# "GENETIC CHARACTERIZATION OF TOLL-LIKE RECEPTOR (TLR)-4 GENE IN INDIGENOUS BREEDS OF GOAT"

# THESIS

SUBMITTED TO THE

## RAJENDRA AGRICULTURAL UNIVERSITY

(FACULTY OF POST - GRADUATE STUDIES)
PUSA ( SAMASTIPUR ), BIHAR


*By*

## Dr. Shashi Bhushan Kumar

Registration No. - M/VBG/13/2007-2008.


In Partial fulfillment of the requirements
**FOR THE DEGREE OF**

## MASTER OF VETERINARY SCIENCE

(Animal Breeding & Genetics)


DEPARTMENT OF ANIMAL BREEDING & GENETICS
BIHAR VETERINARY COLLEGE
PATNA (BIHAR)


2009

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P A T N A (BIHAR)

**2009**

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# CERTIFICATE -I

This is to certify that the thesis entitled " **Genetic characterization to toll-like receptor (TLR)-4 gene in indigenous breeds of goat** " submitted in partial fulfillment of the requirements for the Degree of **Master of Veterinary Science (Animal Breeding & Genetics)** of the faculty of post-graduate studies, Rajendra Agricultural University, PUSA, Samastipur, Bihar is the record of bonafide research work carried out by **Dr. Shashi Bhushan Kumar, Registration No. M/ABG/13/2007-08,** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that the assistance and help received during the course of this investigation and preparation of the thesis have been fully acknowledged.

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We, the undersigned, members of the Advisory Committee of **Dr. Shashi Bhushan Kumar, Registration No. M/ABG/13/2007-08**, a candidate for the Degree of Master of Veterinary Science with major in **Animal Breeding & Genetics** have gone through the manuscript of the thesis and agree that the thesis entitled "**Genetic characterization of toll-like receptor (TLR)-4 gene in indigenous breeds of goat"** may be submitted by **Dr. Shashi Bhushan Kumar** in partial fulfillment of the requirement for the Degree.

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***(Shashi Bhushan Kumar )***

# ABBREVIATION

| | | | |
|---|---|---|---|
| 1. | bp | : | Base Pairs |
| 2. | dsDNA | : | Double standard DNA |
| 3. | dNTP | : | Deoxy nucleotide tri phosphate |
| 4. | ddw | : | Double distilled water |
| 5. | $^0C$ | : | Degree celcius |
| 6. | EDTA | : | Ethylene Diamine Tetra Acetic Acid |
| 7. | EtBr | : | Ethidium Bromide |
| 8. | FAO | : | Food and Agricultural Organization |
| 9. | HCl | : | Hydrogen Chloride |
| 10. | IgG | : | Immunoglobulin G |
| 11. | $KHCO_3$ | : | Potassium bicarbonate |
| 12. | $KH_2PO_4$ | : | Potassium dihydrogen phosphate |
| 13. | KCl | : | Potassium Chloride |
| 14. | $MgCl_2$ | : | Magnessium Chloride |
| 15. | mM | : | Milli Molar |
| 16. | M | : | Molar |
| 17. | mg | : | Milligram |
| 18. | ng | : | Nanogram |
| 19. | nm | : | Nanometer |
| 20. | NaCl | : | Sodium Chloride |
| 21. | $NH_4Cl$ | : | Ammonium Chloride |
| 22. | $NaH_2PO_4$ | : | Sodium dihydrogen phosphate |
| 23. | NaOH | : | Sodium hydroxide |
| 24. | $Na_2HPO_4$ | : | Disodium Hydrogen Phosphate |
| 25. | Pg | : | Picogram |
| 26. | PCR | : | Polymerase Chain Reaction |
| 27. | OD | : | Optical Density |
| 28. | RFLP | : | Restriction Fragment Length Polymorphism |
| 29. | µg | : | Microgram |
| 30. | rpm | : | Rotation per minute |
| 31. | SDS | : | Sodium Dodecyl Sulphate |
| 32. | Taq | : | Taq Polymerase |
| 33. | TBE | : | Tris Borate EDTA |
| 34. | Tris Hcl | : | Tris hydrochloride |
| 35. | µm | : | Micrometer |
| 36. | µl | : | Microlitre |

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# INTRODUCTION

# INTRODUCTION

Livestock play a crucial role in the Indian economy. Despite poor infrastructure, low investments and resource poor stakeholders in animal husbandry sector, livestock has provided strength and sustainability to Agricultural Production as a whole. While the annual growth rate for agricultural crop sector has gradually declined in recent past, the average growth of 2.5% in total agriculture is mainly because there has been a consistent annual growth of more than 4% in the livestock sector. The contributions of Agriculture to National Gross Domestic Product(GDP) have substantially decreased over past decade, but the contribution of livestock remained consistent between 27 to 32% of agriculture GDP. The contribution of milk alone (90,358 crores) is higher than wheat (51,002 crores) and sugarcane (30,988 crores). Animal Husbandry provides self-employment to millions of household in rural India. It is an employment generating sector in Agrarian India. The export earning from livestock sector has shown an annual growth of about 12% (www.scribd.com/doc/7785730/Dr-KS-Dangi: Nov,2008).

India is a repository of livestock diversities, but faces a challenge between plenty and poverty with respect to livestock. According to FAO,India has 29 breeds of goat but Indian Council of Agricultural Research has registered only 20 breeds of goat. In spite of having 130 accredited breeds of documented animals and birds species viz. goats (20), sheep (42), cattle (30), buffalo (10), camel (8), horses (6) and poultry (18), there are hardly any systematic and sustainable breed improvement programme for its conservation in its native tracts. Several breeds with excellence in important traits are, therefore, at the verge of extinction. There is an urgent need to shift our emphasis on assessing the genetic potential of indigenous breeds, which of late have been found to be highly productive

under suitable management and environment. Intensive research work also needs to be undertaken for genetic identification of traits of excellence in Indian breeds, like Jaffarabadi buffalo, Black Bengal goat, Jamunapari goat, Garole sheep as well as Aseel and other indigenous fowl breeds. This is required to identify the functional genomics associated with their traits of excellence. The biodiversity existing in the domestic livestock needs to be investigated using molecular tools involving the transfer of major genes associated with production excellence, tropical adaptability, besides diseases and stress resistance. Further, improving animal health is a major goal in the current animal breeding industry (Soller and Anderson, 1998). Disease related cost and loses can be reduced by genetic control of resistance to pathogens and improvement of the immune capacity of animals.

To control the infection during the first few days of life, our body relies on the evolutionarily ancient and more universal innate immune system. The innate immune system also has an important function in activation and shaping of the adaptive immune response through the induction of co-stimulatory molecules and cytokines (Medzhitov and Janeway, 1997). The innate immune system in mammals permits direct inflammatory and antimicrobial processes that effectively kill pathogens. A key element in the initiation of an innate immune response is the early detection of potentially harmful microbes by recognising components commonly found on these foreign pathogens. These have been referred to as **P**athogen-**A**ssociated **M**olecular **P**atterns **(PAMP)** as reported by Medzhitov and Janeway (1997). Antigen-presenting cells (APC), like macrophages (MΦ) and dendritic cells (DC) express **P**attern **R**ecognising **R**eceptor (PRR), also known as **T**oll **L**ike **R**eceptor (TLR), on their surface. (TLR)- recognise and bind these PAMP (Schnare *et al.*, 2006) to initiate a signalling pathway that stimulates the host defence mechanism through the

induction of Reactive Oxygen Intermediates and Nitrogen Intermediates (ROI and RNI). The TLR–PAMP interaction also initiates adaptive immunity as it activates APC by inducing production of pro-inflammatory cytokines and up-regulating co-stimulatory molecules. Moreover, TLR signalling stimulates the maturation and migration of APC to the draining lymph nodes in some species (i.e. mice), although this migration seems to be more constitutive in ruminants, but can be modulated (Haig *et al.*, 1999).

The literatures suggest that one or more genes that might influence the trait can often be hypothesized based on the prior knowledge and as such, it is a reasonable strategy to test each gene for association with the trait in a phenotyped population. This 'candidate gene' approach has not been used extensively in goat, particularly in indigenous goat breeds, probably due to the paucity of characterized genes and immunological pathways for the majority of diseases. Candidate gene analysis is a powerful approach to detect genes controlling traits of economic importance, such as growth and immune response (Rothschild and Soller, 1997) in farm animals. Although over the past few years considerable progress has been made to know the role of TLRs and their association with disease resistance and susceptibility in man, relatively little is known about how TLRs contribution to successful host defence mechanism in veterinary species. There is a growing evidence from species other than goat, sheep and cattle that the ability of individuals to respond properly to TLR ligands may be affected by single nucleotide polymorphisms (SNPs) within the TLR genes, which consequently lead to an altered susceptibility to infectious or inflammatory diseases (Schroder and Schumann, 2005). There are at least 10 TLRs in mammals, each of which recognizes a different spectrum of PAMPs (Takeda *et al.*, 2003). TLR4 recognizes lipopolysaccharide (LPS) of Gram-negative bacteria (Chow *et al.*, 1999; Lien *et al.*, 2000), and is also involved in the recognition

of other bacterial and viral PAMPs (Lien *et al.*, 2000; Kurt-Jones *et al.*, 2000). A mutation in or a lack of TLR4 can result in altered immune responses to pathogens that produce these PAMPs (O'Brien *et al.*, 1980; Poltorak *et al.*, 1998; Chapes *et al.*, 2001; Abel *et al.*, 2002 and Helmby *et al.*, 2003), are available evidence to indicate association between TLR4 polymorphism and disease resistance (Agnese *et al.*, 2002; Leveque *et al.*, 2003; Schroder *et al.*, 2005 and Mockenhaupt *et al.*, 2006). Variation in TLR4 has been reported in Human (Chen *et al.*, 2005), Mice (Smirnova *et al.*, 2000), Bison (White *et al.*, 2005), Chickens (Leveque *et al.*, 2003), Sheep (Beg, 2002 and Zhou *et al.*, 2007), Cattle (White *et al.*, 2003a/b) and recently in Exotic Goat (Zhou *et al.*, 2008), but not yet in any Indian goat breed. It also need to be accertained whether the Caprine TLR4 gene is polymorphic or not.

Hence, this study was undertaken to know the variation in the caprine TLR4 gene in Beetal, Black Bengal and Jamunapari breeds of goat using gene sequencing and Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) analysis with following objectives:

1. To identify the caprine TLR4 gene in Beetal, Jamunapari and Black Bengal breeds.
2. To characterize any variation found in the gene within or between breeds.
3. To compare serum Lysozyme or IgG levels between breeds.

# REVIEW OF LITERATURE

# REVIEW OF LITERATURES

## 2.1 GENETIC CHARACTERIZATION :

It is now established that biochemical assays of genetic variation at the molecular level can provide rich insights into the genetic structure and evolutionary history of livings. The first technique for estimation of differences at genomic (DNA) level was developed in 1960's, to study the organization of eukaryotic genomes (Britten and Kohne, 1968), which was subsequently applied to the questions of molecular evolution and systematics. This technique known as DNA hybridization is based on the thermodynamic reannealing properties of heterologous single stranded DNA sequences. However, in recent years this technique has lost ground due to theoretical and practical difficulties and has been superseded by more direct DNA sequence-based approaches (Avise, 1994).

In conjunction with the technique of Southern hybridization (Southern, 1975), restriction enzymes have provided a very powerful method to assay genetic variation at the DNA level. Polymorphisms at the DNA sequence level can be visualized as changes in the cleavage patterns of DNA fragments, which when treated with particular restriction enzymes. Botstein *et al.* (1980) termed these polymorphisms as RFLPs The advent of the Polymerase Chain Reaction (PCR) by Mullis *et al.* (1986) dramatically changed this equation and facilitated direct amplification of DNA sequence from a large number of organisms.

## 2.2 Vertebrate immune system:

Vertebrate immunity can be broadly categorized into innate and adaptive immunity (Janeway and Medzhitov, 2002).

### 2.2.1 The Innate Immunity:

To control the infection during the first few days, our body relies on the evolutionarily ancient and more universal innate immune system. Its main functions include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling cascades, and apoptosis (Medzhitov, 2001). The innate immune system also has an important function in activation and shaping of the adaptive immune response through the induction of costimulatory molecules and cytokines (Medzhitov and Janeway, 1997). In contrast to the clonotypic receptors, expressed by B and T lymphocytes, the innate immune system uses nonclonal sets of recognition molecules, called pattern recognition receptors. Pattern recognition receptors bind conserved molecular structures found in large groups of pathogens, termed pathogen-associated molecular patterns (Medzhitov and Janeway, 1997). There are various groups of pattern recognition receptors, which can be secreted, expressed on the cell surface or resident in intracellular compartments (Medzhitov, 2001). The TLRs are one of the most important pattern recognition receptor families.

