MOLECULAR CHARACTERIZATION OF TOLL-LIKE RECEPTOR 9 OF RED KANDHARI CATTLE

T H E S I S

Submitted

In partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE

IN

ANIMAL BIOTECHNOLOGY

BY

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Enrolment No. V/14/053

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(INDIA)

2022

DECLARATION OF STUDENT

I hereby declare that the experimental Research work and interpretation of the thesis entitled **MOLECULAR CHARACTERIZATION OF TOLL-LIKE RECEPTOR 9 OF RED KANDHARI CATTLE** or part thereof has not been submitted for any other degree or diploma of any University, not the data have been derived from any thesis/publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

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We also certify that the thesis or part thereof has not been previously submitted by him for a degree of any other University.

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CERTIFICATE

This is to certify that the thesis entitled **"MOLECULAR CHARACTERIZATION OF TOLL-LIKE RECEPTOR 9 OF RED KANDHARI CATTLE"** submitted by Shri. **DEVKATTE VYANKATRAO SUBHANRAO** to the Maharashtra Animal and Fishery Sciences University in partial fulfillment of the requirement for the degree of **MASTER OF VETERINARY SCIENCE** in the subject of **ANIMAL BIOTECHNOLOGY** has been approved by the Student's Advisory Committee after oral examination in collaboration with the External Examiner.

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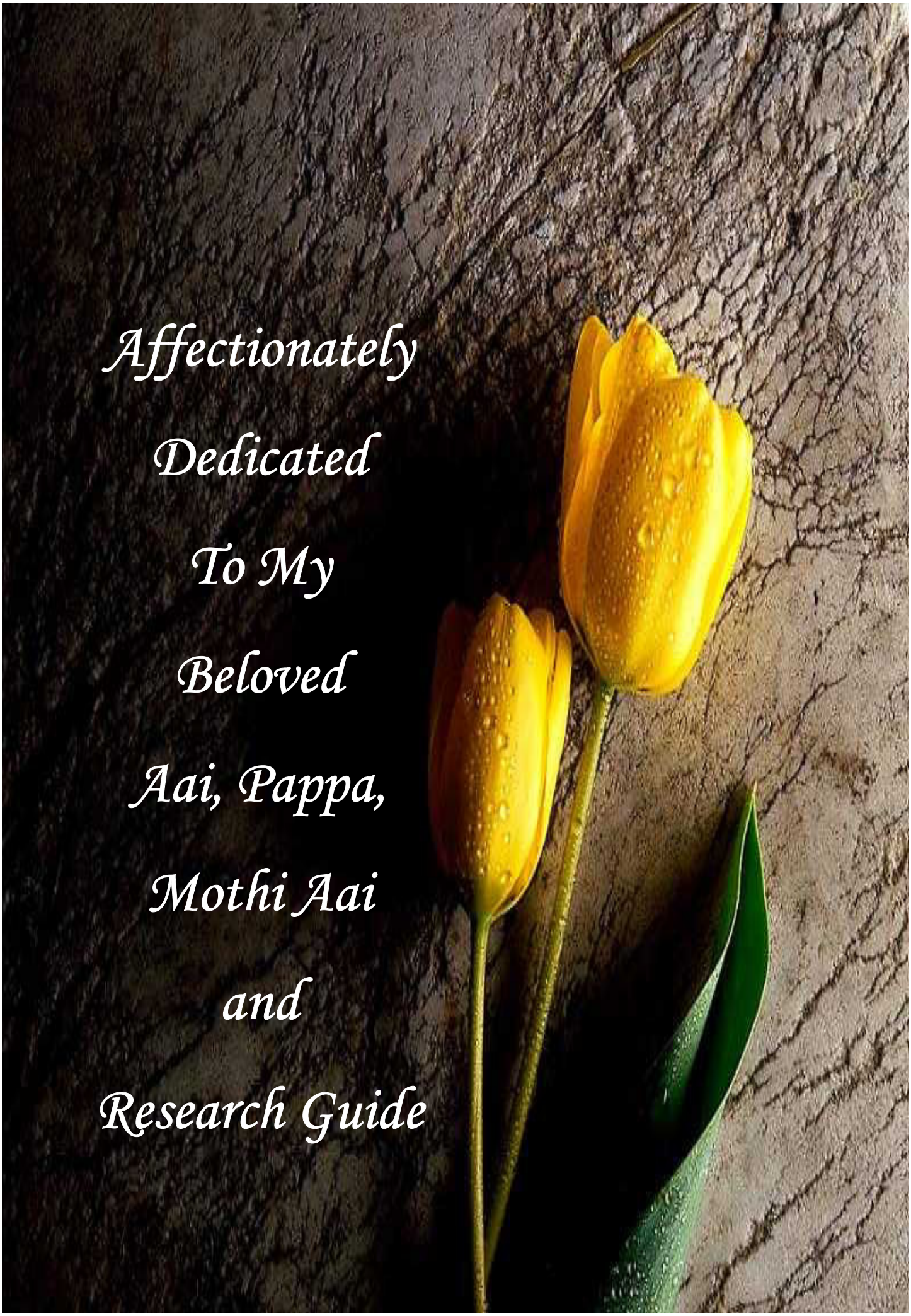
Affectionately
Dedicated
To My
Beloved
Aai, Pappa,
Mothi Aai
and
Research Guide

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Place: Parbhani

Date: / /2022 (Devkatte Vyankatrao Subhanrao)

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List of Abbreviations/ symbols

et al.	and others
i.e.,	That is
@	at the rate of
min.	Minute
e.g.,	For example, / example gratia (Latin)
mL	Millilitre
PCR	Polymerase Chain Reaction
pmol.	Pico Mol
rpm	Revolution Per Minute
EDTA	Ethylenediaminetetraacetic Acid
TAE	Tris Acetate EDTA
TE	Tris EDTA
SDS	Sodium Dodecyl Sulphate
µl	Microliter
bp	Base Pairs
TLR	Toll Like Receptor
LRR	Leucine Rich Repeats
PAMP	Pathogen Associated Molecular Patterns
TIR	Toll Interleukin-1 Receptor
&	And
SSCP	Single Stranded Confirmation Polymorphism
IFN	Interferon
DC	Dendritic Cells
APC	Antigen Presenting Cell
ODN	Oligodeoxynucleotides
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PBMC	Peripheral Blood Mononuclear Cell
SNP	Single Nucleotide Polymorphism
EAE	Experimental Allergic Encephalomyelitis
IDO	Indoleamine 2, 3 deoxygenase

IFNA	Interferon Alpha
LPS	Lipophosphoglycan
CpG	Cytosine Phosphate Guanine
TRIF	TIR-domain-containing adaptor-inducing interferon
TIRAP	TIR Domain Containing Adaptor Protein
THRA	Thyroid Hormone Receptor Activator

Introduction

CHAPTER - I

INTRODUCTION

India has a large livestock population i.e., 535.78 million, with 192.49 million cattle (20th Livestock census 2019). India produces and consumes the most milk of any nation on this planet. In India, cattle are raised for milk and draught purposes. In comparison to exotic animals, indigenous breeds are more disease-resistant to many infections (Savalia *et al.,* 2019).

An organism is in a constant struggle for existence in its environment and one of the challenges it faces is a myriad of pathogens with whom it shares the environment. The line of defense against pathogens begins with immunity. The immune system is a complicated biological system that recognizes and tolerates what belongs to oneself while rejecting what does not. Innate and adaptive immunity are two types of the immune system. The adaptive system is made up of evolved lymphatic cells that are designed to recognise non-self-substances in the presence of self. Out of both, innate immune defences are nonspecific. When pathogens successfully invade an organism, they come into contact with the innate immune system's cells and mechanisms. The pattern recognition receptors recognise components that are conserved across wide microbes, or when damaged, injured, or stressed cells send out alarm signals, many of which are recognised by the same receptors that recognise pathogens, the innate response is triggered (Matzinger, 2002).

This research aims to understand the molecular structure of Toll-like Receptor 9 in Red Kandhari cattle. There are very few molecular characterization reports available for Red Kandhari and indigenous cattle, which could provide vital information for future studies and comprehension of the animals' immunity. Microorganisms that infect a vertebrate host are first identified by the innate immune system, which uses pattern-recognition receptors (PRRs). Toll-like and cytoplasmic receptors are two types of PRRs that directly identify different microbial components and activate immune cells. Toll-like receptors (TLRs) are PRRs that are primarily expressed in antigen-presenting cells (APCs) in the host

and play a critical role in determining the outcome of a pathogenic infection (Akira *et al.,* 2006).

Toll-like receptors and cytoplasmic receptors are two types of PRR that identify different microbial components and activate immune cells directly. TLRs (Toll-like Receptors) are a type of proteins that are important in the innate immune system. They are single-pass membrane-spanning receptors mostly found on dendritic cells (Hemmi *et al.,* 2000).

TLR9 recognises a wide range of pathogen-associated molecular patterns (PAMPs) from bacteria, viruses, and fungi, as well as some host molecules, and activates intracellular signalling cascades that result in the release of pro-inflammatory cytokines, chemokines, and antiviral molecules. TLR9 is a member of the TLR family that plays an important role in both innate and adaptive immunity through ligand detection and signal transduction. TLR9 is a significant sensor for innate defence against bacterial and viral infection. TLRs are important for innate immunity, and knowing the genetic basis of different TLRs receptor expression and function is crucial for the multiple biological endpoints that rely on TLR signalling (Kumagai *et al.*, 2008).

TLR9 was first identified as a receptor for unmethylated cytosine phosphate guanine (CpG) dinucleotides prevalent in bacterial DNA (Hemmi *et al.,* 2000). In man and mice, significant progress has been achieved in representing the TLRs relationship with disease resistance and susceptibility. Full-length sequence data has been reported for 10 TLR genes in human, mouse, pig, cattle, sheep, and chicken, according to sequence information in the public databases. TLR9 is thought to be implicated in the aetiology of autoimmune diseases, in addition to recognising bacterial and viral CpG DNA. The B cell receptor and TLR9 bind the IgG2a–chromatin complex in a sequential manner, resulting in efficient rheumatoid factor generation by auto-reactive B cells (Takeda and Akira, 2005).

Toll-like receptor 9 (TLR9) recognises unmethylated DNA containing CpG-motifs and triggers pleiotropic immune responses. In response to B/K-type

CpG-DNA, macrophages and conventional dendritic cells (cDCs) release proinflammatory cytokines, whereas plasmacytoid DCs create type I interferons in response to A/D-type CpG-DNA and DNA viruses. TLR is made up of leucine-rich repeats (LRRs), a transmembrane domain, and a Toll/interleukin-1 receptor homology (TIR) domain in the cytoplasm. TLR9 is found in the endoplasmic reticulum (ER), endosomes, and lysosomes, an intracellular membrane compartments. TLR9's LRR is on the cytosolic side of the membrane compartment, whereas the TIR domain is on the inside. TLR9 ligand stimulation activates several transcription factors, including nuclear factor B (NF-kB) and activator protein-1 (AP-1) (Kumagai *et al.,* 2008).

The cattle breed Red Kandhari, from the Marathwada region, is predominantly a draught purpose breed with a long history. It is a muscular and robust medium-sized animal with a squarely constructed physique. The body colour is consistently dark red, however there are slight variances ranging from dull red to almost dark brown. This breed is mostly found in Nanded, Latur, Beed, and Parbhani districts in Marathwada region of Maharashtra state. Despite being modest milk producers with lactation milk yields of only 400-600 kg, Red Kandhari cows have excellent breeding efficiency. Bullocks are used for heavy work with well-known draft capacity (Sodhi *et al.,* 2005).

Scanty reports regarding the genetic characterization of Red Kandhari cattle are available in literature databases. These reports characterize different genes like haemoglobin (Vedpathak *et al.,* 2006) and kappa casein (Kishore, *et al*., 2014). A study on genetic differentiation with microsatellite markers is also carried out (Sodhi *et al.,* 2005). The proposed research will characterize DNA encoding the TLR9 gene of Red Kandhari cattle through Sanger sequencing and study the molecular structure of TLR9 protein *in silico*. The work also includes a comparison of the TLR9 gene of Red Kandhari cattle to the available TLR9 genes in cattle and other related species in the NCBI nucleotide database.

Objectives:

1. To determine coding DNA sequence of TLR9 gene of Red Kandhari cattle through Sanger sequencing.

2. To study molecular structure of TLR9 protein *in silico*.

3. To study comparative evolution of the TLR9 gene of Red Kandhari cattle against available TLR genes in NCBI nucleotide and protein database.

Review of Literature

CHAPTER - II

REVIEW OF LITERATURE

2.1 Toll like receptors and innate immunity

Akira & Hemmi, (2003) reviewed and reported that TLRs recognize a wide variety of pathogen-associated molecular patterns (PAMPs) from bacteria, viruses, and fungi as well as some of the host molecules which in turn trigger intracellular signal transduction cascades that result in the expression of pro-inflammatory cytokines, chemokines, and antiviral molecules. Plasmacytoid dendritic cells express TLR7 and TLR9 and respond to TLR7 and TLR9 ligands by producing a large amount of interferon (IFN-α). Thus, it indicates that TLR3, TLR7, and TLR9 may play an important role in detecting and combating viral infections.

Uematsu & Akira, (2006) reviewed Toll-Like Receptors and stated that these are critical sensors of microbial attack and effectors of the TLR dependent innate defense mechanism, enabling the host to eliminate pathogens that otherwise would cause disease or mortality.

Kang *et al.,* (2009) divided TLRs into two groups based on cellular localization. The first group comprises members of the TLR family expressed on the cell surface, such as TLR2 which forms heterodimers with TLR1 or TLR6, recognizing bacterial lipopeptides.

Blasius & Beutler, (2010) said that the second group of TLRs resides in intracellular compartments, with the LRR domains facing organelle lumens and interacting with nucleic acids derived from viral or bacterial pathogens. This group includes TLR3, which recognizes double-stranded RNA (dsRNA); TLR7–8, which interacts with single-stranded RNA (ssRNA) and TLR9, which binds unmethylated CpG DNA.

Shiraki, (2010) said that TLR9 is a pattern recognition receptor that plays a key role in cell survival by recognizing various bacterial components, including

unmethylated CpG-DNA. The expression of TLR 9, in concert with 2, and 4, is the determining factor of innate defense against bacterial infection.

Yoon *et al.,* (2012) studied other members of TLR that are expressed on the cell surface. TLR4 and TLR5 interact with bacterial LPS and flagellin, respectively. TLR4 requires the adaptor molecule MD-2 to recognize its ligand. Indeed, MD-2 interacting with TLR4 molecules provides the major LPS binding site, inducing homodimerization of the TLR4–MD-2 complex. Like TLR4, upon interaction with its cognate ligand, TLR5 also forms a dimer.

Kumar *et al.,* (2012) performed *in silico* analysis of evolutionary divergence of TLR9 transcript in Indian major carp (*Catla catla).* This TLR9 partial cDNA (1155 nucleotides) was reverse transcribed from total RNA isolated from the spleen and finally sequenced. Multiple sequence analysis of the translated amino acid sequence indicated that it is conserved, with insertion of few amino acids in fish as compared to that of mammalian sequence. The estimates of the disparity index per site (DI) for each of the species with regard to the nucleotide sequence of *Catla catla* showed that none of the sequences has evolved through the same pattern of substitution. However, natural selection has played a significant role in purifying TLR9 sequences during evolution.

Vijay, (2018) in his review of Toll-like receptors in immunity and inflammatory diseases, stated that 13 different types of TLRs (TLR1-TLR13) have been discovered and described in mammals since the first discovery of TLR4 in humans in late 1997. 10 TLRs are known to occur in cattle, and the expression of TLR transcripts varies among different mammalian species.

2.2 Role of TLR9 in immunity

Letunic, (2021) has found that TLR9 belongs to a subfamily of receptors that includes TLR7 and TLR8, which are likewise expressed within the endosome and sense pathogen-derived RNA and DNA. The full open reading frame of rat, feline, canine, porcine, ovine, and bovine TLR9 was PCR amplified, cloned, and sequenced using human and mouse TLR9 gene sequences. According to the

mammalian TLR9 sequences, bovine TLR9 has 79 percent identity with human TLR9 and 73 percent homology with murine TLR9. The public profile searching strategies used by the Simple Modular Architecture Research Tool (SMART) computer programme were used to complete protein structure predictions for the 12 sequenced mammalian TLR9s. TLR9 protein structure domains were found to be conserved across all six species, with minimal differences in projected protein length and the number and location of LRR.

