Molecular characterization of *mannose binding lectin gene1 (MBL1)* in *Bubalus bubalis* & its variability in cattle and buffalo genome

THESIS

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OF

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

BY

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Enrolment No. B-1385/14

COLLEGE OF BIOTECHNOLOGY

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Mathura-281001 (UP)

(2020)

CERTIFICATE

This is to certify that the thesis entitled "**Molecular characterization of *mannose binding lectin gene1 (MBL1)* in *Bubalus bubalis* & its variability in cattle and buffalo genome**" submitted by **Manali Baghel**, Enrolment No. **B-1385/14** in partial fulfillment of the requirement for the award of **Doctor of Philosophy** in **Biotechnology** of the **U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan, Mathura (UP)**, India, is a bonafide research work carried out by her under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated: 27/08/2020

(Deepak Sharma)

Major Advisor / Chairman
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CERTIFICATE

It is certified that the thesis entitled "**Molecular characterization of *mannose binding lectin gene1 (MBL1)* in *Bubalus bubalis* & its variability in cattle and buffalo genome**" submitted by **Manali Baghel**, Enrolment No. **B-1385/14** in partial fulfillment of **Doctor of philosophy** in **Biotechnology** at **U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan, Mathura (UP)**, embodies the original work done by the candidate herself. The candidate has carried out her work sincerely and methodically.

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College	:	College of Biotechnology
Title of the thesis	:	Molecular characterization of mannose binding lectin gene1 (MBL1) in Bubalus bubalis & its variability in cattle and buffalo genome
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Manali

Date: 27/08/20 ***(Manali Baghel)***

Place: Mathura

′	Prime
%	Percentage
*	Asterisk
<	Less than
>	Greater than
χ^2	Chi square
°C	Degree Celsius
Σ	Sigma
µ	Micro
µL	Micro liter
µM	Micro molar
A	Adenine
AFC	Age at first calving
Amp^R	Ampicillin resistance
ANOVA	Analysis of variance
bp	Base pair
BLAST	Basic local alignment search tool
C	Cytosine
cDNA	Complementary Deoxyribonucleotide triphosphate
CDS	Coding DNA Sequence
cm	Centimeter
CRS-PCR	Created restriction-site polymerase chain reaction
DOB	Date of birth
DNA	Deoxyribonucleic acid
dNTPs	2´-deoxyribonucleotide tri phosphate
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium bromide
etc.	et cetera/And so forth
et, al.	et alii /alia
F	Forward
Fig.	Figure

g	Gram
G	Guanine
GLM	General linear model
hr	Hour
HCl	Hydrochloric acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kbp	Kilo base pair
KCl	Potassium Chloride
kDa	Kilo Dalton
kg	Kilo gram
L	Liter
LB	Luria Bertani
LFC	Livestock farm complex
LN_2	Liquid nitrogen
LP	Lactation period
M	Molar
MBL	Mannose binding lectin
MASP	MBL-associated serine proteases
MMT	Million metric tonnes
MY300	Milk yield in 300 days
mg	Milligram
Mg^{++}	Magnesium divalent ion
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Milliliter
Na acetate	Sodium acetate
NaCl	Sodium Chloride
NCBI	National center for biotechnology information
ng	Nanogram
O.D.	Optical Density
ORF	Open reading frame
P	Probability value
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction

PY	Peak yield
pH	Concentration of Hydrogen Ion
pmol	Pico mole
QTL	Quantitative Trait Loci
R	Reverse
RBC	Red Blood Corpuscles
RE	Restriction Endonuclease
RFLP	Restriction Fragment Length Polymorphism
rpm	rotation per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SCS	Somatic cell scores
SCC	Somatic cell count
SDS	Sodium Dodecyl Sulphate
S.E.M.	Standard error of mean
sec	Second
S. No.	Serial Number
SNP	Single nucleotide polymorphism
SSCP	Single strand confirmation polymorphism
T	Thymine
TAE	Tris Acetate EDTA
Taq	Thermus aquaticus
TBE	Tris Boric acid EDTA
tdH_2O	Triple distilled H_2O
TE	Tris EDTA
TMY	Total milk yield
U	Unit
UTR	Untranslated Region
UV	Ultraviolet
V	Volts
W/V	Weight by Volume
WBC	White Blood Corpuscles
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside

AMINO ACID ABBREVIATIONS

1-letter abbreviation	3-letter abbreviation	Amino acid
A	Ala	Alanine
R	Arg	Arginine
N	Asn	Asparagine
D	Asp	Aspartic acid
C	Cys	Cysteine
E	Glu	Glutamic acid
Q	Gln	Glutamine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
L	Leu	Leucine
K	Lys	Lysine
M	Met	Methionine
F	Phe	Phenylalanine
P	Pro	Proline
S	Ser	Serine
T	Thr	Threonine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
V	Val	Valine

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INTRODUCTION

The total cattle population in India is 192.49 million out of which indigenous cattle population is 142.11 million and exotic/crossbred cattle population is 50.42 million. The total number of buffalo in India is 109.85 million and they comprise around 56% of the total world's buffalo population (20th Livestock Census, 2019). India has rich repository of well descript breeds of buffalo and cattle with 17 recognized buffalo breeds and 50 registered native cattle breeds (NBAGR, 2020). Among them, Murrah is the best breed of buffalo in the world having high butter fat content (7.83 %) and 1500-2500 kg milk production per lactation (Yadav *et al.,* 2017). One of the best milch breed of cattle in India is Sahiwal, which has the capacity to give high quality milk on an average of 2350 kg/lactation and Hariana is best dual purpose cattle breed of India producing on an average 1700 kg of milk per lactation (NBAGR, 2020).

In spite of having large number of indigenous breeds, unfortunately a number of native breeds are facing fast genetic degradation and dilution because of intensive production system, unplanned use of semen of exotic breed. The germplasm of such well-defined breeds constitute a valuable genetic resource which needs to be conserved on priority basis. According to 20th Livestock Census (2019), the total indigenous cattle population has decreased by 6% and crossbred population has increased by 29.3% in 2019 as compared to previous census. Hence, there is urgent need to conserve indigenous germplasm including Murrah buffalo and Sahiwal and Hariana breeds of cattle along with selection for the desirable production and reproduction traits. It will improve the production potential of our indigenous breeds to cope up with the ever increasing demand of milk and milk products for constantly increasing human population of the country.

India places first among the world's milk producing countries. The milk production has increased from 176.35 million metric tonnes (MMT) in 2018 to 187.8 MMT in 2019 registering a growth of 6.5%. A large population of indigenous cattle/non-descript (142.11 million) contributes only 21% as compared to 27% by a small population of crossbreds/exotic (50.42 million) to total milk production in the country (Basic Animal Husbandry Statistics, DAHD & F, 2019). There has been steady increase in per capita availability of milk since 2004-05 as the per capita availability has increased from 233 gm/day in 2004-05 to 394 gm/day in 2019 (Basic Animal Husbandry and Fisheries Statistics report, DAHD & F, 2019). It is observed that the milk yield per head is low in India and to deal with the growing demand of milk and milk products due to constantly increasing human population of the country, it is

essential to increase the production potential of indigenous livestock especially the cattle and buffalo. Among the numerous logjams in accomplishing the milk production targets, disease occurrence continues to stay the most challenging obstacle. Traditional breeding strategies have been focussed primarily on increasing performance such as milk production, growth rate and reproduction rate but the subject of improvement in the disease resistance and decrease in disease incidence has not been addressed satisfactorily. Hence there is an instantaneous need to embrace disease resistance in selection criteria. Infectious diseases had very harmful impact to animal health lead to reduced durability, productivity and forfeit to livestock industry.

To improve disease resistance, the selection criterion has been shifted from phenotypically expressed disease status to allelic status at the DNA level. The use of DNA markers to define the genetic makeup (genotype) and foretell the performance of an animal is an influential support to animal breeding. Such an approach is known as marker-assisted selection (MAS) which facilitate the exploitation of existing genetic diversity in breeding populations and can be used to improve a whole range of desirable traits. Numerous research projects have been undertaken for molecular genetic characterization of native breeds of livestock, which would be profitable in better understanding of the farm animal biodiversity and formulating action plans on conservation and management of animal genetic resources of our country. Moreover, association of identified genotypes with various economic traits and analysis of gene function can be used to determine which polymorphisms will be useful as markers for desirable traits.

Several researchers identified mannose-binding lectin (*MBL*) gene had a putative clinical role and suggested that genetically determined variation in MBL serum concentration influenced the susceptibility to and course of different types of infections as well as autoimmune, metabolic and cardiovascular diseases. MBL, a collagen like serum protein is one of the important components of innate immunity (Worthely, 2005; Bouwman, 2006). It provides first line of defence by its ability to bind the bacterial surface through its carbohydrate recognition domain and activates the complement pathway leading to lysis of bacteria independent of antibody (Dommett *et al.,* 2006). Previously reported that MBL is a member of the collectin family of proteins, which are prepared in the liver and can opsonize bacteria by tagging the surface of a pathogen to facilitate recognition and ingestion by phagocytes. The collectins got their name on account of having a collagen-like region and a lectin region. It binds to the repeating sugar arrays on many microbial surfaces through multiple lectin domains and that binding is able to activate the complement system *via* MBL associated serum proteases (MASPs). Most mammals have two *MBL* genes, *MBL1* and

MBL2, which encode the MBL-A and MBL-C proteins, respectively. MBL deficiency was relatively common in human, mice, pigs, cattle and other mammals and there was increasing evidence that it had a complex role in many diseases (Turner and Hamvas, 2000; Van de Waterning *et al.*, 2004; Frederiksen *et al.*, 2006; Phatsara *et al.*, 2007). Mutations in *MBL1*gene have been reported to contribute to the variation of animal susceptibility to different infections including dairy mastitis (Lillie *et al.*, 2007; Capparelli *et al.*, 2008; Liu *et al.*, 2011; Juul-Madsen *et al.*, 2011a).

Impaired disease resistance has been associated with single-nucleotide polymorphisms (SNPs) within the coding region of *MBL1* in various breeds of pigs (Lillie *et al.*, 2006a). Birds of low MBL type responded less forcefully to viral and bacterial infections as compared to high MBL type of birds (Schou *et al.*, 2010; Juul-Madsen *et al.*, 2011b). Moreover, in chickens of low MBL type, weight gain rate was reduced after an infection with *Escherichia coli* (Norup *et al.*, 2009). In addition, it has been found that *MBL* in Hu sheep contained a vast number of polymorphisms (SNPs and deletions) influenced the serum MBL concentration (Zhao *et al.*, 2011). A study in mice provided direct evidence that MBL played a key role in restricting the complications associated with *S. aureus* infection (Shi *et al.*, 2004). Three frequently occurring SNPs in the coding region of human *MBL* gene were associated with the abnormal polymerization, decreased serum concentration and strongly impaired function of MBL protein (Sumiya *et al.*, 1991; Madsen *et al.*, 1994; Lipscombe *et al.*, 1995 and Larsen *et al.*, 2004). Accumulating data have revealed that haplotype combinations of human *MBL2* SNPs generate increased susceptibility to various bacterial, viral and parasitic diseases as well as to systemic lupus erythematosus, rheumatoid arthritis, hepatitis, cystic fibrosis and ischemia-reperfusion injury (Eisen and Minchinton, 2003; Holmskov *et al.*, 2003; Takahashi *et al.*, 2005).

Several studies have been reported that *MBL1* gene polymorphisms were associated with milk somatic cell score (SCS) in cattle (Liu *et al.*, 2010; Wang *et al.*, 2011; Liu *et al.*, 2011; Yuan *et al.*, 2013). Milk somatic cell count (SCC) can serve as a surrogate trait for dairy mastitis resistance (Koivula *et al.*, 2005; Sharma *et al.*, 2006; Tal-Stein *et al.*, 2010; Yuan *et al.*, 2011, 2012). The selection of lower SCC and SCS for dairy breeding strategy could reduce the occurrence of dairy mastitis (Lund *et al.*, 1999; Carlen *et al.*, 2004; Yuan *et al.*, 2011, 2012). A case-control study has been demonstrated that polymorphism in *MBL2* locus of water buffalo (*Bubalus bubalis*) was associated with susceptibility and resistance to *Brucella abortus* infection (Capparelli *et al.*, 2008). *MBL1* gene in the porcine and bovine is considered as a candidate gene for mastitis resistance (Koivula *et al.*, 2005; Phatsara *et al.*, 2007; Liu *et al.*, 2011; Wang *et al.*, 2012). It possibly contributed to bacterial infection

resistance and was proposed as an indirect molecular marker of milk production traits to control mastitis and to improve dairy mastitis resistance traits in cattle (Liu *et al.,* 2011; Yuan *et al.,* 2013).

Bovine mastitis is an economically important inflammatory udder disease creating havoc to the dairy industry worldwide (Russell *et al.,* 2012). It is an economically devastating disease of livestock and an inflammatory condition of the mammary gland caused by microorganisms as diverse as bacteria, viruses, mycoplasma, yeasts and algae (Awale *et al.,* 2012) or can be due to thermal, chemical or mechanical injury (Zhang *et al.,* 2009). Bacterial mastitis is the most common (Bansal and Gupta, 2014) which can be due to contagious pathogens or environmental pathogens (Bramley, 1996). Mastitis is characterized by physical, chemical and bacteriological changes in the milk and pathological changes in the glandular tissue of the udder and affects the quality and quantity of milk. The bacterial contamination of milk from the affected cows render it unfit for human consumption and provides a mechanism of spread of diseases like tuberculosis, sore-throat, Q-fever, brucellosis, leptospirosis etc. and has zoonotic importance (Sharma *et al.,* 2011).

Development of animals tolerant to mastitis was always sought after by researchers owing to the economic importance of the disease (Rupp and Biochard, 1999). Mastitis can be manifested as clinical and subclinical forms. The clinical mastitis shows observable symptoms such as red and swollen mammary glands *i.e.,* red swollen udder and production of clotted milk while subclinical mastitis do not reveal any apparent signs but is characterized by high SCC, a normal or elevated body temperature, and milk samples that should be test positive on culture (Awale *et al.,* 2012). Somatic cells are indicators of both resistance and susceptibility of cattle and buffalo to mastitis. It can be used to monitor the level or occurrence of subclinical mastitis in herds and in individual animal (Brien *et al.,* 1999; Leitner *et al.,* 2000 and Pillai *et al.,* 2001). SCC is a useful predictor of intramammary infection and therefore important component of milk in assessment of aspects of quality, hygiene and mastitis (Hamann, 2002; Sharma *et al.,* 2011).

A number of candidate genes identified to be associated with mastitis tolerance of which *MBL1* gene required special attention (Gjerstorff *et al.,* 2004b). A number of variations have been found in both the coding as well as the non-coding regions of the *MBL* gene in human, sheep, porcine and cattle, of which several variations affected the assembly of *MBL*, thus lead to a low level of plasmic MBL and innate immune dysfunctions (Madsen *et al.,* 1998; Lillie *et al.,* 2007; Thiel and Gadjeva, 2009; Juul-Madsen *et al.,* 2011a; Liu *et al.,* 2011; Zhao *et al.,* 2011). Both *MBL1* and *MBL2* mutations have been shown to vary the susceptibility of animals to various infections (Lillie *et al.,* 2005). Recently, several SNPs in

the *MBL1* gene have been searched for its association with mastitis tolerance and a few of them were found to be associated with mastitis (Wang *et al.,* 2011). However, little information is available about the *MBL1* SNPs in bovine and their relationship with various infectious diseases.

The study of DNA polymorphism and its association with SCC or milk production traits will help to define future breeding strategies through MAS (Philipsson *et al.,* 1995; Lund *et al.,* 1999 and Carlen *et al.,* 2004). Currently worldwide most of the researches have been targeted SNP markers that were linked to mastitis tolerance in an attempt to understand the genetics of host resistance to mastitis and this knowledge will be helpful in formulating breeding programmes in an endeavour to control mastitis. These results suggested that *MBL* may play a role in innate immunity against a wide range of microorganisms and buffalo *MBL1* gene may also be a promising genetic marker for selection of improved disease resistance and high quality milk production as buffalo is an economically important resource for the dairy industry. Keeping all these points in view, the present study was carried out in Sahiwal, Hariana cattle and Murrah buffalo with following objectives:

OBJECTIVES:

1. To clone and characterization of *MBL1* CDS in buffalo.
2. To study the DNA polymorphism of *MBL1* gene in cattle and buffalo.
3. To analyze the association of allelic variants with somatic cell count (SCC) or milk production traits.

CHAPTER-2

REVIEW OF LITERATURE

Bovine mastitis is an inflammatory condition of the mammary gland caused by variety of microorganisms (Awale *et al.,* 2012). It is an economically devastating disease of livestock harming to the milk industry worldwide (Russell *et al.,* 2012). Bacterial mastitis is the most common and is characterized by physical, chemical and bacteriological changes in the milk and affects the quality and quantity of milk. Modern breeding strategies had been applied and primarily focussed on the improvement in the disease resistance with decrease in disease incidence. To improve disease resistance, the criterion of selection has been changed from phenotypically expressed disease status to gene level.

Several genes played an important role in disease resistance for mastitis in livestock such as lactoferrin, lysostaphin, bovine inhibin alpha, toll like receptors including *TLR2* and *TLR4*, beta defensin, interleukin, mannose binding lectin (*MBL*) and tumour necrosis factor (*TNF*) etc. Among them, *MBL* gene which is associated with the innate immune response and is a member of the collectin family of proteins (Wang *et al.,* 2008; Huang *et al.,* 2010).

2.1 Mannose-Binding Lectin (*MBL*) gene

Mannose binding lectin (*MBL*), previously known as mannan binding protein (MBP) is a C-type or calcium-dependent serum lectin with primary specificity for sugars such as D-mannose, N-acetylglucosamine (GlcNAc), N-acetylmannosamine (ManNAc) and L-fucose (Kawasaki *et al.,* 1983; Sheriff *et al.,* 1994). Kilpatrick (2002) identified the role of MBL protein in the innate immune response by binding to carbohydrates on the surface of a wide range of pathogens (viruses, bacteria, fungi and protozoa) where it could triggered the complement system or acted directly as an opsonin. *MBL* is a member of the collectin family of proteins, which is mainly prepared in the liver and can opsonize bacteria by tagging the surface of a pathogen to facilitate recognition and ingestion by phagocytes.

Over the past twenty years *MBL* has been the focus of interest for several research groups around the world owing to its clinical role, especially based on epidemiological studies that have suggested that genetically determined variation in MBL serum concentration influences the susceptibility to different types of infections and diseases. The MBL concentration in serum varies widely (0–10.000 ng/ml) but is markedly stable for each individual (Thiel *et al.,* 1992; Steffensen *et al.,* 2000; Saevarsdottir *et al.,* 2001 and Minchinton *et al.,* 2002). MBL deficiency has a complex role in many diseases (Yuan et al., 2012). High MBL levels, on the contrary might be helpful to intracellular organisms, which

take the advantage of C3 opsonization and C3 receptor on monocytes/macrophages to enter their host.

2.1.1 History of *MBL* gene

The original discovery of *MBL* may be traced back to findings made right after the Second World War, when it was discovered that factors in non-immune bovine and murine serums could inhibit influenza virus-induced haemagglutination and lead to influenza virus neutralization (Malhotra and Sim, 1995). These findings have been repeated and it appeared that at least one of these factors might be *MBL* (Anders *et al.,* 1993). However, the clinical interest in *MBL* originated from an observation made several decades ago when a serum-dependent defect of phagocytosis on yeast particles was described (Miller *et al.,* 1968). The defect was subsequently linked to the complement system, because C3 was deposited in lower amounts on yeast surfaces incubated in sera from affected individuals (Turner *et al.,* 1981). However, no abnormality in complement function as such could be shown, suggested that a hitherto unknown serum component was the key mediator of the impaired complement deposition. Independent of these efforts, a protein that could be extracted from rabbit liver using mannan particles from *Saccharomyces cerevisiae* as probe was discovered. This protein was initially given the name mannan-binding protein that we today known as MBL (Kawasaki *et al.,* 1978). Subsequently, it was shown that the originally described phagocytic defect was due to deficiency of MBL (Supar *et al.,* 1989). Further studies by Matsushita and Fujita, 1992 revealed that MBL in serum was associated with a serine protease named MASP-1. However, further studies showed that two other proteases, MASP-2 and MASP-3, and a protein with no protease activity named sMAP or MAP19 were also associated with MBL (Thiel *et al.,* 1997; Takahashi *et al.,* 1999; Strover *et al.,* 1999 and Dahl *et al.,* 2001). MBL may have some importance during the period from the time at which the maternal derived antibodies disappear to the time at which the child's own immune system is being matured and in the lag phase before a primary immune response is generated following microbial challenges. MBL may also function as a disease modifier in some accompanying diseases and immunodeficiency states (Garred *et al.,* 2006). In 1989, the gene structure of *MBL* and the protein were identified (Taylor *et al.,* 1989; Sastry *et al.,* 1989).

2.1.2 *MBL1* and *MBL2* genes

Two forms of the MBL protein (MBL-A and MBL-C) have been detected in most of the species, including mice (Sastry *et al.,* 1991), cattle (Gjerstorff *et al.,* 2004b) and pigs (Agah *et al.,* 2001). MBL-A and MBL-C are encoded by the functional gene *MBL1* and *MBL2*, respectively. *MBL-1* and *MBL-2* have been detected in sera from the rhesus monkey, whereas MBL-C is only represented by *MBL-2* in chimpanzee and in human (Mogues *et al.,*

1996). Ancient *MBL1* and *MBL2* are believed to divagate from a common ancestral *MBL* gene by gene duplication (Sastry *et al.,* 1995). MBL-A has been described to possess a pro-inflammatory function (Takahashi *et al.,* 2002), but whether MBL-A and MBL-C possessed complementary or diverse functions still remained to be elucidated. Both *MBL1* and *MBL2* mutations were predicted to contribute to the variation of animal susceptibility to different infections (Shi *et al.,* 2004; Lillie *et al.,* 2005, 2007; Capparelli *et al.,* 2008).