The innate immune system relies on a vast array of non-clonally expressed pattern recognition receptors for the detection of pathogens. Pattern recognition receptors bind conserved molecular structures shared by large groups of pathogens, termed pathogen-associated molecular patterns. The TLRs are a recently discovered family of pattern recognition receptors which show homology with the *Drosophila* Toll protein and the human interleukin-1 (IL-1) receptor family. Engagement of different TLRs can induce overlapping yet distinct patterns of gene expression that contribute to an inflammatory response. The TLR family is characterized by the presence of leucine-rich repeats and a Toll/interleukin-1 receptor-like domain, which mediate ligand binding and interaction with intracellular signaling proteins,

respectively. Most TLR ligands identified so far are conserved microbial products which signal the presence of an infection, but evidence for some endogenous ligands that might signal other danger conditions has also been obtained. Molecular mechanisms for pathogen-associated molecular pattern recognition still remain elusive but seem to be more complicated than initially anticipated. In most cases, direct binding of microbial ligands to TLRs still has to be demonstrated. Moreover, *Drosophila* TLRs bind endogenous ligands, generated through a proteolytic cascade in response to an infection. In the case of endotoxin, recognition involves a complex of TLR4 and a number of other proteins. Moreover, TLR heterodimerization further extends the spectrum of ligands and modulates the response towards specific ligands. The fact that TLR expression is regulated in both a cell type and stimulus-dependent fashion further contributes to the complexity.

TLRs play a critical role in the early innate immune response to invading pathogens by sensing microorganisms. These evolutionarily conserved receptors, homologues of the Drosophila Toll gene, recognize highly conserved structural motifs only expressed by microbial pathogens, called PAMPs. PAMPs include various bacterial cell wall components such as lipopolysaccharide (LPS), peptidoglycan (PGN) and lipopeptides, as well as flagellin, bacterial DNA and viral double-stranded RNA. Stimulation of TLRs by PAMPs initiates signaling cascades that involves a number of proteins, such as MyD88, TRIF and IRAK (Medzhitov *et al.*,1997). These signaling cascades lead to the activation of transcription factors, such as AP-1, NF-κB and IRFs inducing the secretion of pro-inflammatory cytokines and effector cytokines that direct the adaptive immune response. TLRs are type I transmembrane proteins characterized by an extracellular leucine-rich domain and a cytoplasmic tail that contains a conserved region called the Toll/IL-1 receptor (TIR) domain. TLRs are predominantly expressed in tissues involved in immune function, such as spleen and

peripheral blood leukocytes, as well as those exposed to the external environment such as lung and the gastrointestinal tract. Their expression profiles vary among tissues and cell types. TLRs are located on the plasma membrane with the exception of TLR3, TLR7, TLR9 which are localized intracellularly (Nishiya, T. and De Fraco, A.L., 2004).

Ten human and twelve murine TLRs have been characterized, TLR1 to TLR10 in humans, and TLR1 to TLR9, TLR11, TLR12 (aka TLR11) and TLR13 in mice, the homolog of TLR10 being a pseudogene. TLR2 is essential for the recognition of a variety of PAMPs from Gram-positive bacteria, including bacterial lipoproteins, lipomannans and lipoteichoic acids. TLR3 is implicated in virus-derived double-stranded RNA. TLR4 is predominantly activated by lipopolysaccharide.TLR5 detects bacterial flagellin and TLR9 is required for response to unmethylated CpG DNA. Finally, TLR7 and TLR8 recognize small synthetic antiviral molecules (Jurk *et al.*, 2002) and recently single-stranded RNA was reported to be their natural ligand (Heil *et al.*, 2004),TLR11 has been reported to recognize uropathogenic *E. coli* (Zhang *et al.*, 2004), and a profilin-like protein from *Toxoplasma gondii* (Lauw *et al.*,2005).

The repertoire of specificities of the TLRs is apparently extended by the ability of TLRs to heterodimerize with one another. For example, dimers of TLR2 and TLR6 are required for responses to diacylated lipoproteins while TLR2 and TLR1 interact to recognize triacylated lipoproteins (Ozinsky, 2000). Specificities of the TLRs are also influenced by various adapter and accessory molecules, such as MD-2 and CD14 that form a complex with TLR4 in reponse to LPS (Miyake, 2003).

TLR signaling consists of at least two distinct pathways: a MyD88-dependent pathway that leads to the production of inflammatory cytokines, and a MyD88-independent pathway associated with the stimulation of IFN-

β and the maturation of dendritic cells. The MyD88-dependent pathway is common to all TLRs (Adachi *et al.*,1998). Upon activation by microbial antigens, TLRs induce the recruitment of MyD88 via its TIR domain which activates IRAK-1 by phosphorylation. IRAK-1 then leaves the MyD88-TLR complex and associates temporarily with TRAF6. This association elicits downstream signaling, leading to the activation of NF-κB which in turn induces the production of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-12. Accumulating evidence indicates that IRAK-1 activity is negatively regulated by two separate molecules: Tollip and IRAK-M, which may act as moderators of the inflammatory response following TLR activation (Zhang, G and Ghosh,S., 2002;Kobayashi *et al.*, 2002).

Differences between signaling pathways induced by individual TLRs are emerging. TLR4 and TLR2 signaling requires the adaptor TIRAP/Mal, which is involved in the MyD88-dependent pathway (Horn *et al.*, 2002). TLR3 triggers the production of IFN-b in response to double-stranded RNA, in a MyD88-independent manner, through the adaptor TRIF/TICAM-1(Yamamoto *et al.*, 2002). TRAM/TICAM-2 is another adaptor molecule involved in the MyD88-independent pathway (Zhang *et al.*, 2004) which function is restricted to the TLR4 pathway (Yamamoto *et al.*, 2003).

TLR3, TLR7, TLR8 and TLR9 recognize viral nucleic acids and induce type I IFNs. The signaling mechanisms leading to the induction of type I IFNs differ depending on the TLR activated. They involve the interferon regulatory factors (IRFs), a growing family of transcription factors known to play a critical role in antiviral defense, cell growth and immune regulation. Three IRFs (IRF3, IRF5 and IRF7) function as direct transducers of virus-mediated TLR signaling. TLR3 and TLR4 activate IRF3 and IRF7 (Doyle *et al.*, 2002) , while TLR7 and TLR8 activate IRF5 and IRF7 (Schoenemeyer *et al.*, 2005).

Current knowledge of the TLRs indicates that these receptors are essential elements in host defense against pathogens by activating the innate immunity, a prerequisite to induction of adaptive immunity. The growing interest in TLRs would bring a more complete understanding of the role of TLR-mediated responses and increase our range of weapons to treat infectious and immune diseases.

### 2.2.2 Discovery of the TLR system:

The first member of the TLR family identified was a *Drosophila* protein implicated in dorsoventral patterning during embryonal development (Hashimoto *et al.*, 1988). Gay and Keith were the first to realize that the intracellular domain of *Drosophila* Toll showed striking similarities to the intracellular domain of the mammalian IL-1 receptor (Gay and Keith, 1991) Lemaitre *et al.*, (1996) demonstrated that *Drosophila* Toll was also involved in the immune response of the adult fly. Different human homologues of *Drosophila* Toll were identified and shown to induce activation of the transcription factor nuclear factor-κB (NF-κB) upon overexpression, revealing that TLRs and IL-1 receptors trigger similar signal transduction cascades (Medzhitov *et al.*, 1997 and Rock *et al.*, 1998). Poltorak *et al.*, (1998) discovered by positional cloning that the *lps* gene in the LPS-nonresponsive mouse strain CH3/HeJ encoded a murine member of the TLR family, providing the first clue of a function as pattern recognition receptors for mammalian TLRs.

TLRs are evolutionarily conserved proteins (the oldest TLR identified so far is expressed in *Caenorhabditis elegans* (Rich *et al.*, 2000]), characterized by an extracellular leucine-rich repeat domain and an intracellular Toll/IL-1 receptor-like (TIR) domain (Medzhitov *et al.*, 1997). Leucine-rich repeats are found in both cytoplasmic and transmembrane proteins and are involved in ligand recognition and signal transduction

(Kobe and Deisenhofer, 1995). How leucine-rich repeats mediate ligand recognition is still puzzling, especially as it was demonstrated that 7 out of 10 leucine-rich repeat motifs of the CD14 receptor, a transmembrane protein implicated in LPS recognition, could be deleted without affecting LPS binding (Juan *et al.*, 1995). Interestingly, all these TIR-containing proteins seem to have a function in host defense, making the TIR domain one of the earliest signalling motifs to evolve (Aravind *et al.*, 1999).

### 2.2.3 LIGAND RECOGNITION BY TLRS:

**Pathogen-Associated Molecular Patterns:**

TLRs, like other pattern recognition receptors, recognize so-called pathogen-associated molecular patterns, which are conserved motifs that are unique to microorganisms and are essential for their metabolism and thus survival (Medzhitov and Janeway, 1997). This has three major advantages. First, pathogen-associated molecular patterns are produced only by microbes and not by host cells, enabling the innate immune system to distinguish between self and nonself. Second, as pathogen-associated molecular patterns are essential for microbial survival, mutations in or loss of patterns can be lethal, and therefore these patterns are not subject to high mutation rates. And third, pathogen-associated molecular patterns are invariant between microorganisms of a given class, which implies that only a limited number of germ line-encoded pattern recognition receptors are needed to detect the presence of a microbial infection (Medzhitov and Janeway, 1997).

### 2.2.4 TLRs Implicated in Recognition of Pathogens:

Ten different TLRs which mediate recognition of diverse classes of pathogens have been identified in humans (Chaudhary,1998). Many of the TLR ligands were identified through screening of large numbers of known pathogen-associated molecular patterns in human embryonal kidney

HEK293T cells transiently transfected with one of the TLRs. HEK293T cells provide a valuable transfection model for these studies as they almost completely lack expression of any of the TLRs (Hornung *et al.*, 2002). More recently, gene disruption studies of the different TLR genes confirmed most of the results obtained in HEK293T cells. However, impurities in the commercially available pathogen-associated molecular pattern preparations have been shown to be a problem for the correct interpretation of the data. Thus, there has been confusion if TLR2 is also implicated in LPS signaling, as HEK293T cells overexpressing TLR2 induced NF-κB signaling upon LPS triggering (Yang *et al.*, 1998) , although TLR2-negative cells were still responsive to LPS (Takeuchi *et al.*,1999). Repurification of the commercially available LPS preparations eliminated LPS signaling through TLR2, showing that TLR2 was probably responding to the lipoprotein contaminants and not to LPS itself (Hirshfeld *et al.*, 2000).

**2.2.5 Regulation of TLR expression:**

Several reports suggest that TLR expression is regulated in both a cell type- and stimulus-dependent fashion. Generally, cell surface expression of TLRs is rather low, varying in monocytes from a few hundred to a few thousand molecules per cell (for comparison, there are approximately 3 x $10^5$ molecules of the adhesion molecule CD44 per cell) (Visintin, 2001).

According to their cellular expression pattern, TLRs can be categorized as either ubiquitous (TLR1), restricted (TLR2, TLR4, and TLR5), or specific (TLR3). TLR3 shows the most restricted expression pattern, as it is predominantly detected in immature dendritic cells (Muzio *et al.*, 2000), although a recent report by Zarember *et al.* showed a broader expression pattern. TLR1 shows the most ubiquitous expression pattern, reflecting its possible role as regulator of TLR-mediated signaling (Spitzer *et al.*, 2002).There are numerous data on stimulus-dependent up- and

downregulation of TLRs; Interestingly, expression of TLRs declines with age, which is a possible explanation for the increased susceptibility of elderly people to infections (Renshaw *et al.*, 2002). Furthermore, TLR expression is extremely variable among individuals; e.g., TLR expression appears to be much higher in farmer's children than in non-farmer's children (Lauener *et al.*, 2002), which might again correlate with individual differences in pathogen susceptibility, although different polymorphisms at the locus of TLRs might contribute more to altered host immune responses to pathogens (Aravind *et al.*, 1999 ).

More and more promoter studies of TLR genes are being published, aimed at identifying the gene-regulatory elements that control cell type specificity and inducibility of TLR gene transcription in humans and mice. Interestingly, it has been noted that there are substantial differences between the 5' untranslated regions of TLR promoters in mice and humans, possibly indicative of a high selective pressure on these genes during evolution to adapt to a rapidly changing microbial environment (Rehli *et al.*, 2002).

TLRs also exhibit specific subcellular expression patterns, reflecting the fact that they recognize different microbial ligands, which gain access to the cell at different subcellular sites. Thus, CpG DNA and LPS were shown to activate their respective TLRs in distinct cellular compartments in macrophages (Ahmad-Nejad *et al.*, 2002). This was demonstrated by the use of green fluorescent protein-MyD88 constructs, which were recruited to the plasma membrane upon LPS triggering and to the lysosomes upon CpG triggering, correlating well with the subcellular expression of TLR4 and TLR9, respectively. Inhibition of endocytosis interfered with CpG but not LPS signaling, possibly indicative of the fact that bacterial cell walls must be destroyed in order to liberate DNA, a process which takes place in mature,

acid endosomes, while bacterial cell wall LPS is freely accessible to cell surface-expressed TLR4.

TLR2 has been demonstrated to be recruited to yeast-loaded phagosomes (Underhill *et al.,* 1999). Phagosome expression of TLR2 and probably also other TLRs presumably enables them to sample the contents of vacuoles for different pathogen-associated molecular patterns, which are internalized by other pattern recognition receptors, like the mannose receptor (Underhill *et al.*, 1999). Finally, Gewirtz *et al*, (2001) showed that localized expression of TLR5, the TLR responsible for flagellin recognition, in epithelial cells of the gastrointestinal tract contributes to the differential response to commensal and pathogenic bacteria (Gewirtz *et al.*, 2001). The intestinal epithelium is highly polarized, with two distinct compartments: the apical surface, facing the lumen, and the basolateral surface, facing the underlying lamina propria. It has been known for a long time that commensal bacteria present in the lumen of the gut do not trigger inflammatory responses, while pathogenic bacteria do. Flagellin is a conserved protein that forms bacterial flagella and is produced by both commensal and pathogenic bacteria. Flagellin only induces an immune response when it is in contact with the basolateral membrane of the gastrointestinal epithelial barrier, not when it is secreted in the lumen. This could be attributed to the fact that pathogenic but not commensal bacteria translocate flagellin across epithelia , which triggers activation of TLR5, which is expressed exclusively at the basolateral surface (Gewirtz *et al.*, 2001).