Akira & Hemmi, (2003) studied Toll-like receptors (TLRs) are type I transmembrane proteins involved in innate immunity by recognizing microbial conserved structures. Previous studies have shown that TLR3 recognizes dsRNA, a viral product, whereas TLR9 recognizes unmethylated CpG motifs frequently found in the genome of bacteria and viruses, but not vertebrates. TLR7 recognizes small synthetic immune modifiers including imiquimod, R-848, loxoribine, and bropirimine, all of which are already applied or promising for clinical use against viral infections and cancers. Plasmacytoid dendritic cells express TLR7 and TLR9, and respond to their ligands by producing a large amount of interferon (IFN-a). These results indicate that TLR3, TLR7, and TLR9 may play an important role in detecting and combating viral infections.

Bourke *et al.,* (2003) studied the inducible and selective expression of TLR9 and TLR10 in normal and transformed human B lymphocytes and results showed that normal and neoplastic human B lymphocytes express a distinct TLR repertoire including TLR9 and TLR10 and that expression is increased upon engagement of the antigen receptor complex or TLR9 itself. Regulated expression of selected TLRs in B cells is likely to play an important role in linking innate and adaptive immune responses in normal and pathologic conditions.

Hemmi *et al.,* (2003) classified TLR9 ligands, CpG oligodeoxynucleotides (ODN), into two different subtypes, B/K-type and A/D-type. The B/K-type CpG-ODNs are phosphorothioate-modified throughout the sequence and induce DC maturation and B cell proliferation. On the other hand, A/D-type CpG-ODNs, characterized by a phosphodiester backbone CpG motif and phosphorothioate-modified poly G stretches at the 5′ and 3′ ends, induce type I IFNs in pDCs.

Bafica *et al.,* (2005) showed that TLR9 also recognizes bacterial and viral DNA. Upon *Mycobacterium tuberculosis* infection, TLR9 cooperates with TLR2 to induce innate and adaptive immune responses against the bacterium.

Griebel *et al.,* (2005) demonstrated that the mammalian innate immune system recognises non-methylated CpG motifs in viral and bacterial DNA as one of the numerous pathogen-associated molecular patterns (PAMP). This PAMP is recognised by a unique interaction with the toll-like receptor 9 (TLR9), triggering cytokine responses. Cattle and humans have more sequence homology than cattle and mice, according to a study of the bovine TLR9 gene. Both human and bovine leukocytes were triggered optimally by CpG motifs unique from those that activated mouse leukocytes. Class A CpG ODN were more potent inducers of interferon-alpha (IFN-a) than class B CpG ODN, according to functional tests with CpG ODN stimulated bovine blood leukocytes. Furthermore, cell sorting of bovine blood leukocyte subpopulations implicated dendritic cells in regulating CpG ODN-induced IFN production, but not monocytes. The cellular pattern of CpG ODN-induced responses in cattle thus resembles that of human leukocytes in many ways. TLR9 structure and function were shown to be well conserved in human blood leukocytes, cattle, and other domestic species.

Andersen *et al.,* (2006) studied innate immunity at the mucosal surface: role of TLR3 and TLR9 in cervical epithelial cell responses to microbial pathogens and demonstrated that mucosal epithelial cells express functional TLR3 and TLR9, and suggest that these receptors regulates the proinflammatory cytokine and antiviral environment of the lower female reproductive tract during infection with viral and bacterial pathogens.

Albiger *et al.,* (2007) studied role of TLR9 in host defence against pneumococcal infection and shown that pneumococcal infection activates resident macrophages' phagocytic activity in the airways in a TLR9 and MyD88-dependent manner, resulting in early clearance of germs from the lower respiratory tract.

Zhou & Hickford, (2008) examined unique Single Stranded Conformation Polymorphism (SSCP) patterns reflecting three different nucleotide sequences, according to the sequence analyses. These sequences were highly similar to TLR9 sequences from various species, and one of them was identical to the previously disclosed ovine TLR9 sequence. It indicates that the sequences found in this study are allelic variations of the ovine TLR9 gene. The ovine TLR9 fragment was found to have four SNPs, two of which were nonsynonymous substitutions that resulted in amino acid alterations. Arg/Gln447 and Ala/Ser462 were the amino acid substitutions that resulted in a positive charge to polar and nonpolar to polar alterations, respectively. The residue at position 462 is conserved and occupied by serine in all other species studied. These changes may affect TLR9's structure and/or function and thus disease susceptibility.

Krysko *et al.,* (2011) examined and found that TLR2 and TLR9 are sensors of apoptosis in a mouse model of doxorubicin-induced acute inflammation and observed that the innate immune system detects immunogenic apoptotic cells and TLR-2/TLR-9-MyD88 signalling pathways plays a key role in initiating the acute inflammatory response to this type of immunogenic apoptosis.

Sun *et al.,* (2012) studied functional genetic variants in TLR1 and TLR9 which confers tuberculosis susceptibility or resistance in cattle (bTB). The study found that TLR1 gene variants are linked to bTB susceptibility, but polymorphisms in the TLR9 gene have no such linkage.

Shintani *et al.,* (2014) identified the calcium-transporting ATPase, SERCA2, as a key molecule for the alternative TLR9 signalling pathway. TLR9 stimulation decreases SERCA2 activity, altering $Ca2^{+}$ handling between the SR/ER and mitochondria, resulting in lower mitochondrial ATP levels and activation of cellular protective machinery. These findings show how the same ligand-receptor system can elicit different innate responses in immune and non-immune cells, including cardiomyocytes.

McCarthy *et al.,* (2015) examined circulating mitochondrial DNA and Toll-like receptor 9 association with vascular dysfunction in spontaneously

hypertensive rats. They concluded that circulating mtDNA and impaired deoxyribonuclease activity may activate the innate immune system via TLR9 and contribute to elevated arterial pressure and vascular dysfunction.

Paul *et al.,* (2015) studied the expression of TLR genes in Black Bengal goat (*Capra hircus*) during different seasons and demonstrated that TLR and Heat Shock Protein (HSP) genes could play a role to combat the deleterious effect of thermal stress to maintain homeostasis in Black Bengal goats and as HSPs are the endogenous ligands of TLR2 and TLR4. Thus, they play an essential modulatory role in the activation of the immune system in Black Bengal goats during heat stress.

Badami *et al.,* (2019) studied and reported that TLR9 identifies bacteria's CpG DNA and activates innate and adaptive immune responses. The functional domain (LRR) of the TLR9 gene in mastitis-affected cows was compared to apparently healthy animals for nucleotide variations that could affect the host's sensitivity to illness conditions. TLR9's LRR domain was amplified and single-strand conformational polymorphism was investigated. It was noticed that the SSCP mobility pattern was different. The study found that a synonymous mutation seen in these might be used as a type I DNA marker for mastitis susceptibility studies. Three nucleotide variants were found in each of the two functional domain areas, one of which corresponded to a GC variation at position 2318 of the reference sequence (Accession No. AJ509824), suggesting a possible link to mastitis.

2.3 TLR9 Signalling

Häcker *et al.,* (2000) said that TLRs transmit their signals through a specific interaction with adaptor molecules at their cytosolic TIR domain. MyD88, one of these adaptors containing a TIR domain and a death domain (DD), is essential for initiating TLR9 signaling. MyD88-deficiency abolishes the activation of transcription factors and cytokine production elicited by CpG ODNs through TLR9.

Latz *et al.,* (2004) showed that TLR consists of leucine-rich repeats (LRRs), a transmembrane domain, and a cytoplasmic Toll/interleukin-1 receptor homology (TIR) domain. TLR9 is localized at the intracellular membrane compartment, such as the endoplasmic reticulum (ER), the endosome, and the lysosome.

Tabeta *et al.,* (2006) studied the *Unc93b1* mutation triple D (3d) disrupts exogenous antigen-presenting and signalling via Toll-like receptors 3, 7, and 9. In this regard, localization of TLR9 at the intracellular membrane compartment may be critical for preventing occasional TLR9 signaling and the consequent immune response elicited by self-DNAs. A mouse forward genetics screening identified Unc93b as a molecule essential for TLR3, TLR7 and TLR9 signaling and interacts with a membrane spanning domain of TLR9. A 3d mutation in the Unc93b gene impaired TLR9- mediated signaling without affecting localization of TLR9 implying that the membrane spanning domain of TLR9 plays a role not only in localization, but also in TLR9 signaling itself.

Kawagoe *et al.,* (2007) delved into the essential role of IRAK-4 protein and its kinase activity in Toll-like receptor–mediated immune responses and reported that MyD88, in turn, interacts with interleukin-1 receptor-associated kinase-1 (IRAK-1) and IRAK-4 through its DD. IRAK-4 and its kinase activity are shown to be essential for TLR9-mediated cytokine production.

O'Neill & Bowie (2007) showed that, in contrast to TLR2, TLR3, and TLR4, other TIR domains containing adaptors, such as TRIF, TIRAP and TRAM, are not involved in TLR9 responsiveness.

2.4 Study of TLRs in disease models

Wu & Peng, (2006) studied and reported that TLR9 signaling protects against Murine Lupus by modulating the activity of regulatory T cells. These results are in contrast with findings that implicate TLR-9 in the pathogenesis of anti-DNA responses, based on the part of investigations in incompletely backcrossed TLR-9–deficient MRL/*lpr* mice *in vivo* or transgenic B cells *in vitro*. The present result highlights the need for caution in the assessment of disease

paradigms based on the study of isolated cell populations *in vitro* and *in vivo* studies of knockout animals involving non-ideal genetic models.

Prinz, (2006) studied TLR9 in multiple sclerosis to determine the functional relevance during sterile autoimmunity. He studied the role of different TLRs and their common signaling adaptor MyD88 in the development of Experimental Allergic Encephalomyelitis (EAE). He found that MyD88$^{-/-}$ mice were completely EAE resistant. Surprisingly, this protection is partly due to the engagement of the CpG receptor TLR9. Restricting the MyD88 or TLR9 mutation to host radio-resistant cells, including the cells within the Central Nervous System (CNS), revealed that engagement of radio-resistant cells modulated the disease course and histopathological changes. The data demonstrated that both TLR9 and MyD88 are essential modulators of the autoimmune process during the effector phase of the disease and suggest that endogenous "danger signals" modulate the disease pathogenesis and progression.

Bafica *et al.,* (2006) showed that TLR2 and TLR9 cooperate to control parasite replication and that TLR9 has a primary role in the MyD88-dependent induction of IL-12/ IFN-γ synthesis during infection with *T. cruzi.*

Parroche *et al.,* (2007) studied the role of Toll-like Receptor 9 in malarial and demonstrated that natural hemozoin is coated with malarial but not human DNA. Purified malarial DNA activated TLR9 but only when DNA was targeted directly to the endosome with a transfection reagent. Stimulatory quantities of natural hemozoin contain <1g of malarial DNA; its potency in activating immune responses was even greater than transfecting malarial DNA. Thus, although the malarial genome is extremely AT-rich, its DNA is highly proinflammatory, potentially inducing cytokinemia and fever during disease.

Calcaterra *et al.,* (2008) examined the role of TLR9 in Graft-vs-host disease (GVHD). Using C57BL/6 knockout mice, they evaluated the role of TLR4 and TLR9, which recognize bacterial LPS and DNA, respectively, in the GVHD associated with allogeneic bone marrow transplantation. When myeloablative-irradiated TLR9 knockout (TLR9$^{-/-}$) mice were used as graft recipients, survival

and a clinical score of acute GVHD were improved as compared with the wild-type recipient mice; while no differences were observed using recipient TLR4 knockout ($TLR4^{-/-}$) mice. The reduced mortality and morbidity in $TLR9^{-/-}$ mice related to the reduced stimulatory activity of $TLR9^{-/-}$ spleen APCs after conditioning and reduced proliferation of allogeneic donor T cells. Experiments using $TLR9^{+/+}$ into $TLR9^{-/-}$ and $TLR9^{-/-}$ into $TLR9^{+/+}$ chimeric mice as recipients indicated a critical role for nonhematopoietic $TLR9^{+/+}$ cells interacting with bacterial breakdown products released in myeloablated mice. Altogether, these data reveal an important role of TLR9 in GVHD, a finding that might provide tools to reduce this complication of allogeneic transplantation.

Fallarino *et al.,* (2009) studied TLR9 in autoimmune disease. They evaluated whether TLR3 and TLR9, recognizing microbial dsDNA and CpG-containing DNA sequences, respectively, play a role in protecting from experimental autoimmune diabetes induced in C57BL/6 mice by streptozotocin. In wild-type animals, the disease was accompanied by upregulation of Indoleamine 2,3 dioxygenase (IDO) in pancreatic lymph nodes and would be significantly exacerbated by in vivo administration of an IDO inhibitor. Conversely, the administration of a CpG-containing oligodeoxynucleotide greatly attenuated the disease in an IDO-dependent fashion. TLR9, but not TLR3-deficient mice developed a more robust disease, an event accompanied by lack of IDO induction in pancreatic lymph nodes. Thus, he suggested that the TLR9-IDO axis may represent a valuable target in the prevention/therapy of type 1 diabetes.

Arsenault *et al.,* (2013) studied *M. avium* subsp. *paratuberculosis* exerts a distinct and significant effect on TLR responsiveness. TLR9's ability to activate the traditional TLR signalling pathway is blocked by *M. avium* subsp. *paratuberculosis*. The failure of *M. avium* subsp. *paratuberculosis* to stimulate IL-10 release from infected cells, as well as the lack of TLR9-associated signalling events discovered through kinome analysis, support this hypothesis. Interestingly, this impact appears to be TLR9-specific, as other TLR agonists can still cause *M. avium* subsp. *paratuberculosis*-infected cells to release more IL-10. Although induction of IL-10 production to promote anti-inflammatory responses

is a hallmark of *M. avium* subsp. *paratuberculosis* infection, the kinetics of IL-10 induction is quite slow, with significant levels of IL-10 not emerging until at least 12 hours after infection.

Bharti *et al.,* (2014) studied the role of TLR9 polymorphism in susceptibility to pulmonary tuberculosis and showed that the "C" allele enhances transcription and translation of TLR9 after stimulation with *M. tuberculosis.* The expression of TLR9 is inducible in PBMCs after stimulation with H37Rv wcl, and those individuals having the "C" allele show increased transcriptional activity of TLR9. An increase in TLR9 expression results in enhancement of the type I IFN-inducible gene IP-10, which is a biomarker for active pulmonary tuberculosis. Recently, it has been shown that type I IFNs, which play an indispensable role against viral disease, downregulate the production of protective cytokines such as TNFα, type II IFN (IFNγ) and IL-1β during mycobacterial infection.

2.5 Study of TLRs in animal diseases

Souza, (2012) studied the role of PRRs in Bovine Mastitis and concluded that innate immunity is crucial to maintain mammary gland health, which is mediated through recognition of Pattern Recognition Receptors (PRRs). The interaction of PRRs and PAMPs mediated the inflammatory response characterized by each mastitis-causing pathogen that can contribute to the development of severe acute inflammation or chronic mastitis.

Hosein *et al.,* (2015) studied transcription of TLRs in Canine *Leishmania infantum* infection and results revealed significant downregulation of transcription with disease progression in lymph node samples for TLR3, TLR4, TLR9, IL-17, IL-22, and FoxP3. In spleen samples, significant downregulation of transcription was seen in TLR4 and IL-22. In liver samples, downregulation of transcription was evident with disease progression for IL-22. In the skin, upregulation was seen only for TLR9 and FoxP3 in the early stages of infection. Subtle changes or down regulation in TLR transcription, Th17 cytokines and FoxP3 indicate the silent establishment of infection that Leishmania is renowned for. These observations provide new insights regarding TLR transcription, Th17 cytokines and Foxp3 in

the liver, spleen, lymph node and skin in canine leishmaniasis and highlight possible markers of disease susceptibility.

Lakshmi *et al.,* (2016) studied TLRs in Vechur cattle. They indicated significant variation in the promoter region of TLR2 and 9 genes in Vechur cattle breed and concluded that it might potentially link the influence of the innate immunity response against mastitis disease.

Bhaladhare *et al.,* (2017) investigated Single Nucleotide Polymorphism in TLR9 gene to explore the association of four SNPs in TLR9 gene with susceptibility/resistance against bovine tuberculosis infection in cattle. Three of SNPs under investigation (rs210982793, rs207807011, rs209190268) revealed polymorphism whereas monomorphism was observed in SNP rs55617140. SNP loci rs210982793 and rs207807011 were significantly associated with susceptibility to bovine tuberculosis in the case-control population. Both these SNPs loci were non-synonymous, thus suggestive of their functional role in the immune response against bovine tuberculosis.

2.6 Role of TLR polymorphism in disease resistance

Singh *et al.,* (2015) studied differential Toll-like Receptor and cytokine gene expression profiles in natural caprine brucellosis and showed higher mRNA expression of TLR4, TLR9, IFN-γ and IL12 in SMLN and MG as compared to the uterus of infected goats. However, TLR2 expression was found lower in all three tissues. This indicates that TLR4, TLR9, IFN-γ and IL12 play important roles in disease resistance and its agonist can be used along with vaccines to enhance the immune response. Moreover, elevated levels of IFN-γ and IL12 may be helpful in the early diagnosis of brucellosis in goats.