2.1.3 C-type lectins

C-type lectins referred to as CTLs, which are Ca^{2+}-dependent lectins that contain homologous carbohydrate recognition domains (CRDs) regardless of displaying binding affinity to a variety of substrates (Weis *et al.,* 1998). They comprise 17 different groups that have, during evolution, diversified to interact with a large range of glycan ligands, although some also bind proteins, lipids and inorganic molecules. C-type lectins have a number of functions in vertebrates, including initiation of immune responses, pathogen sensing and homeostasis of serum glycoprotein. The majority of CTL groups outside of collectins do not interact with complement (Mayer *et al.,* 2017).

2.1.4 Collectins

The innate immune system represents the first line of defense against infectious diseases. Pattern recognition receptors (PRRs), a key part of the innate immune system, recognize conserved motifs on pathogens called pattern associated molecular patterns (Janeway, 1989). The collagenous lectins are a subset of membrane-bound and/or soluble, circulating C-type lectins that function as PRRs, recognizing carbohydrate residues on the surfaces of bacteria, viruses, and fungi. The collagenous lectin family includes the collectins and ficolins, which share structural and functional similarities (Hansen and Holmskov, 2002; Gjerstorff *et al.,* 2004a).

The name collectin is derived from the words “collagen” and “lectin.” They are characterized by calcium dependent (C-type) lectin domains attached to collagen-like regions via *a*-helical neck regions (Håkansson *et al.,* 1999). The collectins represent an important group of pattern recognition molecules, which bind preferentially to monosaccharide units of the mannose type, which present two vicinal hydroxyl groups in an equatorial position. High-affinity interactions between collectins and microorganisms depend, on the one hand, on the high density of the carbohydrate ligands on the microbial surface, and on the other hand, on the degree of oligomerization of the collectin. Binding of collectins to microorganisms may facilitate microbial clearance through aggregation, complement activation, opsonisation, activation of phagocytosis, inhibition of microbial growth, apoptotic cell clearance and

modulate the adaptive immune system. Collectins bind selectively to non-self glyco-conjugates on the surface of invading microorganisms (Fujita, 2002; Fujita *et al.,* 2004).

2.1.4.1 Members of collectin family

A total of nine collectins have been discovered till date including the genes encoding mannose-binding lectins A and C (MBL1 and MBL2), surfactant proteins A and D (SP-A and SP-D), collectin-10, also known as collectin liver 1 (CL-10 or CL-L1), collectin placenta 1 (CL-12 or CL-P1), conglutinin, collectins of 43 kDA and 46 kDa (CL-43 and CL-46) and collectin-11, also known as collectin kidney 1 (CL-11 or CL-K1) (Van de Watering *et al.,* 2004). All vertebrates employ collectins in their innate immune defense. Curiously, conglutinin, along with two other collectins, CL-43 and CL-46, are found only in the bovidae (Hansen and Holmskov, 2003; Gjerstorff *et al.,* 2004b). MBL is the most characterised collectin till date as it was the first to be discovered and has a CRD region that recognises the microbial carbohydrates mannose and N-Acetyl-D-glucosamine as well as nucleic acids (in a calcium-dependent manner) (Nakamura *et al.,* 2009). The size of fully assembled collectins ranges from 13 nm for MBL to about 100 nm for SP-D (Lu et *al.,* 1990; Crouch *et al.,* 1994).

2.1.4.2 Structure of collectins

The basic functional unit of collectins is a trimer and its number differs among the various collectins. In the monomeric subunits, four structural domains can be distinguished: an N-terminal cysteine-rich domain, a collagen domain, a coiled-coil neck domain and finally a C-type lectin domain (Weis and Drickamer, 1994; Mogues *et al.,* 1996).

2.1.4.2.1 N-terminal region

The N-terminal region is defined as the segment N-terminal to the first collagenous triple-helix residue. It is a cysteine-rich region which stabilizes the trimers through disulfide bridging (Crouch *et al.,* 1994; Holmskov *et al.,* 1995) and links them together in the collectin oligomers. There seems to be no overall homology between the collectins in this part of the molecule. The only general trend seems to be two cysteine residues separated in sequence by 4–5 amino acids and most of these amino acids are relatively hydrophobic in MBP and SP-D but hydrophilic in SP-A (Håkansson and Reid, 2000).

2.1.4.2.2 Collagenous region

The collagen-like region of the collectins consists of repeating motifs of Gly-X-Y, where X and Y can be any amino acid, but frequently are proline or hydroxyproline. The collagen helices of monomers are coiled around each other to form a stable tensile collagen domain that is relatively resistant to proteases. Another interesting structural feature of the collagen domain is that it can be N-glycosylated or O-glycosylated (Colley *et al.,* 1987;

Crouch *et al.,* 1994). The repeat Gly- X-Y pattern in some collectins is interrupted, which is thought to introduce a kink or region of flexibility into the protein enabling the trimeric subunits to angle away from the central core to form a structure resembling a bouquet of flowers (Lu *et al.,* 1990; Voss *et al.,* 1991).

2.1.4.2.3 α-helical coiled-coil region

The coiled-coil has no absolute amino acid sequence requirement, although proline residues, unable to form amino hydrogen bonds, usually introduce bends in a-helices. The coiled-coil is stabilized by hydrophobic amino acids at every turn of the helix. These hydrophobic amino acids form the interior of the coiled-coil. The structure of several coiled-coils, including the structure of trimeric MBP and SP-D fragments and the related tetranectin molecule (Nielsen *et al.,* 1997) are known from X-ray crystallography (Weis and Drickamer, 1994; Håkansson *et al.,* 1999; Håkansson and Reid, 2000).

2.1.4.2.4 Carbohydrate recognition domain (CRD)

The CRDs of collectins are compactly folded protein modules of 115–130 amino acid residues and are located at the C-terminus of the protein. Selective binding of collectins to specific complex carbohydrates is mediated by their CRDs, and requires the presence of calcium (Weis *et al.,* 1991). CRDs contain several calcium binding sites, although the exact number of ligated calcium ions under physiological conditions is as yet not totally clear. The actual carbohydrate binding site can be found in a shallow groove in the CRD. This region can usually be classified on the basis of four cysteine residues, being involved in disulfide bridging, which are the trademark of this domain type. The framework surrounding this domain type can be very different from its C-terminal location in the collectins. The monomeric and membrane bound selectins have an N-terminal C-type lectin domain that mediates adhesion between certain cell types through carbohydrate binding (Weis *et al.,* 1992; Ng *et al.,* 1996; Hakansson *et al.,* 1999). The disulfide bridge probably plays an important role in stabilizing the structure around the functional carbohydrate binding site (Håkansson and Reid, 2000).

2.1.4.3 Bovine collectins

Bovine collectins are ubiquitously expressed and may serve protective roles in various tissues. It contains conglutinin, along with two other collectins, CL-43 and CL-46. CL-46 is highly expressed in the liver and thymus, but transcripts have also been observed in the mammary gland and various segments of the gastrointestinal tract as well (Hansen *et al.,* 2002). Conglutinin and CL-43 are serum proteins primarily synthesized in the liver (Hansen and Holmskov, 2002). Holmskov *et al.* (1998) observed that serum levels of both conglutinin and CL-43 vary similar to the situation of MBL in humans and that low levels of conglutinin

predispose calves for infectious diseases. The genes encoding bovine SP-A, SP-D, MBL-A, conglutinin, CL-43 and CL-46 form a distinct collectin locus on *Bos taurus* chromosome 28 (BTA28) at position q.1.8–1.9. The study of bovine-specific collectins is important for understanding the innate immune system and to establish breeding schemes with the perspectives of improving the health status in cattle herds (Gjerstorff *et al.,* 2004b).

The genes encoding CL-43 (*COLEC9*), CL-46 (*COLEC13*), conglutinin (*COLEC8*) and bovine SP-D (*SFTPD*) show identities of 75–91%, and their presence are likely the results of multiple duplications of an ancestral *SFTPD*-like gene that took place after the bovidae separated from other mammals (Hansen and Holmskov, 2002). It appears that this additional group of collectins are not restricted to cattle (*Bos taurus*) or the *Bos* subgroup of bovidae, as conglutinin and CL-43 mRNA transcripts have been detected in the liver of sheep (Lu *et al.,* 1993). Since, ruminants rely heavily on symbiosis with microorganisms, immune-protection in gastrointestinal tract may be of great importance. It is likely that collectins expressed in these tissues modulate the inflammatory stimuli induced by the microorganisms, while at the same time controlling their growth (Gjerstorff *et al.,* 2004b).

2.1.5 MBL associated proteases (MASPs)

MBL, as an oligomer (tri- to hexameric), forms complexes with different MASP proteins. All the MASP proteins bind to MBL in homodimeric form (Chen and Wallis, 2001; Teillet *et al.,* 2008). However, the stoichiometry of the different components in the MBL-MASP complex is highly variable. MBL binds to MASP *via* its collagen domain (Super *et al.,* 1992; Kurata *et al.,* 1993). MASPs act as activators of the lectin pathway upon binding of MBL to carbohydrate or acetyl groups on the surface of pathogens or altered self-tissues (Ricklin *et al.,* 2010). So far, five proteins have been identified, including three MASP enzymes (MASP-1, MASP-2 and MASP-3) and two truncated proteins (MAp19 and MAp44) which lacks the serine protease domain and consequently, functional activity (Thiel *et al.,* 1997; Thiel *et al.,* 2009). All MASPs are capable to associate with MBL in the presence of Ca2C, forming a proteolytic complex (Kjaer *et al.,* 2013).

Both MASP-1 and MASP-2 play a crucial role in the activation of lectin pathway. Recent studies showed that MASP-1 can auto- activate and lead to MASP-2 activation (Degn *et al.,* 2012; Héja *et al.,* 2012). MASP-2 can also auto-activate, but under physiological conditions, MASP-1 is the essential MASP-2 activator (Kjaer *et al.,* 2013). MASP-2 is a protease that cleaves very efficiently C4 and C2, generating C3 convertase (Thiel *et al.,* 1997; Kristensen *et al.,* 2007). On the other hand, MASP-3 seems to attenuate the lectin- pathway activity due to competition for MASP binding sites on the recognition molecules (Degn *et al.,* 2010). In addition, MASP-3 occurs pre-dominantly complexed with Ficolin-3 and is thought

to have an inhibitory effect on complement activation and participate in developmental processes (Skjoedt *et al.,* 2010; Degn *et al.,* 2011a). All three MASPs are structurally similar to each other. The roles of MAp19 and MAp44 are still not well understood, but MAp44 was shown to negatively regulate the lectin pathway by competing for the same binding sites of MASP-2 and MASP-1 (Degn *et al.,* 2009; Degn *et al.,* 2011b).

MASP- 1 and MASP-2 are produced mainly in the liver and present in plasma (Ytting *et al.,* 2007; Thiel *et al.,* 2012) while MASP-3 is produced in several other tissues besides the liver (Degn *et al.,* 2010). MASP-1, MASP-3 and MAp44 are codified by *MASP1* gene on chromosome 3q27–q28, while MASP-2 and MAp19 are encoded by *MASP2* gene located on chromosome 1p36.23–31 (Stover *et al.,* 1999; Degn *et. al,* 2009; Skjoedt *et al.,* 2010).

2.1.6 MBL-Pathogen interaction

MBL binds multiple bacterial polysaccharides having terminal monosaccharides such as D-mannose, GlcNAc, ManNAc and L-fucose but not galactose and sialic acids which are present on host cells (Fig 2.1) (Weis *et al.,* 1992; Drickamer, 1992). In fact, some pathogens use the strategy of producing polysaccharide capsule and sialylation of lipopolysaccharide structures to escape MBL binding (Jack *et al.,* 2001, Krarup *et al.,* 2005). MBL has been shown to bind to a wide range of bacteria, viruses, fungi and protozoa (Dommett *et al.,* 2006). However, the binding of MBL to pathogens differs both between and within species (heterogeneous binding patterns) (Neth *et al.,* 2000; Townsend *et al.,* 2001).

Teichoic acid of *Staphylococcus aureus*, a cell surface glycopolymer containing GlcNAc residue, has been shown to be a functional ligand of MBL (Park *et al.,* 2010). In addition to *Staphylococcal aureus*, several bacterial species have been found to bind to MBL including: *Actinomyces israelii*, *Bifidobacterium bifidum*, *Leptotrichia buccalis*, *Proprionibacterium acnes* (Townsend *et al.,* 2001); *Burkholderia cepacia*, *Pseudomonas aeruginosa* (Davies *et al.* 2000); *Chlamydia pneumoniae* (Swanson *et al.,* 1998); *Klebsiella aerogenes*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Escherichia coli*, *Haemophilus influenza*, *Neisseria meningitidis*, *Listeria monocytogenes*, (van Emmerik *et al.,* 1994; Neth *et al.,* 2000); *Mycobacterium avium* (Polotsky *et al.,* 1997); *Mycoplasma pneumonia* (Hamvas *et al.,* 2005); and *Salmonella montevideo* (Kuhlman *et al.,* 1989). Interestingly, other bacteria (anaerobic) that are most commonly implicated in clinical disease such as, Bacteroides and Clostridium, bound little or no MBL. Similarly, the only *Veillonella* species that causes any appreciable disease, *V. parvula*, bound little or no MBL. In contrast, *Vitellariopsis dispar*, *Bifidobacterium bifidum*, *Propionibacterium acnes*, *Leptotrichia buccalis*, which very rarely cause significant infections, bound to MBL. Also, *Fusobacterium*, a rarely isolated organism is bound to measurable amounts of MBL. This suggests that there

may be an inverse relationship between pathogenicity and the level of MBL binding (Townsend *et al.,* 2001). MBL can bind to viruses such as, influenza A, HIV (Hartshorn *et al.,* 1993; Saifuddin *et al.,* 2000; Hart *et al.,* 2002; Ji *et al.,* 2005), and severe acute respiratory syndrome (SARS) coronavirus (CoV) (Ip *et al.,* 2005). A number of clinical studies have suggested that deficiency of MBL is a risk factor for acquiring HIV infection. MBL can bind to purified HIV-gp120 which is likely the target of HIV (Ezekowitz *et al.,* 1989). MBL can also bind to *Aspergillus fumigatus*, *Candida albicans* (Neth *et al.,* 2000) and protozoans such as *Cryptosporidium parvum* (Kelly *et al.,* 2000); *Plasmodium falciparum* (Klabunde *et al.,* 2002) and *Trypanosoma cruzi* (Kahn *et al.,* 1996), to prevent infection.

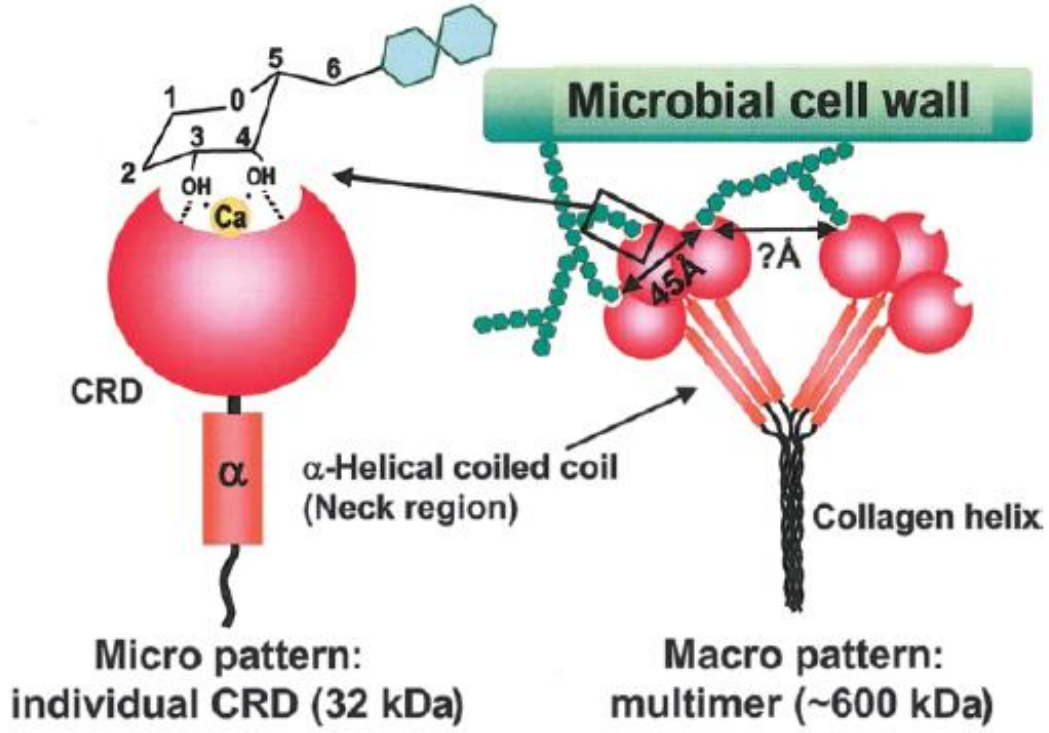


Fig. 2.1: Schematic model of pattern recognition by mannose binding lectins (MBLs). Micro pattern, monosaccharide binding by a single carbohydrate recognition domain (CRD). Macro pattern, polysaccharide binding by a dimerized MBL (Hoffmann *et al.*, 1999).

2.1.7 Functions of *MBL* gene

2.1.7.1 Role of *MBL* in activation of complement system

2.1.7.1.1 Overview of complement system

The immune system is classically divided in two parts, the innate and the adaptive, which are wide-ranging and interconnected. The innate immune system provides an immediate and non-specific first line defense through humoral, cellular, and mechanical

processes, have a vital role in protection against pathogenic challenge (Dunkelberger and Song, 2010). The complement system is an essential part of the innate immune system, with three overlapping roles: defense against infection, clearance of immune complexes and cell debris, and link between innate and adaptive immunity (Walport, 2001).

The activation of complement can take place on the surface of pathogens or damaged/infected cells by three distinct but converging cascade pathways: classical, alternative and lectin (Fig. 2.2). All three pathways are initiated by multiple stimuli independently from each other and subsequently the proteolytic cascades converge toward the activation of the major component C3, which results in the assembly of the membrane-attack complex (MAC) (Fig. 2.1) (Blom *et al.,* 2004). The activation of the classical pathway is initiated on immune complexes by the binding of C1q to Fc portion of IgM or IgG (Chen *et al.,* 2010; Ali *et al.,* 2012). On the other hand, the activation of the alternative pathway occurs by spontaneous hydrolysis of C3 in plasma (Chen *et al.,* 2010). Similarly to the alternative pathway, the lectin pathway may be activated in the absence of immune complexes. It is initiated by the binding of pattern-recognition plasma molecules such as MBL to carbohydrates or acetylated residues present on microorganisms or to aberrant glycocalyx patterns on apoptotic, necrotic, or malignant cells (Ali *et al.,* 2012).

2.1.7.1.2 Lectin pathway of complement activation

The lectin pathway is initiated when pattern-recognition molecules (MBL, CL-K1, and ficolins) bind to the so-called pathogen associated molecular patterns (PAMPs) (D-mannose, N-acetyl-D- glucosamine, or acetyl groups), on the surface of pathogens or to apoptotic or necrotic cells (Degn *et al.,* 2011a). Circulating MBL, CL-K1, and ficolins form complexes with two dimers of MASPs, MASP-1 and MASP-2. After the binding of MBL/MASPs, CL-K1/MASPs, or ficolin/MASPs complexes to their targets, MASP-1 can auto-activate and trigger MASP-2 (Héja *et al.,* 2012), leading to C4 and C2 cleavage. This allows the assembly of the C3 and C5 convertases, with subsequent activation of C3 and C5, respectively, and generation of C3a and C5a, two pro-inflammatory anaphylatoxins that increase the inflammatory response. The fragment C3b binds covalently to hydroxyl and amino groups on the surface of target molecules of all three pathways. In the absence of complement regulatory proteins, a powerful amplification in the number of surface-bound C3b molecules takes place through the alternative pathway. In this amplification loop, factor B binds to the attached C3b and is cleaved by factor D generating the alternative pathway C3 convertase C3bBb, which leads to accelerated C3b formation (Lesavre *et al.,* 1979; Fishelson *et al.,* 1984). C3b tags antigens/pathogens for opsonization and antigen presentation or killing by phagocytes through the interaction with complement membrane receptors CR1, CR2, CR3, and CR4, and the immunoglobulin superfamily member CRIg (He *et al.,* 2008). Finally, the

complement cascade culminates with the formation of the multiprotein complex (C5b, C6, C7, C8, and C9n) known as terminal complement complex or MAC, which are inserted as pores of up to 11 nm into the cell membrane inducing loss of membrane integrity and ultimately cell death (Fosbrink *et al.*, 2005; Gulla *et al.*, 2009) (Fig. 2.2).