### 2.2.6 Critical role of TLR in host defence:

The innate immune system in mammals permits direct inflammatory and antimicrobial processes that effectively kill pathogens. A key element in the initiation of an innate immune response is the early detection of

potentially harmful microbes by recognising components commonly found on these foreign pathogens. These have been referred to as PAMPs (Medzhitov and Janeway, 1997). Antigen-presenting cells (APC), such as macrophages (MF) and dendritic cells (DC), express TLR on their surface, which recognise and bind these PAMP and initiate a signalling pathway that stimulates the host defences through the induction of reactive oxygen and nitrogen intermediates (ROI and RNI, respectively). The TLR–PAMP interaction also initiates adaptive immunity as it activates APC by inducing production of proinflammatory cytokines and up-regulating co-stimulatory molecules. Moreover, TLR signalling stimulates the maturation and migration of APC to the draining lymph nodes in some species (i.e. mice), although this migration seems to be more constitutive in ruminants, but can be modulated (Haig *et al.*, 1999).

Mammalian TLR are a family of receptors that sense a broad range of microbial products (Takeda *et al.*, 2003). Each TLR is a single-pass transmembrane receptor with an extracellular domain, and an intracellular signalling domain that is homologous to the cytoplasmic tail of the IL-1 receptor. Stimulation of TLR initiates the stimulation of a signalling cascade, which directs activation of nuclear transcription factor kappaB (NF-kB) and the interferon response element (IRF) 3 as well as the production of pro-inflammatory cytokines. A vast array of microbial molecules have been shown to stimulate TLRs, including LPS (TLR4), bacterial flagellin (TLR5), double stranded (ds)RNA (TLR3), and bacterial DNA(TLR9). TLR7 and TLR8 have been shown to be important for recognition of single-stranded (ss) viral RNA and the synthetic imidazoquinoline-like molecules imiquimod (R-837) and resiquimod (R-848) (Kawai and Akira, 2005 ). In addition, TLR3, TLR7, TLR8 and TLR9, all of which recognize nucleic acids, are not expressed on the cell surface but are exclusively expressed in endosomal compartments. Thus, after

bacteria and viruses are internalized to be delivered to the endosome, nucleic acids might be released, to be recognized by TLR. In addition to the pro-inflammatory response induced by TLR–ligand interaction, increasing knowledge becomes available regarding the negative regulation of TLR-induced signalling (Liew *et al.*, 2005).

### 2.2.7 LPS Recognition by the TLR4 Complex: TLR4 the Sole LPS Receptor:

TLR4 does not need to heterodimerize with other TLRs to function but forms a complex with several other proteins on the cell surface which are needed for LPS recognition. LPS is bound in the serum by LPS binding protein, which transfers LPS to CD14 molecules (Wright, *et al.*, 1989). CD14 is a glycosylphosphatidylinositol-anchored membrane protein (but also exists in a soluble form) which binds LPS binding protein complexes with high affinity, but lacks an intracellular domain to signal (Wright, *et al.*, 1989). Therefore, it has long been proposed that CD14 functions in a complex with another membrane receptor to transmit LPS signals (Ulevitch, 1993). At the time TLR4 was identified as the long-sought Lps gene, responsible for defective LPS responses in CH3/HeJ mice (Poltorak,1998), and the confirmation that TLR4-/- mice are hyporesponsive to LPS (Hoshino, 1999), it was presumed that CD14 complexes with TLR4 to form a functional LPS receptor complex. However, 4 years after its discovery, physical interaction between CD14 and TLR4 remains to be demonstrated, and biochemical evidence showing direct LPS binding to TLR4 has not been published yet (although genetic evidence strongly suggests that TLR4 binds LPS directly). Furthermore, overexpression of TLR4 (with or without CD14) in HEK293T cells is not sufficient to confer LPS responsiveness on these cells, indicating the need for additional components (Kirschning *et al.*,1998).

One of the first additional components discovered in LPS signaling was MD-2, a homolog of MD-1, which is a B-cell-specific secretory protein that remains tethered to the membrane via interaction with RP105, a B-cell-specific leucine-rich repeat-containing molecule (Miyake *et al.*,1998). As TLRs also express leucine-rich repeats in their extracellular domain, Miyake and colleagues reasoned that additional MD molecules might be necessary to interact with TLRs, leading to the identification of MD-2 (Shimazu *et al.*, 1999). MD-2 and TLR4 interact physically on the membrane, and coexpression of MD-2 with TLR4 in HEK293T cells confers LPS responsiveness on these cells (Shimazu *et al.*,1999). MD-2 knockout mice do not respond to LPS and exhibit an impaired intracellular distribution of TLR4, showing that interaction with MD-2 is essential for proper targeting of TLR4 to the plasma membrane (Nagai *et al.*,2002). Interestingly, B cells deficient in RP-105 or MD-1 show an impaired LPS response, indicating that in B cells both TLR4/MD-2 and RP105/MD-1 clusters are needed for an intact LPS response (Nagai *et al.*, 2002).

Biophysical approaches used to study intramolecular interactions revealed that LPS is associated with non-TLR-related molecules as well, ranging from integrins such as CD11b/CD18 to chemokine receptors, scavenger receptors, and many others (Pfeiffer *et al.*, 2001). Many of these receptors are clustered upon LPS triggering in lipid rafts, suggesting the formation of supramolecular LPS activation clusters. These could vary according to the cell type and the activation state of the cell (Triantafilou *et al.*, 2002).Finally several several report suggest that LPS is not recognized as a free monomer but in the context of its packing in a membrane. E5531 is a synthetic LPS antagonist that resembles LPS but blocks its action in cells. Inversion of all 13 chiral centers of E5531 yields a mirror image, which was found to be an equally active antagonist. This observation argues against the recognition of LPS by a stoichiometric interaction with a stereospecific

binding site in a receptor. In conclusion, the molecular mechanism for LPS recognition still remains elusive but seems to be more complicated than initially anticipated.

### 2.2.8 Mutation and Polymorphism in TLR genes:

Mutations and polymorphisms in TLR, and TLR signalling molecules have revealed the importance of TLR in human defence against disease, leaving patients hypo or unresponsive to TLR ligands (Bochud *et al.*, 2003). Although over the past few years' considerable progress has been made regarding the role of TLRs and their association with disease resistance and susceptibility in man, relatively little is known about how TLRs contribute to successful host defence in veterinary species. There is a growing evidence from species other than goat and sheep that the ability of individuals to respond properly to TLR ligands may be affected by single nucleotide polymorphisms (SNPs) within the TLR genes, which consequently lead to an altered susceptibility to, or course of, infectious or inflammatory disease (Schroder and Schumann, 2005). Similarly, differences in bovine TLR have been described recently (White *et al.*, 2003a,b). Whether these differences may be useful to identify traits enabling the selection of cattle breeds resistant to diseases as described for other molecules of the immune system ( Nagaoka *et al.*, 1999 ;Konnai *et al.*, 2003) is currently under investigation. A mutation in, or a lack of TLR4 can result in altered immune responses to pathogens that produce these PAMPs (O'Brien *et al.*, 1980; Poltorak *et al.*, 1998; Chapes *et al.*, 2001; Abel *et al.*, 2002 and Helmby *et al.*, 2003), and accumulating evidence points to the association between TLR4 polymorphism and disease resistance (Agnese *et al.*, 2002; Leveque *et al.*, 2003; Schroder *et al.*, 2005 and Mockenhaupt *et al.*, 2006). Variation in TLR4 has been reported in humans (Chen *et al.*, 2005), mice (Smirnova *et al.*, 2000), bison (White *et al.*, 2005), chickens

(Leveque *et al.*, 2003), sheep (Beg, 2002; Zhou *et al.*, 2007), cattle (White *et al.*, 2003) and recently in goat (Zhou *et al.*, 2008), but not yet in any Indian indigenous goat breeds.

**2.3 The adaptive immune system:**

Adaptive immune responses are mediated by clonally distributed B and T lymphocytes and are characterized by specificity and memory. Recognition relies on the generation of a random and highly diverse repertoire of antigen receptors, the T- and B-cell receptors, followed by clonal selection and expansion of receptors with relevant specificities. This mechanism accounts for the generation of immunological memory, an important advantage, but has the main limitation that specific clones need to expand and differentiate into effector cells before they can participate in host defence. Therefore, adaptive immune responses are typically delayed for 4 to 7 days (Janeway and Medzhitov, 2002).

The adaptive immune system is composed of highly specialized, systemic cells and processes that eliminate or prevent pathogenic challenges. Thought to have arisen in the first jawed vertebrates, the adaptive or "specific" immune system is activated by the “non-specific” and evolutionarily older innate immune system (which is the major system of host defense against pathogens in nearly all other living things).The adaptive immune response provides the vertebrate immune system with the ability to recognize and remember specific pathogens (to generate immunity), and to mount stronger attacks each time the pathogen is encountered. It is adaptive immunity because the body's immune system prepares itself for future challenges.

The system is highly adaptable because of somatic hypermutation (a process of accelerated somatic mutations), and V(D)J recombination (an irreversible genetic recombination of antigen receptor gene segments). This

mechanism allows a small number of genes to generate a vast number of different antigen receptors, which are then uniquely expressed on each individual lymphocyte. Because the gene rearrangement leads to an irreversible change in the DNA of each cell, all of the progeny (offspring) of that cell will then inherit genes encoding the same receptor specificity, including the memory B cells and memory T cells that are the keys to long-lived specific immunity.

### 2.3.1 Function of adaptive immune system:

Adaptive immunity is triggered in vertebrates when a pathogen evades the innate immune system generates a threshold level of antigen(Janeway *et al.*,2001).

The major functions of the adaptive immune system include:

- The recognition of specific "non-self" antigens in the presence of "self", during the process of antigen presentation.
- The generation of responses that are tailored to maximally eliminate specific pathogens or pathogen infected cells.
- The development of immunological memory, in which each pathogen is "remembered" by a signature antibody. These memory cells can be called upon to quickly eliminate a pathogen should subsequent infections occur.

### 2.3.2 Effector cells:

The cells of the adaptive immune system are a type of leukocyte, called a lymphocyte. B cells and T cells are the major types of lymphocytes. The human body has about 2 trillion lymphocytes, constituting 20-40% of white blood cells (WBCs); their total mass is about the same as the brain or liver(Alberts *et al.*, 2002). The peripheral blood contains 20–50% of circulating lymphocytes; the rest move within the lymphatic system.

B cells and T cells are derived from the same pluripotential hematopoietic stem cells, and are indistinguishable from one another until after they are activated(Janeway *et al.*, 2002). B cells play a large role in the humoral immune response, whereas T-cells are intimately involved in cell-mediated immune responses. However, in nearly all other vertebrates, B cells (and T-cells) are produced by stem cells in the bone marrow (Janeway *et al.*,2002). T-cells travel to and develop in the thymus, from which they derive their name. In humans, approximately 1-2% of the lymphocyte pool recirculates each hour to optimize the opportunities for antigen-specific lymphocytes to find their specific antigen within the secondary lymphoid tissues (microbiology and Immunology On Line Testbook).

In an adult animal, the peripheral lymphoid organs contain a mixture of B and T cells in at least three stages of differentiation:

- naive cells- that have matured, left the bone marrow or thymus, have entered the lymphatic system, but that have yet to encounter their cognate antigen,
- effecter cells- that have been activated by their cognate antigen, and are actively involved in eliminating a pathogen and,
- memory cells- the long-lived survivors of past infections.

**2.3.3 Antigen Presentation:**

Adaptive immunity relies on the capacity of immune cells to distinguish between the body's own cells and unwanted invaders.

The host's cells express "self" antigens. These antigens are different from those on the surface of bacteria ("non-self" antigens) or on the surface of virally infected host cells ("missing-self"). The adaptive response is triggered by recognizing non-self and missing-self antigens.

With the exception of non-nucleated cells (including erythrocytes), all cells are capable of presenting antigen and of activating the adaptive response (Janeway *et al.*,2002). Some cells are specially equipped to present antigen, and to prime naive T cells. Dendritic cells and B-cells (and to a lesser extent macrophages) are equipped with special immuno- stimulatory receptors that allow for enhanced activation of T cells, and are termed professional antigen presenting cells (APC).

Several T cells subgroups can be activated by professional APCs, and each type of T cell is specially equipped to deal with each unique toxin or bacterial and viral pathogen. The type of T cell activated, and the type of response generated depends, in part, on the context in which the APC first encountered the antigen (Janeway *et al.*, 2002).

### 2.3.4 Helper T Cells:

Helper T cells, are immune response mediators, and play an important role in establishing and maximizing the capabilities of the adaptive immune response (Janeway *et al.*, 2002). These cells have no cytotoxic or phagocytic activity; and cannot kill infected cells or clear pathogens, but, in essence "manage" the immune response, by directing other cells to perform these tasks.