2.7 Protein-Protein Interaction in TLR signalling

Rao *et al.,* (2014) reviewed the protein-protein interaction phenomenon and stated that it plays a crucial role in predicting the function of a target protein and drug ability of molecules. To study protein-protein interaction, *in silico* methods like sequence-based approaches, structure-based approaches,

chromosome proximity, gene fusion, *in silico* 2 hybrid, phylogenetic tree, phylogenetic profile, and gene expression-based approaches were adopted to study protein-protein interaction. Elucidation of protein interaction networks also contributes greatly to the analysis of signal transduction pathways. Recent developments have also led to the construction of networks having all the protein-protein interactions using computational methods for signaling pathways and protein complex identification in specific diseases.

Berglund *et al.,* (2015) reviewed the role of protein-protein interactions of TLR functions and proposed that dimerization initiates a signal, either through homodimerization or heterodimerization. The stability of several TLR complexes and their interaction with ligands are dependent upon local pH and the lipid membrane which is likely to influence structure/dynamics and the interaction with membrane proximal adaptor proteins and associated complexes. Likewise, higher-order macromolecular assembly may have biological relevance and combined with receptor clustering (possibly in a lipid raft dependent manner) likely plays a key role in determining receptor cooperativity and hence the cellular signalling output.

Murakami *et al.,* (2017) studied that protein–protein interactions (PPIs) are vital to maintaining cellular homeostasis. Several PPI dysregulations have been implicated in the etiology of various diseases and hence PPIs have emerged as promising targets for drug discovery. Surface residues and hotspot residues at the interface of PPIs form the core regions, which play a key role in modulating cellular processes such as signal transduction and are used as starting points for drug design.

Szklarczyk *et al.,* (2019) stated that STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) is a biological database and web resource of known and predicted protein-protein interactions. For the enrichment analysis, STRING implements well-known classification systems such as Gene Ontology but also offers additional, new classification systems based on high-throughput text-mining as well as on a hierarchical clustering of the association network itself.

2.8 Phylogenetic tree estimation

Felsenstein & Felenstein, (2004) studied inferences of molecular phylogenies and stated that ancestral sequences or node positions could be determined to optimize the joint or marginal likelihood. A joint maximum likelihood assignment corresponds to the global configuration with the highest likelihood. In a marginal maximum-likelihood assignment, individual parameters are assigned to the most likely value after summing or integrating over all other unknown states. On a tree, both of these optimal assignments can be calculated in linear time.

Yang, (2007) studied maximum likelihood phylogenetic analysis programs for phylogenetic analyses of DNA and protein sequences using maximum likelihood (ML). The programs may be used to compare and test phylogenetic trees, and have a rich repertoire of evolutionary models implemented, which can be used to estimate parameters in models of sequence evolution and to test interesting biological hypotheses. Darwinian selection through phylogenetic comparison of protein-coding genes, reconstruction of ancestral genes and proteins for molecular restoration studies of extinct life forms, combined analysis of heterogeneous data sets from multiple gene loci, and estimation of species divergence times incorporating uncertainties in fossil calibrations can be carried out with these methods.

Han *et al.,* (2019) studied phylogenetic analysis of *Mastacembelus armatus* based on TLR genes. He successfully identified 3 members of the TLR7 subfamily from the spiny eel (MaTLR7, MaTLR8 and MaTLR9). The amino acid sequence identities of MaTLR7 and MaTLR8 with *Monopterus albus* TLR7 were 87.2% and 76.5%, respectively and the identity of MaTLR9 with *Seriola lalandi* TLR9 was 74.7%. The phylogenetic analysis revealed MaTLRs showed a close relationship to other species in Synbranchiformes or Perciformes. Evolutionary analysis suggested that the ancestral lineages of teleost TLR8 and TLR9 had been subject to positive selection pressures and multiple Maximum likelihood methods reported 3 positively selected sites in teleost TLR7, 4 in TLR8 and 8 in TLR9.

Domain distribution revealed that most positively selected sites were located in leucine-rich repeat domains.

Kangayan, *et al.,* (2019) studied phylogenetic analysis of TLR7 and TLR8 in Himalayan Kumaon cattle and goats. He reported that protein and extracellular domain (ECD) phylogeny of TLR-8 ruminants, swine, dolphin and alpaca were found more closely related. In the whole protein, ECD and intracellular domain (ICD) phylogeny, interestingly, HK-goat formed a sister taxon with sheep in the ruminant cluster. In addition, dolphin (cetacean) clustered with the even-toed ungulates (artiodactyls) in all the TLR-7 and -8 phylogenies. This observation corroborates the finding that cetaceans such as whales evolved from an aquatic artiodactyl ancestor.

2.9 Toll-like receptor work carried out in India

Vahanan *et al.,* (2008) examined expression profiles of toll-like receptors in a range of water buffalo tissues (*Bubalus bubalis*). The study was carried out to determine the expression profile of toll-like receptors (TLRs) 1–10 in buffalo peripheral blood mononuclear cells (PBMNC), neutrophils, spleen, liver, lung, heart, kidney, ovary and uterus using reverse transcriptase polymerase chain reaction (RT-PCR) with bovine TLR-specific primers. The buffalo TLR partial nucleotide sequences had 95–98% nucleotide homology with bovine TLR sequences available in the GenBank. PBMNC expressed all TLRs except TLR1 and neutrophils expressed all TLRs except TLR3. Expression of all TLRs was observed in spleen, lung and liver tissues. A wide range of TLR mRNA expression was observed in the heart, which lacked the expression of only TLR10. Among the tissues analyzed, kidneys had the least repertoire of TLR expression. The kidney tissue revealed mRNA expression of only TLR2, TLR5, TLR7 and TLR9. Among the reproductive tissues analyzed, the uterus expressed a wide range of TLRs such as 2, 5, 7, 8, 9, and 10 while the ovary expressed all TLRs except TLR1, indicating their immunocompetence.

Doreswamy *et al.,* (2010) studied comparative expression in peripheral blood mononuclear (PBM) Cells of *Capra hircus* and *Antelope cervicapra*

cultured in the presence of TLR9 Agonist. The study characterized Interferon Alpha (IFNA) cDNA and predicted protein sequences in goat and black buck. Response of the PBM cells to TLR9 agonist CpG ODN C and Phorbol Myristate Acetate (PMA) was evaluated by real time PCR. IFNA coding sequences were amplified from leukocyte cDNA and sequenced. Sequence analysis revealed 570 bp, IFNA ORF encoding 189 amino acids in goat and black buck. Black buck and goat IFNA has 92.1% to 94.7% and 93% to 95.6% similarity at the nucleotide level, 86.3% to 89.5% and 70.9% to 91.6% identity at amino acid level with other ruminants, respectively.

Lahiri *et al.,* (2010) studied TLR9 activation in dendritic cells enhances Salmonella killing, it was observed that *Salmonella Typhimurium* growth can be inhibited by the CpG DNA treatment in the murine dendritic cells. This inhibitory effect was mediated by an increased reactive oxygen species (ROS) production. In addition, it was noted that CpG DNA treatment of dendritic cells during Salmonella infection leads to an increased antigen presentation. Further, this increased antigen presentation depended on the enhanced reactive oxygen species production elicited by Toll-like receptor-9 activation. With the help of an exogenous antigen, it was shown that Salmonella antigen could also be cross-presented in a better way by CpG induction. These data collectively indicate that CpG DNA enhances the ability of murine dendritic cells to contain the growth of virulent Salmonella through reactive oxygen species dependent killing.

Srivastava *et al.,* (2013) studied Leishmania expressed lipophosphoglycan (LPG) interacts with TLR2 to decrease TLR9 expression and reduce anti-leishmanial responses. He reported that *in vitro* infection of macrophages with a *L. major* parasite with high expression levels of lipophosphoglycan results in decreased TLR9 expression compared to infection with a *L. major* parasite with lower expression levels of LPG. The addition of anti-LPG as well as anti TLR2 antibodies prevents this reduction of TLR9 expression. Also, addition of purified LPG to macrophages results in a decrease of TLR9 expression, which is shown to be mediated by TGFβ and IL-10. These interactions between LPG and TLR2

reduce anti-leishmanial responses via cytokine-mediated decrease of TLR9 expression.

Manuja, (2013) studied Toll-like Receptor 9 *Bubalus bubalis*. In this study, buffalo TLR9 amino acid sequence revealed a close association of TLR9 proteins within other bovines and small ruminants; but high divergence from other species. Multiple alignment of deduced amino acid sequences of *Bubalus bubalis* TLR9 with other species showed that 156/201 (74.28%) amino acids were conserved in all species. Leucine rich repeat (LRR) motifs in the ectodomain of TLR9 are responsible for molecular recognition of its agonist. The LRR pattern of *Bubalus bubalis* TLR9 protein was predicted towards N-terminal sequence and was found to be conserved among all species except *Rattus norvegicus* and *Equus caballus*. BLAST analysis of buffalo TLR9 sequence with single nucleotide polymorphisms (SNPs) database revealed 13 SNPs out of which 7 were of functional significance.

Kar *et al.,* (2015) worked on influence of common variants of TLR4 and TLR9 on clinical outcomes of *Plasmodium falciparum* malaria in Odisha and found that The TLR9-1237CC genotype was observed at significantly low frequency in MODS, while in heterozygous state (TC) it was proportionately more frequent in SOD as compared to mild malaria. The TLR9T-1486C heterozygote was common in all categories of severe malaria. However, pairwise Linkage Disequilibrium analysis revealed significant linkage between T-1237C and T-1486C, whereas haplotype analysis showed a significantly low frequency of C-T haplotype in CM and high frequency of T-C haplotype in NCSM as compared to mild malaria. Concluded that, although TLR9-1237C could be a risk factor for severe malaria in heterozygous state, negative association of CC genotype with MODS warrants caution of segregating severe malaria into its sub-clinical groups while interpreting data.

Verma *et al.,* (2016) studied ethion exposure alters expression of TLR 9 in lungs of Mice. Lipopolysaccharide resulted in a strong immunopositive TLR9 reaction in septal and airway epithelial cells. Further, acute exposure to ethion alone or in combination with LPS showed intense immunopositive expression of

TLR9. The qPCR analysis revealed that LPS caused 2.76 fold increase in the expression of TLR9 mRNA. Ethion exposure further increased the expression to 6.69 folds. However, ethion followed by LPS resulted in a 9.52 fold increase in TLR9 mRNA expression. The expression TLR9 following acute exposure to ethion suggests that ethion alone or in combination with LPS increases the expression of TLR9 mRNA at protein and mRNA levels.

Lakshmi & Jayavardhanan (2017) conducted research on the relative expression of TLR9 in bovine mastitis caused by *E. coli* and revealed that mRNA expression of TLR9 gene in sub-clinical mastitis was higher (3.22-fold) than clinical case (1.09-fold) when compared to normal bovine case. Relative differences in the expression of the TLR9 gene in sub-clinical and clinical mastitis was found to be statistically significant. During the subclinical stage of infection, the expression of TLR9 gene was at high level, therefore most of the sub-clinical mastitis subsided by itself without precipitating into clinical mastitis. TLR9 mediated signalling cascades will be fundamental to understanding the host immune response against bovine mastitis.

Manuja *et al.,* (2019) studied sequence and functional variability of Toll-like receptor 9 gene in equines and revealed 14% evolutionary divergence between equine and human TLR9, while it was 1% between the *Equus caballus* and *Equus asinus* and less than 1% within *Equus caballus.*

Nath *et al.,* (2020) in their study revealed an association between TLR4/9 and CYP1A1 polymorphisms with increased HPV16/18 infection susceptibility and cervical squamous cell carcinoma risk among the women of Jharkhand state.

Materials and Methods

CHAPTER - III

MATERIALS AND METHOD

The experiment were carried out from August 2021 to October 2021 at the Department of Animal Biotechnology, Department of Veterinary Microbiology and Department of Veterinary Biochemistry in the College of Veterinary and Animal Sciences, Parbhani (Maharashtra Animal and Fishery Sciences University, Nagpur, Maharashtra)

3.1 Chemicals

1. RBC lysis buffer
2. DNA extraction buffer
3. 10% Sodium Dodecyl Sulphate
4. Proteinase K
5. Tris saturated phenol
6. 3M sodium acetate
7. Isopropanol
8. 70% ethanol
9. TE buffer
10. Phenol: Chloroform: Isoamyl Alcohol (25:24:1 V/V)
11. Chloroform: Isoamyl Alcohol (24:1 V/V)
12. Hi-Chrome PCR master mix (HiMedia)
13. Agarose powder molecular biology grade (HiMedia)
14. DNA 100bp marker (HiMedia)
15. Ethidium bromide (HiMedia)
16. 1X TAE buffer (HiMedia)
17. 6X gel loading dye (HiMedia)

3.2 Instruments/Glassware

1. Polypropylene centrifuge tubes
2. Centrifuge
3. Water bath

4. Eppendorf tubes
5. Nanodrop-photometer
6. Polymerase Chain Reaction thermocycler
7. Agarose Gel Electrophoresis
8. Laminar flow
9. UV transilluminator

3.3 Experiment

3.3.1 Collection of samples

The blood samples (2ml) were collected from randomly selected Red Kandhari cattle maintained at Livestock Farm Complex, College of Veterinary and Animal Sciences, Parbhani during a routine clinical examination. These were collected in sterile vacutainers containing EDTA (0.5Mm, pH 8.0) as anticoagulants from the jugular vein from all the selected animals (n=6). Immediately after sample collection, blood samples were transported to the laboratory by maintaining a cold chain. Whole blood samples stored at 4^0C for isolation of DNA. Blood collected during routine blood collection was carried out for clinical examinations of the farm animals.

3.3.2 DNA extraction from blood samples

The standard Phenol-Chloroform extraction method was used to extract DNA from blood samples.

Procedure of isolation of genomic DNA

1. Blood samples stored at 4^0C were thawed at room temperature.
2. Samples were transferred to sterile polypropylene centrifuge tubes.
3. Chilled RBC lysis buffer twice the volume of sample was added and mixed gently and kept in ice for 10 minutes.
4. Sample was centrifuged at 10000 rpm at room temperature for 10 min and the supernatant containing lysed RBCs was discarded by pipetting without disturbing the pellet. This step is repeated 3-4 times till the WBC pellet becomes free of reddish tinge or lysed RBCs.

5. 600µl of DNA extraction buffer was added and vortexed to disperse the WBC pellet gently in the extraction buffer.
6. WBC pellet mixed with DNA extraction buffer was incubated at 37^0C for 30 minutes.
7. Then, 10% SDS was added i.e., 40 µl, and mixed gently by inverting the tubes twice.
8. Proteinase K (20mg/ml) was added in two pulses. In the first pulse, 2µl of Proteinase K was added and mixed gently end to end and incubated at 50^0C for 3 hours. After incubation second pulse i.e., 2µl was added and again incubated at 50^0C overnight.
9. On the second day, contents of the tubes were transferred to a clean sterile autoclaved Eppendorf tube, and then an equal volume of tris-saturated phenol (pH 7.8) was added. Contents were mixed gently for 10 min, and the tubes were centrifuged at 10000 rpm for 10 min at room temperature.
10. The upper aqueous phase containing DNA was transferred to another sterile autoclaved Eppendorf tube with the help of 1ml wide bore microtip (3mm).
11. Similarly, extraction in step 9 and 10 was done once with Phenol: Chloroform: Isoamyl alcohol (25:24:1) and once with Chloroform: Isoamyl alcohol (24:1).
12. Finally, the aqueous solution was transferred to a sterile autoclaved Eppendorf tube, and 3M sodium acetate at 100µl/ml aqueous phase was added by gentle mixing.
13. More than two volumes of chilled isopropanol was added and mixed gently by inverting the tubes once or twice. The tube was left at room temperature to allow precipitation of DNA and the supernatant was discarded without interfering with the DNA pellet.
14. The DNA pellet was then washed twice with 300 to 400 µl of 70% ethanol.
15. Finally, the DNA pellet was air dried for 1 hour to remove traces of ethanol and subsequently dissolved in a 200µl TE buffer.

16. The Eppendorf tubes were kept in a water bath at 60^0C for 2 hours to inactivate the DNAse activity and dissolve the pellet properly in the TE buffer.
17. After 2 hours of incubation in a water bath, DNA was cooled and stored at -20^0C for further use.

The composition and preparation of buffers and solutions used in the procedure are given in the Appendix I.

3.3.3 Quality check and quantification of DNA

The quality and quantity of DNA were assessed by Nanodrop-photometer by using the following procedure.