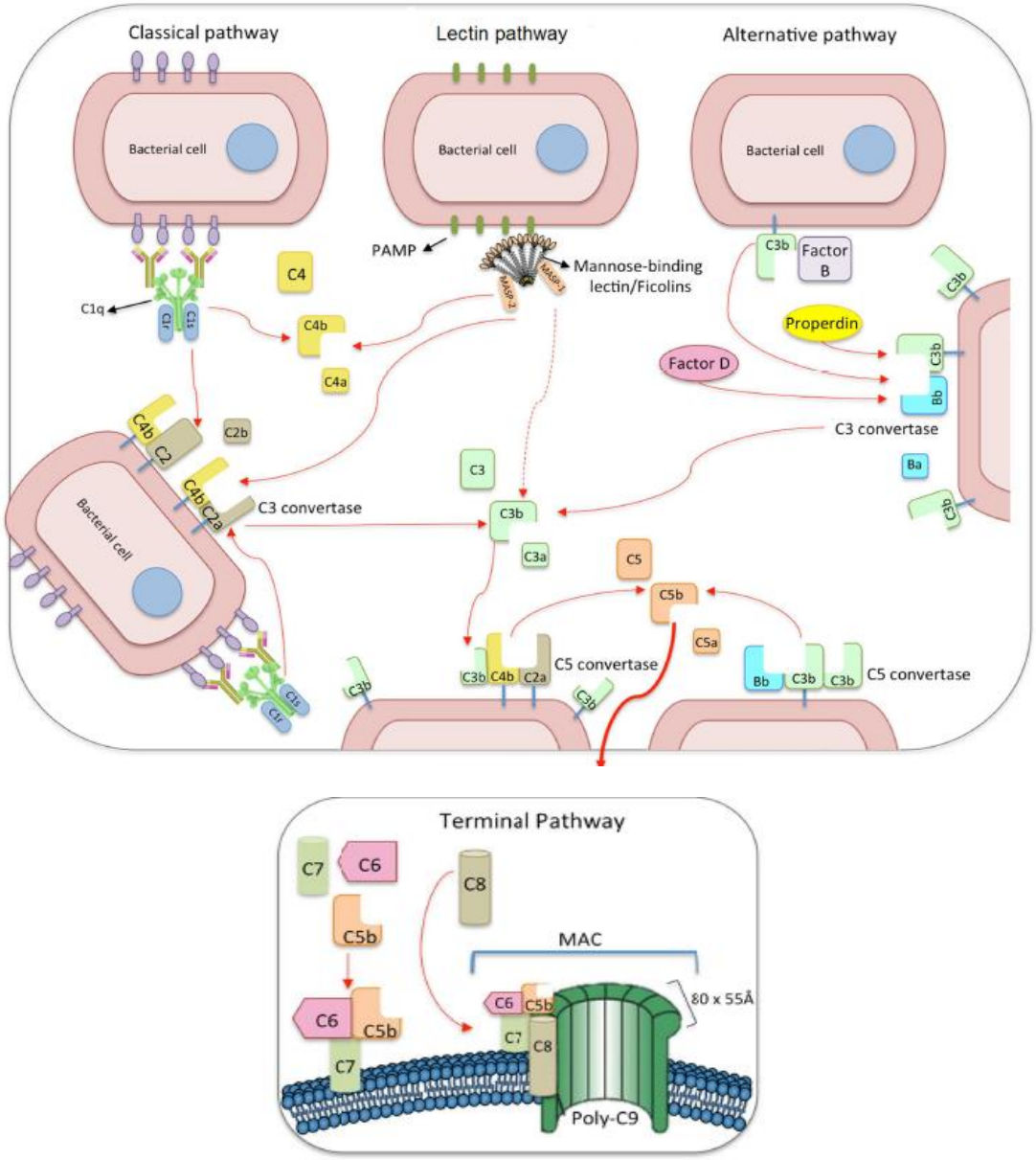


Fig. 2.2: The three pathways of complement activation: classical, lectin, and alternative pathways. The three pathways converge to the common terminal pathway, culminating with cell lysis and death (Beltrame *et al.*, 2015).

2.1.7.2 Opsonisation and phagocytosis

MBL can function directly as an opsonin by binding to pathogen, or indirectly by producing opsonins like C3b (Fig. 2.3). These opsonized pathogens/particles are recognized by a number of putative binding proteins/phagocytic receptors including, calreticulin/CD91

(cC1qR/LRP-1) (Ogden *et al.,* 2001; Malhotra *et al.,* 1990), C1QR1 (C1qRp, CD93) (Tenner *et al.,* 1995) and complement receptor type-1 (CR1, CD35) (Ghiran *et al.,* 2000). Calreticulin, an endoplasmic reticulum (ER) protein that acts as a chaperone during protein assembly, can be recruited to the cell surface during phagocytic recognition (Gagnon *et al.,* 2002). Low levels of serum MBL were found to be associated with defects in C3b opsonization on yeast surfaces and recurrent infections in children, which imply a role for MBL in host defense in humans (Turner *et al.,* 1981; Super *et al.,* 1989). MBL was able to interact directly with cell surface receptors and promoted opsonohagocytosis (Kuhlman *et al.,* 1989). MBL can opsonize Human immunodeficiency virus 1 (HIV-1) but does not induce neutralization at the levels at which it is normally present in serum. However, binding and opsonization of HIV by MBL may alter virus trafficking and viral antigen presentation during HIV infection (Ying *et al.,* 2004). MBL and C1q modulate monocyte activation and chemokine responses during the clearance of oxidized (Ox) LDL. MBL has been reported to directly bind OxLDL and enzymatically modified forms of LDL (E-LDL) in OxLDL-loaded monocytes and human monocyte derived macrophages (HMDM) and can therefore enhance cholesterol efflux (Fraser and Tenner, 2010).

2.1.7.3 Recognition and clearance of altered self

Role for MBL in the clearance of apoptotic cells was suggested through antibody blockade studies that showed that inhibition of calreticulin and CD91 blocked collectin mediated uptake of apoptotic cells by macrophages (Ogden *et al.,* 2001). MBL was found to bind directly to apoptotic cells that expose terminal sugars of cytoskeletal proteins, thereby permitting their recognition and directly facilitating their phagocytosis by macrophages (Ip *et al.,* 2009). Changes in cell surface structures during oncogenic transformation appear to promote binding of MBL to cancer cells (Hakomori, 2001), wherein the protein can mediate cytotoxic effects including MBL dependent cell mediated cytotoxicity (Ma *et al.,* 1999; Nakagawa *et al,.* 2003). MBL was reported to be known to mediate clearance of apoptotic debris through binding of nucleic acids displayed on the surface of apoptotic cells (Palaniyar *et al.,* 2004). MBL binds nucleic acids via its CRD in a Ca^{2+} dependent manner and binds more avidly to dsDNA than ssDNA or ssRNA. It was postulated that binding of nucleic acid displayed on apoptotic cell surfaces promotes phagocytosis, thereby curtailing autoimmunity and helping to maintain tissue homeostasis (Nakamura *et al.,* 2009).

2.1.7.4 Modulation of inflammation

MBL plays an important role in modulating inflammation, by releasing cytokines and interleukins. MBL was found to be involved in the binding of cryptococcal mannoprotein (MP2) to human peripheral blood mononuclear cells (PBMCs) and the release of tumor

necrosis factorα (TNFα) (Chaka *et al.*, 1997). Likewise, PBMCs from HIV infected patients when bound to MBL, increased cytokine production and viral replication (Heggelund *et al.*, 2005). Monocytes secreted higher levels of TNFα, interleukin6 (IL6) and IL1β, when infected with MBL opsonised *Neisseria meningitides* (Jack *et al.*, 2001) or *Leishmania chagasi* promastigotes (Santos *et al.*, 2001), as compared to non-opsonized bacteria.

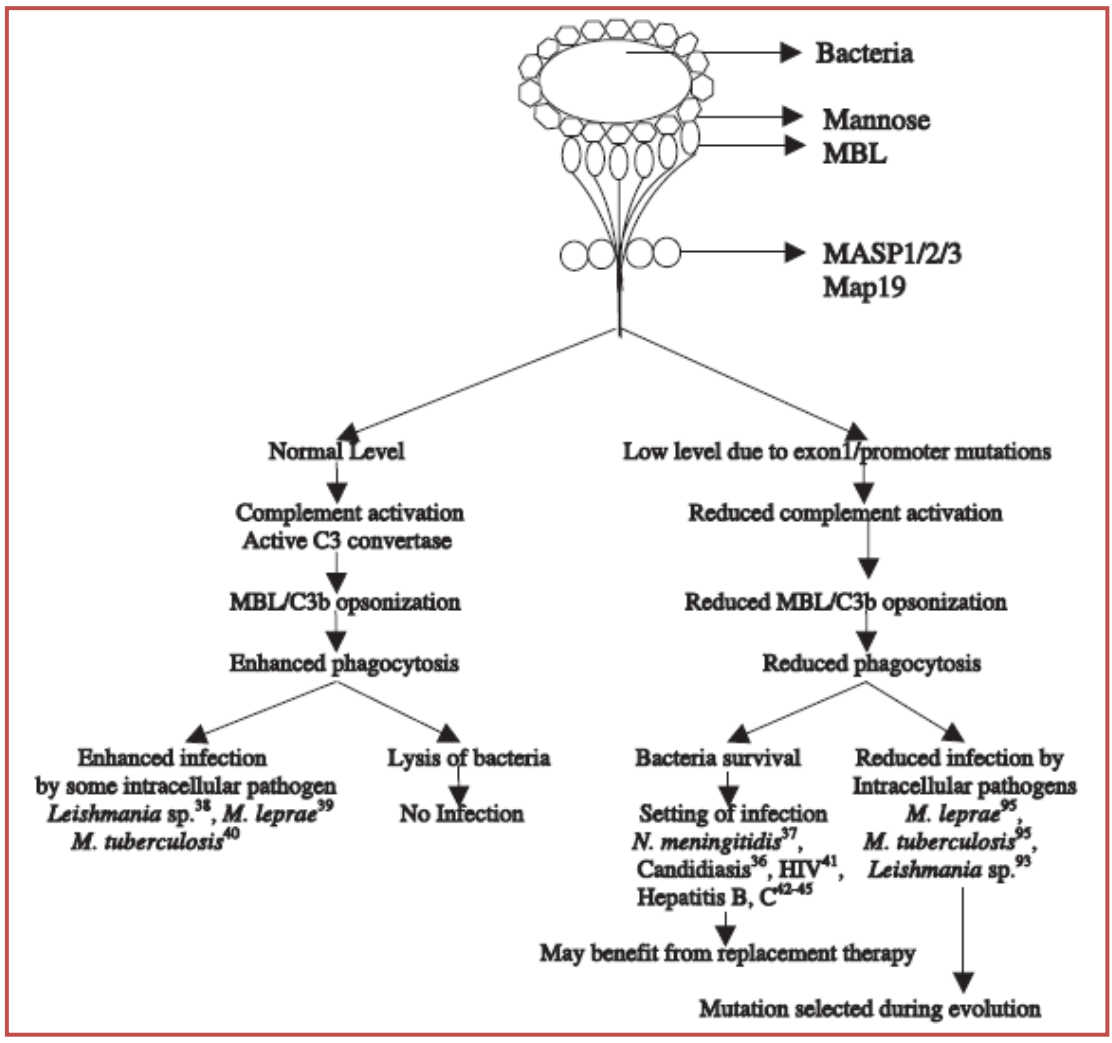


Fig. 2.3: Schematic representation of MBL pathway of complement activation and its role in clearance or infectivity by various pathogens (Gupta *et al.*, 2006).

2.1.8 Organization and localization of *MBL*1 gene

MBL-A is encoded by the functional gene *MBL1* on chromosome 14 in mice (White *et al.*, 1994), chromosome 28 in cattle (Gjerstorff *et al.*, 2004b), chromosome 14 in pigs (Phatsara *et al.*, 2007). MBL-C is encoded by the functional gene *MBL2* on chromosome 19 in mice and chromosome 26 in cattle. Both genes in pigs are located on chromosome 14 (Phatsara *et al.*, 2007). In human, *MBL1* has been characterised as pseudogene (Seyfarth *et al.*, 2005) and is positioned close to the *MBL2* gene on chromosome 10. Only one form of *MBL* found on chromosome 6 has been described in chickens, and evolutionary research has

indicated that the occurrence of two *MBL* genes happened after the divergence of avian and mammalian lineages (Hughes, 2007).

In humans, the different regions of the protein are encoded by five exons (Laursen *et al.,* 1998a). The signal peptide, the N-terminal and part of the collagen-like region are encoded by exon 1. The rest of the collagen-like region is encoded by exon 2. The neck region is encoded by exon 3, and the CRD is encoded by exon 4. In humans, an alternative exon (exon 0) was identified 1 kb upstream of the *MBL2* exon 1 (Naito *et al.,* 1999). This exon encodes a 5′ untranslated region (5′-UTR) (Seyfarth *et al.,* 2005). The gene encodes two major transcripts by alternative transcription, resulting in different lengths of mRNA transcripts. Transcription may initiate either at exon 1 or at an additional, non-coding 1.0 kb upstream located, exon 0 (Sastry *et al.,* 1989; Taylor *et al.,* 1989; Naito *et al.,* 1999). Approximately 10–15% of the liver-produced MBL seems to originate from exon '0' transcription (Seyfarth *et al.,* 2006; Heitzeneder *et al.,* 2012). Such an exon has also been found in the mouse (Laursen and Nielsen, 2000), cattle (Wang *et al.,* 2011) and pig (Lillie *et al.,* 2007). The promoter region of the *MBL* gene contains a number of regulatory elements, which affect transcription of the protein (Dommett *et al.,* 2006). Both, exon '0' and exon 1, promoter regions possess a TATA box for transcription initiation. In both, the binding sites for transcription factors include response elements to IL-6. This finding was assumed to underlie the regulation of MBL synthesis as an acute phase protein. In addition, the promoter region of exon 1 comprises a glucocorticoid responsive element (Gabolde *et al.,* 1999).

The chromosomal location of the bovine *MBL1* genes encoding MBL-A was identified by linkage analysis using a bovine/hamster radiation hybrid panel (Williams *et al.,* 2002). The bovine *MBL1* gene was found to be located at *Bos taurus* chromosome 28 (BTA28) at position q1.8–1.9 (Gallagher *et al.,* 1993; Gjerstorff *et al.,* 2004a). The bovine *MBL1* gene span over 5223 base pairs (bp) long and contains five exons and four introns (Fig. 2.4), encoding 248 amino acid (aa) (NCBI Accession No. AC_000185). Along with *MBL1* gene, the genes encoding bovine SP-A, SP-D, conglutinin, CL-43 and CL-46 form a distinct collectin locus on *Bos taurus* chromosome 28 (BTA28) at position q.1.8–1.9 (Gjerstorff *et al.,* 2004b).

Gjerstorff *et al.* (2004b) assembled BAC clones into a contig spanning 330–1150 kb, which includes the bovine genes encoding the collectins SP-A (*SFTPA*), SP-D (*SFTPD*), mannan-binding lectin A (*MBL1*), CL-43 (*COLEC9*), CL-46 (*COLEC13*) and conglutinin (*COLEC8*). In the same contig, they also identified a gene that potentially encodes a novel conglutinin-like collectin (*COLEC14*). Screening of the radiation hybrid panel linked *SFTPA* and *MBL1* to the genomic markers IDVGA8 and ILSTS099, located proximal to *COLEC8*,

COLEC9, *COLEC13* and *SFTPD* at *B. taurus* chromosome 28 (BTA28) at position q1.8–1.9 (Gallagher *et al.*, 1993; Hansen *et al.*, 2002, 2003; Gjerstorff *et al.*, 2004a).

The arrangement of *STFPA*, *SFTPD* and *MBL1* was homologous to the organization found in humans and mice, whereas the bovidae-specific collectin genes, *COLEC8*, COLEC9 and *COLEC13*, extend from *SFTPD*. Homologous arrangements of collectin genes in human, mouse and cow, suggest that an ancestral collectin locus, comprising *SFTPA*, *SFTPD*, *MBL1* and *MBL2*, was present in early mammals. The translocation of *MBL2* in mouse and cattle to other chromosomes, and the creation of additional collectin genes in both humans (one additional *SFTPA* gene) and cattle, are likely to represent chromosomal rearrangements that took place after the divergence of these species from other mammals (Gjerstorff *et al.*, 2004b).

2.1.9 Molecular structure of MBL-A protein

Various oligomeric structures ranging from dimers to hexamers have been reported for MBL. These oligomers are based on subunits comprising three identical peptide chains of 32 kDa as evaluated by SDS-PAGE. Each chain comprises four distinct regions: (1) N-terminal cysteine-rich region involved in oligomerization by formation of intraand inter-subunit disulphide bonds, **(**2**)** a collagen-like domain consisting of tandem repeats of Glycine-Xaa-Yaa (except repeat 8, which consists of only Glycine-Glutamine) that account for the long stalk of the molecule, (3) a α-helical, hydrophobic coil–coil neck domain, which is crucial for initiating the oligomerization and (4) a C-terminal carbohydrate-recognition domain (Fig. 2.5.) (Larsen *et al.*, 2004). Three polypeptide chains form a triple helix through the collagenous region, stabilized by hydrophobic interaction and interchain disulphide bonds within the N-terminal cysteine-rich region (Sheriff et al., 1994). This trimeric form is the basic structural subunit of all circulating forms of MBL. In serum, MBL consists of oligomers of the subunit, ranging from dimers to hexamers. It has become apparent that MBL of higher order oligomers (e.g. tetramers to hexamers) are the effective forms in terms of the protein functions, for examples, the glycan interaction and complement activation on microbial surfaces. (Lu *et al.*, 1990; Yokota 1995).

It is believed that the repetitive patterns of sugar groups decorating microbial surfaces constitute ideal targets for MBL interactions since the three lectin domains clustered into each subunit array offer a relatively flat binding platform with a constant 45Å separation distance between the three sites in human MBL (Sheriff *et al.*, 1994). Although the affinity of each lectin–sugar interaction is only 10−3M (Iobst *et al.*, 1994), the fact that the protein is able to bind at multiple sites simultaneously ensures a high functional avidity for such binding.

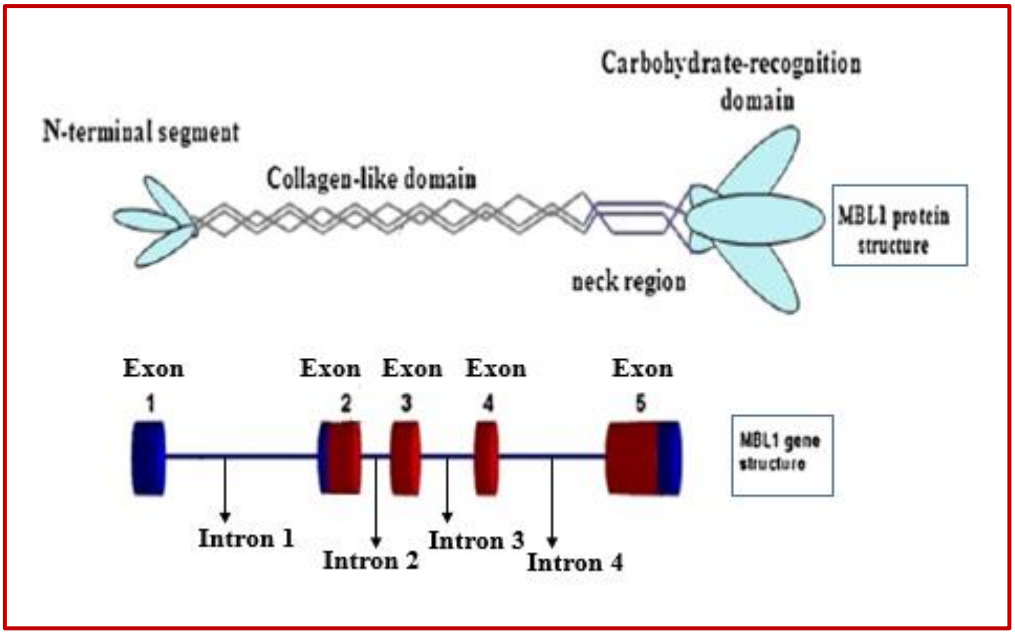


Fig. 2.4. Structure of *MBL1* gene and its protein; coding and non-coding sequences are indicated with red and blue, respectively. (Wang *et al.*, 2011).

2.1.10 Major sites of *MBL* expression and its concentration

MBL is synthesized in the liver and circulates in the serum (Wild *et al.*, 1983). Both *MBL* genes (*MBL1 and MBL2*) were highly expressed in liver of porcine (Phatsara *et al.*, 2007). *MBL1* was also found to be expressed (low concentration) in the lung, testis and brain, whereas low expression of *MBL2* was detected in the testis and kidney. However, extra-hepatic expression of *MBL* also observed (Nonaka *et al.*, 2007). The expression of functional MBL peptide is largely genetically determined. MBL is considered as an acute phage reactant protein (serum levels increases during inflammation) (Ezekowitz *et al.*, 1988). However, unlike other lectin proteins which increase drastically, MBL increases only 23 fold.

2.1.11 Regulation of MBL activity

Hepatocyte gene expression and plasma levels of MBL are stimulated by peroxisome proliferator-activated receptor α (PPARα) and fenofibrate (used to reduce cholesterol levels in humans at risk of cardiovascular disease). This evidence links PPARα to regulation of innate immunity and complement activation in humans, and suggests a possible role of MBL in lipid metabolism (Rakhshandehroo *et al.*, 2012). The salivary scavenger and agglutinin (SALSA, gp340), binds to both pathogen surface and MBL. This interaction (when SALSA is bound to the surface) activates the lectin pathway, while soluble SALSA inhibits MBL function (Reichhardt *et al.*, 2012). Thus, SALSA can protect host tissues from complement induced damage. MBL binding to biglycan, an extracellular matrix proteoglycan, inhibits the lectin

pathway (Groeneveld *et al.,* 2005). Thyroid and growth hormones have a significant effect on regulating MBL synthesis (Frakking *et al.,* 2006; Riis *et al.,* 2005).

2.1.12 MBL deficiency and regulation of concentration

MBL serum levels were reported to be comparatively constant in an individual and may increase 2–3 folds upon infections and inflammatory challenges (Thiel *et al.,* 1992). The level of MBL in plasma is genetically determined, and deficiency was found to be associated with frequent infections in childhood, and possibly also in adults (Turner, 1996). Large molecular mass complexes (200–700 kDa) of MBL circulate in serum, which are probably stabilized by interaction through the cysteine-rich, amino-terminal regions of adjacent trimeric subunits (Lipscombe *et al.,* 1995). The serum concentration of MBL varies, from 0 to 10 μg/ml with a median around 1 μg/ml (Steffensen *et al.,* 2000; Saevarsdottir *et al.,* 2001; Minchinton *et al.,* 2002). Recently, using antibodies of human collectin kidney 1 (COLEC11) (Yoshizaki *et al.,* 2012) the MBL concentration in blood was established as 1.72 ± 1.51 μg/ml. Enzyme-linked immunosorbent Assays (ELISAs) was used to measure MBL concentration in cerebrospinal fluid was found to be 0.0016-0.056 μg/ml (Kwok *et al.,* 2012). Low levels of MBL (< 1 μg/ml) were mostly caused by three point mutations in exon 1 of the human MBL gene (in codons 52, 54, and 57) that disrupt the assembly of the oligomers, and also by a promoter polymorphism that was associated with low MBL production. The combination of structural gene and promoter polymorphisms results in a dramatic variation in MBL concentration in apparently healthy individuals of up to 1000- fold (Ezekowitz *et al.,* 1988). MBL levels vary with age, increase within the first months of life and subsequently decline (Lau *et al.,* 1995; Sørensen *et al.,* 2006; Sallenbach *et al.,* 2011). High levels of MBL (>1 mg/ml) were associated with a greatly decreased risk of myocardial infraction (MI) in hypercholesteromic individuals (Saevarsdottir *et al.,* 2005).

