ line encoded receptors of the innate immune system that recognize of a wide variety of pathogens, inducing a fast and appropriate host defense reaction. The heat shock response confers transient thermal tolerance, in part due to the expression of HSPs. HSPs are responsible for maintaining the balance between survival and an effective immune system in the organisms in order to acclimatize the stress.

The present investigation was carried out to demonstrate expression profile of TLR and HSP genes during different seasons in Black Bengal goats. Blood samples were collected from eighteen healthy Black Bengal goats healthy goats during peak winter, moderate season and peak summer.

Goats were divided in three (n=6 in each group) age groups- Group I: up to 2 years, Group II- 2 to 5 years and Group III - above 5 years. The PBMCs was collected density gradient centrifugation using Histopaque-1077. Total RNA isolation was done by Trizol reagent. The purity and concentration of total RNA was checked using nanodrop reading. RNA samples showing the OD 260: OD 280 values more than 1.8 were used further. The integrity of the total RNA was checked using 1.0 % agarose gel electrophoresis and bands of 28s and 18s with smearing indicated good quality and intactness of RNA.

The RNA was reverse transcribed into cDNA by taking 1µg of total RNA and stored at -20°C for further use. The integrity of the cDNA was checked by PCR with GAPDH primers and desired band of 82 bp is obtained. qPCR was performed for the TLR and HSP genes with Ssofast™ Eva Green® qPCR kit following standard procedure in stratagene mx3005P (Agilent technologies, USA) real time machine. Efficiencies of primers were determined by amplification of a standardized dilution series of cDNA. Finally relative mRNA expression was calculated by Pfaffl equation by 2 Way ANOVA. Moderate season values were used as calibrator. During present study animals were exposed to ambient temperature beyond the comfort zone during winter and summer, so they were under thermal stress in both the seasons.

The study shows a wide variation in the expression of different TLR genes during different season and as well as in different age group also. The mRNA expression of almost all the TLR1-10 & HSP genes were significantly higher ($P<0.05$) in different age groups during peak summer season as compared to peak winter season. Moreover, lower expression of all the TLR genes except TLR3 & TLR8 was observed in winter compared to both moderate and summer season. The mRNA expression of HSP60, HSP70 and HSP90 was higher in all age groups during peak summer season as compared to peak winter season in Black bengal goats.

Conclusion:

1. TLR1-10 genes are expressed in PBMCs of Black Bengal goat constitutively in all the age groups.
2. Thermal stress during summer and winter season differentially regulates their expression.
3. HSPs could possibly play a significant role to combat the deleterious effect of thermal stress so as to maintain homeostasis in goats
4. As HSPs are the endogenous ligand of TLR2 and TLR4 thus, they play a essential modulatory role in activation of immune system in Black Bengal goat during heat stress.

Mini Abstract

MINI ABSTRACT

The stress response is a very complex mechanism that involves a series of behavioral, physiological, metabolic, and immunological reactions that the body uses to redistribute the demands placed on it, adapt to stressors, and survive. Despite animal's physiological and behavioural response to ameliorate the discomfort of thermal stress, there may be some molecular mechanisms to maintain their cellular homeostasis. It has been well established from various earlier studies that thermal stresses trigger a complex program of gene expression and biochemical adaptive responses.

TLRs are a family of at least 10 proteins that function as central mediators of the innate immune response to diverse pathogens as well as to endogenous molecules released by injured or dying cells. HSPs are the molecular chaperons that prevent the misfolding of proteins in the cell during heat stress. The present study has demonstrated the expression of TLR1-10 genes and HSP60, HSP70 and HSP90 in PBMCs during different seasons in three different age groups of Black Bengal goats. Real-time PCR was applied to investigate mRNA expression of examined factors. Specificity of the desired products was documented using analysis of the melting temperature and high resolution gel electrophoresis to verify that the transcripts are of the exact molecular size predicted. The study shows a wide variation in the expression of different TLR genes during different season and as well as in different age group also. The mRNA expression of almost all the TLR1-10 & HSPs were significantly higher ($P<0.05$) in different age groups during peak summer season as compared to peak winter season. Moreover, lower expression of all the TLR genes except TLR3 & TLR8 was observed in winter compared to both moderate and summer season. HSP70 mRNA expression was higher in both summer & winter as compared to moderate season in below 2 yr age group. In conclusion our findings may provide a basis for establishing the role of HSPs to ameliorate deleterious effect of heat & cold stress more concretely. Moreover, as HSPs are the endogenous ligand of TLR2 & TLR4 thus they play a essential modulatory role in activation of immune system in Black Bengal goat during heat stress.