1. An integrated vortex was used to mix the sample well to achieve homogeneity of the sample.
2. Blank was set by using the buffer solution used to store DNA samples.
3. Sample volume 1µl was dropped onto the center of the measuring window, and the lid was fitted exactly onto the positioning supports mounted to the body of the cell, and measurements were taken.
4. Sample residues were removed from the measurement window and the mirror in the lid and they were cleaned with a slightly wet fluff-free tissue by using distilled water.
5. Procedure repeated for all the samples and measurements were recorded.
6. Samples showing 260/280 ratio in between 1.7 to 2.0 OD and DNA concentration above 30ng/µl were selected for quality checking through agarose gel electrophoresis.

3.3.4 Oligonucleotide sequences for PCR amplification

Oligonucleotide primers (Table 3.1) to amplify the TLR9 genes in Red Kandhari cattle were used as per reference nucleotide sequence with XM-027523062 as an accession number (Cargill & Womack 2007).

Table 3.1 Primer sequences for amplification TLR9

TLR9 Primers			Location as per XM-027523062	Nucleotide length
TLR9 – 1	F	GTTTGTGCTCTGATGGTGCT	1625 to 1644	718bp
	R	CCGTGTTTCTCTCCATCACT	2323 to 2342	
TLR9 - 2	F	TTCTCACTTCCTCTGATCTCT	2125 to 2174	1292bp
	R	TTCTCTCTCCAGTGCCCATC	3133 to 3152	
TLR9 – 3	F	AGATTGCAGGTCTCAGGATG	3037 to 3056	1152bp
	R	CGGAACCAATCTTTCTCTAGTT	4167 to 4188	
TLR9 – 4	F	CCTGACACCTTCAGTCACCT	4103 to 4122	1097bp
	R	AGGGTGTGCAGATGGTTCTC	5180 to 5199	
TLR9 - 5	F	GGGAGACCTCTATCTCTGCTTT	5119 to 5140	1215bp
	R	CGGTTATAGAAGTGACGGTTG	6313 to 6333	

3.3.5 PCR amplification

The TLR9 coding gene sequence was amplified through Polymerase Chain Reaction (PCR). PCR was performed as with modifications described by Cargill & Womack, (2007) with cyclic conditions as depicted in Table 3.2. PCR was carried out in a final volume of 25µl. The reaction contained 12.5 µl Hichrome PCR master mix (containing dNTPs- 0.4 mM each, Taq polymerase 0.05U/µl, magnesium chloride-4 mM), 1 µl forward primer (10 pmoles), 1 µl reverse primer, 1 µl Template DNA and 9.5 µl Nuclease free water. The master mix was prepared for one additional sample without a template to control pipetting error. All reactions were carried out in 0.2 ml thin-walled PCR tubes. PCR tubes containing the reaction mixture were tapped gently and quickly centrifuged at 3000 rpm for a few seconds. The tubes were placed in a thermal cycler (Eppendorf) and subjected to PCR. The PCR product was evaluated by Agarose Gel Electrophoresis.

Table 3.2 PCR program for amplification of TLR9 in Red Kandhari cattle

Step	Temperature	Time	No of Cycles
Initial denaturation	95^0C	3 min	1
Denaturation	95^0C	30 sec	35
Annealing	55^0C	30 sec	
Extension	72^0C	1 min	
Final extension	72^0C	10 min	1

The PCR product was evaluated by Agarose Gel Electrophoresis. Thereafter the gel was transferred to a UV transilluminator and visualized for expected band size.

3.3.6 Agarose Gel Electrophoresis

The PCR product was evaluated by Agarose Gel Electrophoresis. One gram of agarose gel dissolved in 1x TAE buffer to final volume up to 100ml. Then it was heated in the microwave oven for 90 sec and allowed to cool. 1% of Ethidium Bromide was added at 10µl/100 ml of gel solution and this melted gel was poured in the gel casting tray. Once the gel solidified, it was transferred to a gel electrophoresis tank filled with a 1x TAE buffer with maintaining level of 1 cm above the gel. The time and temperature for gel run was kept 55 min and 80 V respectively. 10 µl of PCR product and 5 µl of 100bp DNA ladder (HiMedia MBT-130) were used for gel run. The gel was transferred to a UV transilluminator and visualized for expected band size.

3.3.7 Sanger's Sequencing

All positive PCR products were subjected to Sanger's sequencing for reverse and forward strands. The sequencing services were hired from geneOmbio Technologies Pvt. Ltd. Baner, Pune, India 411045. The quality control curated chromatograms were inspected visually. The sequences obtained were assembled and aligned using the Lasergene version 5.00 (http://www.dnastar.com/) (Burland, 2002). The final sequence was finalised using overlapping region assembly of individual fragments. Sequencing files obtained as chromatograms were

submitted to the research repository database available at Zenodo (https://zenodo.org/) (Peters *et al.,* 2017).

3.3.8 List of Online Resources Used in the Study

Due to increasing computational powers, new bioinformatics tools are being deployed as web servers and can be accessed from a browser window over the internet. Following web servers and applications were used during the study (Table. 3.3).

Table 3.3 List of Online Resources used in the Study

Sr. No	Name	Address	Utility
1	Zenedo	https://zenodo.org/	Uploading sequencing chromatograms
2	BLAST	https://www.ncbi.nlm.nih.gov/	To identify related TLR9 nucleotide sequences
3	ExPASy	https://web.expasy.org/translate/	Six frame translation, Nucleotide translation
4	SMART	http://smart.embl.de	Domain structure prediction
5	STRING	https://string-db.org/	TLR9 Protein-Protein Interactions, Enrichment network analysis, Gene ontology
6	SWISS-MODEL	https://swissmodel.expasy.org/	To predict 3D protein model
7	EMBOSS cons	https://www.ebi.ac.uk/Tools/msa/emboss_cons/	To determine consensus of a sequence
8	Lasergene version 5.0	http://www.dnastar.com/	DNA Sequence analysis
9	MEGA X	https://www.megasoftware.net/	Multiple sequence alignment of TLR9, substitution model estimation, phylogeny construction
10	PyMOL	https://pymol.org/	3D model visualization
11	Figtree	http://tree.bio.ed.ac.uk/software/figtree/	Phylogenetic tree visualization

3.3.9 Sequence Analysis

The coding region of TLR9 of Red Kandhari cattle was successfully amplified using specific primer pairs in fragments and referred to as regions 1 to 5. The sizes of amplified products were verified using agarose gel electrophoresis. Sequences of the TLR9 gene were analysed using the Lasergene software package (DNASTAR, London, UK) software. BLAST was used to identify related TLR9 nucleotide sequences within the non-redundant nucleotide database (https://www.ncbi.nlm.nih.gov/) by comparison. The multiple alignment of TLR9 coding sequences from Red Kandhari was performed using the program MUSCLE alignment in MEGA X (https://www.megasoftware.net/). Protein sequence was deduced from Expasy six-frame translation (https://web.expasy.org/translate/). Nucleotide sequences for the coding region of TLR9 gene were deposited in NCBI GenBank at National Library of Medicine, Bethesda, MD, USA.

The Simple Modular Architecture Research Tool (SMART) available at (http://smart.embl.de/) (Letunic & Bork, 2018) was employed to predict the domain structure of Red Kandhari. In this study, the LRR finder was used to detect the signal peptide sequence, LRRs present, and TIR domain in the deduced amino acid sequences of TLR9 of Red Kandhari cattle.

3.3.10 Protein-protein interaction prediction

We performed protein interaction prediction based on the TLR9 sequence of Red Kandhari cattle in a STRING database version 11 (Zklarczyk *et al.,* 2021). STRING is a database of known and predicted protein-protein interactions. The interactions include direct (physical) and indirect (functional) associations; they stem from computational prediction, from knowledge transfer between organisms, and interactions aggregated from other (primary) databases. The resource is available at https://string-db.org/ (Mering *et al.,* 2003). The amino acid sequence was submitted to the above server with the available organism as *Bos taurus* and default settings of network node, confidence and size cutoff. The network view summarizes the network of predicted associations for a particular group of

proteins. The network nodes are proteins. The edges represent the predicted functional associations. Edge may be drawn with up to 7 differently colored lines. Enrichment analysis of gene ontology was also done, showing terms in databases that are more enriched in the set of proteins in the network than the background. The confidence in the gene ontology network is measured with enrichment p-value. The expected number of edges gives how many edges to be expected if the nodes were selected at random. A small PPI enrichment p-value indicates that the nodes are not random and that the observed edges are significant.

3.3.11 Three Dimensional (3D) structure

The three-dimensional structure of TLR9 protein of Red Kandhari cattle was modelled based on homology available at https://swissmodel.expasy.org/ (Kiefer *et al.,* 2009). The protein sequence of TLR9 was entered as input. Top reported template with a sequence identity of 98.09 % was used for homology modelling. The output was visualised using PyMOL (Yuan *et al.,* 2017). The similarity of the predicted 3D model to that of the reported was compared(https://pymol.org/).

The similarity of the predicted 3D model to that of the reported was compared with the help of QMEAN Z-score to decide quality. The Ramachandran plot was used to analyse the suitability of amino acids in energetically allowed regions which show the torsional angles of the amino acids in a peptide, phi (φ) and psi (ψ) (Safi *et al.,* 2021).

3.3.12 Phylogenetic tree construction

A phylogenetic tree is a branching diagram or a tree diagram showing the evolutionary relationships among various biological species or entities based upon similarities and differences in their physical or genetic characteristics. For molecular phylogenetic tree construction, all sequences are aligned together, and the best fit model for its substitution is found. This best fit model is used to construct phylogenetic tree construction.

Table 3.4 Accession number list of TLR9 sequences from 21 species used in the phylogenetic analyses

Sr. No.	Protein	Species	GenBank accession number
1	TLR9	Isolate 1	Submitted
2		Isolate 2	Submitted
3		Isolate 3	Submitted
4		Isolate 4	Submitted
5		Isolate 5	Submitted
6		Isolate 6	Submitted
7		*Bos indicus*	ABN71657.1
8		*Bos indicus*	XP019840413.1
9		*Bos indicus*	ACL5098.5.1
10		*Bos indicus*	APQ40216.1
11		*Bos taurus*	ABN71650.1
12		*Bos grunniens*	AHY88398.1
13		*Bubalus bubalis*	XP_006044084.3
14		*Capra hircus*	NP_001272615.2
15		*Ovis aries*	NP_001011555.1
16		*Camelus ferus*	XP_032314257.1
17		*Camelus dromedarius*	KAB1264415.1
18		*Sus scrofa*	NP_999123.1
19		*Eschrichtius robusts*	MBV96971.1
20		*Physeter catodon*	XP_007105994.2
21		*Hyaena hyaena*	XP_039099596.1
22		*Suricata suricatta*	XP_029771902.1
23		*Panthera tigris*	XP_042835011.1
24		*Panthera leo*	XP_042785505.1
25		*Equus asinus*	XP_014682103.1
26		*Elephus hippelaphus*	OWK02682.1
27		*Muntiacus muntjak*	KAB0358093.1

To infer the phylogenetic position of the Red Kandhari cattle based on the TLR9 sequences, we used TLR protein sequences of six isolates under study. We retrieved TLR9 protein sequences related to cattle and few dissimilar species (eighteen) from GenBank given in Table 3.4. Amino acid sequences were used for phylogenetic analyses. The assembled sequences of six isolates along with sequences to be compared were aligned using the MUSCLE algorithm in MEGA X with the default setting. The best fit model of amino acid substitution was determined in MEGA X with 500 bootstraps (Russo *et al.,* 2018). The phylogenetic analysis was carried out using the Maximum Likelihood (ML) method (Yang, 2007). Accuracies and statistical tests of phylogenetic trees were measured by the bootstrap method. The obtained tree in Newick format was visualised using Figtree software available at http://tree.bio.ed.ac.uk/software/ figtree/ (Rambaut, 2009).

Results and Discussion

CHAPTER - IV

RESULTS AND DISCUSSION

The findings of **"Molecular Characterization of Toll-like Receptor 9 of Red Kandhari cattle"** are presented in this section as per the objectives of the study.

4.1 Selection of animals

For current research work, Red Kandhari animals are randomly selected from the Livestock Farm Complex of College of Veterinary and Animal Sciences, Parbhani. As per the university mandate, the farm has maintained pure line Red Kandhari animals for decades (Wankar *et al.,* 2021). Medium sized, strong, and deep dark red coloured animals with the ideal matching of apparently phenotypic characters of Red Kandhari animals were preferred. Total of six animals were selected with two cows, two bulls, and two calves ranging from 2 to 6 years. Blood samples were collected during routine clinical examination, in sterile vacutainers containing EDTA (0.5Mm, pH 8.0) as anticoagulant. Immediately after sample collection, blood samples were transported to the laboratory by maintaining a cold chain.

4.2 DNA isolation

The standard Phenol-Chloroform extraction method was used to extract genomic DNA from blood samples. The purity and concentration of DNA obtained were determined through 260/280 nm absorbance measured using NanoDrop Lite Spectrophotometer (Thermo Scientific) (Table 4.1). The DNA concentration of isolated samples was in the range of the ideal value of DNA purity according to the NanoDrop Lite Spectrophotometer user manual.

Table 4.1 Absorbance ratio values for DNA Quantification of Isolates by NanoDrop Lite Spectrophotometer

Sr. No.	Isolate	A260/280	A260(10nm)	ng/µl
1	1	1.93	1.383	69.2
2	2	2.13	11.022	551.1
3	3	1.76	1.787	89.4
4	4	1.58	0.598	29.9
5	5	1.98	8.988	449.4
6	6	1.72	3.010	450.5
7	Blank	1.72	-0.004	-0.2

4.3 Polymerase chain reaction

PCR is a fast and easy approach to amplify particular DNA fragments from very little amounts of source DNA material, even the original DNA is of low quality. It does not require the usage of radioisotopes or hazardous chemicals. The TLR9 gene of Red Kandhari cattle was amplified through PCR as per standard protocol with minor deviations. The final adopted protocol is given in Materials and Method (chapter III). The amplified PCR products after 35 cycles were subjected to agar gel electrophoresis on 1 % agarose gel for visualization of bands that revealed expected amplicon product size for regions 1, 2, 3, 4, 5 was 718bp, 1292bp, 1152bp, 1097bp and 1215bp, respectively. The tentative amplicon size was based on the reference nucleotide sequence (XM-027523062) based on which primers were based. The amplicon product length was determined by comparison with 100 bp Himedia DNA ladder. These results are shown in Plate No. 4.1. The size of amplicons was in the range of expected length of the region. On this basis, PCR amplicons were sent for Sanger's sequencing.