2.1.13 Polymorphism in *MBL-1* gene and its association with different traits

Polymorphisms of both *MBL1* and *MBL2* genes have been reported to correlate with infection resistance to different bacteria and viruses, such as *Staphylococcus aureus* in mice (Shi *et al.,* 2004), *Haemophilus parasuis*, *Streptococcus suis*, *Mycoplasma* spp. and porcine circovirus 2 in pigs (Lillie *et al.,* 2006b; Lillie *et al.,* 2007), *Brucella abortus* in water buffalos (Capparelli *et al.,* 2008) and *Meningococcus* in humans (Eisen and Minchinton, 2003). MBL deficiency in humans is associated with higher susceptibility to viral as well as bacterial infections. A number of SNPs have been identified in the collagen-like domain of the human *MBL* gene, of which several are strongly associated with decreased concentrations of MBL in serum. Association studies of SNPs with mastitis tolerance/susceptibility have resulted in building a strong foundation on the niche of research on mastitis enabling new areas of focus

in selection of animals. Since bacteria are the most common causes of mastitis in bovine, analysis of bovine *MBL* SNPs may contribute to the discovery of mastitis resistance related genetic traits.

2.1.13.1 In cattle

Liu *et al*. (2010) carried out a study in Chinese Holstein cattle to identify polymorphisms in intron 1 and exon 2 of *MBL1* gene and their correlation with mastitis and milk quality. One SNP in the intron1 G855A and two SNPs in the exon2 G2651A (Val24Ile), T2686C (Ala35Ala) were found. At locus G2651A, the cows with genotype AA showed higher SCS than those with genotypes GG and GA ($P<0.05$), but no correlation was found between the SNP G855A, T2686C and SCS, fat rate, protein rate and 305 d milk yield. However, they found 8 haplotypes and 19 haplotype combinations among which cows with haplotype combination H2H2 had the highest protein rate and SCS. Haplotype combination H3H7 had the lowest SCS. The cows with haplotype combinations H4H4 had the highest 305 d milk yield. They concluded that *MBL1* gene H3H7 was a favourable haplotype combinations to the mastitis resistance selection and H4H4 can be chosen as the molecular markers for choosing high milk yield.

Liu *et al.* (2011) investigated influence of *MBL1* haplotypes on serum MBL-A concentration, complement activity and milk production traits in Chinese Holstein cattle. They reported three novel SNPs in the promoter region and screened two previously reported SNPs in exon 2 of the *MBL1* gene. The g.2651G>A SNP in exon 2 affected the serum MBL-A concentrations and the serum CH50 values, whereas the g.1330G>A SNP significantly affected CH50 and the SCS. It was revealed that cows with the ATGGC/ACAAC and AAGGT/ACGGT combined genotype exhibited the lowest and highest SCSs, respectively. They also conducted serum antibacterial activities to verify the effect of the SNPs on resistance to mastitis pathogens. It was revealed that the liver of cows with clinical mastitis exhibited a higher *MBL1* expression compared with healthy animals ($P<0.05$) which indicated a possible role of *MBL1* gene in bacterial infection resistance and it can be used as a molecular marker of milk production traits to control mastitis.

Wang *et al.* (2011) reported three SNPs of the *MBL1* gene in Chinese native cattle and analyzed their associations with milk traits. SNP g.855G>A was located within intron I, the other two SNPs resided in the exon 2 region with one mutation being non-synonymous (GTT (Val)>ATT (Ile)) and the other synonymous (GCT (Ala)>GCC (Ala)). They detected 8 different haplotypes and 19 genotype combinations. No correlation was found between either g.855G>A or g.2686T>C and SCS, however a significant association was found between g.2651G>A and SCS, suggesting a possible role of this SNP in the host response against

mastitis. They also suggested that the combined genotypes of GGC/AAC with the lowest SCS, AAT/AAT with the highest protein content and AGC/AGC with the highest 305-d milk yield were favorable combinations for mastitis resistance and milk production traits.

Zhao *et al.* (2012) reported four new SNPs in *MBL2* gene of Chinese Holstein cows. The g.1164 G>A SNP was predicted to substitute arginine with glutamine at the N-terminus of the cysteine-rich domain. SNPs g.1197 C>A and g.1198 G>A changed proline to glutamine in the collagen-like domain, whereas SNP g.1207 T>C was identified as a synonymous mutation. They found that the g.1197 C>A marker was significantly correlated to SCS, and the g.1164 G>A locus had significant effects on SCS, fat content, and protein content ($P<0.05$), suggesting possible roles of these SNPs in the host response against mastitis. They found 9 haplotype pairs corresponding to the loci of the 4 novel SNPs. Haplotype pairs MM, MN, and BQ were correlated with the lowest SCS; MN with the highest protein yield; MM with the highest protein rate, and MN with the highest 305 d milk yield.

Wang *et al.* (2012) reported four SNPs in the exon 1 of the bovine *MBL2* gene and observed their relationship with milk production traits, mastitis, serum MBL-C levels and hemolytic complement activity in both classical pathway (CH50) and alternative pathway (ACH50) in Chinese Holstein cattle. The SNP g.201 G>A was identified as a non-synonymous mutation (Arg31Gln) at the N-terminus cysteine rich domain and the SNPs g.234 C > A and g.235 G>A substituted Pro42Gln at the 1st Gly-X-Y repeat of the collagen-like domain, while the SNP g.244 T>C was identified as a synonymous mutation (Asn45Asn) at the 2th Gly-X-Y repeat of the collagen-like domain. They found that the SNPs (g.201 G > A, and g.234 C > A) were significantly correlated with SCS ($P<0.05$). They identified six combinations of different haplotypes from the four SNPs and revealed that cows with the haplotype combination H4H5 exhibited the lowest SCS. The CH50 value of H4H5 and H5H5 cows were significantly higher than H2H5 haplotype combination ($P<0.05$).

Yuan *et al.* (2013) identified three SNPs (c.1252G>A in intron I, c.2534G>A and c.2569T>C in exon 2) in cattle. The correlation analysis of *MBL1* gene polymorphisms with milk SCS showed a significant association in c.2534G>A, individuals with the GG genotype was significantly ($P<0.05$) lower SCS than those with GA and AA genotype. However, c.1252G>A and c.2569T>C animals were not found to be significantly associated with milk SCS.

Asaf *et al.* (2014a) investigated a SNP, rs110326717 (g.2651G>A) of *MBL1* gene in Vrindavani crossbred (Holstein Friesian/ Brownswiss/Jersey × Hariana) cattle. They found polymorphism in affected as well as unaffected groups of cattle and non-significant association with the clinical mastitis.

Asaf *et al.* (2014b) also found two distinct genotypes *viz.*, GG (311 bp and 272 bp fragments) and GA (588 bp, 311 bp and 277 bp fragments) with SNP "rs109231409 in a different study. Again they have reported non-significant association between targeted SNP" with clinical mastitis in Vrindavani crossbred cattle.

Kamaldeep *et al.* (2017a) screened g.2686T>C point mutation in exon 2 of *MBL1* gene in Sahiwal cattle and analysed its association with SCS. They found three genotypes *i.e.*, TT (274, 127 bp), TC (274, 184, 127 and 90 bp), and CC (184, 127 and 90 bp). Their study indicated that the targeted variant of *MBL1* gene in indigenous cattle had no association with SCS and mastitis resistance.

Kamaldeep *et al.* (2017b) screened g.2686T>C point mutation in exon 2 of *MBL1* gene in Sahiwal cattle and analysed its association with age at first calving (AFC) and first calving interval (FCI). Their study indicated that the targeted variant of *MBL1* gene in indigenous cattle had no association with AFC and FCI. However, TT genotype animals revealed lowest AFC (1208.27± 98.35 days) compared to TC (1215.00±89.85 days) and CC (1261.43±78.30) whereas, TC genotype animals revealed lowest FCI (420.78±79.30) compared to TT (484.33±85.82) and CC (515.21±66.48) genotype animals.

Aksel *et al.* (2019) reported *MBL1* polymorphism in different cattle breeds of Turkey. They found SNPs in intron I (1252 G>A) and exon 2 (2534 G>A, 2569 T>C) regions of *MBL1* gene. It was found that Hardy Weinberg equilibrium (HWE) broke down in Eastern Anatolian Red and Simmental breeds ($P<0.05$) in terms of 1252 G>A coded SNP; in Anatolian Black and Black Switzerla races ($P<0.001$ and $P<0.01$, respectively) in terms of 2534G>A coded SNP. It was determined that all breeds are in HWE in terms of 2569 T>C coded SNP.

2.1.13.2 In buffalo

Capparelli *et al.* (2008) identified four alleles in the exon 1 of the *MBL* gene (A, B, C, D), two alleles (H, L) at position −550 and two more at −221 (X, Y) of the *MBL* promoter in a case-control study of water buffalo. They stated that the haplotype pair HYA/HYA was associated with resistance to *Brucella abortus* infection ($P<10\ (-7)$) and the haplotype pairs LYD/LYD with susceptibility to the same pathogen ($P<10\ (-7)$). The serum from genetically resistant cases displayed in vitro significantly higher antibacterial activity compared to the serum from genetically susceptible cases, lending biological significance to the results from the association study. Inhibition of the antibacterial activity following heat treatment of the serum, addition of specific MBL inhibitors (EDTA, mannose, Nacetyl- D-glucosamine) or anti-human MBL antiserum provide convincing evidence that the antibacterial activity present in the serum results from the interaction between MBL and B. abortus.

Kamaldeep *et al.* (2019) conducted a study on Murrah Buffalo to identify polymorphism in exon 2 region of *MBL1* gene and its association with mastitis. Genotype analysis using PCR-RFLP revealed a monomorphic CC banding pattern (184, 127 and 90 bp). The result indicates highly conserved DNA sequence in Murrah buffalo. The study suggested that the targeted region of *MBL1* gene in water buffalo has no significant association with mastitis resistance.

2.1.13.3 In sheep and goat

Zhao *et al.* (2011) investigated association of SNPs in *MBL* gene with serum concentration of MBL protein in Hu sheep. SSCP analyses of PCR amplicons from a 194-bp section of the exon 1 region revealed four patterns: A, B, C and D. It was observed that pattern A has a 3-bp deletion, a 6-bp deletion and 42 SNPs. Pattern B has 3 SNPs, pattern C has 2 SNPs, whereas pattern D is identical to the reference sequence. Twenty-four of the 47 SNPs of the four patterns were synonymous whereas the other 23 SNPs were non-synonymous. The two deletions in the pattern A resulted in deletions of amino acids but there were no frame shifts in the putative MBL protein. The concentration of MBL protein in serum ranged from 1571 to 3657 g/L. Their statistical analyses showed that patterns A and B were associated with reduced MBL protein level in serum, whereas pattern C was associated with increased MBL protein level in serum ($P < 0.05$).

Cosenza *et al.* (2012) detected polymorphisms in the goat and sheep *MBL2* gene. The sequences obtained from sheep showed 13 polymorphic sites, six in the promoter and seven in exon 1, four of which were of the missense type. While in the goats, 12 polymorphic sites were detected, five intronic, five in the promoter, and one exonic. The exon site was responsible for an amino acid change. Mutations detected at the *MBL2* locus in the sheep were responsible for the alterations of gene expression.

Zhai *et al*. (2019) analysed three introns of the *MBL* gene in Hu sheep and 6 new SNP mutation sites were found, g.288T>A in intron I, g. 1091 T>C, g.1096A>C, g.1770G>C in intron II, g.2297C>T, g.2331G>A in intron III. Analysis of MBL serum level and its relationship with different genotypes revealed that the MBL serum level of AA genotype was higher than BB genotype ($P<0.05$) in intron I, CC genotype was higher than DD, GG genotype was higher than HH ($P<0.05$) in intron II. So, AA, CC and GG genotypes may be related to disease resistance; BB, DD and HH genotypes be related to disease susceptibility. The point mutation in intron III couldn't lead to the change of MBL serum level.

2.1.13.4 In pig

Lillie *et al.* (2006a) analysed both porcine *MBL* genes (*MBL1 and MBL2*) for SNPs that might impair disease resistance. Three SNPs within the coding region of *MBL1* in various

breeds of pigs were reported. One non-synonymous SNP that substituted cysteine for glycine in the collagen like domain of pig MBL-A was found with allele frequencies ranging from 1.4 to 46.4%. No SNPs were detected in the coding region of porcine *MBL2* but the expression of MBL-C in the liver was widely variable in comparison to the expression of MBL-A, GAPDH, PigMAP and haptoglobin. These results indicated that some pigs had a miscoding defect in MBL-A and a possible expression defect in MBL-C, which were analogous to coding and promoter polymorphisms that affect human MBL-C.

Lillie *at al.* (2007a) compared the 5′ flanking regions of porcine *MBL1* (1907 bp) and *MBL2* (1880 bp) in normal and diseased pigs with low or high hepatic expression of *MBL2*. Hepatic expression of MBL-C was very low in all pigs submitted for post mortem diagnosis. A G1081A substitution was linked to very low hepatic MBL-C expression, and was more frequent in diseased pigs of various European pig breeds. A C251T substitution with less influence on MBL-C expression was more common in various breeds but was not associated with disease. *MBL2* polymorphisms were associated with some disease groups and with the presence of some etiologic agents. These findings indicated that some promoter polymorphisms impair MBL-C expression in pigs and may increase their susceptibility to disease.

Lillie *et al.* (2007b) investigated for mutations that impair MBL-C function in pigs in which MBL-A is normally expressed. Expression of MBL-C was markedly reduced in some healthy pigs, but low in most pigs diagnosed with common infectious diseases. Four SNPs associated with reduced MBL-C expression were identified in the 5′-fanking region of pig MBL-C. They reported a G1081A mutation with the largest effect on MBL-C expression in both the GA and AA genotypes. The G1081A promoter defect was significantly more common among pigs with various common infections compared with various healthy populations. These results demonstrated that a promoter polymorphism that impairs production of MBL-C was an immunodeficiency in pigs with normal MBL-A expression.

Phatsara *et al.* (2007) reported two synonymous SNPs at the positions 579 (G to A) and 645 (G to A) of porcine *MBL2* cDNA which were located at codons 193 (AAG) and 215 (GTG) of predicted amino acid sequence but did not affect amino acid composition in the translated protein (Lys and Val at codon 193 and 215, respectively). The SNP (at codon 215) affecting an *Ade*I restriction site was found to be segregating in the F2 DUMI resource population. The study thus promoted the porcine *MBL* genes as functional and positional candidate gene for complement activity.

Juul-Madsen *et al.* (2011a) identified 14 SNPs, eight of which were found in exons and six in introns of *MBL1*. Four of the eight exon SNPs were non-synonymous. They

identified four haplotypes from sequence data of pigs. One of the identified haplotypes was associated with low concentration of MBL-A in serum. The concentration of MBL-A in serum was further assessed to analyse its correlation with disease frequency. The MBL-A concentration in Duroc boars showed one single population, whereas Landrace boars showed four distinct populations for MBL-A concentration. No association between *MBL* and disease incidence was found in this study.

Bergman *et al.* (2012) reported a G949T SNP in *MBL1* gene which was found to be associated with low MBL-A concentration in serum and detected at different frequencies in various European pig populations. The T allele of G949T was present among the BC2 animals while T allele of this SNP was not detected in the Austrian and Japanese samples and was thus unlikely to be an original feature of wild boars. They observed a co-variation between the presence of the T allele and low MBL-A concentration in serum.

Bergman *et al.* (2014) found that certain SNPs in porcine *MBL1* and *MBL2* affected mRNA expression, serum concentration and susceptibility to disease. They analysed *pMBL1* and *pMBL2* alleles, combined *pMBL* haplotypes, and MBL-A serum concentration in pigs. Moreover, the combined *pMBL* haplotypes of 89 crossbred pigs were studied and the genotypes of 67 crossbreds challenged with *Escherichia coli* were compared to their individual disease records. Three non-synonymous SNPs and a two-nucleotide deletion were detected in the coding sequence of *pMBL2* in purebred animals. The two-nucleotide deletion was present at a frequency of 0.88 in the Landrace pigs and 0.90 in the Duroc pigs, respectively. In the crossbreds, the T allele of the SNP G949T in *pMBL1* was detected in 47 % of the pigs. An association was also found between low producing *MBL* genotypes and low body weight on the day of weaning in the same animals.

2.1.13.5 In chicken

Laursen *et al.* (1998b) investigated the level of MBL in different chicken strains during embryogenesis, early and adult life. The MBL concentrations in 14 different strains, showed a unimodal distribution profile with a mean concentration of 5.8 μg/ml (range 0 4-37.8 μg/ml). They could not demonstrated any difference between the strains and did not found any MBL deficient chicken. Ontogenetic studies disclosed that MBL was already detectable in embryos at a gestational age of 10 days. At hatching, the MBL level was comparable to the level found in adult chickens. This level was fairly stable during the first weeks of life but a deficiency state developed at 4 weeks of age, thereafter the level was normalized again at 5 weeks of age. They bred chickens with relatively low or high MBL levels with cockerels having similar MBL levels which resulted in F generations having significantly different MBL levels suggesting that the protein level is genetically influenced.

Juul-Madsen *et al.* (2007) conducted a study to understand the function of *MBL* in relation to infectious viral diseases. They selected two chicken lines for high and low concentrations of MBL in serum for several generations. Offspring from the two sub-lines were exposed to infection with infectious bronchitis virus (IBV) in order to determine their genetic susceptibility to the virus. Results suggested that MBL plays a role in the innate immunity against IBV in the way that it performs an acute phase response, is able to activate complement, and inhibits the propagation of the virus in the trachea.

Schou *et al*. (2010) determined the genetic serum MBL concentration and examined its association with specific *Pasteurella multocida* humoral immune response during an experimental infection. Additionally, they examined the association of the genetic serum MBL concentration with systemic infection and found that the chickens with systemic infection had a statistically significant lower mean serum MBL concentration than the rest of the chickens which suggested that MBL plays an important role against *P. multocida*. Also found a statistically significant negative correlation between the specific antibody response and the genetic serum MBL concentration for both breeds. The study indicated that MBL in chickens was capable of acting as the first line of defence against *P. multocida* by diminishing the infection before the adaptive immune response takes over.

Kjaerup *et al*. (2013) selected two inbred chicken lines (L10L and L10H) for low and high MBL levels in serum and four other experimental chicken lines for polymorphism detection in the *MBL* gene. The differences in the serum concentrations of MBL were found to be of transcriptional origin. Several SNPs were discovered in the promoter and the 5′-UTR of the chicken *MBL* gene which resulted in the identification of six different alleles. SNPs that could affect the MBL serum concentration were identified by mapping of regulatory elements in the promoter region. One SNP, found to be located in a TATA box, was altered in one of the six alleles only. This allele was associated with low MBL serum concentration.

Kjaerup *et al.* (2014) assessed the transcription activity of a 670-bp promoter region covering all 14 SNPs from the four *MBL* promoter alleles A1 to A4 using a dual-luciferase assay. Of the analysed alleles A1 showed the highest transcription activity although this allele is frequently found in chickens with low *MBL* mRNA expression.

2.1.13.6 In murinae

Takahashi *et al.* (2002) created a MBL-A null mice that were MBL-C sufficient in order to evaluate the relative roles of two forms of *MBL*. It was found that MBL-A null mice had enhanced survival in a septic peritonitis model compared to wild-type mice and complement 3 null mice ($P<0.05$). Reconstitution of these mice with human MBL reversed the phenotype. Surviving mice had significantly decreased TNF-α and IL-6 levels in the blood

and peritoneal cavity (*P<0.01*). The study indicated that bacteria opsonized with MBL-A-deficient serum induced significantly less cytokine by peritoneal macrophages compared to those with wild-type serum. Their results suggested that MBL-A was a modulator of inflammation in vivo and in vitro in the mouse and that the role of MBL may extend beyond its role as an opsonin.

Shi *et al.* (2004) reported circumstantial evidence *in vitro* and *in vivo* suggests that *MBL* plays a key role in first line host defense. They tested this contention directly *in vivo* by generating mice that were devoid of all MBL activity and found that 100% of MBL-null mice died 48 h after exposure to an intravenous inoculation of *S. aureus* compared with 45% mortality in wild type mice. They demonstrated that neutrophils and MBL were required to limit intraperitoneal infection with *S. aureus*. This study provided direct evidence that *MBL* plays a key role in restricting the complications associated with *S. aureus* infection in mice and indicated that the *MBL* gene may act as a disease susceptibility gene against *staphylococci* infections in humans.

Phaneuf *et al.* (2007) identified Sequence comparisons identified a total of 15 structural SNPs in *MBL1* in two strains, 27 SNPs in *MBL2* in five strains of mice among which most of the non-synonymous SNPs were occurred in the CRD region and some resulted in altered residues close to known ligand binding sites.

CHAPTER-3

MATERIALS AND METHODS

3.1 Glasswares and Plasticwares

Routine glasswares like flask, measuring cylinder, beaker, test tubes, pipettes, etc., used for the present study were of Borosilicate made. All the glasswares and plasticwares were used after proper sterilization. Plastic centrifuge tubes (15 ml), eppendorf tubes (1.5 ml), PCR tubes (200 µl) and tips (Volex), mortar and pestle made of ceramic for tissue homogenization and 90 mm petri dishes (HiMedia) were used in the present study. The glasswares were cleaned as per standard procedures.

All plasticwares used in the present study were either certified to be free from DNase and RNase activity or were treated with DEPC and subsequently autoclaved to render them DNase, RNase and DEPC free. During RNA work, the working bench was sprayed with RNAZAP (MP Biomedicals, France) to make it free from the effect of RNase.