लघु सारांश

लघु सारांश

तनाव प्रतिक्रिया एक बहुत जटिल तंत्र है जो जीवित तथा अनुकुलित रहने के लिए, व्यावहारिक, शारीरिक, चयापचय, और प्रतिरक्षा प्रतिक्रियाओं का उस पर रखा मांगों को फिर से विभाजित कर उपयोग करता है. थर्मल तनाव की असुविधा को सुधारने के लिए जानवरों में शारीरिक और व्यवहार प्रतिक्रिया के बावजूद, उनके कोशिकीय होमीयोस्टॅसिस को बनाए रखने के लिए कुछ आणविक तंत्र हो सकता है. पहले के विभिन्न अध्ययनों से यह अच्छी तरह से स्थापित किया गया है कि थर्मल तनाव एक जटिल जीन अभिव्यक्ति और जैव रासायनिक अनुकुलित कार्यक्रम को शुरू करता है.

टीएलआर कम से कम १० प्रोटीन के एक परिवार के हैं जो विविध रोगजनकों के लिए सहज प्रतिरक्षा प्रतिक्रिया मारे कोशिकाओं द्वारा उत्पन्न केंद्रीय मध्यस्थों के रूप में कार्य करता है. एचएसपी गर्मी तनाव के दौरान कोशिकाओं में प्रोटीन की विकारीय वलन को रोकने कि आणविक संरक्षिका हैं. वर्तमान अध्ययन ब्लैक बंगाल बकरी के तीन विभिन्न आयु समूहों में विभिन्न मौसमों के दौरान पीबीएमसी में टीएलआर-१० जीन और एचएसपी-६०, एचएसपी-७० और एचएसपी-९० की अभिव्यक्ति का प्रदर्शन किया. जांच कारकों की एमआरएनए अभिव्यक्ति की जांच करने के लिए वास्तविक समय पीसीआर लागू किया गया था. वांछित उत्पादों की विशिष्टता टेप भविष्यवाणी सटीक आणविक आकार के होते हैं, यह सत्यापित करने के पिघलने के तापमान और उच्च संकल्प जेल वैद्युतकणसंचलन का विश्लेषण का उपयोग कर लिखा गया था. यह अध्ययन विभिन्न आयु वर्ग में और साथ ही अलग -अलग मौसम के दौरान विभिन्न टीएलआर जीनों की अभिव्यक्ति में एक व्यापक बदलाव को दर्शाता है. शिखर सर्दियों के मौसम की तुलना में एमआरएनए की अभिव्यक्ति लगभग सभी टीएलआर १-१० और एचएसपी शिखर गर्मी के मौसम में विभिन्न आयु वर्ग में (पी <0.05) काफी अधिक थे. इसके अलावा, टीएलआर ३ और टीएलआर८ कर सभी टीएलआर जीनों अभिव्यक्ति मध्यम और गर्मी के मौसम दोनों की तुलना में सर्दियों में कम पाया गया. २ छोड़ वर्ष आयु से नीचे समूह में एचएसपी ७० एमआरएनए अभिव्यक्ति मध्यम मौसम की तुलना में गर्मियों और सर्दियों दोनों में अधिक था. अंत में हमारे निष्कर्ष बताते हैं कि अधिक गर्मी और ठंड तनाव के हानिकारक प्रभाव में एचएसपी की भूमिका की स्थापना के लिए एक आधार प्रदान कर सकता है. इसके अलावा गर्मी तनाव के दौरान ब्लैक बंगाल बकरी में टीएलआर-2 और टीएलआर-4 की अंतर्जातीय लिगेंड के रूप में एचएसपी एक प्रकार से प्रतिरक्षा प्रणाली के सक्रियण में एक आवश्यक अधिमिश्रकीय भूमिका निभाते हैं.

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Appendix

APPENDIX-I

I. SOLUTIONS USED FOR PBMC ISOLATION:

1. PBS 0.01M, 7.4 pH

$Na_2HPO_4:2H_2O$	1.86 gram/litre
KH_2PO_4	0.43 gram/litre
NaCl	7.20 gram/litre

2. 0.9% Nacl

0.9gm Nacl in 100 ml Distilled water

4. 0.01M Citrate buffer. (MW 294.1)

For 10 ml take 29.41 mg

II. REAGENTS USED IN AGAROSE GEL ELECTROPHORESIS

1. Tris-acetate-EDTA (TAE) buffer 50 X

Tris base	242 g
Glacial acetic acid	57.1 ml
Sodium EDTA (pH 8.0)	37.2 g

Distilled water was added to make up to a final volume of 1000 ml. A working solution of 1X was used.

2. Safe view DNA stain

3 µl of safe view DNA stain was added in 40 ml 2% agarose solution.

3. Loading dye (6X)

Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)

Sucrose 40% (w/v) Store at 4° C.

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