Helper T cells express T-cell receptors (TCR) that recognize antigen bound to Class II MHC molecules. The activation of a naive helper T-cell causes it to release cytokines, which influences the activity of many cell types, including the APC that activated it. Helper T-cells require a much milder activation stimulus than cytotoxic T-cells. Helper T-cells can provide extra signals that "help" activate cytotoxic cells. ( Janeway *et al.*,2002).

### 2.3.5 Th1 and Th2:Helper T Cell Responses:

The Th1 response is characterized by the production of Interferon-gamma, which activates the bactericidal activities of macrophages, and induces B-cells to make opsonizing (coating) antibodies, and leads to "cell-mediated immunity" (Janeway *et al.*, 2002). The Th2 response is characterized by the release of Interleukin 4, which results in the activation of B-cells to make neutralizing (killing) antibodies, leading to "humoral immunity" (Janeway *et al.*, 2002). Generally, Th1 responses are more effective against intracellular pathogens (viruses and bacteria that are inside host cells), while Th2 responses are more effective against extracellular bacteria, parasites and toxins.

HIV is able to subvert the immune system by attacking the CD4+ T cells, precisely the cells that could drive the destruction of the virus, but also the cells that drive immunity against all other pathogens encountered during an organisms' lifetime (Janeway *et al.*, 2002).

A third type of T lymphocyte, the regulatory T cells (Treg), limits and suppresses the immune system, and may control aberrant immune responses to self-antigens; an important mechanism in controlling the development of autoimmune diseases.T cells never act as antigen-presenting cells (Janeway *et al.*, 2002).

### 2.3.6 B Lymphocyte and antibody production:

B Cells are the major cells involved in the production of antibodies that circulate in blood plasma and lymph, that provide humoral immunity. Antibodies (or immunoglobulin, Ig), are large Y-shaped proteins used by the immune system to identify and neutralize foreign objects. In mammals there are five types of antibody: IgA, IgD, IgE, IgG, and IgM, differing in biological properties, each has evolved to handle different kinds of antigens.

Upon activation, B cells produce antibodies, each of which recognizes a unique antigen and neutralize specific pathogens.

Like the T cell receptor, B cells express a unique B cell receptor (BCR), in this case, an immobilized antibody molecule. The BCR recognizes and binds to only one particular antigen. A critical difference between B cells and T cells is how each cell "sees" an antigen. T cells recognize their cognate (or specific) antigen in a processed form - as a peptide in the context of an MHC molecule, while B cells recognize antigens in their native form (Janeway *et al.*, 2002).

Once a B cell encounters its cognate (or specific) antigen (and receives additional signals from a helper T cell (predominately Th2 type)), it further differentiates into an effecter cell, known as a plasma cell (Janeway *et al.*, 2002).

Plasma cells are short lived cells (2-3 days) which secrete antibodies. These antibodies bind to antigens, making them easier targets for phagocytes, and trigger the complement cascade (Janeway *et al.* 2002). About 10% of plasma cells will survive to become long-lived antigen specific memory B cells. Already primed to produce specific antibodies, these cells can be called upon to respond quickly if the same pathogen re-infects the host; while the host experiences few, if any, symptoms.

Although the classical molecules of the adaptive immune system (e.g. antibodies and T cell receptors) exist only in jawed vertebrates, a distinct lymphocyte-derived molecule has been discovered in primitive jawless vertebrates, such as the lamprey and hagfish. These animals possess a large array of molecules called variable lymphocyte receptors (VLRs) that, like the antigen receptors of jawed vertebrates, are produced from only a small number (one or two) of genes. These molecules are believed to bind

pathogenic antigens in a similar way to antibodies, and with the same degree of specificity (Alder *et al.*, 2005).

### 2.3.7 Immunological memory:

When B cells and T cells are activated some will become memory cell throughout the lifetime of an animal these memory cells form a database of effective B lymphocytes. Upon interaction with a previously encountered antigen, the appropriate memory cells are selected and activated. In this manner, the second and subsequent exposures to an antigen produce a stronger and faster immune response. This is "adaptive" because the body immune system prepares itself for future challenges. Immunological memory can either be in the form of passive short-term memory or active long-term memory.

### 2.3.8 Passive memory:

Passive memory is usually short-term, lasting between a few days and several months. Newborn infants have had no prior exposure to microbes and are particularly vulnerable to infection. Several layers of passive protection are provided by the mother. In utero, maternal IgG is transported directly across the placenta, so that at birth, human babies have high levels of antibodies, with the same range of antigen specificities as their mother (Janeway *et al.*, 2002). Breast milk contains antibodies that are transferred to the gut of the infant, protecting against bacterial infections, until the newborn can synthesize its own antibodies (Janeway *et al.*, 2002). This is passive immunity because the fetus does not actually make any memory cells or antibodies, it only borrows them. Short-term passive immunity can also be transferred artificially from one individual to another via antibody-rich serum.

Active immunity is generally long-term and can be acquired by infection followed by B cells and T cells activation, or artificially acquired by vaccines, in a process called immunization.

### 2.3.9 Immunisation:

Infectious disease has been the leading cause of death in the human population. Over the last century, two important factors have been developed to combat their spread; sanitation and immunization (Janeway *et al.*,2002).Immunization is the deliberate induction of an immune response, and represents the single most effective manipulation of the immune system mankind has developed (Janeway *et al.*,2002). Immunizations are successful because they utilize the immune system's natural specificity as well as its inducibility.

The principle behind immunization is to introduce an antigen, derived from a disease causing organism, that stimulates the immune system to develop protective immunity against that organism, but which does not itself cause the pathogenic effects of that organism. An antigen (short for *anti*body *gen*erator), is defined as any substance that binds to a specific antibody and elicits an adaptive immune response (Alberts *et al.*,2002).

Most viral vaccines are based on live attenuated viruses, while many bacterial vaccines are based on acellular components of micro-organisms, including harmless toxin components (Alberts *et al.*,2002). Many antigens derived from acellular vaccines do not strongly induce an adaptive response, and most bacterial vaccines require the addition of adjuvants that activate the antigen presenting cells of the innate immune system to enhance immunogenicity (Janeway *et al.*,2002).

### 2.3.10 Immunological Diversity:

Most large molecules, including virtually all proteins and many polysaccharides, can serve as antigens (Janeway *et al.*, 2002). The parts of an antigen that interact with an antibody molecule or a lymphocyte receptor, are called epitopes. Most antigens contain a variety of epitopes and can stimulate the production of antibodies, specific T cell responses, or both.

An antibody is made up of two heavy chains and two light chains. The unique variable region allows an antibody to recognize its matching antigen. A very small proportion (less than 0.01%) of the total lymphocytes are able to bind to a particular antigen, which suggests that only a few cells will respond to each antigen (Janeway *et al.*, 2002).

For the adaptive response to "remember" and eliminate a large number of pathogens the immune system must be able to distinguish between many different antigens, (Alberts *et al.*,2002). and the receptors that recognize antigens must be produced in a huge variety of configurations, essentially one receptor (at least) for each different pathogen that might ever be encountered. Even in the absence of antigen stimulation, a human is capable of producing more than 1 trillion different antibody molecules (Janeway *et al.*, 2002). Millions of genes would be required to store the genetic information used to produce these receptors, but, the entire human genome contains fewer than 25,000 genes (International Human Genome Sequencing Consortium , 2004).

This myriad of receptors are produced through a process known as clonal selection. (Janeway *et al.*, 2002).According to the clonal selection theory, at birth, an animal will randomly generate a vast diversity of lymphocytes (each bearing a unique antigen receptor) from information encoded in a small family of genes. In order to generate each unique antigen receptor, these genes will have undergone a process called combinatorial diversification, in which one gene segment recombines with other gene

segments to form a single unique gene. It is this assembly process that generates the enormous diversity of receptors and antibodies, before the body ever encounters antigens, and enables the immune system to respond to an almost unlimited diversity of antigens. Throughout the lifetime of an animal, those lymphocytes that can react against the antigens an animal actually encounters, will be selected for action, directed against anything that expresses that antigen.

It is important to note that the innate and adaptive portions of the immune system work together and not in spite of each other. The adaptive arm, B and T cells, would be unable to function without the input of the innate system. T cells are useless without antigen-presenting cells to activate them, and B cells are crippled without T-cell help. On the other hand, the innate system would likely be overrun with pathogens without the specialized action of the adaptive immune response.

# MATERIALS AND METHODS

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## 3.1 Experimental animals:

Altogether 27 healthy goats, 9 from each of the three indigenous breeds i.e. Beetal, Jamunapari and Black Bengal, maintained at Composite Instructional Livestock Farm, Bihar Veterinary College, Patna were taken as the experimental animal for DNA studies in this investigation.

## 3.2 Chemicals and equipments:

Chemicals, reagents, buffers and equipments used in the present study are listed in Appendix: Most of the reagents and chemicals used in this study were of molecular biology grade.

## 3.3 Blood collection:

Five ml. of blood was collected from jugular vein of each of the 27 goats selected, in heparinized syringe for genomic DNA extraction.

- Two ml. of blood was also collected from each goat without any anti-coagulant to isolate serum for the estimation of Serum Lysozyme and total Immunoglobulin G (IgG).

The blood sample were stored in the deep freeze at $-20^0$C for the further use.

## 3.4 Isolation of genomic DNA from goat peripheral blood samples

**Materials, equipments and other materials used:**

- Weighing balance
- Centrifuge tube
- Micropipette
- Centrifuge machine
- Vortex mixer

- Water bath
- Ice pack

➢ **Reagent used:**

- Phosphate Buffer saline
- RBC lysis buffer
- DNA extraction buffer
- SDS (10%)
- Proteinase K (20 mg/ml)
- Tris saturated phenol ($p^H$ 7.4)
- Phenol
- Chloroform
- Isoamyl alcohol
- Sodium Chloride
- Isopropyl alcohol or absolute alcohol

Genomic DNA was isolated by modified Salting Out method detailed by Miller *et al.* (1988). 5 ml of blood from each animal was used for genomic DNA isolation.

1. After decanting supernant, the Buffy coat was collected by centrifuging 5 ml blood samples and 5 ml of Phosphate Buffer Saline at 1800 rpm for 20 minutes in 15 ml centrifuge tube.
2. Buffy coat was transferred into new 15 ml tube and centrifuged with 5ml PBS at 2000 rpm for 5 minutes.

3. 3 ml of chilled RBC lysis buffer was added to tube containing washed 1.5 ml buffy coat and after gentle mixing it was kept on ice for 10 minutes.

4. After 10 minutes incubation on ice, tubes were centrifuged at 2000 rpm, for 20 minutes and the supernatant was carefully removed.

5. Tubes were again washed with 5ml PBS for 20 minutes to remove hemolyzed black tary coloured RBS by pipetting so that WBC pellet became free of reddish tinge colour. The similar washing procedure was repeated thrice to get clear WBC pellet.

6. Now, 2 ml of DNA extraction buffer, 100 µl of 10% sodium dodecyl sulfate (SDS) and 10 µl of Proteinase K (20 mg/ml) were added to each tube.

7. The contents were mixed gently by swirling in vortex and the tubes were incubated overnight at 55°C in water bath.

8. 3 ml mixture was extracted by gentle mixing with 3ml of Tris saturated Phenol (pH 7.4) followed by extraction with 1.5 ml of 1:1 mixture of Phenol and Chloroform-Isoamyl alcohol (24:1).

9. Chloroform Isoamyl Alcohol extraction was performed once again and in each step 1ml. supernatant was transferred to a new tube.

10. Sodium chloride (5M solution) was added to a final concentration of 1.5 M and adding 2ml of Isopropyl alcohol or absolute alcohol, DNA was allowed to be precipitated.

11. The precipitated DNA was washed with 0.75ml 70 % ethanol and air dried for 10 minutes without over drying to remove the traces of alcohol and finally it was dissolved in 400 µl of DNase free distilled water.

12. The purity and concentration of isolated DNA was verified by spectrophotometry.

## 3.5 Preparation of aqueous suspension of DNA:

1. The Isopropyl alcohol used as preservative in precipitated DNA was decanted carefully.
2. The precipitated DNA was transferred into 2 ml eppendrof tube by micropipette.
3. 0.5 ml of 70% Ethanol was added into the eppendrof tube containing precipitated DNA and spinned at 10,000 rpm for 1 minute in spinnix to dissolve sodium acetate and other salts.
4. Absolute alcohol was decanted carefully so that the precipitated DNA is allowed to remain in eppendrof tube. It was air dried for 10 minutes without over drying to remove the traces of alcohol.
5. 400 µl of DNase free distilled water was put into the dried DNA in eppendrof tube to dissolve DNA and was stored at $-20^0$C in deep freezer for future analysis.