3.2 Equipments

The following equipments were used in the present study: Autoclave (Scientech), Hot air oven (Uday), Triple Glass Distillation plant (Borosilicate), Incubator (Sonar), Digital pH meter (Scientech), Digital balance (Kern), Deep freeze (Blue star), Refrigerated centrifuge (Remi), Microcentrifuge (Genei, Banglore), UV trans-illuminator (GeNei, Banglore), Vortex (GeNei, Banglore), Waterbath (Scientech), Thermocycler (BioRad, USA), Gel documentation (Alpha Innotech), Electrophoresis apparatus (Genei, Banglore), Power supply power pack 1000v (GeNei, Banglore), Refrigerator (Kelvinator), Microwave oven (IFB), Laminar air flow (Sonar), Orbital shaking incubator (Remi) and Nanodrop (Eppendorf, Germany).

3.3 Buffers, media and reagents

The composition and preparations of media, reagents and buffers used in present study are listed in Annexure. The following chemicals, enzymes and biologicals were utilized during the present study:

3.3.1 Chemicals

Tris hydrochloride (HiMedia), Triton-X (HiMedia), EDTA (CDH), Sucrose (SRL), Agarose (HiMedia), Sodium chloride (RFCL), Magnesium chloride (GeNei, Banglore), Ethidium bromide (GeNei,Banglore), Potassium chloride (SRL), Phenol (HiMedia), Absolute

ethanol (Changshu Yangyuan Chemical, China), Bromophenol blue (RFCL), Chloroform (RFCL), Glacial acetic acid (HiMedia), Hydrochloric acid (HiMedia), Isopropanol (SRL), Isoamyl alcohol (SRL), Sodium acetate trihydrate (CDH), Boric acid (SRL), RNAlater (Invitrogen; Thermo Fisher Scientific), DEPC (PUREGENE), β-mercaptoethanol (HiMedia), Liquid Nitrogen (LN2), Ampicillin sodium salt (HiMedia), X-gal (Thermo Fisher Scientific) and IPTG (Thermo Fisher Scientific).

3.3.2 Enzymes and biologicals

Proteinase-K (GeNei, Banglore), *Taq* DNA polymerase (New England Biolab), *Hae*III (New England Biolab), *Apa*I (New England Biolab), *Sty*I (New England Biolab), dNTP solutions (dATP, dGTP, dCTP, dTTP) (Thermo Fisher Scientific), GeneJET RNA purification kit (Thermo Fisher Scientific), RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific), PUREGENE-Quickclone PCR cloning kit (Genetix Biotech Asia) and GeneJET plasmid miniprep kit (Thermo Fisher Scientific).

3.3.3 Miscellaneous items

Adhesive tapes, parafilm paper, scissors, cello tape, thermometer, tissue paper, stickers and racks, inoculation loop and L-shape spreader were also used in current study.

3.4 Gene cloning and characterization of *MBL1* CDS

3.4.1 Sample collection

For cloning and characterization of *MBL1* CDS, liver tissue sample from adult female buffalo (n=2) of Murrah breed was collected randomly from slaughter house. Sample was collected in 1.5 ml eppendorf tubes containing 500 µl RNAlater as a stabilization solution which is an aqueous, nontoxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA.

3.4.2 Isolation of total RNA

The total RNA was isolated from freshly collected liver tissue using GeneJET RNA purification kit (Thermo Fisher Scientific) following the manufacturer's instructions. However, the method is described briefly as follows:

1. Approximately 30 mg of tissue was placed in liquid nitrogen (LN_2) and grinded thoroughly using DEPC treated mortar and pestle to make the powder of tissue.
2. The tissue powder was transferred immediately into a 1.5 ml microcentrifuge tube containing 300 µl of lysis buffer that was supplemented with 14.3 M β-mercaptoethanol.
3. The above contents were vortexed for 10 sec to mix thoroughly.

3. 600 µl of diluted Proteinase K (10 µl of Proteinase K was diluted with 590 µl of TE buffer) was added, vortexed shortly to mix thoroughly and incubated at 15-25°C for 10 min.
4. The contents were centrifuged at 12000 x g for 10 min at 4°C temperature.
5. The supernatant was carefully transferred into a fresh RNase free microcentrifuge tube, 450 µl of 96-100% ethanol was added and mixed by pipetting.
6. Up to 700 µl of lysate was transferred to a purification column placed in a collection tube and centrifuged at 12000 x g for 1 min.
7. The flow-through solution was discarded and purification column placed back into the collection tube. This was repeated (twice) until all of the lysate has been transferred into the column and centrifuged at 12000 x g for 1 min.
8. The collection tube containing the flow-through solution was discarded and column was placed into a new 2 ml collection tube.
9. 700 µl of wash buffer 1 (supplemented with ethanol) was added to the purification column and centrifuged 12000 x g for 1 min. The flow-through was discarded and purification column placed back into the collection tube.
10. 600 µl of wash buffer 2 (supplemented with ethanol) was added to the purification column and centrifuged 12000 x g for 1 min, the collection tube containing the flow-through solution was discarded and purification column transferred to the to a sterile 1.5 ml RNase-free microcentrifuge tube.
11. 50 µl of nuclease free water was added to the centre of the purification column membrane and centrifuged at 12000 x g for 1 min to elute RNA. The purification column was discarded and purified RNA then aliquoted and stored at -20°C for further use.
12. The quality and integrity of the total RNA was checked using agarose gel (1%) electrophoresis and visualization under UV light. Two intact bands of 28s and 18s indicated the good quality and intactness of RNA.
13. The purity of total RNA was checked by taking the spectrophotometric reading at OD_{260} and OD_{280} using Nanodrop (Eppendorf, Germany). The RNA samples showing the OD_{260}:OD_{280} value approximately 1.8-2.0 were considered having no protein and used for further process.
14. The concentration of total RNA was also estimated using Nanodrop.

3.4.3 Reverse Transcription PCR for cDNA Synthesis

The first strand cDNA was synthesized using Revert Aid First strand cDNA Synthesis Kit (Thermo Fisher Scientific) by following manufacturer's instructions as described below:

1. All the components of cDNA synthesis kit were thawed, mixed thoroughly, centrifuged briefly (excluding the enzymes) and were placed on ice. Following components were added to a 200 µl PCR tube on ice:

Table 3.1: Components and their quantity for cDNA synthesis reaction mixture

Component	Volume (µl)
RNA sample (0.1 ng to 5µg total RNA)	1
Oligo $(dT)_{18}$ primer	1
Nuclease-free water	10
5X reaction buffer	4
Ribolock RNase inhibitor (20 U/µl)	1
10 mM dNTP mix	2
RevertAid M-MuLV RT (200 U/µl)	1
Total volume	**20**

2. Above contents were mixed gently and centrifuged briefly, incubated for 60 min at 42ºC. Reaction was terminated by heating at 70ºC for 5 min to inactivate the reverse transcriptase. cDNA product was stored at -20ºC for further use.

3.4.4 Primer designing for characterization of *MBL1* gene

To amplify complete CDS region of *MBL1* gene, specific primer pair was designed using VNTI software and commercially synthesized (Imperial life sciences Pvt. Ltd, Gurugram, Haryana) on the basis of *MBL1* gene sequence of *Bos taurus* available in the GenBank (Acc. no. NM_001010994). The primer sequences are as follows:

MBLcDNA F: 5′-GATGTTGCTGAGGCATCCGC-3′

MBLcDNA R: 5′-TCCAGAGCAGCAGGGAGACA-3′

3.4.5 PCR Amplification of single strand cDNA

Complete coding region of *MBL1* gene was amplified using a pair of gene specific primer (MBLcDNA F: 5′-GATGTTGCTGAGGCATCCGC-3′ and MBLcDNA R: 5′-TCCAGAGCAGCAGGGAGACA-3′). The PCR was carried out in a total volume of 25 µl containing 1 µl of first strand of cDNA, 10 X PCR buffer (New England Biolab), 2 mM $MgCl_2$, 2 mM of dNTPs, 5 pmoles of each primer and one unit of *Taq* DNA polymerase (New England Biolab) and autoclaved double distilled water. PCR profile consisted of a denaturation step at 94ºC for 50 sec, an annealing step at 65ºC for 50 sec and an elongation step at 72ºC for 1 min for a total of 34 cycles, followed by a final extension of 10 min at 72ºC. The PCR product was

checked by agarose (1%) gel electrophoresis containing ethidium bromide in 1X TBE buffer. The gel was visualized under UV light and photographed with automated gel documentation system.

3.4.6 Cloning of *MBL1* PCR product in Vector pTZ57R/T

Amplified products were cloned into pTZ57R/T cloning vector. The cloning vector was linearized and ddT tailed for direct use in cloning of PCR products, generated with DNA polymerase mixture which added extra adenines to the ends of PCR products. Un-purified PCR fragments were ligated into the PCR cloning plasmid vector and the ligation reaction product was transformed into *Escherichia coli* DH5α host cells by following manufacturer's (PUREGENE-Quickclone PCR cloning kit (Genetix Biotech Asia) instructions.

3.4.6.1 Preparation of LB-broth-ampicillin and LB-agar-ampicillin plates

The cloning plasmid vector pTZ57R/T contained Ampicillin-resistance gene (Amp^R) as a positive selectable marker gene. Therefore, for selective growth of *E.coli* cells, which harboured the cloning vector (recombinant or non-recombinant) ampicillin was consistently included in LB-broth and LB-agar at a level of 50 µg/ml. For use in liquid media, 100 ml of autoclaved LB-broth media allowed to cool to ~55ºC and was supplemented with 100 µl of ampicillin stock solution (50 mg ampicillin-sodium salt/ ml sterilized Milli-Q water). 5-6 ml of media was poured in 10 ml glass tubes and stored at 37ºC. Similarly for use in solid media, 100 ml of autoclaved LB-agar medium, allowed to cool to ~55ºC and mixed with 100 µl of ampicillin stock solution. 30-35 ml of medium was poured into 90 mm petri dishes and allowed to solidify at room temperature and stored at 37ºC.

Medium preparation for transformation of ligation reaction into host *E.coli* DH5α and for isolation of recombinant clones is given in annexure.

3.4.6.2 Preparation of LB–ampicillin agar plates for blue/white selection of recombinant clones

The multiple cloning site in the plasmid vector pTZ57R/T is located in β-lactamase gene under the transcriptional control of LacZ promoter, to serve as screenable marker gene for recombinant plasmids in the presence of its chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactoside) and inducer IPTG (isopropyl-β-D-thiogalactopyranoside) in the medium. Therefore, the solidified LB-Ampicillin-agar medium in plates was uniformly smeared upon with 40 µl of X-gal solution (20 mg/ml) and 40 µl of IPTG (100 mM) using a sterile L-shaped spreader.

3.4.6.3 Revival of *E.coli* strain DH5α

E. coli DH5α strain was freshly streaked on LB agar plate with the help of inoculation loop. LB agar plate having DH5α cells was incubated 37ºC for overnight and observed next day for growth of single white colonies. Round shaped white colony of DH5α was picked up from the agar plate and inoculated in T-medium (kit component).

3.4.6.4 Ligation of PCR product into cloning vector pTZ57R/T

The ligation of unpurified PCR amplified fragment into cloning vector was performed in a 1.5 ml microcentrifuge tube with 30 µl ligation reaction as given in Table 3.2.

Table 3.2: Components and their quantity for ligation reaction mixture

Component	Volume (µl)
pTZ57R/T Vector (0.17 pmol ends)	3 µl
5X Ligation Buffer	6 µl
PCR product (unpurified)	1 µl
Nuclease-free Water	to 29 µl
T4 DNA Ligase	1 µl
Total volume	**30 µl**

The ligation mixture was briefly vortexed and centrifuged for 3-5 sec. Then, ligation mixture was incubated overnight at 4ºC and used next day to transform *E.coli* DH5α cells.

3.4.6.5 Preparation of Competent Cells and Transformation

Competent cells were prepared chemically using kit components given for transformation *viz.* T-medium, Solution A and Solution B. The day before transformation a single colony of DH5α was inoculated in 5 ml eppendorf tube containing 2 ml of T-medium and incubated overnight at 37º in shaker incubator under shaking conditions of 200 rpm.

On the day of transformation, 2 ml tubes containing the 1.5 ml of T-medium (1.5 ml for each two transformations) were pre warmed at 37ºC for 20 min. All the components of the kit were allowed to thaw on ice. 250 µl of Solution A and 250 µl of Solution B were mixed together to prepare 500 µl T-solution on ice which then used for preparation of competent cells. Meanwhile, LB agar plates supplemented with ampicillin, X-Gal and IPTG were also pre-warmed in a 37ºC incubator for at least 20 min before plating. The preparation of competent cells and transformation were performed as per manufacturer's protocol explained below:

1. 150 µl of the overnight bacterial culture was added to 1.5 ml of pre-warmed T-medium and the mixture was incubated at 37ºC for 20 min.
2. Bacterial cells were pelleted by centrifuging at 8000 rpm for 1 min and supernatant was discarded.
3. 300 µl of T- solution was added to pelleted cells and incubated on ice for 5 min. Centrifuged for 1 min in a microcentrifuge and supernatant was discarded.
4. 120 µl of T- solution was added to the pelleted cells and incubated for 5 min on ice.
5. 2.5 of ligation mixture was taken in a new microcentrifuge tube to which 50 µl of prepared cells were added. The contents were mixed and incubated on ice for 5 min.
6. Then, the mixture (ligation mixture and prepared cells) was plated immediately on pre-warmed LB-ampicillin X-Gal/IPTG agar plate to enable blue-white screening and incubated overnight at 37ºC.
7. The recombinant clones were identified from the transformed bacterial colonies using blue (non-recombinant) and white (recombinant) colony selection.

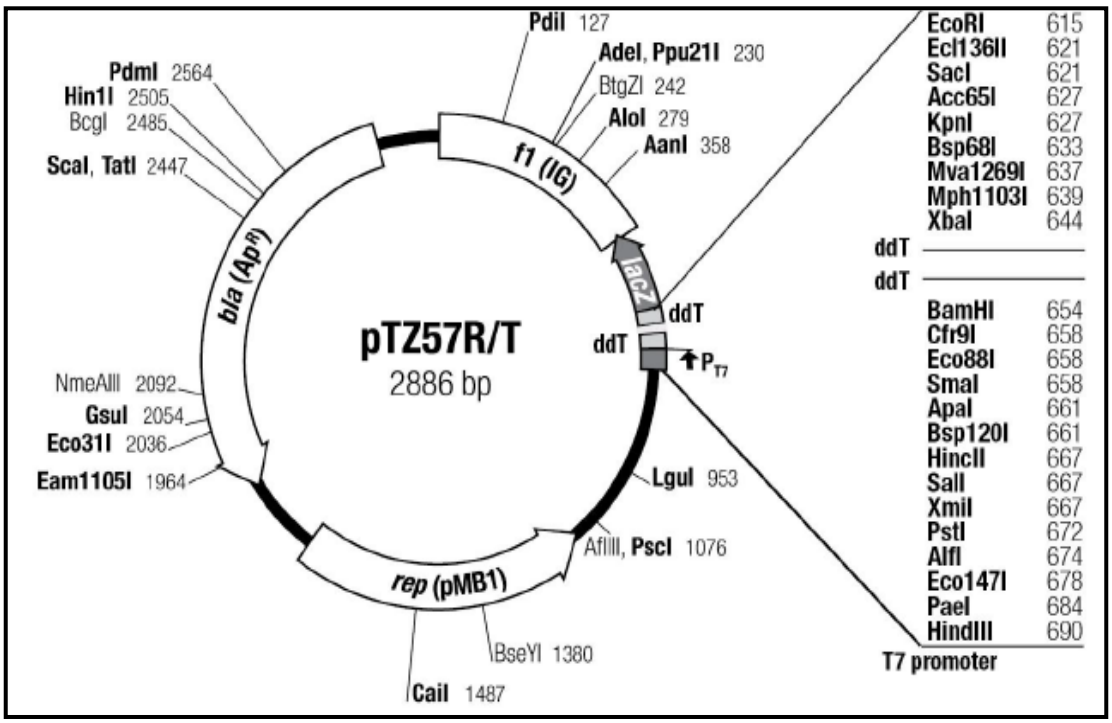


Fig. 3.1: Map of the pTZ57R/T cloning vector with unique restriction sites.

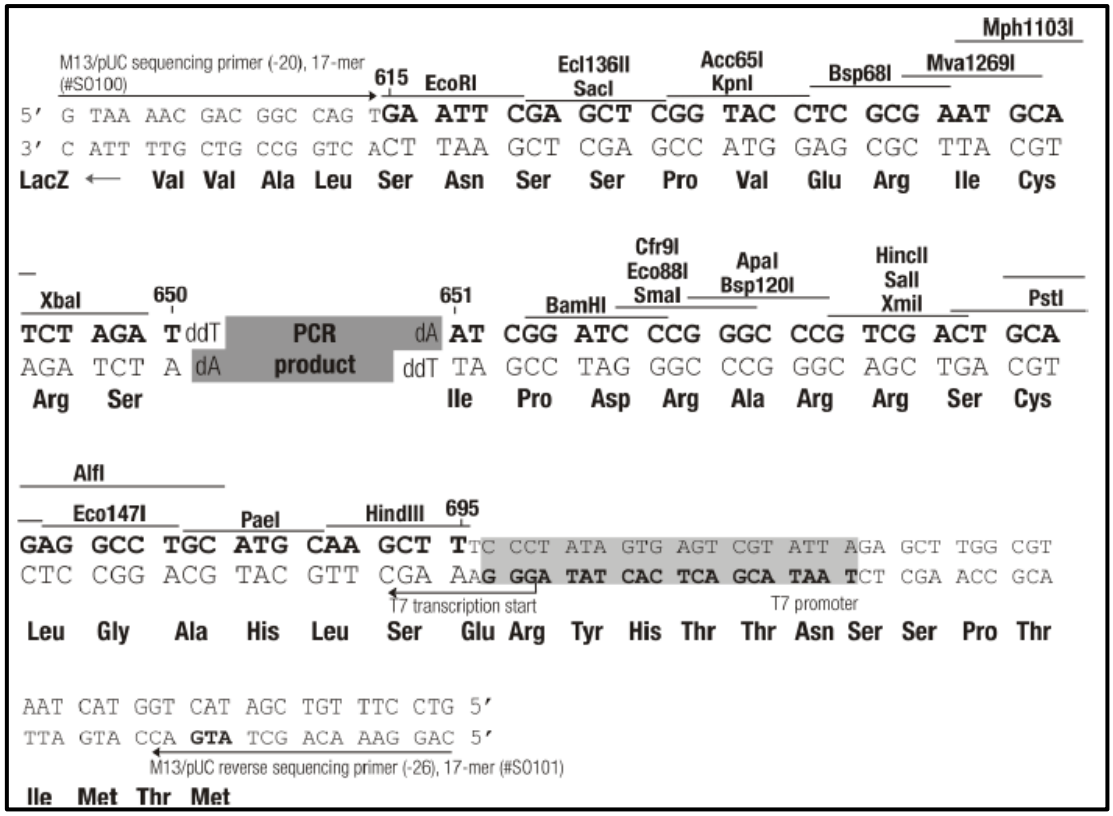


Fig. 3.2: DNA sequence of pTZ57R/T having multiple coning sites region.

3.4.6.6 Isolation of recombinant plasmid DNA from recombinant clones of *E. coli*

Two individual recombinant white clones from plate were picked up with the help of a sterile inoculation loop, inoculated into culture tubes containing 5 ml LB-Ampicillin broth and the tubes incubated overnight at 37ºC under shaking conditions (200 rpm).

Above DH5α bacterial culture derived from cloning was harvested the by centrifugation at 8000 rpm (6800 × g) in a microcentrifuge for 2 min at room temperature. Supernatant was discarded and pelleted cells were used for plasmid isolation. Then, plasmid purification was carried out using GeneJET plasmid miniprep kit (Thermo Fisher Scientific) following the manufacturer's instructions as per the protocol described below:

1. 250 µl of the Resuspension Solution (supplemented with RNase A) was added to the tube containing the pelleted cells and mixed. Cell suspension was transferred to a microcentrifuge tube. The bacteria was resuspended completely by vortexing and pipetting up and down until no cell clumps remain.
2. 250 µl of the Lysis Solution was added and mixed thoroughly by inverting the tube 4-6 times until the solution became viscous and slightly clear.
3. 350 µl of the Neutralization Solution was added and mixed immediately and thoroughly by inverting the tube 4-6 times to avoid localized precipitation of bacterial cell debris. The neutralized bacterial lysate became cloudy.

4. The above mixture was centrifuged for 5 min to pellet cell debris and chromosomal DNA.
5. Supernatant was transferred to the spin column assembled with a collection tube by pipetting without disturbing or transferring the white precipitate.
6. The column containing the supernatant was centrifuged for 1 min. Flow-through was discarded and column was placed back into the same collection tube.
6. 500 µl of the Wash Solution (diluted with ethanol) was added to the spin column and centrifuged for 30-60 seconds. Flow-through was discarded and column was placed back into the same collection tube.
7. Wash procedure (above step) was repeated by adding 500 µl of the Wash Solution. Flow-through was discarded and centrifuged for an additional 1 min to remove residual Wash Solution.
8. Spin column was transferred into a fresh 1.5 mL microcentrifuge tube and 50 µl of the elution buffer was added to the centre of spin column membrane to elute the plasmid DNA avoiding the contact of pipette tip with the membrane.
9. Incubated for 2 min at room temperature and centrifuged for 2 min. Column was discarded and purified plasmid DNA was stored at -20°C for further use.
10. The concentration and purity of plasmid DNA was determined spectrophotometrically at OD_{260} and OD_{280}.

3.4.6.7 Preparation of glycerol stocks

For preparing glycerol stocks, two individual recombinant (white) clones from agar plate were picked up with a sterile loop, inoculated into culture tubes containing 5 ml of LB-Ampicillin broth and the tubes incubated overnight at 37°C under shaking conditions (200 rpm) so that both are moderately turbid from bacterial growth. 400 µl of broth for each sample was transferred into a 1.5 ml microcentrifuge tubes containing 100 µl of 80% glycerol, mixed and freezed on ice. These aliquots were stored at -20 as the future source of the desired transformants.