4.4 Sanger's Sequencing

All positive PCR products were subjected to Sanger's sequencing for reverse and forward strands. The sequencing services were hired from geneOmbio Technologies Pvt. Ltd. Baner, Pune, India 411045. The sequences obtained as

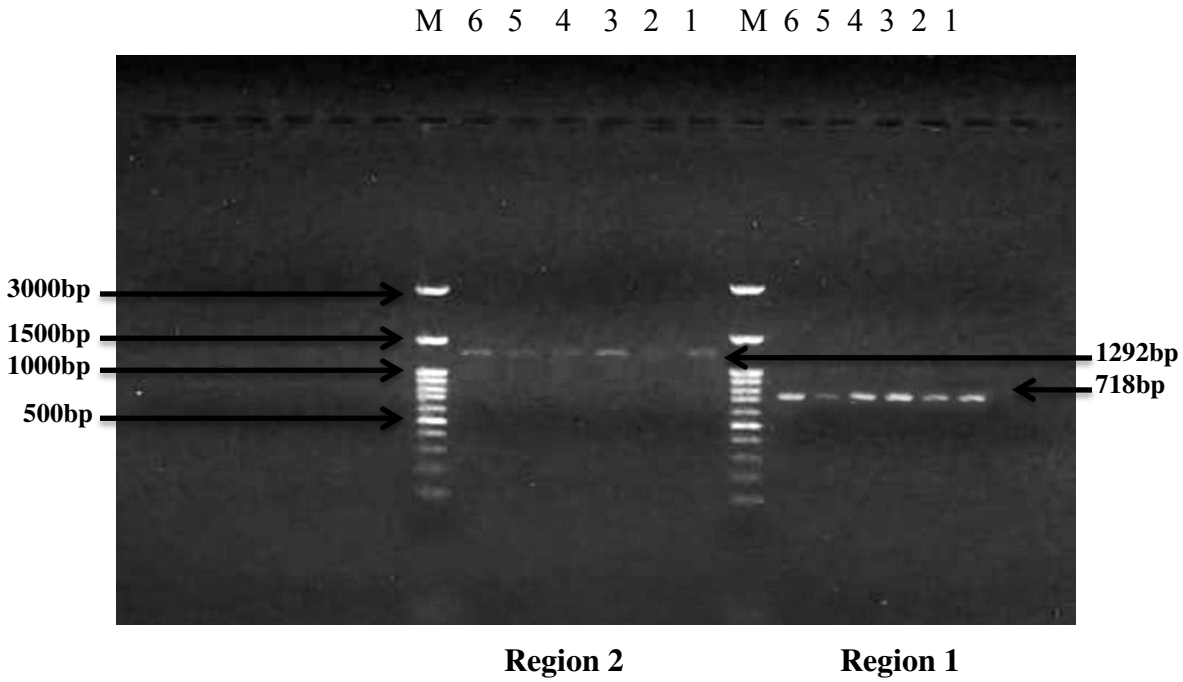


Plate No. 4.1(a): PCR amplification of TLR9 gene of Red Kandhari cattle Isolate 1-6 with primer pair 1-2 and comparison with 100bp DNA ladder

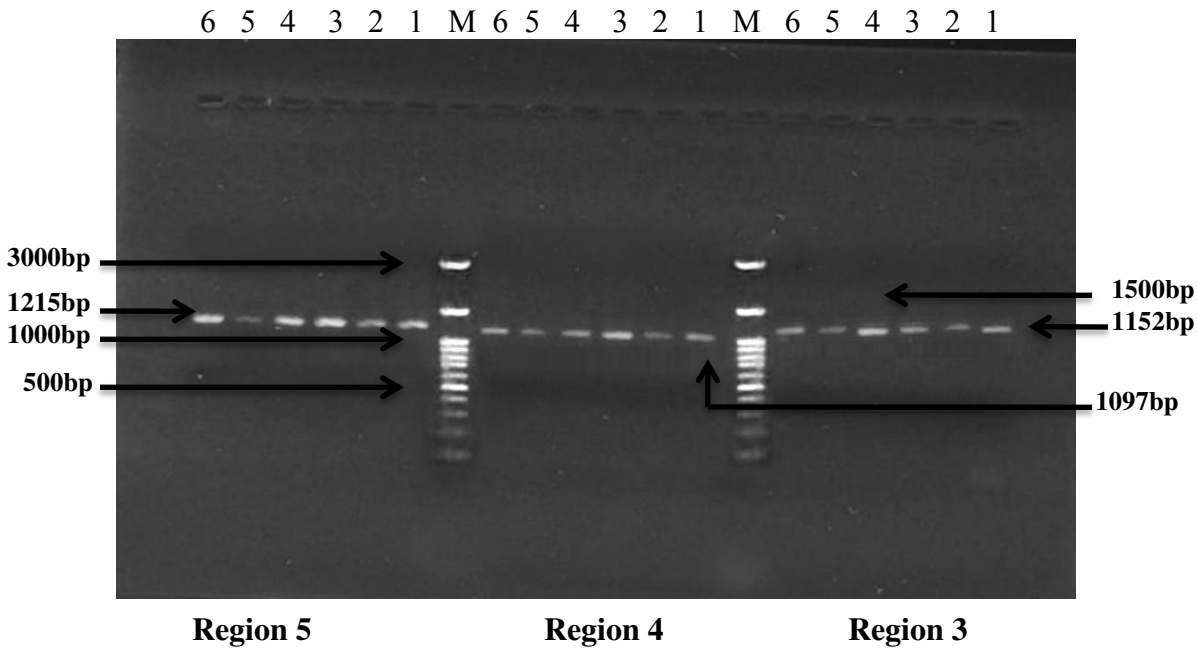


Plate No. 4.1(b): PCR amplification of TLR 9 gene of Red Kandhari cattle Isolate 1-6 with Primer pair 3, 4, 5 and comparison with 100bp DNA ladder

1 = Isolate 1
2 = Isolate 2
3 = Isolate 3
4 = Isolate 4
5 = Isolate 5
6 = Isolate 6

Region 1 (718bp) = Primer 1F & 1R
Region 2 (1292) = Primer 2F & 2R
Region 3(1152) = Primer 3F & 3R
Region 4 (1097) = Primer 4F & 4R
Region 5 (1215) = Primer 5F & 5R
M = 100 bp DNA Marker

chromatograms Plate No. 4.2 were inspected visually and curated using the Lasergene version 5.0 (http://www.dnastar.com/). These sequences were copied to a text file and overlapping regions were identified and the final sequence was constructed. For posterity, the chromatogram files were uploaded at zenedo (https://zenodo.org/) which is a research database repository (Devkatte, 2022).

4.5 Sequence Analysis

Sequence analysis is the process of subjecting a DNA, RNA, or peptide sequence to a wide range of analytical methods so that we can understand its features, function, structure, or evolution (Durbin *et al.,* 1998). Methodologies used include sequence alignment, searches against biological databases. The coding region of TLR9 of Red Kandhari cattle amplified using overlapping primer pairs in fragments were assembled and compared with the sequences in nucleotide databases. Sequences of the TLR9 gene were analysed using the Lasergene software package (DNASTAR, London, UK) software. BLAST was used to identify TLR9 nucleotide sequences within the non-redundant nucleotide database (https://www.ncbi.nlm.nih.gov/) by comparison with related sequences obtained NCBI, Resource Coordinators, 2016). The sequences were annotated through NCBI BankIt on its online interface. When sequences were submitted to the NCBI GenBank submission system, they were approved for inclusion in NCBI nucleotide database and following Accession numbers (Table 4.2) were assigned to all the isolates of the TLR9 coding region of Red Kandhari cattle under study. They will be released in the public domain on Jun 9, 2023. The details of this submission are available in Annexure III.

Table 4.2 List of isolates for which NCBI nucleotide accession numbers were assigned

Sr.No.	Isolate	Accession No.
1	Isolate 1	OL780023
2	Isolate 2	OL780024
3	Isolate 3	OL780025
4	Isolate 4	OL780026
5	Isolate 5	OL780027
6	Isolate 6	OL780028

Conceptual protein translation of each isolate of the coding region of the sequence was carried out with Expasy six frame translation (https://web.expasy.org/translate/). To determine consensus sequence, the multiple sequence alignment of TLR9 coding sequences from Red Kandhari was performed using the MUSCLE alignment algorithm in MEGA X. The single consensus protein sequence was obtained using EMBOSS cons (https://www.ebi.ac.uk/Tools/msa/emboss_cons/) (Mullan & Bleasby, 2002) which used multiple aligned protein sequences as input and is presented in tabular format at Table 4.3.

Henceforth, the single consensus protein sequence will be referred to as TLR9 protein of Red Kandhari cattle.

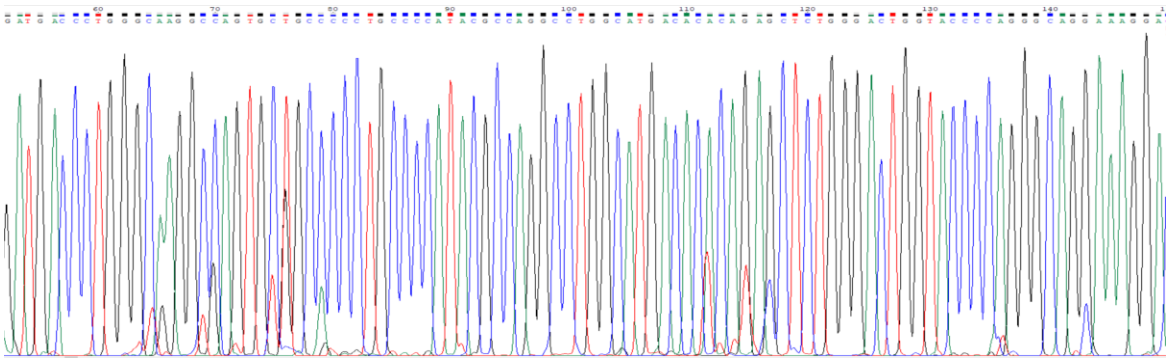


Plate No. 4.2(a): Representative chromatogram of sequencing of TLR9 gene of Red Kandhari cattle Isolate 1 with Primer 1F

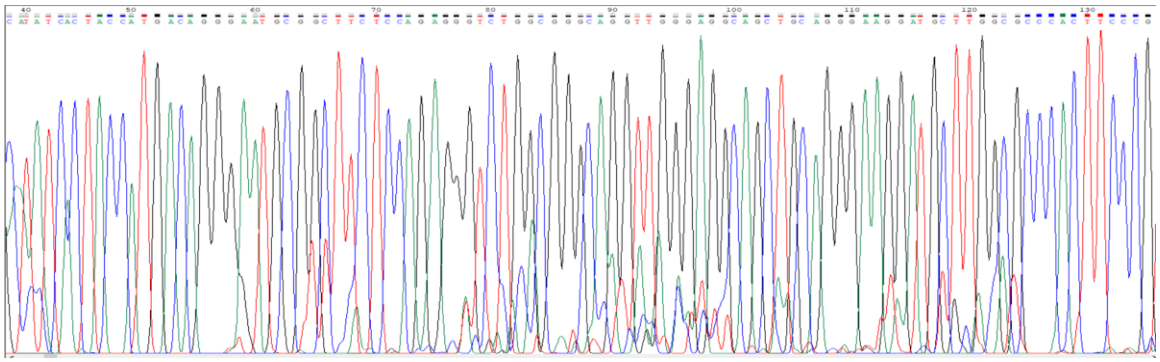


Plate No. 4.2(b): Representative Chromatogram of sequencing of TLR9 gene of Red Kandhari cattle Isolate 1 with Primer 1R

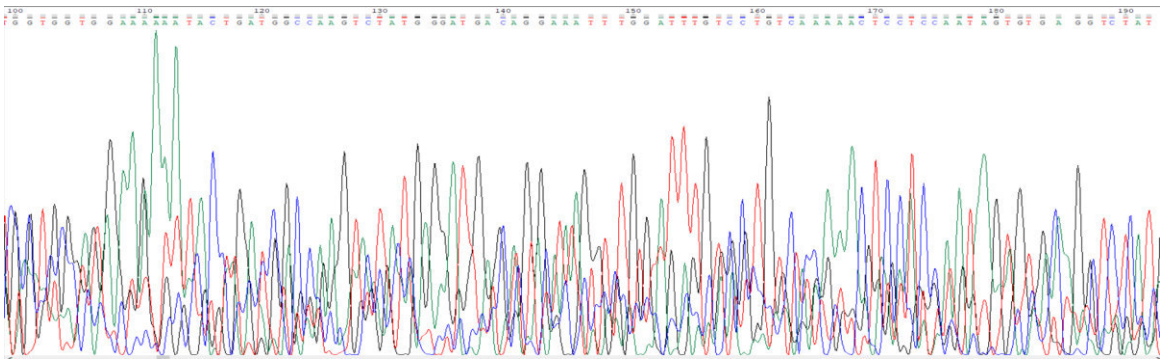


Plate No. 4.2 (c): Representative Chromatogram of sequencing of TLR9 gene of Red Kandhari cattle Isolate 1 with Primer 2F

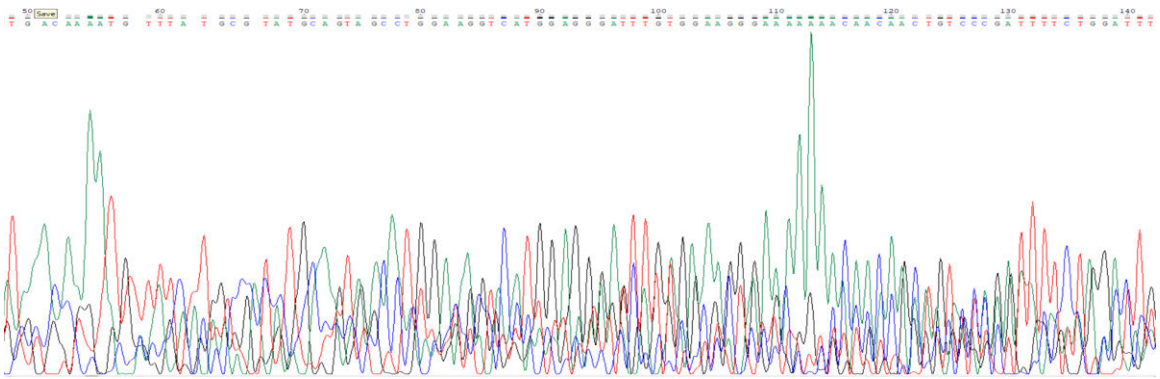


Plate No. 4.2 (d): Representative Chromatogram of sequencing of TLR9 gene of Red Kandhari cattle Isolate 1 with Primer 2R

Table 4.3 Consensus protein sequence of TLR9 of Red Kandhari cattle

Particular	Protein Sequence
consensus protein sequence of TLR9 of Red Kandhari cattle (TLR9 protein)	MGPYCAPHPLSLLVQAAALAAALAEGTLPAFLPCELQPHGQVDCN WLFLKSVPHFSAGAPRANVTSLSLISNRIHHLHDSDFVHLSNLRV LNLKWNCPPAGLSPMHFPCRMTIEPNTFLAVPTLEELNLSYNGIT TVPALPSSLVSLSLSHTSILVLGPTHFTGLHALRFLYMDGNCYYM NPCPRALEVAPGALLGLGNLTHLSLKYNNLTEVPRRLPPSLDTLL LSYNHIVTLAPEDLANLTALRVLDVGGNCRRCDHARNPCRECPKN FPKLHPDTFSHLSRLEGLVLKDSSLYKLEKDWFRGLGRLQVLDLS ENFLYDYITKTTIFNDLTQLRRLNLSFNYHKKVSFAHLHLASSFG SLVSLEKLDMHGIFFRSLTNITLQPLTRLPKLQSLRLQLNFINQA QLSIFGAFPSLLFVDLSDNRISGAATPAAALGEVDSRVEVWRLPR GLAPGPLDAVSSKDFMPSCNLNFTLDLSRNNLVTIQQEMFTRLSR LQCLRLSHNSISQAVNGSQFVPLTSLRVLDLSHNKLDLYHGRSFT ELPQLEALDLSYNSQPFSMQGVGHNLSFVAQLPSLRYLSLAHNGI HSRVSQKLTSASLRALDdSGNsLSQMWAEGDLYLCFFKGLRNLVQ LDLSENHLHTLLPRHLDNLPKSLRQLRLRDNNLAFFNWSSLTVLP RLEALDLAGNQLKALSNGSLPPGIRLQKLDVSSNSIGFVIPGFFV RATRLIELNLSANALKTVDPSWFGSLAGTLKILDVSANPLHCACG AAFVDFLLERQEAVPGLSRRVTCGSPGQLQGRSIFTQDLRLCLDE TLSLDCFGLSLLMVALGLAVPMLHHLCGWDLWYCFHLCLAHLPRR RRQRGEDTLLYDAFVVFDKVQSAVADWVYNELRVQLEERRGRRAL RLCLEERDWLPGKTLFENLWASVYSSRKTMFVLDHTDRVSGLLRA SFLLAQQRLLEDRKDVVVLVILRPAAYRSRYVRLRQRLCRQSVLL WPHQPSGQGSFWANLGIALTRDNRHFYNRNFCRGPTTAE

4.6 Molecular Composition of Amino acids of TLR9

Following Table 4.4 indicated amino acid composition of TLR9 protein of Red Kandhari cattle.

Table 4.4 Amino acid composition of TLR9 protein of Red Kandhari cattle

Amino Acid	Numbres	Percent
Ala (A)	69	6.7%
Arg (R)	76	7.4%
Asn (N)	56	5.4%
Asp (D)	49	4.8%
Cys (C)	25	2.4%
Gln (Q)	38	3.7%
Glu (E)	34	3.3%
Gly (G)	55	5.3%
His (H)	37	3.6%
Ile (I)	24	2.3%
Leu (L)	**191**	**18.6%**
Lys (K)	26	2.5%
Met (M)	13	1.3%
Phe (F)	50	4.9%
Pro (P)	57	5.5%
Ser (S)	**90**	**8.7%**
Thr (T)	45	4.4%
Trp (W)	14	1.4%
Tyr (Y)	22	2.1%
Val (V)	58	5.6%
Pyl (O)	0	0.0%
Sec (U)	0	0.0%

The TLR9 protein of Red Kandhari cattle under study is composed of 1029 amino acids with molecular weight of 115.4 Kda. The theoretical isoelectric pH of protein is 9.05. Total number of negatively charged residues (Asp + Glu) and positively charged residues (Arg + Lys) are 83 and 102, respectively. The protein has the highest proportion of Leucine (18.6 %), followed by serine (8.7%) amino acid, highlighted in Table No.4.4. The high % of leucine in the protein gives leucine-rich repeats and is a characteristic of this class of protein. Goldammer's study showed that the nucleotide sequence encoding the bovine TLR9 is 83.5 and 76.9% similar to the homologous genes from humans and mice, respectively. Conceptual translation of the bovine sequence shows that the bovine protein comprises 1,029 amino acid (aa) residues, and hence, is shorter by three aa residues than the homologous receptors from both other species. The entire protein sequence of the bovine receptor is 79.5% and 73.5% similar to those of humans and mice, respectively (Goldammer *et al.,* 2004). Raja also found a similar amino acid composition of TLR9 gene of *Bubalus bubalis,* which codes for 1029 amino acids belonging to the TIR superfamily. The amino acid sequence of TLR3 and TKR9 has a great degree of similarities among different mammalian species (Raja *et al.,* 2011). Alignment of the hTLR9 protein (Accession No. NP_059138) with the bovine TLR9 protein (Accession No. NP_898904) reveals 80% similarity between the sequences and hTLR9 amino acid position 545 corresponds to bovine TLR9 amino acid position 543 (Cargill and Womack, 2007).