## 3.6 Agarose gel electrophoresis to check the quantity of isolated genomic DNA:

**Equipments and plasticware used:**

- Horizontal gel electrophoretic machine
- UV spectrophotometer
- Gel casting plate
- Comb
- Oven
- Beaker (500ml)
- Micropipette (5 and 10 ml variable)

**Reagents**

- 1 x TBE
- Ethidum bromide
- Loading dye.
- Agarose

Agarose gel electrophoresis is the method of separating and analysing charged biomolecules like DNA, RNA and proteins. Starch, polyacrilamide and agarose are used as supporting media to enhance resolution particularly for nucleic acids and proteins. The location of DNA within the gel can easily be detected by staining with ethidium bromide. Even very small amount of DNA (1-10 ng) can be detected by this method. Larger sized DNA molecules face a large frictional force, as they move through the gel, and so have lower mobility as compared to the smaller sized DNA fragments that experience lesser frictional force. A mixture of DNA molecules, therefore, separates into discrete bands during electrophoresis. Frozen stock of genomic DNA samples of 9 Beetal , 9 Jamunapari , and 9 Black Bengal goats were diluted by adding 0.5 ml milli Q water. Their quantity and quality were assessed by 1% agarose gel electrophoresis using 1xTBE buffer. Based on the result of electrophoresis the selected samples were further diluted. The detailed protocol is as under.

### 3.6.1 Dissolution of agarose:

Two types of running buffers are commonly used for DNA gel electrophoresis in agarose : Tris Acetate EDTA (TAE) and Tris-Borate EDTA (TBE). TBE buffer was used in the present study.

1. 20 ml of 5XTBE was diluted to make 100 ml with distilled water from milipore Q in a 250 ml Erlenmeyer flask to make final concentration as 1XTBE.

2. 200 mg of agarose power was weighed and added to the 20 ml 1 XTBE buffer to form 1% agarose gel.
3. The slurry was autoclaved on a oven until agarose was completely dissolved. Solution was gently swirled during heating cycles to release trapped air and re-suspend any agarose caught on the side of the flask.
4. The agarose solution was cooled until it reached at temperature of approximate 50-55$^0$C (Slightly hot to the hand). The beaker was swirled occasionally to keep the contents at uniform temperature and prevent agarose from gelling at the bottom of the beaker. Ethidium bromide stock solution (10 mg /ml in water) was added to the final concentration of 0.5 µl/ml.

**3.6.2 Casting of Gel:**

1. The comb was placed at the desired position on the gel tray i.e. 0.5-1.0 mm above the surface of the gel plate so that the complete well is formed.
2. The warm agarose solution was poured into the mold until its thickness becomes 3–5 mm. Care was taken to avoid the air bubbles under or between the teeth of comb.The bubbles formed were removed with pointed end of micropipette tips before the setting of gel.
3. After the gel was completely set (30-40 minutes at room temperature) comb was removed carefully by wriggling back and forth gently and the lifting up.

**3.6.3 Gel loading:**

The gel along with gel tray was placed in the electrophoretic tank. Fresh running buffer (1 XTBE) prepared by diluting 5 XTBE was added to

cover the gel up to the height of about 1-2 mm. The 3 µl DNA samples were mixed with 3 µl of gel loading dye before loading into wells. The loading dye serves three purposes:

(i) It increases the density of the samples.

(ii) Ensures that the DNA drops evenly into the wells.

(iii) Adds colour to the samples, thereby simplify the loading process. Gel loading dye contains dyes that in an electric field move towards the anode at predictable rates.

Bromophenol blue migrates through agarose gel approximately 2.2 fold faster than cyanol regardless of agarose concentration. Bromophenol blue migrates through the agarose gel run in 1XTBE at approximately the same rate as linear dsDNA of 300 bp in length. Whereas, xylene cyanol migrates approximately at the same rate as linear dsDNA of 4,000 bp in length. The mobility is not affected by the concentration of agarose provided concentration varies between 1–2%. After loading the DNA samples, lid of the gel tank was closed, attached to the electric leads and power supply was turned on so that DNA migrated towards anode. Initialy the Voltage was kept at 100 and 45-50 mAmp. So that loaded DNA and loading dye move foward leaving the well. Then Voltage as well as Current were adjusted to 8 Volt/cm of gel glass length, so that resolution of DNA band may become clear. The gel was run until the bromophenol blue and xylene cyanol migrated the approximate distance through the gel.

### 3.6.4 Determination of purity and concentration of DNA:

1. The concentration and purity of DNA preparation was determined using UV spectrophotometer absorbance at 260 and 280 nm.
2. The integrity of DNA was assessed by UV visualization of intact DNA bands on 1% agarose gel.

3. The possible traces of DNase was removed from genomic DNA by incubation at 65°C for 10 minutes.
4. The DNA samples were stored at –20°C for further use.

### 3.6.5 Design of primers for TLR-4 gene:

1. The primers were designed for exon 3 region of *Capra hircus* TLR-4 gene from the published caprine TLR-4 sequence (GenBank accession number EF409989) using online GeneTool software.
2. Specificity of primers was checked using NCBI Genbank database BLAST program (http://www.ncbi.nlm.nih.gov/entrez/).Gene specific forward and reverse primers were prepared as one synthesized commercially.

### 3.6.6 PCR amplification of exon 3 region to TLR-4 gene

**Equipments used:**

- Programmable multi-gradient PCR
- PCR tubes (autoclaved)
- Thermoseal
- Micropipette
- Eppendorf tubes

**Reagents and other materials used:**

- Genomic DNA
- 10X buffer
- dNTP
- Primers (Forward and Reverse)
- Taq DNA polymerase

- PCR grade water
- Ice flakes

Polymerase chain reaction is an *in vitro* method of synthesizing enzymatically known sequence of DNA. The reaction uses two oligonucleotide primers that hybridize to opposite strand at the specific temperature and flank the target DNA sequence to be amplified. A heat stable DNA polymerase such as Taq polymerase (*Thermus aquaticus*) catalyses the elongation of the primers. Template denaturation, primer annealing and extension of the annealed primers by the polymerase results in exponential accumulation of a specific DNA fragment. The primer extension products are synthesized in the next cycle.

**Optimisation of PCR Conditions:**

Various PCR parameter like concentration of genomic DNA template dNTP, Taq polymerase. $MgCl_2$ and annealing temperature of primers were optimised to obtain a specific amplified product in significant quantity (25µl volume) which was kept constant for all reactions.

**Table-1: Details of PCR reaction**

| Components | Quantity | Final concentration |
|---|---|---|
| Distilled Water | 15.5 µl | |
| 10x buffer | 2.5 µl | 10x |
| dNTP | 1.0 µl | 10 mM |
| F. primer | 1µl | 10pmole |
| R. Primer | 1 µl | 10pmole |
| Taq polymerase | 0.5 µl | 1 u/µl |
| $MgCl_2$ | 2.5 µl | 25 mM |
| Genomic DNA(Diluted) | 1.0 µl | 80-100 ng/µl |

**Cycling parameter in PCR:**

The PCR tubes were vortexed for proper mixing of DNA and other reagents. The PCR tubes were set in PCR machine. Amplification protocol used was as follow:

**Table No-2: Protocol of PCR reaction**

| Steps | Temp. primer | Time |
|---|---|---|
| 1 Initial denaturation | $94^0$C | 10 min. |
| 2 Denaturation | $94^0$C | 1 min. |
| 3 Annealing | $56^0$C | 1 min. |
| 4 Extension | $72^0$C | 1 min. |
| Step 2 to 4 repeated for 30cycles | | |
| 5 Final extension | $72^0$C | 10 min. |

**Methods for the detection of PCR product by Agarose gel electrophoresis:**

After completion of PCR programme,the PCR products were checked on 2% Agarose for amplification.For this 5µl PCR product was mixed with 1 µl loading dye and run under constant voltage and current (100 volt and 45-50 mAmp) till the two dyes got separated. Amplified products appeared as sharp orange colour bands under UV light due to the intercalation of Ethidium Bromide in the DNA. The amplified samples were identified and results were recorded for further analysis.

### 3.7 PCR-RFLP for detection of restriction site of Alu1 R.E and pattern

**Table No-3:** R.E digestion protocol of amplified PCR product

| | |
|---|---|
| *Water* | 8.0 µl |
| Buffer | 2.0 µl |
| Alu1enzyme | 0.2 µl |
| PCR product | 10.0 µl |
| Total | 20.2 µl |

1. Reagents as mentioned in protocol were taken in eppendrof tubes.
2. Eppendrof tubes containing reagents were kept in waterbath at $37^0$C over night.
3. Two percent Agarose gel was prepared by adding 1g Agarose in 50 ml. 1xTBE and 2 µl of Ethidium Bromide.
4. The gel was loaded with 10 µl of restriction endonuclease to digest product of PCR with 2 µl of gel loading dye along with 7 µl of Puc19/Msp1 digest DNA marker in separate wells.
5. Loaded gel was initially run at 100 Volt and 60 mAmp current till DNA and gel loading dye migrate from well and there after the voltage was reduced to 50 Voltage at 45 mAmp.
6. Location of DNA within the gel along with Puc19/Msp1 was detected by observing on UV-spectrophotometer.

### 3.8 Immunological investigation:

1. Blood serum were isolated from 2 ml. of blood collected without any anti- coagulant from 9 Beetal, 9 Jamunapari and 9 Black Bengal goats selected for this study.
2. Serum IgG and Lysozyme levels were compared between breeds.

### 3.9 Serum Lysozyme level:

1. The serum lysozyme was estimated by lysoplate method as described by Lie *et al.* (1986).
2. The Standard curve was prepared using known concentrations of Egg White Lysozyme (EWL)
3. One percent Agar was prepared in dibasic buffer (0.066M; pH 6.3).
4. While preparing the gel, *Micrococcus lysodiekticus* (50µg/ml of gel solution) was added and thoroughly mixed when the gel solution was cool to approximately 65 °C.
5. The gel was spread on clean and defated glass plate.
6. Wells were cut in the gel after solidification and 10 µl of individual concentration of Lysozyme standard was added in each well.
7. The serum samples were also loaded in different wells.
8. The plate was incubated at 37°C in humidified chambers.
9. After 18 hours of incubation the lysis of bacteria appeared in the form of clear circular zone around each well.
10. The diameter (mm) of circular clear zone was measured with the help of vernier caliper.
11. The standard curve was plotted between the known concentrations of standards and respective diameters of circular clear zone. The

diameter of rings of unknown samples were also measured and read on standard curve.

### 3.10 Total serum IgG level:

1. Serum IgG level was assayed by Single Radial Immuno Diffusion (SRID) test (Manicini *et al.*, 1965) as modified by Fahey and Mckelevey (1965). The Standard curve was prepared using known concentrations of IgG standard.
2. Solution of different dilutions of IgG (40, 20, 10, 5 & 2.5 mg/ml) were prepared in Tris-HCl buffer (0.1 M; pH 7.4).
3. The 2% Agar solution was prepared in Tri-HCl buffer and boiled properly.Anti chicken IgG antiserum @1.750ml/50ml of gel solution was added and mixed thoroughly when the gel solution cooled to 55-60°C.
4. The gel solution was spread on defated and clean glass plate.
5. Wells were cut in the gel after solidification and in each well 10 µl of individual concentration of IgG standards was added.
6. The serum samples diluted 1:5 times was also loaded in different wells.
7. The plate was incubated at 37°C in humidified chambers.
8. After 18 hours of incubation the antigen- antibody reaction appeared in the form of ring around each well.
9. The diameter (mm) of ring around each well was measured with the help of vernier caliper.
10. The standard curve was plotted between the known concentrations of standards and respective ring diameters measured

11. The diameter of ring around unknown samples were also measured and read on standard curve.

**3.11 Statistical analysis:**

Standard statistical methods were followed to compare allelic frequency, serum IgG and lysozyme levels between different goat breeds (Snedecor and Cochran, 1984).

**Alleles Scoring:**

For each PCR-RFLP Marker the alleles were scored manually and denoted as A and B. Genotype of each individual was recorded for further statistical analysis.

Allele number is the total number of alleles for a given marker/locus in a population, which is counted with a non-zero frequency. A locus can have many different alleles.

**Allele Size Estimation:**

Size of each allele was determined by measuring the distance traveled by the allele in the gel as compared to standard marker Puc19/MsP1 digester. Data were analyzed using online Gene Tool software for size estimation.

**Allele Frequency Estimation:**

The frequency of an allele 'P' is the number of 'A' alleles in the population divided by the total number of alleles/genes (Smith, 1998). It gives an indication of the most or least prevalent alleles in the population. The allele frequency is affected over time by forces such as genetic drift. mutation and migration etc.

Further, the following Predication equation was used for estimation of serum IgG and Lysozyme.

$$Y = a + bx$$

The values of constant $a = y - bx$

$$Y' = \overline{Y} + byx\,(X - \overline{X})$$

Where Y is to be predicated and the value of x is given

$$\overline{Y} = \sum y / n$$

$$\overline{X} = \sum x / n$$

$$byx = \frac{\sum xy - \frac{(\sum x)(\sum y)}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}}$$

n = paired number of observation

# RESULTS AND DISCUSSION

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**Purity and Concentration of genomic DNA:**

Isolated genomic DNA samples were subjected to 1% Agarose gel electrophorosis in order to assess the quality. After electrophoresis, the gel was observed under UV transilluminator for the presence of DNA band. Freshly extracted genomic DNA showed intense orange colour due to intercalation of Ethidium Bromide with genomic DNA (Fig-1). The quality of extracted DNA of all the samples was found to be excellent (Fig-1).

**PCR products of TLR-4 gene with Puc19/Msp1 digest DNA marker:**

After obtaining excellent quality DNA, the DNA samples were amplified through PCR machine. Amplified PCR products along with Puc19/Mspl DNA marker were subjected to horizontal 2% Agarose gel electrophoresis and the gel was observed under UV trans -illuminator for the presence of DNA band pattern. The amplified genomic DNA fragment was found to be of 494 bp (Fig-2).