3.4.6.8 Confirmation of gene insert presence by Plasmid-PCR

The presence of the gene insert was confirmed by PCR amplification of insert using recombinant plasmid as a template. *MBL1* gene insert was amplified using gene specific primers. The PCR was carried out in a total volume of 25 µl containing 1 µl of recombinant plasmid, 10X PCR buffer (New England Biolab), 2 mM $MgCl_2$, 10 mM of dNTPs, 5 pmoles of each primer and one unit of *Taq* DNA polymerase (New England Biolab) and autoclaved double distilled water. PCR profile consisted of an initial denaturation step for 5 min at 95^{o}C for 1 cycle, denaturation step at 94^{o}C for 50 sec, an annealing step at 65^{o}C for 50 sec and an elongation step at 72^{o}C for 50 sec for a total of 34 cycles followed by a final extension step of

10 min at 72°C. The PCR product of 951 bp was checked by agarose (1%) gel electrophoresis containing ethidium bromide in 1X TBE buffer. The gel was visualized under UV light and photographed with automated gel documentation system.

3.4.7 Sequencing and analysis of sequence

The positive clones were sequenced commercially (Eurofins genomics India Pvt Ltd, Bangalore) by automated sequencer using standard cycle conditions by Sanger′s dideoxy chain termination method with standard sequencing primers. The sequences obtained were subjected to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) to ascertain whether the obtained sequences corresponded to *MBL1*. The nucleotide sequences of Murrah buffalo *MBL1* mRNA/CDS was aligned with Indian and other non-Indian cattle and buffalo breeds and other species available in the GenBank database using the Clustal method of MegAlign programme of Lasergene software (DNASTAR, USA) and BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). A phylogenetic and molecular evolutionary analysis was conducted using MEGA version 4.0 (Tamura *et al.*, 2007).

The *MBL1* Coding sequences of murrah buffalo breed was compared with American buffalo (*Bison bison* or American Bision, XM_010834154), *Bubalus bubalis* (Italian Mediterranean buffalo, XM_006064773), *Bos taurus* (Mixed breed, NM_001010994), *Bos taurus* (Crossbred x Angus, BC109674), *Bos taurus* (AB178774), *Bos indicus* (Nelore, XM_019954216), hybrid *Bos indicus x Bos taurus* (Angus x Brahman F1 hybrid, XM_027530780), *Bos mutus* (Wild Yak, XM_005909163), goat (San Clemente, XM_005699265), sheep (Ramouillet, XM_004021518 and domestic sheep, LT852560), pig (York shire, NM_001007194 and Landrace, EU421730), *Camelus bactrianus*/Bactrian camel (Alxa, XM_010963110) and *Camelus dromedaries*/Arabian camel (Breed African, XM_010984099).

3.5 Somatic cell count (SCC)

SCC is the gold standard to measure mastitis and is used as an indicator of udder health and mastitis. Milk samples (5 ml) of the lactating cattle and buffalo population under polymorphic study were collected and analysed using LACTOSCAN somatic cell counter (MILKOTRONIC LTD) based on florescent microscope technique of counting cells. Since the distribution frequency of somatic cell count is usually skewed (Ali and Shook, 1980), we calculated the SCC (cells/µl) based parameter SCS using the following equation:

$$SCS = \log_2 (SCC/100) + 3 \text{ (Rupp and Boichard, 1999)}$$

3.5.1 Working principle of LACTOSCAN somatic cell counter

Unique, 3D, multi-image, patent application protected, sequential scanning process, based on a precise fluorescent optics and low magnification, images analysis software, LACTOSCAN SCC is fast, precise and reliable counter of somatic cells. Via automatic displacement of the mechanism on axles X–Y and liquid lens Z, the device is capturing maximum 60 images. After capturing, the images are being processed by the embedded software and the average result, calculated by using the formula from IDF/ISO 13366, of all the filmed images is displayed. The whole process, after placing the LACTOCHIP in the cartridge, is automatic and the analysis was done as per manufactures' protocol described below:

1. Fresh raw milk samples were collected in 15 ml tubes from genotyped cattle and buffaloes maintained at Livestock Farm Complex (LFC), DUVASU, Mathura (U.P.) and the raw milk samples were stirred using the Mini vortex mixer.
2. 100 µL raw milk was taken in micro-tube containing the SOFIA GREEN lyophilized dye
3. Micro-tube containing the SOFIA GREEN dye and milk sample was closed and the solution was stirred for 1-2 seconds in Mini vortex. The stirring process was repeated 8-9 times carefully so that solution did not reach the cap of the micro-tube.
4. The solution was incubated at room temperature for 1 min to allow the interaction of milk with dye.
5. Stirring of the micro-tube containing the sample was done again in stirrer Mini vortex for 1-2 sec and repeated 2-3 times paying attention during the stirring process that the sample not to reach the cap of the container.
6. 8 µL sample was pipetted from the micro-tube and placed in the micro-fluidic chamber of the LACTOCHIP x4.
7. Sample loaded LACTOCHIP x4 was placed in the cartridge of the LACTOSCAN SCC and analysis was done using the LACTOSCAN SCC software.

3.6 PCR-RFLP assay for polymorphism study

3.6.1 Experimental animals and sample collection

Randomly, a total of 150 blood samples (5 ml) from adult females of Sahiwal (n=50), Hariana (n=50) breed of cattle and Murrah breed of buffalo (n=50) were collected in vacutainer tubes for PCR-RFLP study. All of them maintained at Livestock Farm Complex (LFC), DUVASU, Mathura (U.P.), were utilized in the present investigation.

3.6.2 Isolation of Genomic DNA

Genomic DNA was isolated by using standard phenol-chloroform DNA isolation protocol (Sambrook and Russell, 2001).

1. The blood samples which were stored at 4°C were taken out and thawed to make a uniform solution.
2. The centrifuge tubes were filled with five volume of chilled RBC lysis buffer (1X), mixed end to end, incubated in ice for 10 mins and centrifuged at 3-4000 rpm for 15-20 mins at room temperature (RT).
3. The reddish tinged supernatant containing plasma and lysed RBC was discarded by simple inversion of the tubes or by pipetting.
4. Two volumes of chilled RBC lysis buffer were added, the tube ends were tapped to disperse the pellet of WBC or RBC (if left) and centrifuged at 3-4000 rpm for 15-20 mins.
5. The black tarry coloured supernatant containing lysed RBC was discarded by simple inversion.
6. The steps 4-5 were repeated till the WBC pellet appeared nearly white in colour.
7. The tubes were added with DNA extraction buffer (@ 3ml/10 ml of blood), tapped to disperse the WBC pellet in the extraction buffer and kept in incubator for half an hr. (It was ensured that the buffy coat is completely suspended so that the cells are accessible to SDS and Proteinase K).
8. 10% SDS was added @ 200µl/10 ml of blood and the contents were gently mixed by inverting (The contents of the tube appeared viscous indicating lysis of WBC. The samples were handled gently from now on to avoid shearing of DNA).
9. The tubes were added with Proteinase 'K' (@25 µl of 20 mg Proteinase 'K'/ml of tdH_2O for 10 ml blood) in two pulses i.e., half the requirement was added to the tubes in 1st pulse, mixed gently end to end and kept in water bath at 50°C. After 3-4 hrs the second pulse of the remaining amount of Proteinase 'K' was added and tubes were incubated at 50°C overnight.
10. Next morning, the contents were transferred into a clean, sterile, autoclaved polypropylene tubes, added with equal amount of equilibrated phenol (Tris saturated phenol pH>7.8), mixed by inverting gently for 15 mins till a light coffee coloured uniform solution without any balls of phenol was formed and centrifuged at 3-4000 rpm for 15-20 mins.
11. The upper aqueous phase containing DNA was transferred into fresh 15 ml, clean, sterile, autoclaved polypropylene tubes by means of a 1 ml tip whose tip has been cut to widen the bore. Upper aqueous phase is very viscous and care was taken during transfer of aqueous phase so that lower organic phase (containing phenol, cell lysate, proteins etc.) and white interface (containing proteins) are not disturbed.
12. Similar extractions were done (as in steps 10-11) once with equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) and with chloroform: isoamyl alcohol (24: 1).

13. To the final aqueous phase obtained, 3M Na acetate (pH-5.2) @ 100 µl/1ml of aqueous phase ($1/10^{th}$ the volume) was added and mixed gently.
14. Two volumes of ethanol (chilled)/isopropanol (at room temp) were added, tubes were mixed gently by inversion and kept at RT to allow precipitation of DNA.
15. DNA along with 500 µl of ethanol /isopropanol was transferred into fresh eppendorfs by the means of wide bore tips and centrifuged in microcentrifuge @ 10,000 rpm for 10 mins.
16. The supernatant was discarded by gentle inversion where DNA pellet is intact, otherwise aspirated (when DNA pellet is loose).
17. The DNA pellet was washed twice in 500µl of 70% ethanol i.e. 500µl of 70% ethanol was added and eppendorfs were centrifuged @10000 rpm for 10 mins at RT.
18. Finally the DNA pellet was air dried after inverting on blotting paper so that last traces of ethanol were lost. However the pellet should not be over dried or else dissolution becomes difficult.
19. Approximately 200 µl of TE buffer was added and put in water bath at 60°C for 2 hrs to inactivate DNase or other enzymes.
20. The tubes were stored, at 4°C for a week so that DNA is dissolved and subsequently at -20°C indefinitely.

3.6.3 Checking of concentration, purity and quality of DNA

3.6.3.1 Concentration of DNA

The concentration of DNA was estimated using Nanodrop in ng/µl.

3.6.3.2 Purity of DNA

The OD value at 260 nm gives the amount of nucleic acids present in a given samples whereas OD value at 280 nm gives the amount of protein present in the sample. Based on this, purity of DNA was checked using Nanodrop by taking reading of the ratio of optical density values at 260 and 280 nm. The samples having OD ratio (260/280) lies between1.75 to 1.90 were used in subsequent experiment.

3.6.3.3 Quality of DNA

Horizontal submarine agarose gel electrophoresis was performed to check the quality of DNA. Agarose of 1.0% w/v was dissolved in 1X TBE buffer by heating in microwave oven. The agarose solution was cooled to 45-50°C and poured into casting gel tray after adding ethidium bromide (EtBr; 0.5 µg/ml). After gel solidification, gel casting tray was submerged in gel tank having 1X TBE buffer. For loading, 2µl of DNA samples was diluted in 8µl of autoclaved distilled water and 2µl of loading dye were mixed and then loaded in the well. Electrophoresis was performed at 5 V/cm. Then gel was visualized under UV

transilluminator. The genomic DNA having good quality (intact without smearing) was used for further analysis.

3.6.4 Amplification of different regions of *MBL1* gene using PCR

3.6.4.1 Primer sequences used to amplify different regions of *MBL1* gene

A set of three primer pairs was used to amplify different SNP containing regions (intron I and exon 2) of *MBL1* gene (Wang *et al.,* 2011). The sequences of the primers are presented in Table 3.3.

Table 3.3: Primer sequences used to amplify different regions of *MBL1* gene

SNPs	Region of MBL1 gene	Primer Name	Primer Sequence (5′→3′)	Amplicon Size
g.855G>A	Intron I	MBLINT F	5′ CCCTTCCAACTCATTGCTTC 3′	588 bp
		MBLINT R	5′ AGTCCCAACCACCCTCA 3′	
g.2686T>C	Exon 2	MBL EXII F	5′ GCAGAGGTGGTGGCAAATGT 3′	401 bp
		MBL EXII R	5′ CATCTTTAGAGAGAATGCCCC 3′	
g.2651G>A	Exon 2	MBL Ex2 F	5′ GGTGGCAAATGTTGGCTA 3′	162 bp
		MBL Ex2 R	5′ GTCTTCTGAGCATCCTCCA 3′	

3.6.4.2 Setting up PCR reaction

An aliquot of 5µl genomic DNA was taken in 200µl PCR tubes and kept on ice. After that 20 µl triple distilled water was added, subsequently master mixture was prepared by adding all the reaction components except genomic DNA. The reaction mixture was carried out in final volume of 25µl. The reaction mixture comprised of the following components:

Table 3.4: PCR reaction mixtures for all 3 primers

S. No.	Component	Given Concentration	Required concentration	Amount
1	Autoclaved tdH_2O	----	----	18.8 µl
2.	*Taq* Buffer with 25mM $MgCl_2$	10 X	1 X	2.5 µl
3.	$dNTP_S$ mix	2mM each	2.5mM each	2.5 µl
4.	Primer F	10 pmol/µl	5 pmol	0.5µl
6.	Primer R	10 pmol/µl	5 pmol	0.5µl
7.	*Taq* DNA Polymerase	5 unit/ µl	1 unit	0.2 µl
8.	Genomic DNA	50-60 ng /µl	50-100 ng	2.0 µl

The reaction components were added for master mix in order of Autoclaved triple distilled water→ *Taq* buffer with $MgCl_2$→ dNTPs mix → Forward primer → Reverse primer → finally *Taq* DNA polymerases. Then diluted genomic DNA was added in master mix. All the above procedures were carried out on ice and master mix was mixed properly by tapping and followed by short spinning. Finally master mix was added to 200 µl PCR tubes containing 2µl genomic DNA followed by gentle tapping and short spinning to mix all the components properly. Finally, the PCR tubes were kept in a pre-programmed thermocycler for amplification.

3.6.4.3 Amplification profiles

The amplifications were done in thermocycler (BioRad). The amplification profiles comprised of following steps:

Table 3.5: PCR conditions for the amplifications of primer sequences of intron I and exon 2 regions of *MBL1* gene

STEPS		1	2			3	4
		Initial denaturation	35 Cycles of			Final extension	Storage
			Denat.	Anne.	Exten.		
Intron I (g.855G>A)	**Temp.**	94°C	94°C	62°C	72°C	72°C	4°C
	Time	5 min	30 sec	30 sec	30 sec	10 min	∞
Exon 2 (g.2686T>C)	**Temp.**	94°C	94°C	59°C	72°C	72°C	4°C
	Time	5 min	30 sec	30 sec	30 sec	10 min	∞
Exon 2 g.2561G>A	**Temp.**	94°C	94°C	53°C	72°C	72°C	4°C
	Time	5 min	20 sec	20 sec	20 sec	5 min	∞

Where: Denat: Denaturation; Anne: Annealing; Exten: Extension

3.6.4.4 Resolution of amplified products on agarose gel

The weighed quantity of agarose (1.2 g) was dissolved in required volume of 1X TBE (120 ml) and the solution was heated by microwave for complete dissolution of agarose. The hot solution was cooled to 40-50°C and 1.2 µl EtBr was added. The agarose solution was gently poured on to the gel casting platform by avoiding air bubble formation. Gel was allowed to solidify for 20-30 mins. The gel casting platform was submerged in electrophoresis tank containing 1X TBE solution. Amplified products (5µl) were loaded into wells after adding loading dye and electrophoresis was performed at 5 V/cm for 2 hrs. After electrophoresis, the gel was examined under UV light and photographed for documentation.

3.6.5 PCR-RFLP assay of different regions of *MBL1* gene by restriction enzymes

About 10 µl of amplified product was digested with 10 units of restriction endonucleases enzyme overnight at suitable temperature in water bath (Table 3.6).

Table 3.6: Different RFLP assays with their Recognition sequence and cut site

	Intron I	**Exon 2**	**Exon 2**
RE	***Apa*I**	***Hae*III**	***Sty*I**
Recognition sequence	5′ GGGCCC 3′ 5′ GGGCCC 3′	5′ GGCC 3′ 3′ CCGG 5′	5′ CCWWGG 3′ 5′ GGWWCC 3′
Restriction cut site	5′ GGGCC↓C 3′ 3′ C↑CCGGG 5′	5′ GG↓CC 3′ 3′ CC↑GG 5′	5′ C↓CWWGG 3′ 5′ GGWWC↑C 3′
Incubation Temperature	25°C	37°C	37°C
Incubation Time	Overnight	Overnight	Overnight

Table 3.7: Composition of PCR-RFLP reaction mixtures for different regions of *MBL1* gene

Reaction Components	**Amount**		
	***Apa*I/intron I**	***Hae*III/exon 2**	***Sty*I/exon 2**
PCR Product	7.0 µl	7.0 µl	7.0 µl
10X RE Buffer	1.5 µl	1.5 µl	1.5 µl
Restriction Enzyme (10 unit/ µl)	1.0 µl	1.0 µl	1.0 µl
Autoclaved TDW	5.5 µl	5.5 µl	5.5 µl

The *Apa*I/intron I, *Hae*III/exon 2 and *Sty*I/exon 2 PCR-RFLP assay should have following possible genotypes as presented in figures 3.3, 3.4 and 3.5 respectively:

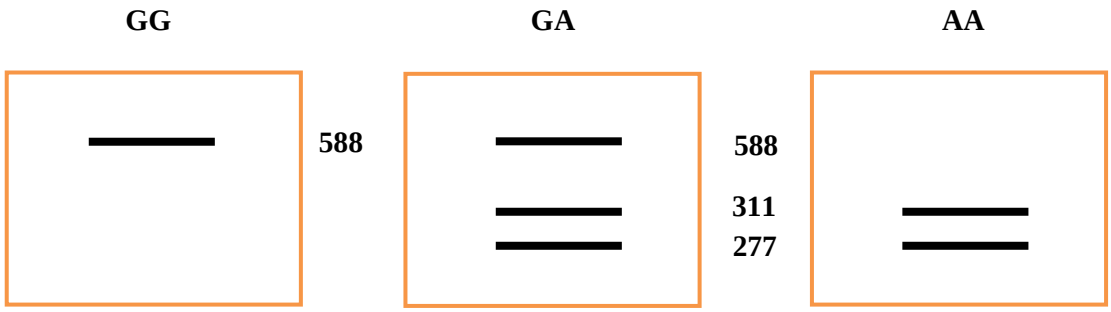


Fig 3.3: Possible genotypes (GG, GA and AA) generated using *Apa*I/intron I PCR-RFLP assay.

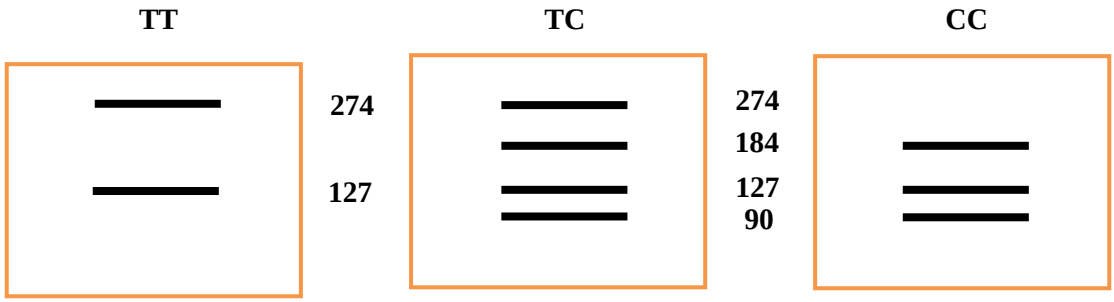


Fig 3.4: Possible genotypes (TT, TC and CC) generated using *Hae*III/exon 2 PCR-RFLP assay.

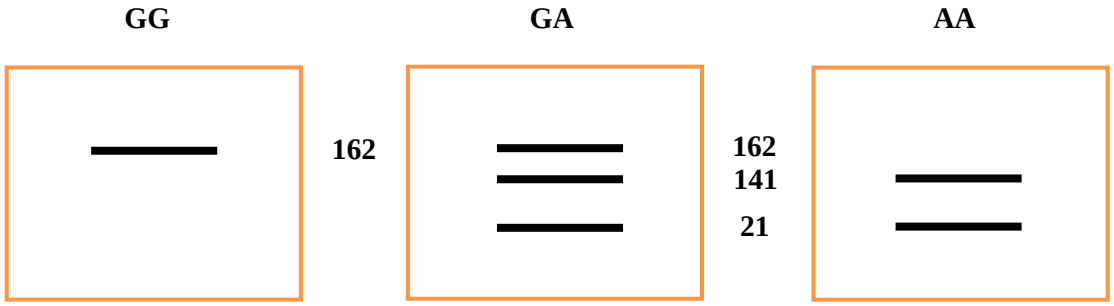


Fig 3.5: Possible genotypes (GG, GA and AA) generated using and *Sty*I/exon 2 PCR-RFLP assay.

3.7 Milk production data

Milk production data of genotyped animals was used for association studies between different genotypes and milk production traits. The milk production data such as age at first calving, lactation period, total milk yield and milk yield at 300 days were taken from the History sheet records maintained at Livestock Farm Complex (LFC), DUVASU, Mathura (U.P.). Milk production records were obtained by totalling daily milk yield of individual cow after completion of their lactation. The cows were milked manually using full hands method twice a day in morning and evening shift and the milk was weighed on digital weighing scale

in liters. Incomplete lactations for any recorded reason or ending with abortion or other anomaly were deleted.

3.8 Statistical analysis

3.8.1 Genotypic and allelic frequencies

The data was generated by estimating the frequency of different *MBL1* genotypes. The Genotypic and allelic frequencies were estimated by standard procedure (Falconer and Mackay, 1996) using following formulae:

$$\textbf{Genotypic frequency} = \frac{\text{Total no. of individual of particular genotype}}{\text{Total no. of individuals of all genotype}}$$

$$\textbf{Allelic/gene frequency} = \frac{(2D + H)}{2N}$$

Where, D is number of homozygote of particular gene; H is number of heterozygote having that gene and N is total no. of individuals

3.8.2 Chi Square (χ^2) analysis

The chi square (χ^2) test ($P \leq 0.05$) was also performed to test whether the distribution of the genotype frequencies was in the Hardy-Weinberg equilibrium (Snedecor and Cochran, 1989) as following:

$$\chi 2 = \Sigma \frac{(\text{Observed} - \text{Expected})^2}{\text{Expected}}$$

The calculated Chi-square values were compared to the tabulated values at specific degree of freedom.

3.8.3 Association study with somatic cell count

Association study between *Apa*I/intron I, *Hae*III/exon 2 and *Sty*I/exon 2 genotypes and somatic cell count of milk was carried out to analyse the correlation between genotype and mastitis.