The percent amino acid identity of the Red Kandhari TLR9 protein with those of TLR9 proteins from selected few other species is shown in Table 4.5.

Table 4.5 Comparison of amino acid identity percentage of TLR9 of Red Kandhari with other species

Isolate	Species	AA identity percentage	Accession Number
TLR9 Red Kandhari	*Bos indicus*	99.61	APQ40216.1
	Bos taurus	98.54	ABN71650.1
	Bubalus bubalis	96.99	XP_006044084.3
	Capra hircus	94.27	NP_001272615.2
	Ovis aries	94.27	NP_001011555.1
	Camelus ferus	83.80	XP_032314257.1
	Sus scrofa	83.88	NP_999123.1
	Panthera tigris	76.96	XP_042835011.1
	Panthera leo	76.87	XP_042785505.1
	Equus asinus	82.42	XP_014682103.1
	Elephus hippelaphus	91.30	OWK02682.1
	Hyaena hyaena	64.80	XP_039099596.1

Comparison of Red Kandhari TLR9 protein sequence with other mammalian TLR9 sequences showed the highest sequence identity to *Bos indicus* (99.61%) followed by *Bos taurus* (98.54%) identical to, *Bubalus bubalis* (96.99%), *Capra hircus* (94.27%), *Ovis aries* (94.27%). In a related work, the promoter sequence of TLR9 gene of Vechur cattle showed 99% similarity to *Bos taurus* sequence (Lakshmi *et al.,* 2016). Zhou and Hickford (2008) analysed TLR9 in sheep and found that those sequences shared high homology to TLR9 sequences from a variety of species and one of them was identical to the published ovine TLR9 sequence and suggested that isolated sequences represent allelic variants of the ovine TLR9. The bovine vrTLR9 sequences are highly similar to sequences from other mammalian species, 85%, 81%, 86% to human, mouse, and dog respectively (Cargill and Womack, 2007). The mammalian TLR9 sequences revealed that the bovine TLR9 shared 79% homology with human TLR9 and 73% homology with murine TLR9 (Griebel *et al.,* 2005).

4.7 Molecular Architecture of TLR9 protein of Red Kandhari cattle

Simple Modular Architecture Research Tool (SMART) was employed to predict the domain structure of the protein of Red Kandhari cattle available at (http://smart.embl.de) SMART (Simple Modular Architecture Research Tool) is a web resource for the identification and annotation of protein domains and the analysis of protein domain architectures. SMART contains manually curated models for more than 1300 protein domains, with a topical set of 68 new models added (Letunic *et al.,* 2021). The TLR9 protein sequence was submitted to the server and the pictorial presentation of domains are shown in Fig 4.1.

This tool detected the 18 number of LRRs present in the deduced amino acid sequences of TLR9 of Red Kandhari cattle. Similar findings observed by Cargill and Womack (2007) with 18 LRRs in TLR9 of cattle and Banerjee *et al.,* (2011) found that 17 LRRs in TLR9 of *Bubalus bubalis.* TIR domains bind the cytoplasmic adaptor proteins MyD88 and TOLLIP, which link with different kinases to initiate signalling cascades when they are active (Cargill and Womack, 2007).

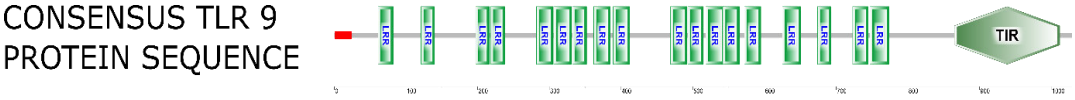


Fig 4.1 Domain analysis of TLR9 of Red Kandhari cattle.

TIR domain starts from 865 amino acids and ends at 1012 amino acids (Table 4.6). Signal sequence is located from the first to 24th amino acid.

Similar work of TLR9 protein in common carp and prediction of protein domains revealed that the putative amino acid sequence consisted of 14 leucine-rich repeat (LRR) domains, a leucine-rich repeat C-terminal (LRR-CT) domain, and a 150-amino acid Toll-interleukin-1 receptor (TIR) domain. Results of a sequence homology search using BLASTP displayed 80% identity to the zebrafish (*Danio rerio*) ortholog, 56% to another piscine and 38% to human orthologs. The amino acid sequence identities and similarities between TIR

domains were approximately 10 to15% higher than the entire ORF identities and similarities (Kongchum *et. al.,* 2011).

Table 4.6 Two dimensional molecular domain analysis of Red Kandhari TLR9

Name	Start	End	E-value
signal peptide	1	24	N/A
LRR	61	82	539
LRR	121	140	0.93
LRR	197	216	138
LRR	218	238	224
LRR	282	305	116
LRR	306	331	129
LRR	332	352	327
LRR	362	385	247
LRR	389	412	39.8
LRR	469	492	192
LRR	493	515	153
LRR	518	544	24.8
LRR	542	565	240
LRR	572	595	539
LRR	625	651	90.9
LRR	674	693	147
LRR	723	745	44.5
LRR	747	774	14.4
TIR	865	1012	0.00284

Zhang *et al.,* (2008) reported that equine TLR9 is highly homologous to other mammalian orthologs and has a conserved TIR domain and three LRR motifs. Similar to canine and human TLR9. A TIR domain that is functionally important for TLR signal transduction was identified in the cytoplasmic domain

of Japanese flounder TLR9. The TIR domain of zebrafish TLRs have three boxes that are important for TLR functions (Jault *et al.,* 2004).

4.9 Protein-Protein interaction study

As per STRING analyses following three types of roles of protein are described: Process, Function, and component—the larger processes, or 'biological programs' accomplished by multiple molecular activities. Molecular function terms describe activities at the molecular level, such as "catalysis" or "transport". The component relative to cellular structures in which a gene product performs a function, either cellular compartments (*e.g.*, mitochondrion), or stable macromolecular complexes of which they are parts (*e.g.*, the ribosome). Unlike the other aspects of GO, cellular component classes refer not to processes but rather cellular anatomy in STRING databases.

The TLR9 protein is found to interact with TLR2, TLR4, Myd88, TIRAP, TICAM1, TNF, HMGB1, UNC93B1, and LY96 Plate No. 4.3. Among these, TLR2 and TLR4 are related to Toll-like receptor molecules. The MYD88 gene provides instructions for making a protein involved in signaling within immune cells. The MyD88 protein acts as an adapter, connecting proteins that receive signals from outside the cell to the proteins that relay signals inside the cell. TIRAP is involved in bridging MyD88 to the receptor complex for TLR-2 and TLR4 signaling in response to bacterial infection. TICAM1 gene encodes an adaptor protein containing a Toll/interleukin-1 receptor (TIR) homology domain, an intracellular signaling domain that mediates protein-protein interactions between the Toll-like receptors (TLRs) and signal-transduction components. Tumor necrosis factor (TNF, cachexin, or cachectin; often called tumor necrosis factor-alpha or TNF-α) is an adipokine and a cytokine. HMGB1, UNC93B1, and LY96 perform related functions to innate and adaptive immunity (Chaturvedi and Pierce 2009).

Enrichment network analysis done in STRING database indicates the role of TLR9 mainly in defense response through various processes like TLR signalling, regulation of other molecules involved in various functions. The

following Table 4.7 describes these roles in relation to biological process, function and component. The strength indicated statistical support to the description compared to the background. Term ID indicates the Gene Ontology term as per The Gene Ontology Consortium available at http://geneontology.org/ (Gene Ontology Consortium, 2019).

Table 4.7 Gene ontology network of Red Kandhari TLR9

Term ID	Term description	Observed gene count	Background gene count	Strength	False discovery rate
Biological Processes					
GO:0002224	Toll-like receptor signaling pathway	9	44	2.57	1.90E-18
GO:0045087	Innate immune response	10	559	1.51	1.26E-11
GO:0006952	Defense response	11	1044	1.28	2.30E-11
GO:0006954	Inflammatory response	9	369	1.65	3.23E-11
GO:0006955	Immune response	11	1099	1.26	3.23E-11
GO:0002237	Response to molecule of bacterial origin	7	200	1.8	3.39E-09
GO:0009617	Response to bacterium	8	459	1.5	1.02E-08
GO:0071219	Cellular response to molecule of bacterial origin	6	135	1.91	3.64E-08
GO:0032103	Positive regulation of response to external stimulus	6	278	1.59	1.96E-06
GO:0043123	Positive regulation of i-kappab kinase/nf-kappabsignaling	5	131	1.84	3.33E-06
GO:0032648	Regulation of interferon-beta production	4	41	2.25	3.83E-06
GO:0034162	Toll-like receptor 9 signaling pathway	3	6	2.96	5.20E-06

GO:0031347	Regulation of defense response	6	398	1.44	1.32E-05
GO:0031349	Positive regulation of defense response	5	179	1.71	1.32E-05
Biological Function					
GO:0035325	Toll-like receptor binding	4	12	2.78	2.65E-07
GO:0038187	Pattern recognition receptor activity	3	20	2.44	0.00032
GO:0005102	Signaling receptor binding	7	1406	0.96	0.0022
GO:0001875	Lipopolysaccharide immune receptor activity	2	4	2.96	0.003
GO:0035662	Toll-like receptor 4 binding	2	4	2.96	0.003
GO:0045322	Unmethylated cpg binding	2	9	2.61	0.0074
GO:0050135	NAD(P)+ nucleosidase activity	2	14	2.41	0.0139
GO:0061809	NAD+ nucleotidase, cyclic ADP-ribose generating	2	14	2.41	0.0139
Biological Component					
GO:0030139	Endocytic vesicle	4	128	1.75	0.00087
GO:0046696	Lipopolysaccharide receptor complex	2	4	2.96	0.0031
GO:0031410	Cytoplasmic vesicle	7	1719	0.87	0.004
GO:0045335	Phagocytic vesicle	3	71	1.89	0.004
GO:0032009	Early phagosome	2	13	2.45	0.0062

4.8 Three Dimensional (3D) structure

In the 3D model depicted, the red colour shows helix structure, yellow colour sheet structure and green colour loop structure Plate No. 4.4 This horseshoe shaped structure is similar to that of previously reported TLR structures (Safi *et al.,* 2021). LRR family proteins share conserved tandem modular repeats

(Bella, J. *et al.* 2008) and horseshoe-like 3D structures, with common structural patterns recognized within their crystal structures (Werling *et al.,* 2009). This LRR repeats also recognises TLR9 agonists (Matsushima et al., 2015). The N-terminal LRR domains in TLR8 and TLR9 contain three tandem repeats of a super motif of *STT* in which "*S*" is a "Bacterial" LRR unit and "*T*" is a "Typical" LRR unit, as seen in the family of small leucine rich repeat proteoglycans (SLRPs) such as biglycan and decorin or the FLRT family (Scott *et al.,* 2006).

The Ramachandran plot can be used to assess the fitness of the model depicted. It is a plot of the torsional angles - phi (φ)and psi (ψ) - of the amino acids contained in a peptide. In our model most of the amino acids were found to be in favoured regions Plate No. 4.5, showing that the protein model is of good stereochemical quality (Safi *et al.,* 2021).

The QMEAN Z-score is an estimate of the "degree of nativeness" of the structural features observed in a model by describing the likelihood that a model is of comparable quality to high-resolution experimental structures. Benkert *et al.,* 2011 studied QMEAN Z-score analysis. Z-scores around 0.0 therefore reflect a "native-like" structure and, as a rule of thumb, a "QMEAN" Z-score below -4.0 indicates a model with low quality. This is illustrated by the "Comparison" plot. In our model we report an identity match of 99.31%. to the template. Predicted local similarity to target was more than 0.6 for the complete protein region except from 400 to 450 amino acid patches where it was 0.2. Our model showed a Z-score of 0.5 to 1, indicating that the model is of good quality (Plate No. 4.5).

4.10 Phylogenetic analysis

A phylogenetic or evolutionary tree is a branching diagram that depicts the assumed evolutionary connections between distinct biological species or entities based on physical or genetic similarities and differences. There are different methods for phylogenetic tree construction, but Maximum Likelihood (ML) methods are currently the most commonly used ones in phylogenetic tree construction—as long as sufficient computation power and time are available. For maximum likelihood tree construction, nucleotide/ amino acid substitution models

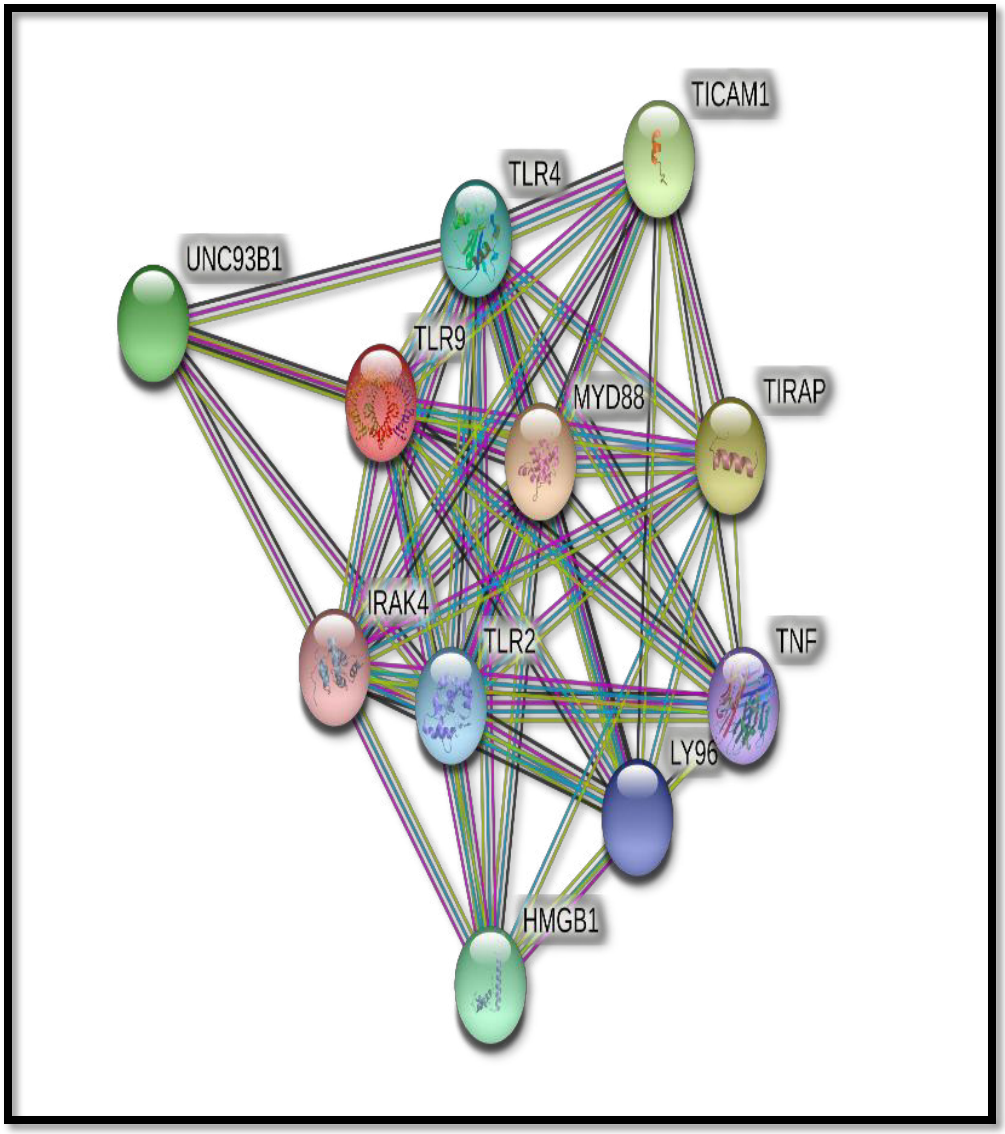
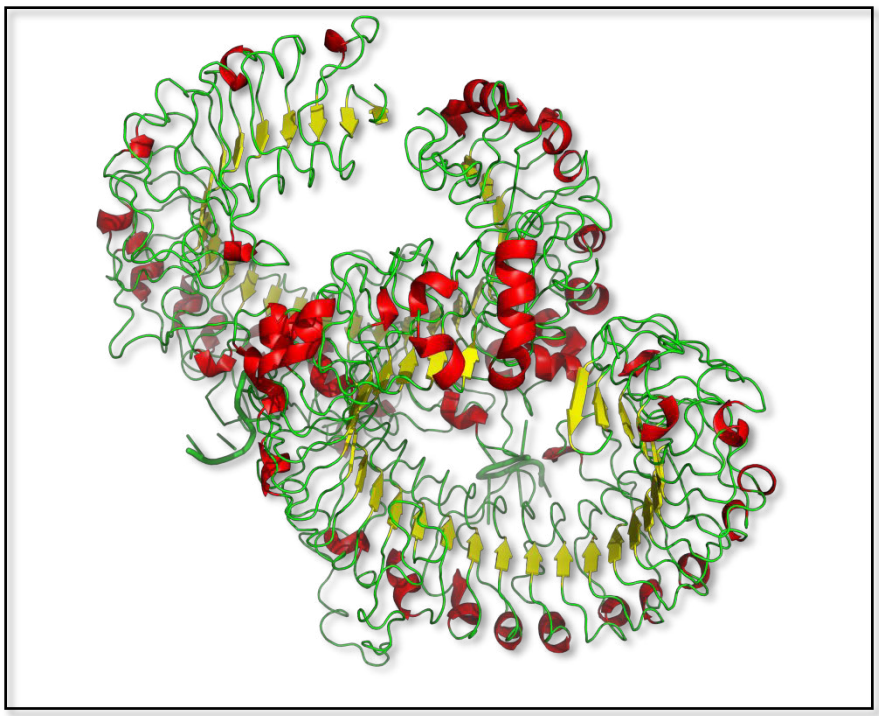
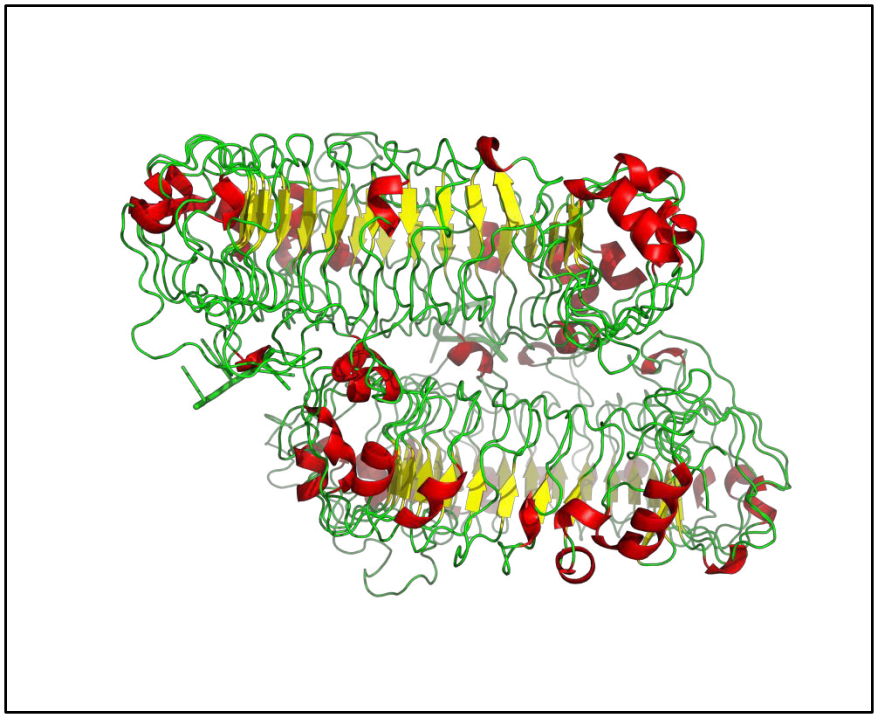