PCR-primers= fwd-name: GTATTCAAGGTCTGGCTGGTT

Rev-name :ATCATTGAAGCTCAGATCTAA

BASE COUNT ORIGIN 147 a 90 c 87 g 148 t

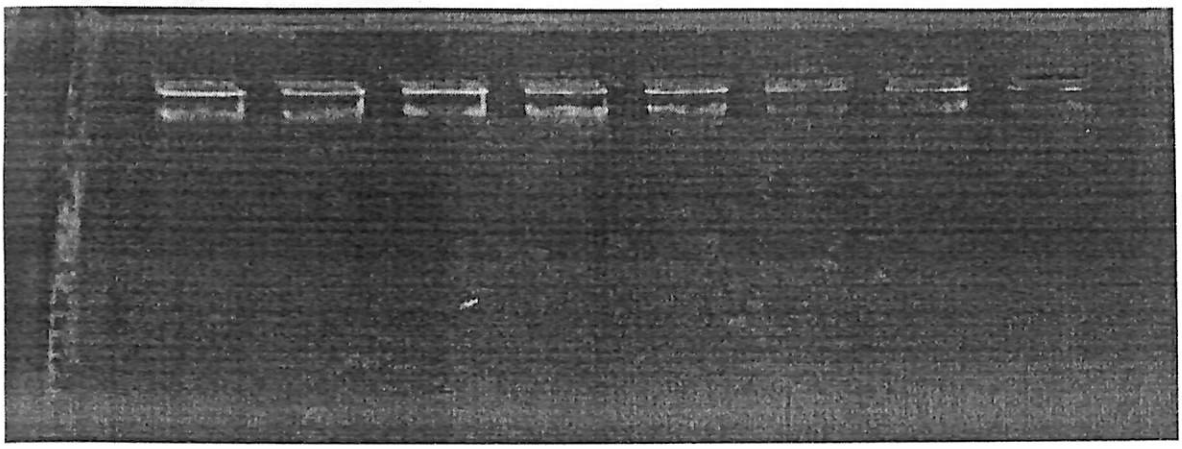

**Fig -1: Showing purity and concentration of genomic DNA**

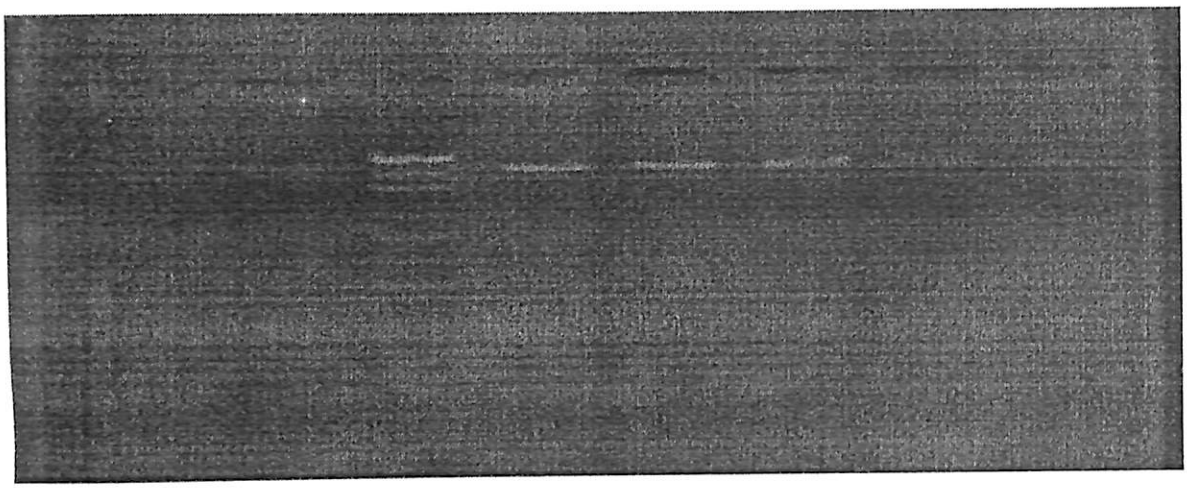

**Fig -2: Showing PCR of TLR-4 gene with Puc19/Msp1 digest DNA marker**

1 GTATTCAAGGTCTGGCTGGTTGATATGAGTTTTAAAAATGAAAGAG
AAGTTGCAAAAATT

61 TGACAGCACTCTTGCCTGGAGGGACTGTGCAACCTGACCATTGAGCA
ATT CCGGATAGCG

121 TACTTGAACAAATTCTCACGGAACGATACAGACTTATTTAATTGTTTG
GC AAATGTTTCT

181 ATGATTTCTCTGTTGAGTATACCTTTAGGAAGTCTACAAGCCCTTCTTA
AAGATTTTAGA

241 TGGCAACACTTAGAAATGATTAACTGTGACTTTGATAAGTTTCCTGCA
CT GGAGCTCCGT

301 TCTCTCAAAAAGTTTGTTTTCACAGACAACAAAGATGTAAGCAGTTTT
AC TAAACTGACT

361 ACCAACCTTCATATCTAATCTCAAAAAAAATCACTTGAGTTTCAAGAC
CT GCTGTTCTCA

421 CACTGATTTTGGGACAACCAACCTGAAGCATTTAGATCTGA
GCTTCAATG AT

Nucleotide BLAST of this sequences showed 99% identity with the sequences of accession number EF409988.1. In general, the toll-like receptor 4 (TLR4) of *Capra hircus* was reported to have TLR4*04 allele, exon 3 and complementary DNA strand length.

Score = 791 bits (428), Expect = 0.0 Identities = 438/442 (99%), Gaps =4/442 (0%) Strand=Plus/Plus

GenBank accession numbers for nucleotide sequences:

GQ150396-150398

http://www.ncbi.nlm.nih.gov

**PCR-RFLP of TLR-4 GENE**: Amplified DNA products digested over-night with AluI restriction endonuclease were run on 2% Agarose gel electrophoresis and compared with Puc19/MspI DNA molecular marker.

The types of bands observed on UV transilluminator have been depicted in fig: 3, 4 and 5. Two types of band pattern i.e. AA and AB were observed in case of Beetal breed of goats whereas, only AA genotype was observed in Jamunapari and Black Bengal goats. The allelic frequencies of A and B alleles in Beetal were reckoned to be 0.67 and 0.33 respectively while the frequency of A allele in Jamunapari and Black Bengal was 1.0. Hence, Beetal is found to be polymorphic. Among the nine animals from Beetal, six were found to have AA genotype while three showed BB genotype pattern (Table-4). The frequency of A allele was reckoned to be 0.67 while that of B was 0.33 (Table-5). In Jamunapari and Black Bengal goats all the nine goats of each breed, included in the present investigations, showed AA genotype pattern (Fig.-4), the frequency of A allele being unity.No specific reference could be found pertaining to Caprine TLR4 gene.However, Zhou H,*et al.* (2007) observed variation in the nucleotide sequence of the ovine TLR4 gene in an amplified DNA fragment containing a putative ligand-binding region using PCR followed by Single-Strand Conformational Polymorphism (PCR-SSCP) analysis and DNA sequencing. Four novel SSCP patterns,representing four different sequences were identified. Either one or two different sequences were detected in individual sheep and all the sequences identified shared high homology to the TLR4 sequences from a variety of species, suggesting that these sequences represent allelic variants of the ovine TLR4 gene. Fourteen Single Nucleotide Polymorphisms (SNPs) were detected, and 79% (11 of 14) of SNPs were non-synonymous substitutions resulting in amino acid changes. Variation detected here might have an impact on pattern recognition and hence affect the immune response to pathogen. In the present investigation also the variation in nucleotide sequence of caprine TLR4 gene was investigated by amplification of a fragment of DNA containing a putative ligand-binding region using PCR followed by (PCR-RFLP ) analysis and DNA sequencing. Two novel RFLP

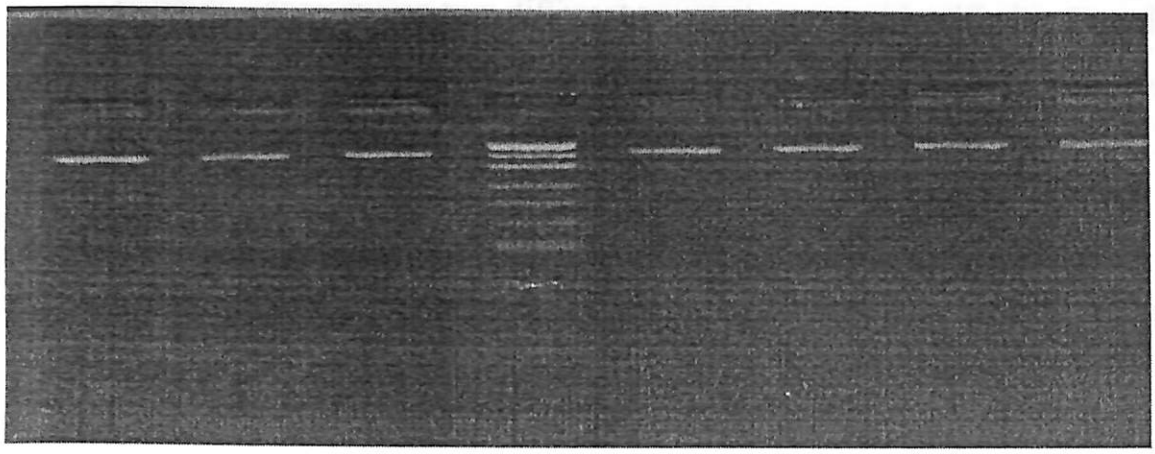

Fig -3: Showing resolution of PCR-RFLP of TLR-4 GENE in Beetal goat breeds at 2% of agarose gel electrophoresis

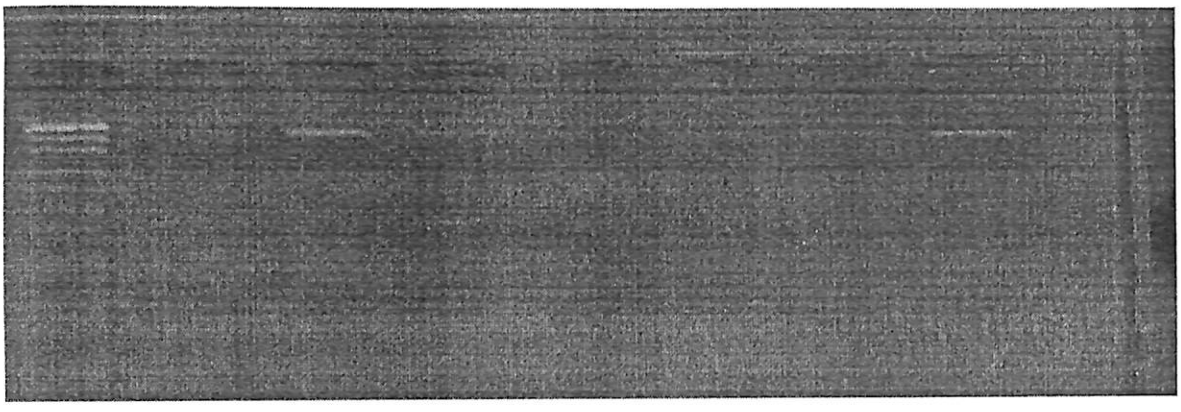

Fig -4: Showing resolution of PCR-RFLP of TLR-4 GENE in Jamunapari goat breeds at 2% of agarose gel electrophoresis

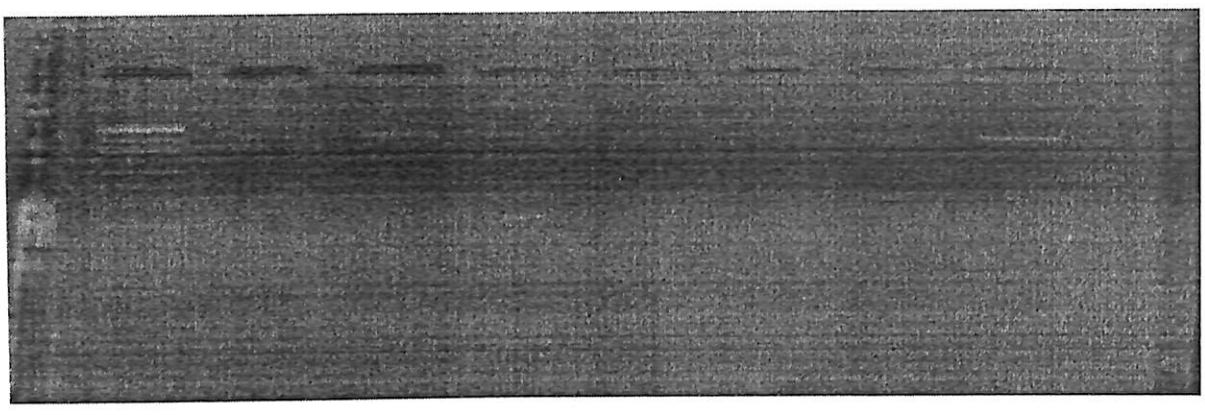

Fig -5: Showing resolution of PCR-RFLP of TLR-4 GENE in Black Bengal goat breeds at 2% of agarose gel electrophoresis

patterns, representing two different sequences, were identified in Beetal goat while in Jamunapari and Black Bengal goats only one pattern representing one sequence was identified.