3.8.4 Association study with milk production traits

The association study of *Apa*I/intron I, *Hae*III/exon 2 and *Sty*I/exon 2 genotypes with following milk production traits: Age at first calving (AFC = date of 1st calving – date of birth; DOB), Lactation period (LP = date of drying – date of calving), Total milk yield (TMY = calculated by totalling of daily milk records of individual cow after completion of their lactation.), Milk yield at 300 days (MY300 = calculated by totalling of daily milk records of individual cows up to 300 days of lactation)Association study was performed and analyzed by one way ANOVA with the help of SPSS (version 16.0) statistical software. Statistical analysis

of milk production and reproduction traits in relation to *MBL1* genotypes was carried out using the General Linear Model (GLM) using SPSS software. The following linear model was applied:

- $Y_{ij} = \mu + G_i + e_{ij}$

Where Y_{ij}– observed trait value in animal; μ – mean trait value; G_i – effect of genotype; e_{ij}– random error. Significant differences among least square means of different genotypes were calculated using Duncan's multiple-range test, and P values of 0.05 were considered statistically significant.

CHAPTER-4

RESULTS

In the present study, *MBL1* gene CDS in Murrah buffalo was cloned and characterized. Additionally, cattle (Hariana and Sahiwal) and Murrah buffalo population screened for reported single nucleotide polymorphisms *viz.* g.855G>A located within intron I region, g.2686T>C and g.2651G>A located within exon 2 region of *MBL1* gene, respectively. Genotyped animals were analysed for association study with milk production traits and SCC.

4.1 Cloning and characterization of *MBL1* CDS

4.1.1 RNA isolation and cDNA synthesis

The quality of total RNA isolated from liver tissue sample of Murrah buffalo was checked on 1.0% agarose gel electrophoresis. RNA was visualized under gel documentation system and revealed two bands *viz.,* 28S and 18S of rRNA (Fig. 4.1).

The concentration and purity of the isolated RNA was estimated by using Nanodrop. The concentration of isolated RNA was observed in the range of 71.2 ng/µl. Purity and contamination was checked by OD_{260}/OD_{280} ratio value. The OD_{260}/OD_{280} ratio was 1.84 indicating purity of RNA without contamination of phenol and protein.The cDNA was synthesized from total RNA by reverse transcription PCR using Oligo $(dT)_{18}$ primer.

4.1.2 PCR amplification of *MBL1* CDS

Complete coding sequence of *MBL1* gene was successfully amplified using a specifically designed primers and with optimized PCR condition. A 951 bp amplicon was obtained by PCR amplification using cDNA as template (Fig. 4.2).

4.1.3 Cloning of *MBL1* PCR product

PCR amplicon of 951 bp was directly used in ligation reaction and cloned using plasmid vector pTZ57/RT. The cloning vector was linearized and ddT tailed for direct use in cloning of PCR products generated with *Taq* DNA polymerase mixture, which added extra adenines to the ends of PCR products. The ligation reaction product transformed into *Escherichia coli* DH5α host cells. White recombinant clones were isolated from LB-Amp-X-gal agar plates using blue-white screening (Fig. 4.3). Two white recombinant clones were picked up using inoculation loop and transferred in LB broth media containing ampicillin antibiotic solution which were kept overnight at 37ºC.

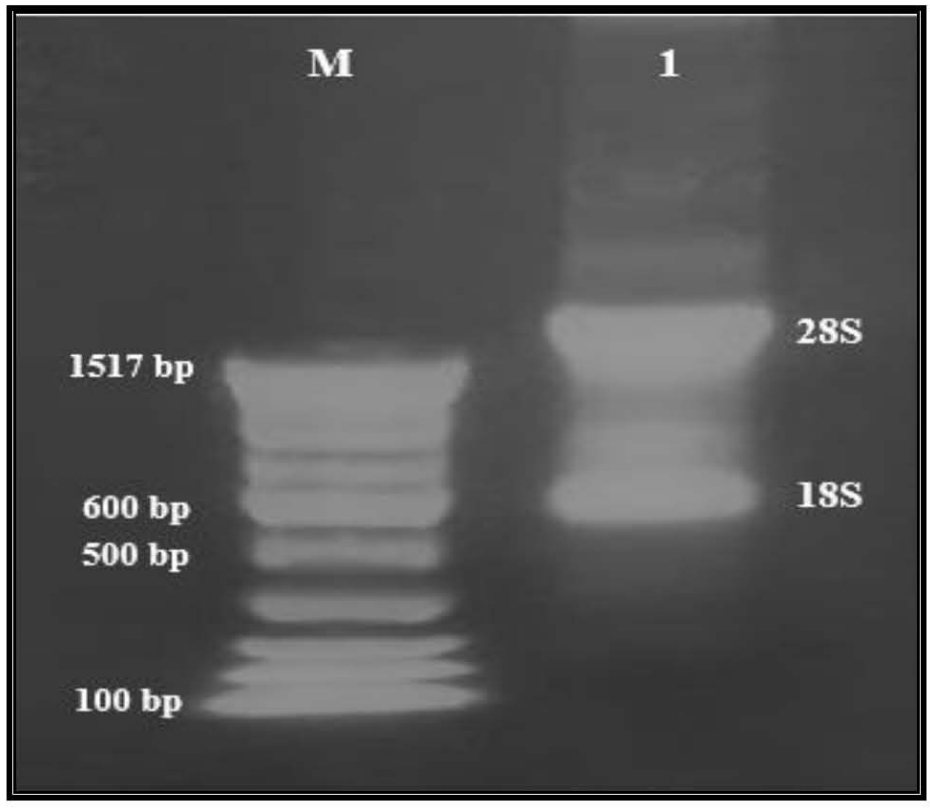


Fig. 4.1: Agarose (1%) gel electrophoresis showing bands of RNA. Lane M: Marker (100 bp DNA ladder), Lane 1: 28S and 18S band of RNA.

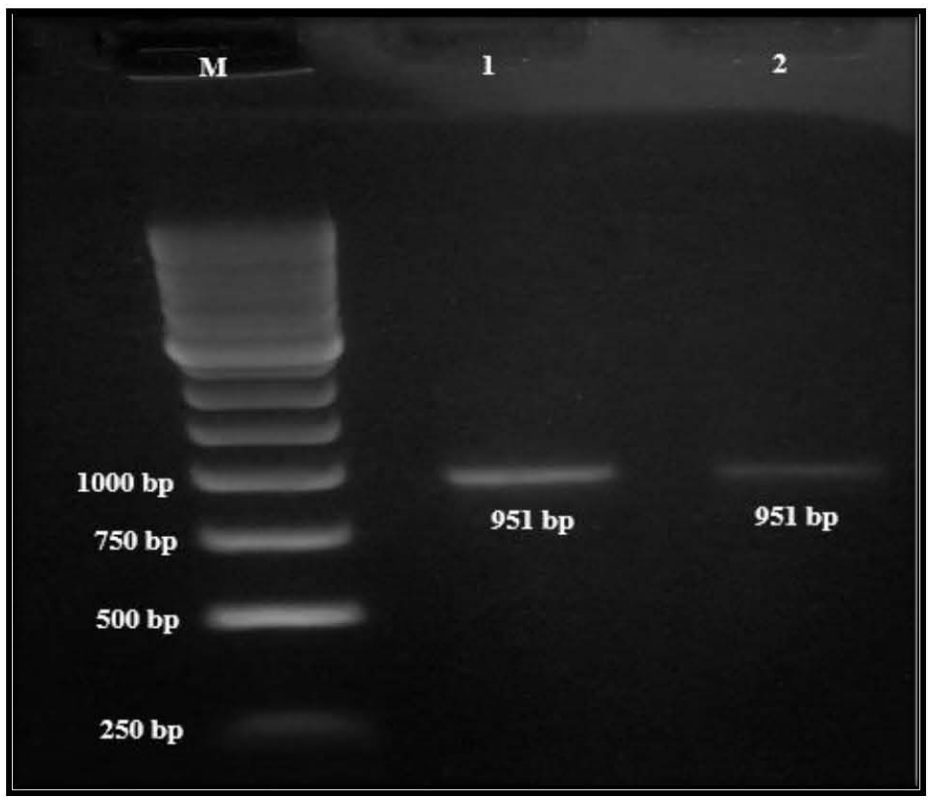
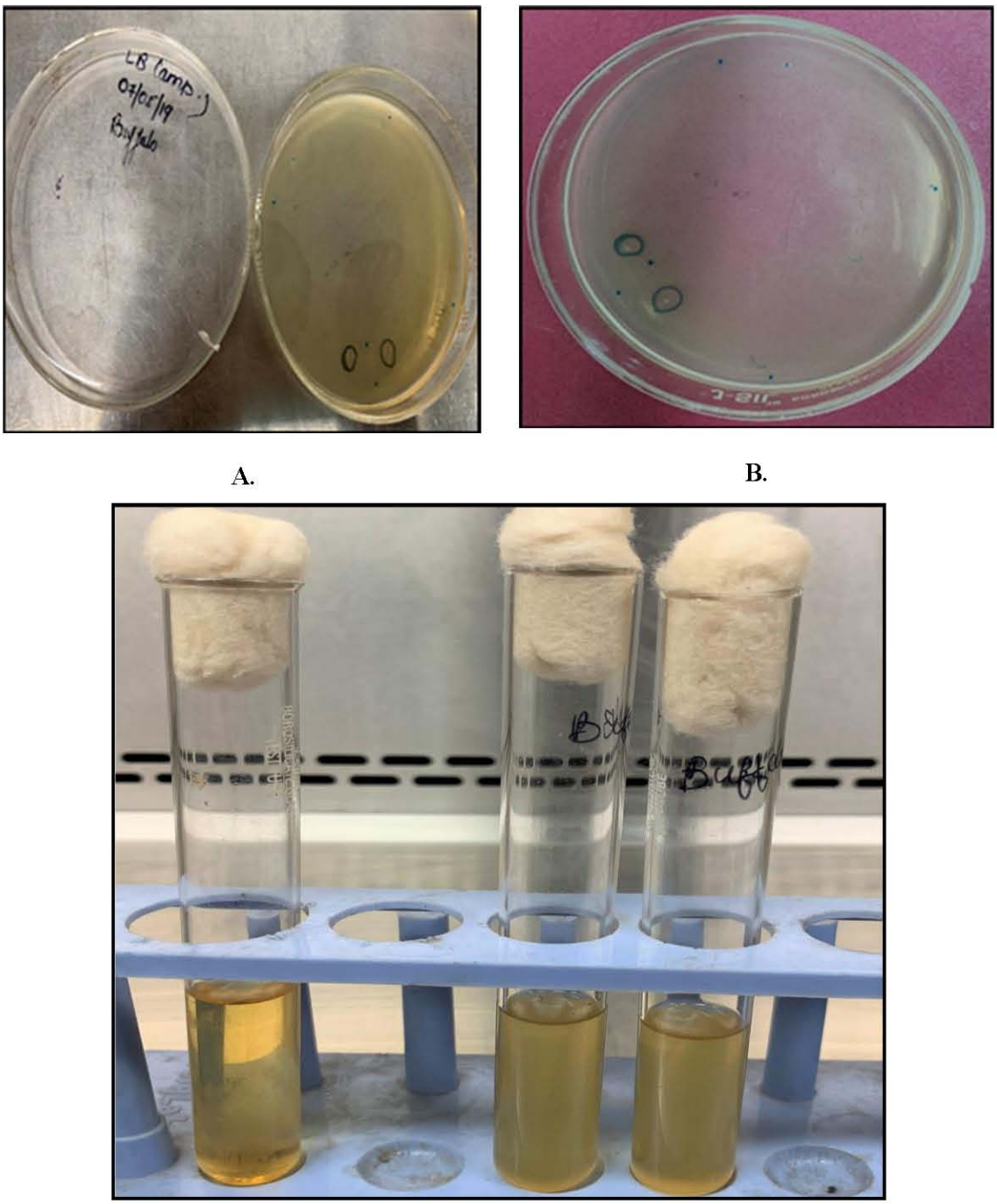


Fig. 4.2: Agarose (1%) gel electrophoresis showing *MBL1* coding region amplicon. Lane M: Marker (250 bp DNA ladder), Lane 1-2: PCR product of *MBL1* coding region.



A. B.

C.

Fig. 4.3: A. LB-Amp-X-gal agar plate showing blue (non-recombinant) and white colonies (recombinant clone), B. Close up picture of LB-Amp-X-gal agar plate showing white colonies (recombinant clones), C. One glass tube having LB-Amp broth and two tubes having turbidity showing growth of recombinant clones kept overnight at 37 ℃.

4.1.4 Plasmid purification for analysing the presence of *MBL1* gene insert

For analysis of true identity of cloned fragment, the recombinant plasmid DNA was isolated from two individual clones kept in broth media culture tubes overnight at 37°C. Concentration of plasmid was estimated by using Nanodrop and quantified to be 54.50 ng/µl and 86.40 ng/µl for clone 1 and clone 2, respectively.

4.1.5 PCR amplification of insert DNA from recombinant plasmid DNA

The amplification of insert fragment of candidate gene in recombinant plasmid vector was performed using *MBL1* gene specific primers that were previously used to amplify *MBL1* CDS region using cDNA as template. PCR amplification of recombinant plasmid DNA resulted into fragments of expected size (Fig. 4.4). The size of the amplicon was further confirmed by the nucleotide sequencing. The recombinant clone with desired insert size true to identity of correspondingly cloned amplicon DNA for the amplified candidate gene fragment was selected for sequencing. Recombinant plasmid having higher concentration *viz.* 86.4 ng/µl was used for sequencing.

4.1.6 Sequencing of the cloned *MBL1* gene fragment

The size of the gene insert/amplicon was further confirmed by commercial sequencing of recombinant plasmid DNA containing gene insert of 951 bp. After sequencing, a sequence similarity search was carried out in GenBank database using NCBI BLAST tool. The BLAST result showed the most resemblance to *MBL1* sequences from other buffalo/cattle breeds, confirming that the nucleotide sequence obtained in the present study was exactly the expected CDS encoding *MBL1*. The CDS sequence of the Murrah buffalo *MBL1* was submitted in GenBank database with accession no. MN990687.

4.1.7 Sequence analysis

The sequence analysis of nucleotide as well as amino acid sequence by Lasergene software (DNASTAR, USA) revealed that the complete CDS of buffalo *MBL1* consisted of 747 base pairs and encodes a protein of 248 amino acids. Among the total base count, it contains adenine (A) =194 (25.97%), guanine (G) =216 (28.92%), thymine (T) =152 (20.35%) and cytosine (C) =185 (24.77%). The *MBL1* CDS sequence had, A+T =346 (46.32%) and C+G =401 (53.68%). Among the 248 amino acids, 29 were strongly basic (+) amino acids (K and R), 27 were strongly acidic (-) amino acids (D and E), 72 were hydrophobic amino acids (A, I, L, F, W and V) and 64 were polar amino acids (N, C, Q, S, T and Y). The isoelectric point of the protein was analyzed to be 7.91 and charge was 2.04 at pH 7.0. Six open reading frames (ORF) were present in the *MBL1* CDS.

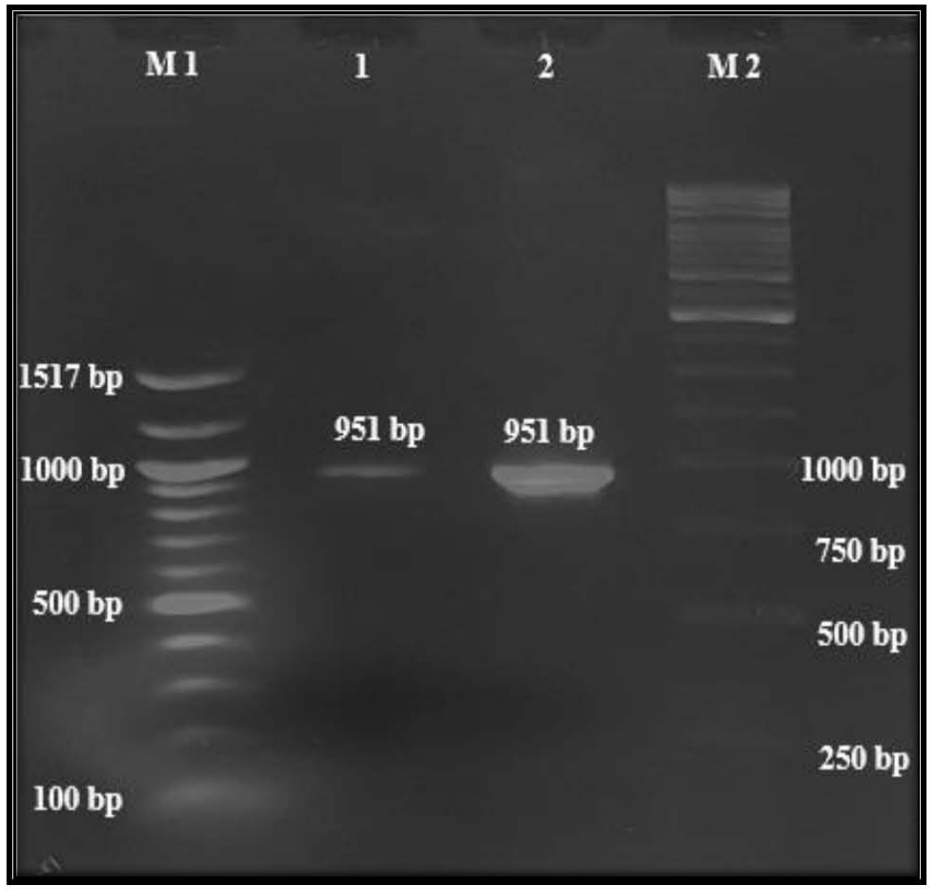


Fig. 4.4: Recombinant plasmid based PCR products of 951 bp. Lane M1: Marker (100 bp DNA ladder), Lane 1-2: PCR products of 951 bp obtained using plasmid of 54.5 ng/µl and 86.4 ng/µl, Lane M2: Marker (250 bp DNA Ladder).

In present study, a 951 bp sequence was obtained. It contained a long 158 bp (part of exon 1 and exon 2) 5′-untranslated region (5′-UTR) followed by 57 bp analogous to a signal peptide of 19-aa residues, 690 bp analogous to the mature protein and a sort 46 bp 3′-UTR (Fig. 4.5). It consisted five exons including 149 bp long first non-coding exon 1 (1-149) that was transcribed but untranslated, 190 bp long exon 2 (150-339), 117 bp long exon 3 (340-456), 75 bp long exon 4 (457-531) and 420 bp long incomplete exon 5 but only 374 bp (from 532 to 905) of exon 5 were the part of CDS while the remaining nucleotides (46 bp) were noncoding (part of 3′ UTR). The non-coding exon 1 was present before the putative start of exon 2. At the start of exon 2, there were 9 noncoding nucleotides (150-158) of 5′ UTR. Exon 2 encoded the signal peptide (1-19 aa), a cysteine rich N-terminus region (20-42 aa) and a part of collagenous region (43-60 aa), exon 3 encoded the remainder of the collagenous region (61-98 aa), exon 4 encoded an α-helical coiled-coil structure known as 'neck region' (99-124 aa) and exon 5 encoded the CRD (125-248 aa) (Fig. 4.6).

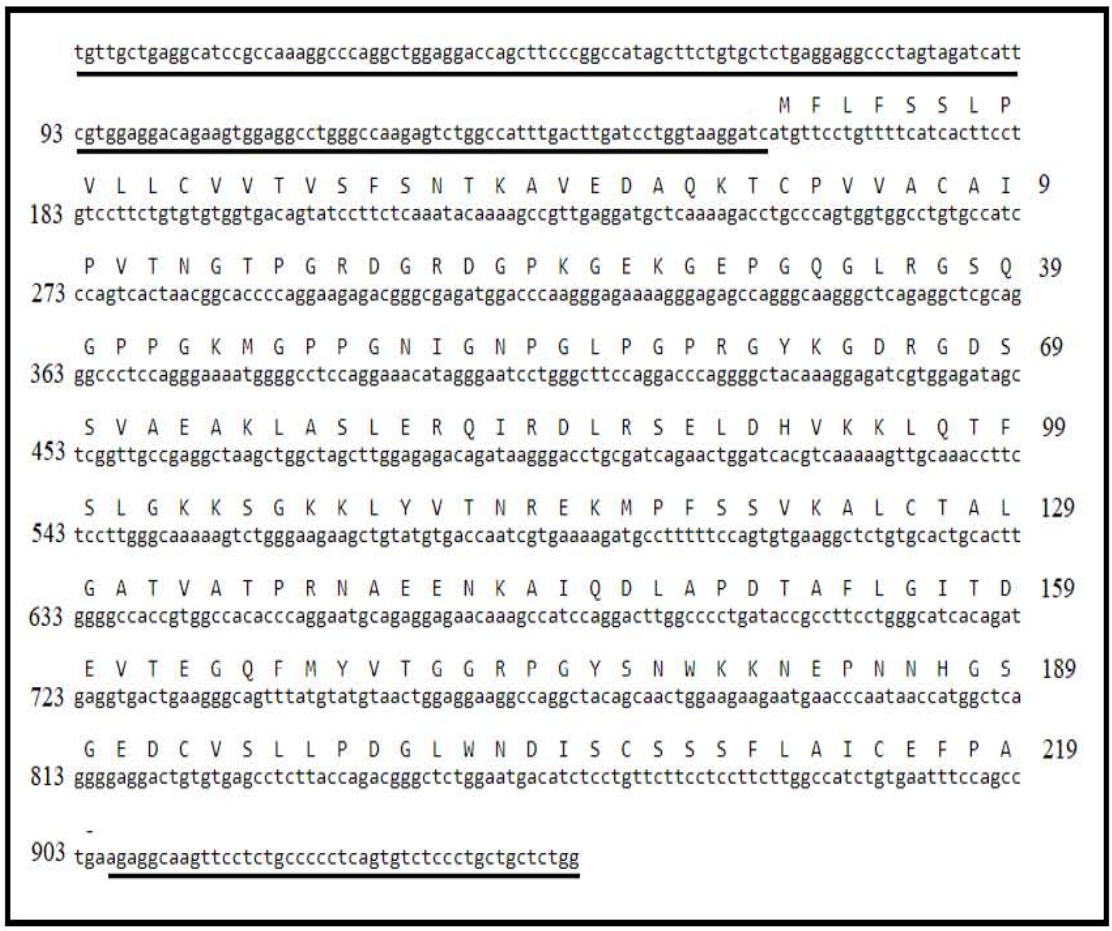


Fig. 4.5: Full-length sequence (951 bp) of MBL1 cDNA encoding the Murrah buffalo MBL-A protein with predicted amino acid translation. Numbers indicate amino acid (right side) and nucleotide (left side) position. The under lined sequences are non-coding regions of exon 1, 5′UTR and 3′UTR.