Plate No. 4.3 Protein–Protein interaction network of TLR9 of Red Kandhari cattle visualized by STRING. In this view, the colour saturation of the edges represents the confidence score of a functional association and protein molecules UNC93B1, IRAK4, HMGB1, LY96, TNF, TIRAP, TICAM1, MYD88, TLR2, TLR4, and TLR9



a) Front view of TLR 9



b) Side view of TLR 9

Plate No. 4.4 Horse shoe shaped 3D structure of TLR 9 of Red Kandhari cattle indicating helices (Red), sheets (Yellow) and loops (Green)

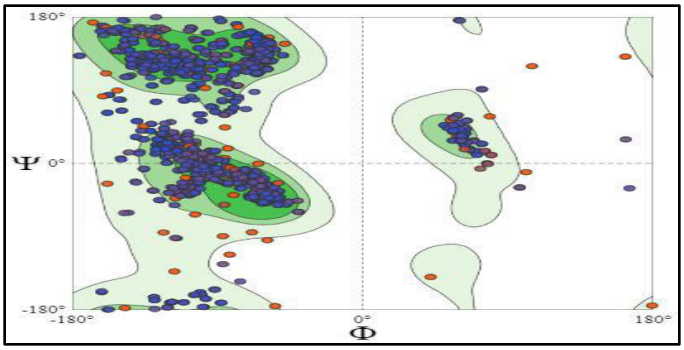


Plate No. 4.5 (a) Ramachandran plot revealing most amino acids in expected region which indicates good stereochemical properties of the model

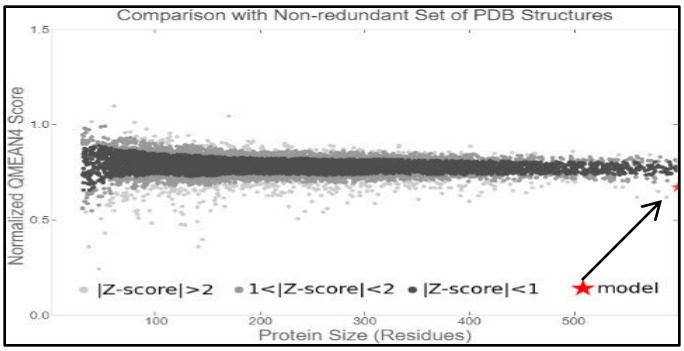


Plate No. 4.5 (b) Plot showing the spread of Z-score (indicating overall model quality) range for all known protein having solved structure. Template sequence having identity 99.31%.Predicted Local similarity to target having more than 0.6, except from 400 to 450 having nearly 0.2.

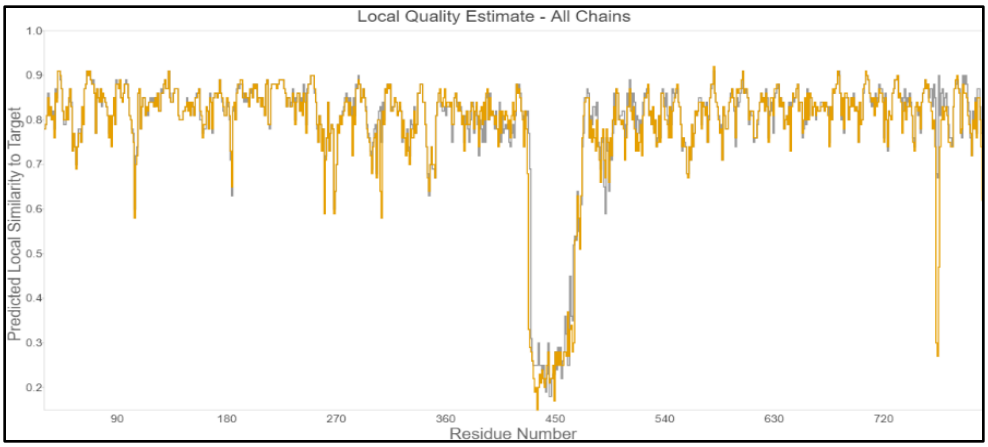


Plate No. 4.5Local quality estimate of residue number in mostly in range of 0.8 to 0.9 except from 400 to 450 residue number having nearly 0.2.

can be tested. This considers different models and selects the one that best fits the input sequence data.

To define the evolutionary relationship of TLR9 of the Red Kandhari cattle and other mammalian species, we constructed phylogenetic trees using the Maximum Likelihood method based on the canonical protein sequences. Three major TLR9 clades were recognized in the tree. TLR9 gene from related mammalian species was grouped together. Aligned sequences were tested for best amino acid substitution model. JTT+G (Jones-Taylor-Thornton) amino acid model was found to be the most favourable amino acid substitution model. Phylogenetic analysis was inferred using distance-based (maximum likelihood) with the above substitution model and 500 bootstrap replicates.

This tree is constructed with the sequences isolated in study and the representative sequences available in the NCBI databases. All six isolates from the study are clustered together in the same cluster indicating a close phylogenetic relationship. The closest relatives to these isolates are the members that belong to the Bovidae family (*Bos taurus, Bos grunniens, Bubalus bubalis, Capra hircus, Ovis aries*). All major clades have good bootstrap support (more than 69.6%)**.** The second cluster includes members from Camelids (*Camelus ferus, Camelus dromedarius)*, Suidae (*Sus scrofa),* Balaenopteridae *(Eschrichtius robustus),* Physeteridae *(Physeter catodon),* Hyaenidae *(Hyaena hyaena),* Herpestidae (*Suricata suricatta),* Felidae *(Panthera tigris, Panthera leo),* Equidae *(Equus asinus)* and third cluster includes members from Cervidae family (*Cervus elephus hippelaphus, Muntiacus muntjac).* The results showed the isolates under study belong to the cluster where other *Bos spp.* are included. This concludes that TLR9 of Red Kandhari cattle belongs to the *Bos indicus* cluster in this representative phylogenetic tree (Fig 4.2).

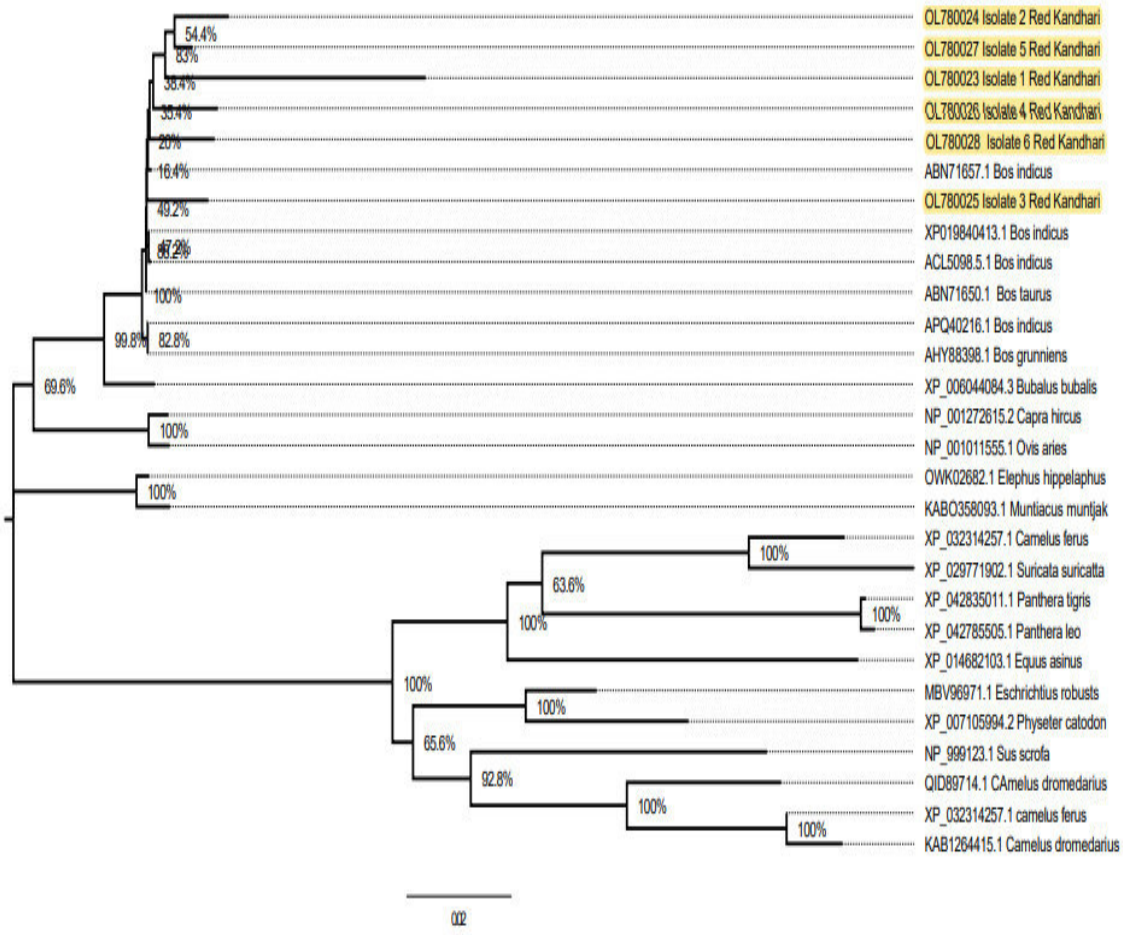


Fig 4.2 Phylogenetic tree of TLR9 of Red Kandhari cattle with nodes indicating bootstrap values

Similar work of Banarjee showed that the phylogenetic tree of *Bubalus bubalis* reveals the similarity with *Bos taurus* and *Bos indicus* amino acid sequences. The tree joined other ruminants like *Ovis aries* and *Capra hircus,* followed by other livestock species (Banerjee *et al.,* 2011). Phylogenetic analysis of TLRs of goats showed a closer relationship to sheep and cattle than other mammals (Raja *et al.,* 2011).

Summary and Conclusions

CHAPTER – V

SUMMARY AND CONCLUSION

The present study was undertaken to determine the coding DNA sequence of the TLR9 gene of Red Kandhari and understand the molecular structure of TLR9 *in silico.* Comparative evolution of TLR9 of Red Kandhari cattle was accomplished against available and related TLR9 genes in NCBI protein databases.

The Toll-like Receptor 9 is linked to the innate animal immunity of animals. TLR9 is an important receptor expressed in immune system cells including dendritic cells, macrophages, natural killer cells, and other antigen-presenting cells. TLR9 preferentially binds DNA in bacteria, viruses and triggers signaling cascades that lead to a pro-inflammatory cytokine response against pathogens.

For the study, animals were selected from the Livestock Farm Complex of the College of Veterinary and Animal Sciences, Parbhani. Blood samples were collected among selected animals. The DNA was extracted from blood samples with standard procedures. The purity and concentration of DNA was accessed. TLR9 gene coding sequences of Red Kandhari animals were amplified through Polymerase Chain Reaction using oligonucleotide primers synthesised as per previously reported reference. Then, Sanger's Sequencing was carried out for all the amplicons. Sequencing results obtained as a chromatogram were analysed and converted as a string of nucleotides. Nucleotide sequence alignment of the TLR9 gene was performed. Overlapping regions of the sequences were identified. The protein sequence was deduced with conceptual protein frame translation. The representative consensus Red Kandhari TLR9 protein sequence was obtained with a Bioinformatics tool using multiple aligned sequences.

The domain structure of TLR9 of Red Kandhari cattle was predicted. The TLR9 protein is found to contain 18 Leucine-Rich Receptors (LRRs) with a Toll Interleukin Receptor (TIR) domain in the range of 865 to 1012 amino acids. The signal sequence is upto 24^{th} amino acid from the first one. Comparison of

TLR9 amino acid sequence with other mammalian TLR9 sequences showed the highest sequence identity to *Bos indicus* (99.61%) followed by 98.54%identical to *Bos taurus, Bubalus bubalis* (96.99%). Less deviation was found in amino acids of TLR9 as compared to related species. This deviation in amino acids may contribute to a specific function of TLR9 of Red Kandhari. This variation may also be a contributing factor to the innate immune resistance which is generally found in indigenous animals.

Enrichment network analysis indicates the role of TLR9 mainly in defense response through various processes like TLR signalling, regulation of other molecules involved in various functions. Three-dimensional structure of this protein was modelled based on homology modelling. This structure is very similar to that of its closest relatives, so there is no variation as per modular structure.

The work is first report of a complete coding sequence of TLR9 in Red Kandhari cattle of the Marathwada region of Maharashtra state. Further studies of TLR9 associated network genes may elucidate their important role in innate immunity variation among Red Kandhari cattle and related breeds of cattle which may influence animals' immune response against pathogens. It is concluded that the TLR9 gene of Red Kandhari cattle is nearly identical to related species.

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Appendices

APPENDIX I

RBC lysis buffer (1X)

Ammonium chloride	8.3gm
Potassium bicarbonate	1.0gm
0.5M EDTA (pH 8.0)	299µl
Double distilled water (up to)	100ml

Adjust pH 8.0 using NaOH pellets.

Sterilized by autoclaving and store at room temperature.

DNA extraction buffer

1M tris buffer (pH 8.0)	5ml
5M NaCl	40ml
0.5M EDTA	2ml
Double distilled water (up to)	100ml

Autoclave in batches of 100ml and store at room temperature.