## Table No-4:

**Genotypic frequencies of Beetal,Jamunapari and Black Bengal goat**

| Genotype | AA | BB |
|---|---|---|
| Beetal | 0.67 | 0.33 |
| Jamunapari | 1.0 | - |
| Black Bengal | 1.0 | - |

## Table–5:

Allelics frequencies of Beetal, Jamunapari and Black Bengal goat

| Alleles | A | B |
|---|---|---|
| Beetal | 0.67 | 0.33 |
| Jamunapari | 1.0 | - |
| Black Bengal | 1.0 | - |

**Serum IgG :**

Mean ±SE and CV% of serum IgG of different breeds of goats have been presented in table-6. Serum concentration of IgG was found to be ranging from 22.36 ±1.27 (mg/ml) in Jamunapari to 27.88±0.95 in Beetal (Table-6).The analysis of variance revealed significant ($P<0.01$) effect of breeds on serum concentration of IgG (Table-7). Serum concentration of IgG in Beetal was significantly ($P<0.01$) higher by 5.52 mg/ml and 4.22

mg/ml than the Jamunapari and Black Bengal goats respectively. This might be reflection of the fact that Beetal goat may have better general immune response than the Jamunapari and Black Bengal goats. However, the difference between Jamunapari and Black Bengal was non-significant.

**Serum Lysozyme:**

Mean±SE and CV% of serum Lysozyme have been presented in table-6 and analysis of variance for the effect of breeds on serum lysozyme is presented in table-7. The average estimates of serum lysozyme were found to be ranging from 61.69 ± 4.55 µg/ml in Jamunapari to 68.27 ± 4.71 µg/ml in Beetal. Although Beetal had higher concentration of serum lysozyme than the Janmunapari and Back Bengal but did not differ significantly among each other. Thus, effect of breed might have no significant influence on immune system in goat, if taken in terms of serum lysozyme level.

**Table - 6:**

Mean±SE and CV% of serum IgG and Lysozyme in different breeds of goats.

| IgG (mg/ml) | | | Serum Lysozyme (µg/ml) | |
|---|---|---|---|---|
| **Breeds** | **Mean ± SE** | **CV%** | **Mean ± SE** | **CV%** |
| Jamunapari | $22.36^{b} \pm 1.27$ | 16.99 | 61.69 ± 4.55 | 22.13 |
| Beetal | $27.88^{a} \pm 0.95$ | 10.22 | 68.27 ± 4.71 | 20.69 |
| Black Bengal | $23.66^{b} \pm 1.27$ | 16.05 | 64.76 ± 3.94 | 18.27 |

Values with same superscript did not differ significantly

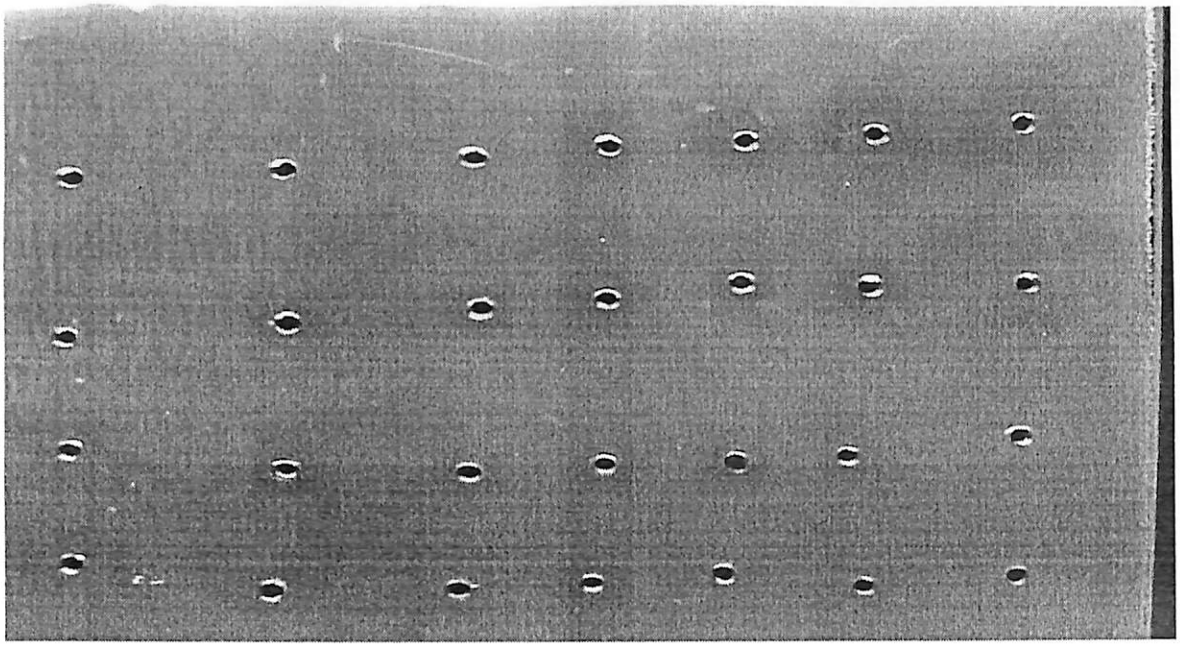

Fig -6: Showing serum IgG estimation using Single Radial Immunodiffusion assay in Beetal, Jamunapari and Black Bengal goat breed .

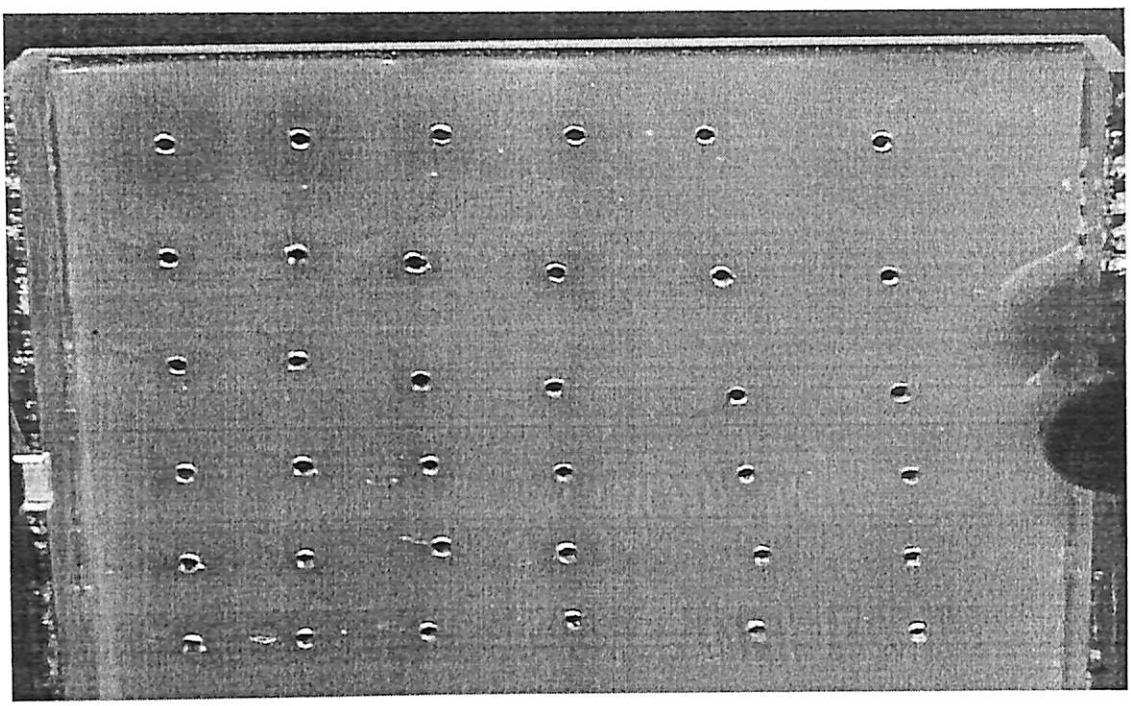

Fig -7: Showing serum Lysozyme estimation by Lysoplate method in Beetal, Jamunapari and Black Bengal goat breeds

## Table -7:

Analysis of variance for the effect of breeds on concentration of serum IgG and serum Lysozyme.

| Source of variation | Df | MS | |
|---|---|---|---|
| | | IgG | Serum Lysozyme |
| Between breeds | 2 | 72.7** | $97.43^{NS}$ |
| Error | 24 | 12.33 | 175.33 |

** = Significant at P<0.01

## Table-8:

Showing Serum IgG and Serum Lysozyme estimation of Beetal Goat

| Serum IgG | | | | Serum Lysozyme | | | |
|---|---|---|---|---|---|---|---|
| Known Concentr -ation (mg/ml) | Known Diameter (cm) | Unknown Diameter (cm) | Unknown Concentration (mg/ml) | Known Concentration (µg/ml) | Known Diameter (cm) | Unknown Diamet -er (cm) | Unknown Concentr -ation (µg/ml) |
| 40 | 1.475 | 1.35 | 28.44 | 100 | 1.80 | 1.75 | 80.53 |
| 20 | 1.275 | 1.30 | 25.68 | 50 | 1.50 | 1.70 | 76.59 |
| 10 | 1.075 | 1.40 | 31.19 | 25 | 1.25 | 1.75 | 80.53 |
| 5 | 0.95 | 1.40 | 31.19 | 12.5 | 0.85 | 1.60 | 68.70 |
| 2.5 | 0.8 | 1.28 | 24.58 | 6.25 | 0.70 | 1.30 | 45.06 |
| 40 | 1.475 | 1.35 | 28.44 | 100 | 1.80 | 1.75 | 80.53 |
| 40 | 1.475 | 1.40 | 31.19 | 50 | 1.50 | 1.60 | 68.70 |
| 20 | 1.275 | 1.30 | 25.68 | 25 | 1.25 | 1.30 | 45.06 |
| 10 | 1.075 | 1.28 | 24.58 | 12.5 | 0.85 | 1.60 | 68.70 |
| **Total** | | | **250.97** | | | | **614.4** |
| **Average** | | | **27.88** | | | | **68.27** |

## Table-9:

Showing Serum IgG and Lysozyme estimation of Jamunapari goat

| | Serum IgG | | | | Serum Lysozyme | | | |
|---|---|---|---|---|---|---|---|---|
| Animal No | Known concentrat-ion (mg/ml) | Known diameter (cm) | Unknown diameter (cm) | Unknown concentration (mg/ml) | Known concentrati-on (µg/ml) | Known diameter (cm) | Unknown diameter (cm) | Unknown concentration (µg/ml) |
| 1 | 5 | 0.95 | 1.19 | 19.63 | 6.25 | 0.7 | 1.55 | 64.76 |
| 2 | 2.5 | 0.8 | 1.19 | 19.63 | 100 | 1.8 | 1.7 | 76.59 |
| 3 | 20 | 1.275 | 1.15 | 17.43 | 50 | 1.5 | 1.55 | 64.76 |
| 4 | 40 | 1.475 | 1.30 | 26.68 | 25 | 1.25 | 1.3 | 45.06 |
| 5 | 20 | 1.275 | 1.28 | 24.58 | 12.5 | 0.85 | 1.75 | 80.53 |
| 6 | 10 | 1.075 | 1.15 | 17.43 | 6.25 | 0.7 | 1.3 | 45.06 |
| 7 | 5 | 0.95 | 1.28 | 24.58 | 100 | 1.8 | 1.6 | 68.70 |
| 8 | 2.5 | 0.8 | 1.28 | 24.58 | 50 | 1.5 | 1.55 | 64.76 |
| 9 | 10 | 1.075 | 1.30 | 26.68 | 25 | 1.25 | 1.3 | 45.06 |
| **Total** | | | | **201.22** | | | | **555.22** |
| **Average** | | | | **22.36** | | | | **61.69** |

## Table – 10:

Serum IgG and Lysozyme estimation of Black Bengal

| al | Known concentrat-ion (mg/ml) | Known diameter (cm) | Unknown diameter (cm) | Unknown concentrat-ion (mg/ml) | Known concentrat-ion (µg/ml) | Known diameter (cm) | Unknown diameter (cm) | Unknown concentration (µg/ml) |
|---|---|---|---|---|---|---|---|---|
| | 40 | 1.475 | 1.19 | 19.63 | 12.5 | 0.85 | 1.7 | 76.59 |
| | 20 | 1.275 | 1.30 | 25.68 | 6.25 | 0.7 | 1.3 | 45.06 |
| | 10 | 1.075 | 1.28 | 24.58 | 100 | 1.8 | 1.7 | 76.59 |
| | 5 | 0.95 | 1.30 | 25.68 | 50 | 1.5 | 1.6 | 68.70 |
| | 2.5 | 0.8 | 1.28 | 24.58 | 25 | 1.25 | 1.6 | 68.70 |
| | 5 | 0.95 | 1.19 | 19.63 | 12.5 | 0.85 | 1.6 | 68.70 |
| | 40 | 1.475 | 1.35 | 28.44 | 6.25 | 0.7 | 1.55 | 64.76 |
| | 20 | 1.275 | 1.30 | 25.68 | 100 | 1.8 | 1.6 | 68.70 |
| | 10 | 1.075 | 1.19 | 19.63 | 50 | 1.5 | 1.3 | 45.06 |
| **Total** | | | | **212.9** | | | | **582.85** |
| **Average** | | | | **23.66** | | | | **64.76** |

# SUMMARY CONCLUSION AND RECOMMENDATIONS

# SUMMARY, CONCLUSION AND RECOMMENDATIONS

India is a repository of livestock diversities, but faces a challenge between plenty and poverty with respect to livestock. Despite poor infrastructure, low investments and resource poor stakeholders in Animal Husbandary, livestock has provided strength and sustainability to Agricultural Production as a whole. While the annual growth rate for crop sector has been even negative for some years in the last decade, the overall average of 2.5% growth in total Agriculture is mainly because there has been a consistent annual growth of more than 4% in the livestock sector. The contribution of Agriculture to National Gross Domestic Product have substantially decreased over past decade, but the contributions of livestock remained consistent between 27 to 32% of Agriculture GDP. Animal Husbandry provides self-employment to millions of household in rural India. The export earning from livestock sector has shown an annual growth of 12%.