0.5M EDTA, pH 8.0

To 5ml of distilled water added with 1.46125gm of Ethylenediamine tetraacetic acid and was shaked vigorously on magnetic stirrer for hours. Then pH was adjusted to 8.0 with NaOH pellets, dispensed into aliquots and sterilized by autoclaving.

1M tris buffer

To prepare 1M of tris buffer 5ml of distilled water added with 0.6057gm of tris buffer and shaked vigorously on magnetic stirrer for hours. Then the solution sterilized by autoclaving.

5M NaCl

To prepare 5M of NaCl 40ml of distilled water added with 2.33gm of NaCl and was shaked vigorously on magnetic stirrer for hours. Then pH was adjusted to 8.0 with NaOH pellets, dispensed into aliquots and sterilized by autoclaving.

Agarose solution (1%)

1 gm of agarose was dissolved in 100 ml of 1X TAE.

Ethanol (70%)

In 70 ml of absolute ethanol 30 ml of double glass distilled water was added.

Ethidium bromide solution

Stock solution was prepared by using 5mg ethidium bromide per ml of TBE working solution. Working solution was prepared in concentration range of 0.5-1 mg/ml using TBE working solution.

APPENDIX II

Instrument	**Manufacturer**
Weighing machine	Contech, India
Incubator	Himedia Pvt. Ltd, India
Laminar flow	Macro scientific, India
Nanodrop lite spectrophotometer	Thermo scientific, USA
UV transilluminator	Genaxy, India
-20^0C Freezer	Mitashi, India
PCR machine	Eppendorf, Germany
PCR tubes	Eppendorf, Germany
Vortex machine	Tarsons, India
Centrifuge machine	Remi CM-12 plus, India
Micropipette	Eppendorf, Germany
Disposable micropipette tips	Eppendorf, Germany
Mini freezer	HiMedia

APPENDIX III

Details of nucleotide sequences submission of TLR 9 Red Kandhari cattle to NCBI database with Accession numbers.

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Isolate 2	OL780024
Isolate 3	OL780025
Isolate 4	OL780026
Isolate 5	OL780027
Isolate 6	OL780028

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  AUTHORS   Devkatte,V., Suryawanshi,P.R., Nandedkar,P.V., Ghorpade,P.B. and
            Gaikwad,S.S.
  TITLE     Molecular Characterisation of coding sequence of TLR 9 in Red
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  JOURNAL   unpublished
REFERENCE   2  (bases 1 to 4743)
  AUTHORS   Devkatte,V., Suryawanshi,P.R., Nandedkar,P.V., Ghorpade,P.B. and
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  TITLE     Direct Submission
  JOURNAL   Submitted (02-DEC-2021) Dept. of Vety. Biotechnology Education and
            Research Cell, College of Veterinary & Animal Sciences, Inside MAU
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COMMENT     Bankit Comment: TOTAL # OF SEQS:6

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  TITLE     Molecular Characterisation of coding sequence of TLR 9 in Red
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  JOURNAL   unpublished
REFERENCE   2  (bases 1 to 4743)
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DEFINITION  Bos indicus breed Red Kandhari.
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            Mammalia; Eutheria; Laurasiatheria; Artiodactyla; Ruminantia;
            Pecora; Bovidae; Bovinae; Bos.
REFERENCE   1  (bases 1 to 4948)
  AUTHORS   Devkatte,V., Suryawanshi,P.R., Nandedkar,P.V., Ghorpade,P.B. and
            Gaikwad,S.S.
  TITLE     Molecular Characterisation of coding sequence of TLR 9 in Red
            Kandhari breed of Bos indicus
  JOURNAL   unpublished
REFERENCE   2  (bases 1 to 4948)
  AUTHORS   Devkatte,V., Suryawanshi,P.R., Nandedkar,P.V., Ghorpade,P.B. and
            Gaikwad,S.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-DEC-2021) Dept. of Vety. Biotechnology Education and
            Research Cell, College of Veterinary & Animal Sciences, Inside MAU
            campus, Parbhani, Maharashtra 431402, India
COMMENT     Bankit Comment: TOTAL # OF SEQS:6

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            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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DEFINITION  Bos indicus breed Red Kandhari.
ACCESSION   Isolate4
VERSION
KEYWORDS    .
SOURCE      Bos indicus (Bos taurus indicus)
  ORGANISM  Bos indicus
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            Pecora; Bovidae; Bovinae; Bos.
REFERENCE   1  (bases 1 to 4948)
  AUTHORS   Devkatte,V., Suryawanshi,P.R., Nandedkar,P.V., Ghorpade,P.B. and
            Gaikwad,S.S.
  TITLE     Molecular Characterisation of coding sequence of TLR 9 in Red
            Kandhari breed of Bos indicus
  JOURNAL   unpublished
REFERENCE   2  (bases 1 to 4948)
  AUTHORS   Devkatte,V., Suryawanshi,P.R., Nandedkar,P.V., Ghorpade,P.B. and
            Gaikwad,S.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-DEC-2021) Dept. of Vety. Biotechnology Education and
            Research Cell, College of Veterinary & Animal Sciences, Inside MAU
            campus, Parbhani, Maharashtra 431402, India
COMMENT     Bankit Comment: TOTAL # OF SEQS:6

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VERSION
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SOURCE      Bos indicus (Bos taurus indicus)
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REFERENCE   1  (bases 1 to 4743)
  AUTHORS   Devkatte,V., Suryawanshi,P.R., Nandedkar,P.V., Ghorpade,P.B. and
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  TITLE     Molecular Characterisation of coding sequence of TLR 9 in Red
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  JOURNAL   unpublished
REFERENCE   2  (bases 1 to 4743)
  AUTHORS   Devkatte,V., Suryawanshi,P.R., Nandedkar,P.V., Ghorpade,P.B. and
            Gaikwad,S.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-DEC-2021) Dept. of Vety. Biotechnology Education and
            Research Cell, College of Veterinary & Animal Sciences, Inside MAU
            campus, Parbhani, Maharashtra 431402, India
COMMENT     Bankit Comment: TOTAL # OF SEQS:6

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LOCUS       Isolate6                4846 bp    DNA     linear   MAM 02-DEC-2021
DEFINITION  Bos indicus breed Red Kandhari.
ACCESSION   Isolate6
VERSION
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SOURCE      Bos indicus (Bos taurus indicus)
  ORGANISM  Bos indicus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Laurasiatheria; Artiodactyla; Ruminantia;
            Pecora; Bovidae; Bovinae; Bos.
REFERENCE   1  (bases 1 to 4846)
  AUTHORS   Devkatte,V., Suryawanshi,P.R., Nandedkar,P.V., Ghorpade,P.B. and
            Gaikwad,S.S.
  TITLE     Molecular Characterisation of coding sequence of TLR 9 in Red
            Kandhari breed of Bos indicus
  JOURNAL   unpublished
REFERENCE   2  (bases 1 to 4846)
  AUTHORS   Devkatte,V., Suryawanshi,P.R., Nandedkar,P.V., Ghorpade,P.B. and
            Gaikwad,S.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-DEC-2021) Dept. of Vety. Biotechnology Education and
            Research Cell, College of Veterinary & Animal Sciences, Inside MAU
            campus, Parbhani, Maharashtra 431402, India
COMMENT     Bankit Comment: TOTAL # OF SEQS:6

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Vitae

VITAE

The author **Devkatte Vyankatrao Subhanrao** is born on 22nd July, 1995 and blossomed at Post. Sendursana, Tq. Aundha Nagnath, Dist. Hingoli in Maharashtra state, India.

He has passed SSC board examination with distinction and excellent academic record from Swami Vivekanand Vidyalaya, Shirur Tajband, Tq. Ahemdpur, Dist. Latur in the year 2011 and HSC examination in the year 2013 from Narhar Kurundkar Junior College, Kurunda, Tq. Basmath, Dist. Hingoli.

As a dignified and noble profession, he joined Veterinary education at College of Veterinary and Animal Sciences, Parbhani in 2014 and successfully completed B. V. Sc. and A. H. degree course in second division from Maharashtra Animal and Fishery Sciences University, Nagpur in 2019. He later joined Master's Degree programme and completed coursework in Animal Biotechnology from College of Veterinary and Animal Sciences, Parbhani under Maharashtra Animal and Fishery Sciences University, Nagpur.

He is registered member of Maharashtra State Veterinary Council, Nagpur. He is an active member of National Service Scheme and participated voluntary in several camps during his graduation and post-graduation studies.

With persistence guidance from his guide, he is committed to pursue the research application of knowledge in Animal Biotechnology learnt in the master's curriculum.

Name: Dr. Devkatte Vyankatrao Subhanrao

e-Mail ID: vnaik0404@gmail.com

Thesis Abstract

THESIS ABSTRACT

a.	Title of the thesis (In Capital letters)	:	**MOLECULAR CHARACTERIZATION OF TOLL LIKE-RECEPTOR 9 OF RED KANDHARI CATTLE.**
b.	Full name of student	:	**DEVKATTE VYANKATRAO SUBHANRAO**
c.	Name and address of Major Advisor	:	**Dr. S. S. GAIKWAD,** Assistant Professor, Department of Animal Biotechnology, COVAS, Parbhani.
d.	Degree to be awarded	:	**M. V. Sc.**
e.	Year of award of degree	:	**2022**
f.	Major subject	:	**Animal Biotechnology**
g.	Total number of pages in the thesis	:	**50**
h.	Number of words in the abstract	:	**316**
i.	Signature of Student	:	
j.	Signature, Name and address of forwarding authority (HOD/SH)	:	**Dr. P. R. Suryawanshi,** HOD, Department of Veterinary Microbiology, COVAS, Parbhani

ABSTRACT

Toll-like receptors (TLRs) detect conserved pathogen-associated molecular patterns (PAMPs) on a range of microbes, leading to innate immune activation and orchestration of the adaptive immune responses. The current study aimed to determine the coding DNA sequence of TLR9 gene of Red Kandhari and study of the molecular structure of TLR9 *in silico.* Comparative evolution of TLR9 of Red Kandhari cattle was accomplished against available and related TLR9 proteins in NCBI protein databases. Blood samples were collected from Red Kandhari cattle, maintained at Livestock Farm Complex, College of Veterinary and Animal Sciences, Parbhani. The genomic DNA was extracted, and a polymerase chain reaction was carried out to amplify TLR9. The amplified product of TLR9 was sequenced by Sanger sequencing. Deduced protein sequence inferred from conceptually translated nucleotide sequence was studied to understand TLR9 structure *in silico*. TLR9 protein of Red Kandhari cattle has 1029 amino acids with a molecular weight of 115.4 Kda. It has the highest proportion of Leucine (18.6%), followed by Serine (8.7%). Comparison of TLR9 amino acid sequence with related mammalian TLR9 sequences showed the highest sequence identity 99.61% identical to *Bos indicus* followed by *Bos taurus* (98.54%), *Bubalus bubalis* (96.99%), *Capra hircus* (94.27%), *Ovis aries* (94.27%). Eighteen LRR domains were found, which is a characteristic of this class of proteins. The 3D structure of TLR9 prepared by homology modeling looks like a horseshoe is identical to its closest relatives and showed good stereochemical quality according to the Ramachandran plot. Protein interaction predictions and gene ontology network study of Red Kandhari TLR9 protein revealed its role in innate immune activation and orchestration of other processes. This is the first study of the coding gene sequence of TLR9 of Red Kandhari cattle, which is important in understanding innate immunity phenomenon in cattle of the Marathwada region of state Maharashtra cattle. The study will further facilitate elucidation of the immune gene networks of Red Kandhari cattle.

प्रबंध सारांश

a)	**प्रबंधाचे शीर्षक**	:	लाल कंधारी गायींच्या टोल सारख्या संवेदक ९ चे आण्विक गुणवर्णन
b)	**विद्यार्थ्याचे पूर्ण नाव**	:	देवकत्ते व्यंकटराव सुभानराव
c)	**प्रमुख मार्गदर्शकाचे नाव व पत्ता**	:	डॉ. एस. एस. गायकवाड सहायक प्राध्यापक, पशु जैवतंत्रज्ञान विभाग, पशुवैद्यक व पशुविज्ञान महाविद्यालय, परभणी.
d)	**प्रदान करण्यात येणारी पदवी**	:	एम. व्ही. एससी.
e)	**पदवी प्रदान करण्याचे वर्ष**	:	२०२२
f)	**मुख्य विषय**	:	पशु जैवतंत्रज्ञान
g)	**प्रबंधातील पानांची एकूण संख्या**	:	५०
h)	**सारांशामधील शब्दांची संख्या**	:	२९३
i)	**विद्यार्थ्यांची स्वाक्षरी**	:	
j)	**पाठविणाऱ्या अधिकाऱ्याची स्वाक्षरी, नाव व पत्ता (विभाग प्रमुख)**	:	

डॉ. पी. आर. सूर्यवंशी,
विभाग प्रमुख,
पशुवैद्यकीय सुक्ष्मजीवशास्त्र
विभाग.

सारांश

टोल समान संवेदक हे प्राण्यांच्या जन्मजात प्रतिकारशक्तीमध्ये महत्त्वाची भूमिका बजावतात कारण ते रोगजनक संबंधित आण्विक प्रकारां द्वारे सूक्ष्मजंतू ना शरीरात रोखण्यास मदत करतात ज्यामुळे जन्मजात रोगप्रतिकारक्षमता चांगल्या प्रकारे विकसित होते. सदरील संशोधनाद्वारे लाल कंधारी प्राण्यांच्या टोल समान संवेदक ९ या जनुकाच्या आण्विक संरचनेचा संगणकीय प्रणालीद्वारे, इतर प्राण्यांचे समवेत तुलनात्मक उत्क्रांतीचा अभ्यास करण्यात आला. लाल कंधारी प्राण्यांच्या टोल समान संवेदक ९ ची तुलनात्मक उत्क्रांती, राष्ट्रीय जैवतंत्रज्ञान माहिती केंद्राच्या (NCBI) डेटाबेसमध्ये उपलब्धअसणाऱ्या इतर पशु सोबत करण्यात आली आहे. सदर संशोधनासाठी परभणी येथील पशुवैद्यक व पशुविज्ञान महाविद्यालय अंतर्गत असणाऱ्या पशुधन प्रक्षेत्रावरील लाल कंधारी प्राण्यांचे रक्त नमुने गोळा करण्यात आले व त्यापासून डीएनए वेगळा करण्यात आला आणि पी. सी. आर. चाचणी करण्यात आली. टोल समान संवेदक ९ चे सँगर सिक्वेन्ससिंग तंत्राद्वारे अनुक्रमिक निर्धारित करण्यात आले आणि अनुक्रम ठरवण्यात आला. टोल समान संवेदक ९ ची रचना समजून घेण्यासाठी टोल समान संवेदक ९ ची थ्रीडी प्रारूपहोमोलॉजी मॉडेलिंगद्वारे करण्यात आले. लाल कंधारी गुरांच्या टोल समान संवेदक ९ प्रथिनांच्या अमिनो आम्लं रचनेमध्ये ल्युसीन (१८.६%) चे प्रमाण सर्वाधिक आहे त्यानंतर सेरीन (८.७ %) आहे. या प्रथिनामध्ये ल्युसीन समृद्ध पुनरावृत्ती ची उच्च टक्केवारी हे या वर्गातील प्रथिनांचे वैशिष्ट्य आहे. इतर सस्तन प्राण्यांच्या टोल समान संवेदक ९ अनुक्रमांशी लाल कंधारी च्या टोल समान संवेदक ९ अमिनो आम्लांची तुलना केल्यावर देशी गाय (९९.६१%), विदेशी गाय (९८.५४), म्हैस (९६.९९%), शेळी (९४.२७%), व मेंढी (९४.२७%) इतके आढळून आले. संकल्पनात्मक प्रथिनांची त्रिमीतीय रचना लालकंधारी प्राण्यांच्या जवळ/कुळातील असणाऱ्या इतर प्राण्यांसारखे आहे. लाल कंधारी TLR9 प्रथिनांच्या परस्परसंवादाची आणि जीन ऑन्टोलॉजी नेटवर्क अभ्यासाने जन्मजात रोगप्रतिकारक सक्रियता आणि इतर प्रक्रियांच्या कार्यामध्ये त्याची भूमिका स्पष्ट होते. संकल्पनात्मक त्रिमीतीय रचनेची गुणवत्ता रामचंद्रन आलेखानुसार चांगली आहे. लाल कंधारी गुरांच्या टोल समान संवेदक ९ च्या कोडींग जनुकाचा हा पहिलाच अभ्यास आहे, जो महाराष्ट्र राज्याच्या मराठवाडा भागातील गुरांमध्ये जन्मजात रोगप्रतिकारक शक्ती समजण्यासाठी महत्त्वाचा आहे. सदरील संशोधनामुळे लाल कंधारी गायींच्या जन्मजात प्रतिकारशक्ती समजून घेणे अधिक सुलभ होईल.