The 'candidate gene' approach has not been used extensively in goat, particularly in indigenous goat breeds, most likely due to the paucity of characterized genes and immunological pathways for the majority of diseases. Candidate gene analysis is a powerful approach to detect genes controlling traits of economic importance such as growth, production and immune response in farm animals, (Rothschild and Soller, 1997). Although over the past few years' considerable progress has been made in knowing the role of TLRs in disease resistance and susceptibility in different species of livestock, but relatively little is known about contribution of TLRs towards successful host defence in goats. There is growing evidences from species other than goat, sheep and cattle that the ability of individuals to respond properly to TLR ligands may be affected by SNPs within the TLR

genes, consequently leading to an altered susceptibility to, or course of infectious or inflammatory diseases. TLR4 recognizes LPS of Gram-negative bacteria and is also involved in the recognition of other bacterial and viral PAMPs. A mutation in or a lack of TLR4 can result in altered immune responses to pathogens that produce these PAMPs. Accumulating evidences support association between TLR4 polymorphism and disease resistance.

Indian breeds of goat being reared in different agro-climatic conditions are efficient converter of scrub material into milk, meat, wool and fur. Beetal and Jamunapari are well known as efficient milk producer. Black Bengal breed is favoured for their lean and juicy meat,proliferacy and skin. The glacid kid leather of Black Bengal goat is quite famous in foreign market. The present study was undertaken to investigate the role of TLR4 in disease resistance or immune responses in Beetal, Jamunapari and Black Bengal breeds of Indian goats with an objective to accertain polymorphism in caprine TLR4 gene using gene sequencing and PCR-RFLP analysis.

1. To identify the caprine TLR4 gene in Beetal, Jamunapari and Black Bengal breeds.
2. To characterize any variation found in the gene within or between breeds.
3. To compare serum Lysozyme or IgG breeds.

Blood samples from altogather 27 healthy animals, 9 each from Beetal, Jamunapari and Black Bengal breeds maintained at Composite Livestock Instructional Farm of Bihar Veterinary College, Patna,were collected from jugular vein of each individual in heparinized syringe for genomic DNA extraction and DNA analysis. Genomic DNA was isolated by

modified Salting Out method by Miller *et.al.* (1988). The concentration and purity of isolated DNA was determined using UV spectrophotometer absorbance at 260 and 280 nm. The samples having A260/A280 ratio of >1.8 were used for further studies. The integrity of DNA was assessed by UV visualization of intact DNA bands on 1% Agarose gel.

The primers were designed for exon 3 region of *Capra hircus* TLR-4 gene from the published caprine TLR-4 sequence (GenBank accession number EF409989) using online GeneTool software. Specificity of primers were checked using NCBI Genbank database-BLAST program (http://www.ncbi.nlm.nih.gov/entrez/). Gene specific forward and reverse primers were synthesized commercially.

TLR-4 gene was amplified from genomic DNA samples by Polymerase Chain Reaction. The PCR protocol comprised of initial denaturation of DNA at 94°C for 10 min. followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55.2 °C for 60 second, extension at 72 °C for 60 second and final extension at 72 °C for 10 min. The 494 bp amplicons of the gene amplified by PCR were checked by comparing with Puc19/Msp1 digest DNA marker by running PCR product on 2% Agarose gel and observed on uv-trasluminar. Subsequently, PCR-RFLP study was carried out to identify different PCR-RFLP genotypic pattern of the goats.Two PCR-RFLP patterns i.e. AA and BB were observed in samples from Beetal goat and only AA pattern was found in Jamunapari and Black Bengal goats. Patterns AA and BB recorded in the samples of Beetal breed had frequencies of 0.67 and 0.33 respectively. Whereas, pattern AA observed in those of Jamunapari and Black Bengal goats had frequency of 1.0.

Blood samples were also collected without any anti-coagulant to isolate serum for the estimation of serum Lysozyme and total IgG. Serum IgG level was assayed by Single Radial Immunodiffusion (SRID) test (Manicini *et.al.* 1965) as modified by Fahey and Mckelevey (1965). Serum concentration of IgG was found to be ranging from 22.36 ±1.27 (mg/ml) in Jamunapari to 27.88±0.95 in Beetal. The analysis of variance revealed significant (P<0.01) effect of breeds on serum concentration of IgG. Serum concentration of IgG in Beetal was significantly (P<0.01) higher by 5.52 mg/ml and 4.22 mg/ml than the Jamunapari and Black Bengal goats respectively.The serum Lysozyme was estimated by Lysoplate method as described by Lie *et al.* (1986). The average estimates of serum lysozyme were found to be ranging from 61.69 ±4.55 µg/ml in Jamunapari to 68.27 ±4.71 µg/ml in Beetal. Although Beetal had higher concentration of serum lysozyme than the Janmunapari and Back Bengal but did not differ significantly among each other.Thus, effect of breed might have no significant influence on immune system in goat, if taken in terms of serum lysozyme level.

## CONCLUSION

1. TLR-4 gene was present in all the three indigenous breeds of goats viz Beetal, Jamunapari and Black Bengal.
2. There was no restriction site for Alu1 in Jamunapari and Black Bengal while it was present in Beetal.
3. Polymorphism was found only in Beetal breed of goat.
4. The average value of serum IgG and Lysozyme in case of Beetal was higher as compare to Jamunapari and Black Bengal due to polymorphism.

5. The goat breeds having high serum IgG and Lysozyme level may be selected for relatively better disease resistance against lipopolysaccharide (Gram negative) bacteria in particular as well as Gram positive bacteria and virus in general.

On the basis of the finding of this study it could be concluded that in the tropical and sub tropical agro-climatic conditions the Beetal goats were the animal of choice followed by Black Bengal and Jamunapari for their higher milk yield, meat and prolificacy, if interference of disease conferred by TLR4 gene is taken into account.

## RECOMMENDATIONS

The indigenous goat breeds which are about 20 in number and possess excellence in one or more economic traits should be saved from extinction by undertaking systematic studies on their phenotypic and molecular characterization including genomic mapping and identification of functional genomic association with expression level of their trait of production excellence. Characterization of candidate gene having information on their adaptability in tropical environmental stress and diseases, measured in terms of immune response in goats should also be the subject of investigation. Besides that, extensive programmes are required to be formulated and implemented for sustainable propagation, maintenance and improvement of Indian goats in their native tracts.

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# APPENDIX

# APPENDIX -I

| Sl.No. | Name of Chemicals/Reagents | Name of company |
|---|---|---|
| 1. | Tris | SIGMA |
| 2. | Boric acid | SRL |
| 3. | EDTA (pH 8.0) | SRL |
| 4. | Ethidium Bromide | LOBA CheNIE |
| 5. | Distilled water | GeNeiTM |
| 6. | NaCl | LOBA CHEMIE |
| 7. | $KH_2PO_4$ | LOBA CHEMIE |
| 8. | $NaH_2PO_4$ | LOBA CHEMIE |
| 9. | $Na_2HPO_4$ | LOBA CHEMIE |
| 10. | KCl | LOBA CHEMIE |
| 11. | $NH_4Cl$ | LOBA CHEMIE |
| 12. | $KHCO_3$ | LOBA CHEMIE |
| 13. | Tris HCL | SIGMA |
| 14. | NaOH | LOBA CHEMIE |
| 15. | S.D.S | LOBA CHEMIE |
| 16. | Proteinase K | GeNei™ |
| 17. | Chloroform | S.d.Fine_CHEM LIMITED |
| 18. | Isoamyl alcohol | S.d.Fine_CHEM LIMITED |
| 19. | Phenol saturated | SRL |
| 20. | Sodium acetate | LOBA CHEMIE |
| 21. | Agarose | GeNei™ |
| 22. | Gel loading buffer | GeNei™ |
| 23. | Isopropyl alcohol | S.d.Fine_CHEM LIMITED |
| 24. | 10 X Assay Buffer | GeNei™ |
| 25. | Primer (BVC 1 and BVC 2) | GeNei™ |
| 26. | $MgCl_2$ | GeNei™ |
| 27. | Taq DNA polymerase | GeNei™ |
| 28. | dNTPs mix (10 mM) | GeNei™ |
| 29. | Alu1 Enzyme | GeNei™ |
| 30. | Puc19 DNA/Msp1 Digest | GeNei™ |
| 31. | Standard Lysozyme | SIGMA |
| 32. | Micrococcus Lysodecticus bacteria | SIGMA |
| 33. | Heparin | Biological E.Limited |
| 34. | Absolute Ethanol | S.d. Fine ChEM Limited |
| 35. | Glacial Acetic acid | Mercle |
| 36. | Nuclease Free water | GeNei™ |
| 37. | Water | GeNei™ |
| 38. | Sodium carbonate anhydrous | LOBA CHEMIE |

# APPENDIX – II

## Constituents of different buffers and other solutions.

1 5XTBE (1000 ML)

| | |
|---|---|
| Tris | 54 gm |
| Boric acid | 27.5 gm |
| 0.5 M EDTA (pH 8.0) | 200 ml |
| Autoclaved distilled water upto | 1000 ml |

Sterilize by autoclaving at $120^0$C, 15 lbs pressure for 30 minutes

2. Ethidium bromide (10 mg/ml)

| | |
|---|---|
| Ethidium bromide | 20 mg |
| Distilled water | 1 ml |

3. 0.5 M EDTA (pH 8.0)

| | |
|---|---|
| Disodium Ethylene Diamine Tetra Acetate | 181.1 gm |
| Distilled water upto | 1000 ml |

Dissolved EDTA in about 800 ml of autoclaved distilled water by keeping it on magnetic stirrer for one hour. Adjusted the pH by NaOH pellets before making to volume 1000 ml. Autoclaved and stored at room temperature.

4. Normal saline solution (N.S.S)

| | |
|---|---|
| NaCl | 8.5 gm |
| Distilled water | 1000 ml |

5. Phosphate Buffer Saline (PBS pH 7.4)

| | |
|---|---|
| NaCl | 8.0 gm |
| $KH_2P0_4$ | 0.20 gm |
| $Na_2HPO_42H_20$ | 1.16 gm |
| KCl | 0.20 gm |
| Distilled water | 1000 ml |

6. RBC lysis buffer (1000 ml)

| | |
|---|---|
| Double distilled water | 1000 ml |
| Ammonium chloride | 8.26 gm |
| EDTA | 0.037 gm |
| $KHCO_3$ | 1.0 gm |

7. 1 M Tris (pH 8.0)

| | |
|---|---|
| Tris HCl | 151.60 gm |
| Distilled water | 1000 ml |

Adjusted pH by adding NaOH to pH 8.0

8. 5 M NaCl (100 ml)

| | |
|---|---|
| NaCl | 29.22 gm |
| Distilled water | 100 ml |

9. DNA extraction buffer (500 ml)

| | |
|---|---|
| 1 M Tris (pH 8.0) | 5 ml |
| 5 M NaCl | 40 ml |
| 0.5 M EDTA | 2 ml |
| Distilled water upto | 500 ml |

10. 10% Sodium Dodecyl Sulphate (SDS) (1000 ml)

| | |
|---|---|
| SDS | 100 gm |
| Distilled water | 1000 ml |

Adjusted pH 7.2 using cone HCl

Heated in water bath at $60^0$C for 30 minutes.

11. 0.1 M Tris HCl buffer (pH 7.4) (100 ml)

| | |
|---|---|
| Tris buffer | 1.2114 g |
| DW | 100 ml |
| HCl | 0.7 ml |

First taken 80 ml distilled water in which added 1.2114 g Tris buffer, thereafter adjusted pH to 7.4 by adding 0.7 ml HCl. Volume is adjusted to 100 by adding distilled water.

# APPENDIX -III

| Sl.No. | Name of the Equipments | Company Name |
|---|---|---|
| 1. | Centrifuge Machine | REMI |
| 2. | Micropipette | UNIQUE |
| 3. | Vortex Machine | SPINIX |
| 4. | Weighing Balance | OWAUS |
| 5. | UV-transilluminator | UVtec |
| 6. | PCR Machine | TECHNE |
| 7. | Gel casting glass plate | GENEI |
| 8. | Water bath incubator shaker | Labotech |
| 9. | Electrophoresis Machine | GENEI |
| 10. | Deep Frezeer | Blue star |
| 11. | Refrigerator | WHIRLPOOL |
| 12. | Water purification System | Milipore |
| 13. | Magnetic Stirrer | Labotech |
| 14. | Microfuge | Spinwin |