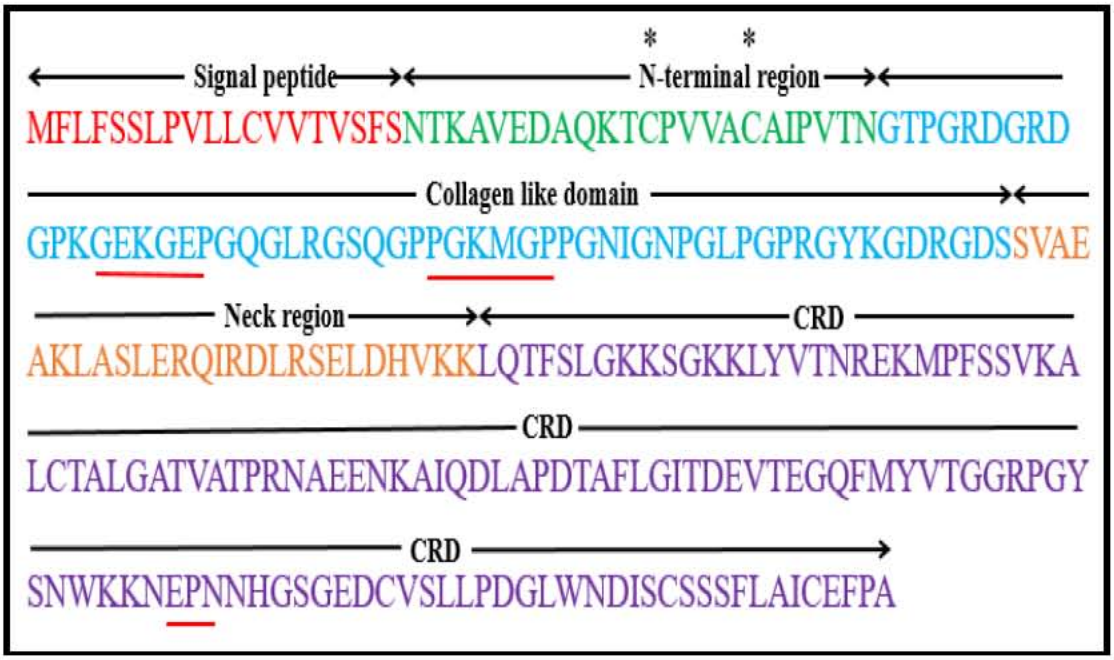


Fig. 4.6: Location of putative structural domains based on exon structure and amino acid sequence. Signal peptide—aa 1–19; N-terminal domain—aa 20–42; collagen domain—aa 43–99; neck region—aa 100–124; CRD—aa 125–248. Asterisks indicate cysteine residues in the N-terminal region. Underlined in red showing conserved sequences of MBL1/MBL-A protein.

The deduced amino acid sequence of the CDS of Murrah buffalo *MBL1* was found to have conserved sequences required for specific MBL functions. Like all other reported mammalian MBL, the amino acid sequence of Murrah buffalo *MBL1* was found to possess two cysteine residues (at position 31 and 36) in the short N-terminal domain, a GEKGEP sequence (at amino acid position 55-60) in the collagen domain, a PGKXGP sequence (PGKMGP at amino acid position 71-76) at the C-terminal end of the collagen like domain and a Glu–Pro–Asn (EPN) motif (the canonical mannose binding site) in its CRD. In addition, the collagen-like domain consisted of numerous Gly-X-Y motifs (X and Y representing any amino acid) and one Gly-X-Gly (a characteristic of MBL) from amino acid position 43 to 98. There was an interruption in the collagen Gly-X–Y triple helix which reported to introduce a kink needed for the sertiform structure of MBLs.

4.1.8 Sequencing comparison with other buffalo/cattle breeds and species

The comparative analysis of sequence included complete CDS (747 bp) of sub family bovinae such as American buffalo (*Bison bison* or American Bision, XM_010834154), *Bubalus bubalis* (Italian Mediterranean buffalo, XM_006064773), *Bos taurus* (Mixed breed, NM_001010994), *Bos taurus* (Crossbred x Angus, BC109674), *Bos taurus* (AB178774), *Bos indicus* (Nelore, XM_019954216), hybrid *Bos indicus x Bos taurus* (Angus x Brahman F1

hybrid, XM_027530780) and *Bos mutus* (Wild yak, XM_005909163), sub family Caprinae such as *Capra hircus* (San Clemente, XM_005699265), *Ovis aries* (Ramouillet, XM_004021518) and *Ovis aries* (Domestic sheep, LT852560). Two breeds of *Sus scrofa* or pig belongs to family suidae were also included in comparative analysis which were Yorkshire (NM_001007194) and Landrace (EU421730). Animals belongs to family camelidae were also included such as *Camelus bactrianus*/Bactrian camel (Alxa, XM_010963110) and *Camelus dromedaries*/Arabian camel (Breed African, XM_010984099).

For the purpose of comparison of *MBL1* CDS sequence of Murrah buffalo with exotic buffalo and cattle breeds and other related species, sequence distances in form of percent identity among nucleotide sequences and a multiple sequence alignment were performed at both nucleotide and amino acid level.

4.1.8.1 Sequence identity analysis at nucleotide level

The percent identity of Murrah buffalo *MBL1* CDS was compared with that of other buffalo and cattle breeds, ruminant and non-ruminant species available in the GenBank database (Fig. 4.7). Comparison of *MBL1* CDS sequences revealed that Murrah buffalo *MBL1* sequence of present study was more similar to that of exotic Mediterranean buffalo (99.6%) than another (partial) Murrah buffalo (99.1%) and American buffalo (*Bison bison*) (98.1%) sequence. Murrah buffalo *MBL1* sequence was more similar to exotic cattle breeds (98.3-98.5%) than American buffalo (98.1%). Among all the cattle breeds, *MBL1* sequence of Murrah buffalo showed more similarity with Nelore cattle (*Bos indicus*, Brazilian origin) (98.5%). It showed 98.4% similarity with its wild relative *Bos mutus* (yak). Murrah buffalo *MBL1* sequence showed 96.1% and 95.2% similarity with sequences of goat and sheep, respectively. It showed less similarity with pig breeds (Landrace pig and Yorkshire pig) (83.5%) and camel breeds (Alxa Bactrian camel and Arabian camel) (83.5-83.7%), respectively.

4.1.8.2 Sequence identity analysis at amino acid level

The deduced amino acid sequence from Murrah *MBL1* CDS was compared with that of other buffalo and exotic cattle breeds and other related species available in the GenBank database (Fig. 4.8). On comparison of MBL1 amino acid sequences, it was revealed that Murrah buffalo MBL1 sequence of present study was equally similar (98.8%) to another Murrah buffalo and exotic Mediterranean buffalo. It was least similar to exotic American buffalo (*Bison bison*) (96.8%) among buffalo breeds. Amino acid sequence of Murrah buffalo MBL1 was more similar to exotic cattle breeds (96.8-97.2 %) than American buffalo (96.8%).

Among all the cattle breeds, amino acid sequence of Murrah buffalo MBL1 showed more similarity with Nellore cattle (*Bos indicus*, Brazilian origin) (97.2%). It showed 97.6% similarity with its wild relative *Bos mutus* (yak). Amino acid sequence of Murrah buffalo MBL1 showed 95.2% and 94.4% similarity with sequences of goat and sheep, respectively. On the other hand, it showed less similarity with pig breeds (79.9%) and camel breeds (81.9%), respectively.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
1		99.2	99.6	98.1	98.3	98.3	98.5	98.3	98.3	96.1	95.2	98.4	83.5	83.5	83.5	83.7	1
2	0.8		99.6	98.5	98.1	98.1	98.4	98.1	98.1	96.1	95.2	98.8	83.2	83.2	83.6	83.8	2
3	0.4	0.4		98.5	98.7	98.7	98.9	98.7	98.7	96.1	95.2	98.8	83.4	83.4	83.8	83.9	3
4	1.9	1.4	1.5		99.3	99.3	99.3	99.3	99.3	96.4	95.2	99.7	83.3	83.3	83.0	83.1	4
5	1.8	1.8	1.4	0.7		99.7	99.7	100.0	99.7	96.1	94.9	99.3	83.1	83.1	83.3	83.4	5
6	1.8	1.8	1.4	0.7	0.3		99.7	99.7	100.0	96.4	94.9	99.3	83.4	83.4	83.1	83.3	6
7	1.5	1.5	1.1	0.7	0.3	0.3		99.7	99.7	96.1	94.9	99.3	83.3	83.3	83.1	83.3	7
8	1.8	1.8	1.4	0.7	0.0	0.3	0.3		99.7	96.1	94.9	99.3	83.1	83.1	83.3	83.4	8
9	1.8	1.8	1.4	0.7	0.3	0.0	0.3	0.3		96.4	94.9	99.3	83.4	83.4	83.1	83.3	9
10	3.9	3.6	3.7	3.3	3.7	3.4	3.7	3.7	3.4		98.7	96.8	84.5	84.5	83.5	83.7	10
11	4.7	4.4	4.6	4.4	4.9	4.9	4.9	4.9	4.9	1.4		95.6	84.3	84.3	82.7	82.9	11
12	1.6	1.1	1.2	0.3	0.7	0.7	0.7	0.7	0.7	3.0	4.2		83.7	83.7	83.3	83.4	12
13	15.9	16.0	15.8	16.1	16.3	16.1	16.3	16.3	16.1	14.7	14.7	15.8		100.0	85.8	85.9	13
14	15.9	16.0	15.8	16.1	16.3	16.1	16.3	16.3	16.1	14.7	14.7	15.8	0.0		85.8	85.9	14
15	17.0	17.0	16.8	17.9	17.5	17.7	17.7	17.5	17.7	17.1	18.0	17.5	13.8	13.8		99.9	15
16	16.8	16.8	16.6	17.7	17.3	17.5	17.5	17.3	17.5	17.0	17.8	17.4	13.6	13.6	0.1		16
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	

1 Murrah Buffalo MN990687.seq
2 Murrah Buffalo_KM087783.seq
3 Mediterranean Buffalo XM006064773.seq
4 American Buffalo XM010834154.seq
5 AngusXBrahman Cattle XM027530780.seq
6 CrossbredXAngus Cattle BC109674.seq
7 Nelore Cattle_XM019954216.seq
8 Bos taurus Cattle AB178774.seq
9 Mixed Breed Cattle NM001010994.seq
10 San clemente Goat XM005699265.seq
11 Rambouilet Sheep XM004021518.seq
12 Yak XM005909163.seq
13 Landrace Pig EU421730.seq
14 Yorkshire Pig NM001007294.seq
15 Alxa Bactrian Camel XM010963110.seq
16 Arabian Camel XM010984099.seq

Fig. 4.7: Percent identity of *MBL1* CDS of Murrah buffalo breed with other breeds of buffalo/cattle and other related species on the basis of nucleotide sequences (above diagonal) and percent divergence (below diagonal) estimated using DNASTAR.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
1		98.8	98.8	96.8	97.2	96.8	97.6	97.2	96.8	95.2	94.4	97.6	79.9	79.9	81.9	81.9	1
2	1.2		99.6	97.6	98.0	97.6	98.4	98.0	97.6	95.2	94.4	98.4	79.8	79.8	82.3	82.3	2
3	0.8	0.4		97.6	98.0	97.6	98.4	98.0	97.6	95.2	94.4	98.4	79.5	79.5	81.9	81.9	3
4	2.9	2.5	2.0		99.2	98.8	98.8	99.2	98.8	94.8	94.0	98.8	79.1	79.1	80.3	80.3	4
5	2.5	2.1	1.6	0.4		99.2	99.2	99.6	99.2	95.2	94.4	99.2	79.5	79.5	80.7	80.7	5
6	2.9	2.5	2.0	0.8	0.4		98.8	99.2	99.6	95.6	94.4	98.8	79.5	79.5	80.7	80.7	6
7	2.0	1.6	1.2	0.8	0.4	0.8		99.2	98.8	94.8	94.0	98.8	79.1	79.1	80.7	80.7	7
8	2.5	2.1	1.6	0.4	0.0	0.4	0.4		99.2	95.2	94.4	99.2	79.5	79.5	80.7	80.7	8
9	2.9	2.5	2.0	0.8	0.4	0.0	0.8	0.4		95.6	94.4	98.8	79.5	79.5	80.7	80.7	9
10	4.6	5.0	4.6	5.0	4.6	4.1	5.0	4.6	4.1		98.4	95.6	80.7	80.7	81.1	81.1	10
11	5.4	5.9	5.4	5.9	5.4	5.4	5.9	5.4	5.4	1.2		94.8	80.7	80.7	80.7	80.7	11
12	2.0	1.6	1.2	0.8	0.4	0.8	0.8	0.4	0.8	4.1	5.0		79.9	79.9	81.1	81.1	12
13	21.4	22.0	21.9	22.4	21.9	21.9	22.4	21.9	21.9	20.3	20.3	21.4		99.6	81.1	81.1	13
14	21.4	22.0	21.9	22.4	21.9	21.9	22.4	21.9	21.9	20.3	20.3	21.4	0.0		81.1	81.1	14
15	20.3	20.4	20.3	22.4	21.9	21.9	21.9	21.9	21.9	21.4	21.9	21.4	19.8	19.8		99.6	15
16	20.3	20.4	20.3	22.4	21.9	21.9	21.9	21.9	21.9	21.4	21.9	21.4	19.8	19.8	0.0		16
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	

1 Murrah Buffalo_MN990687.pro
2 Murrah Buffalo_KM087783.pro
3 Mediterranean Buffalo_XM006064773.pro
4 American Buffalo_XM010834154.pro
5 Angus X Brahman Cattle_XM027530780.pro
6 Crossbred X Angus Cattle_BC109674.pro
7 Nelore Cattle_XM019954216.pro
8 Bos taurus Cattle_AB178774.pro
9 Mixed Breed Cattle_NM001010994.pro
10 San clemente Goat_XM005699265.pro
11 Rambouilet Sheep_XM004021518.pro
12 Yak_XM005909163.pro
13 Landrace Pig_EU421730.pro
14 Yorkshire Pig_NM001007294.pro
15 Alxa Bactrian Camel_XM010963110.pro
16 Arabian Camel_XM010984099.pro

Fig. 4.8: Percent identity of MBL1 CDS of Murrah buffalo breed with other breeds of buffalo/cattle and other related species on the basis of amino acid sequences (above diagonal) and percent divergence (below diagonal) estimated using DNASTAR.

4.1.9 Identification of Single nucleotide polymorphisms (SNPs) in nucleotide sequence

The multiple sequence alignment of the nucleotide sequences of MBL1 gene Murrah buffalo breed with other buffalo and cattle breeds revealed identification of several SNPs, as presented in Table 4.1. MBL1 CDS of Murrah buffalo contained three nucleotide changes including a synonymous nucleotide change at position 87G→A of exon 1 as well as two non-synonymous nucleotide changes at position 538T→C and 611T→C of exon 4, respectively. Similar nucleotide change (538T→C) was also present in goat, sheep, pig and camel breeds when compared with ruminant and non-ruminant species. Apart from these nucleotide changes found in studied *MBL1* CDS of Murrah buffalo, previously reported partial CDS of Murrah buffalo had one non-synonymous nucleotide change at position 451A→G of exon 4. Additionally, a non-synonymous nucleotide change was found in American buffalo at position 178G→A of exon 1 and two synonymous nucleotide changes were found in both (previously reported Murrah buffalo and American buffalo) at positions 420T→C and 576G→C of exon 4, respectively. All the cattle breeds contained a synonymous nucleotide change at position 103G→A. All the nucleotide changes are shown in Fig. 4.9.

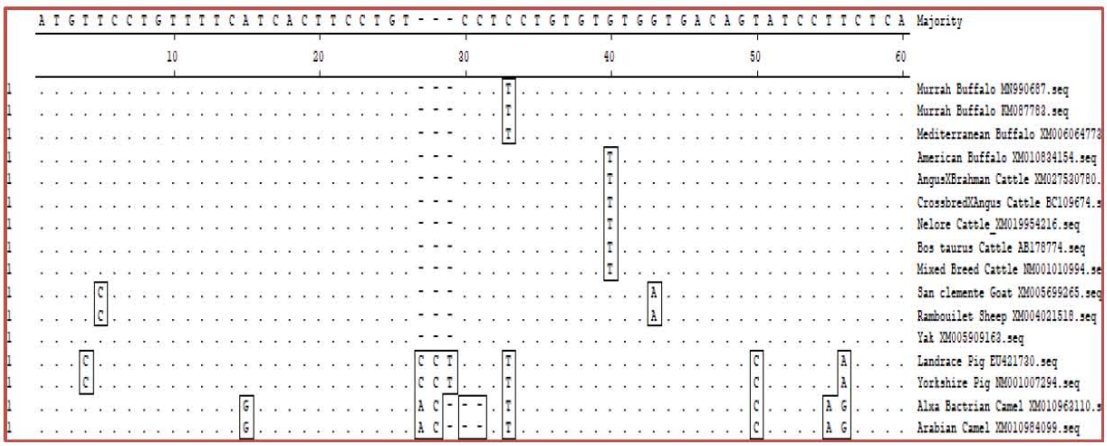
4.1.10 Identification of amino acid substitutions

Deduced amino acid sequence of MBL1 CDS of Murrah buffalo was compared (Fig. 4.10) with other buffalo and cattle breeds as given in Table 4.1. Studied Murrah buffalo MBL1 CDS had "Pro" at positions 180 and 204 in CRD region of amino acid sequence, different to other buffalo breeds and cattle breeds ("Ser" at position 180 and "Leu" at position 204). Previously reported Murrah buffalo had "Gly" at position 151 in CRD region which was different from other buffalo and cattle breeds ("Ser" at position 151). Moreover, American buffalo showed variation at position 60 by having "Lys" (K) in collage like domain while other buffalo and cattle breeds had "Glu" at position 60. On comparison with other ruminant and non-ruminant species, all the species had a gap at position twelve of N-terminal region of amino acid sequence except pig breeds which had "Leu" residue. All the amino acid substitutions are shown in Fig. 4.10.

Table 4.1: Different polymorphic sites in Indian buffalo breed and exotic buffalo/cattle breeds

Nucleotide	87	611	538	451	178	420	576	103
Amino acid	29	204	180	151	60	140	192	34
Region of *MBL1* CDS	Exon 1	Exon 4	Exon 4	Exon 4	Exon 1	Exon 4	Exon 4	Exon 1
Region of MBL-A protein	Mat_P (N-terminal domain)	Mat_P (CRD)	Mat_P (CRD)	Mat_P (CRD)	Mat_P (CLD)	Mat_P (CRD)	Mat_P (CRD)	Mat_P (N-terminal domain)
Codon Indian buffalo breed	CAA	CCT	CCT	GGC	GAG	TAT	ACT	GTG
Codon exotic buffalo/cattle breeds	CAG	CTA	TCT	AGC	AAG	TAC	ACC	GTA
AA Indian buffalo breed	Q	P	P	G	E	T	T	V
AA exotic buffalo/cattle breeds	Q	L	S	S	K	T	T	V
Breed	Murrah buffalo	Murrah buffalo	Murrah buffalo	Murrah buffalo	American buffalo	American buffalo and Murrah buffalo	American buffalo and Murrah buffalo	All cattle breeds

Nucleotide: position of the polymorphism in the nucleotide sequence; Amino acid: Affected amino acid site; Region of *MBL1* CDS: Coding region in which the polymorphic site is located; Region of MBL-A protein: Protein region in which the amino acid substitution occurred; Mat_P: Mature Peptide; AA: Amino acid; CRD: Carbohydrate Recognition Domain; CLD: Collagen Like Domain.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Murrah Buffalo KM087783.seq
Mediterranean Buffalo XM006064773
American Buffalo XM010834154.seq
AngusXBrahman Cattle XM027530780.
CrossbredXAngus Cattle BC109674.s
Nelore Cattle_XM019954216.seq
Bos taurus Cattle AB178774.seq
Mixed Breed Cattle NM001010994.se
San clemente Goat XM005699265.seq
Rambouilet Sheep XM004021518.seq
Yak XM005909163.seq
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Yorkshire Pig NM001007294.seq
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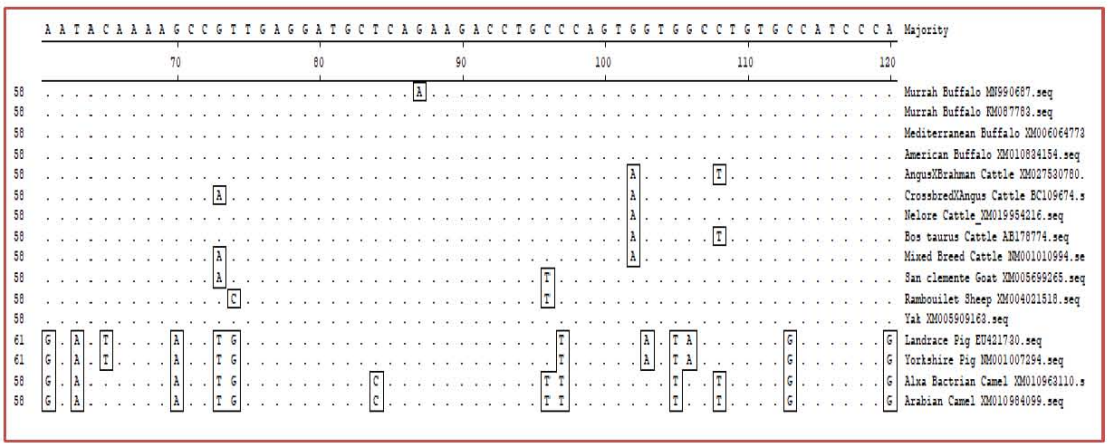
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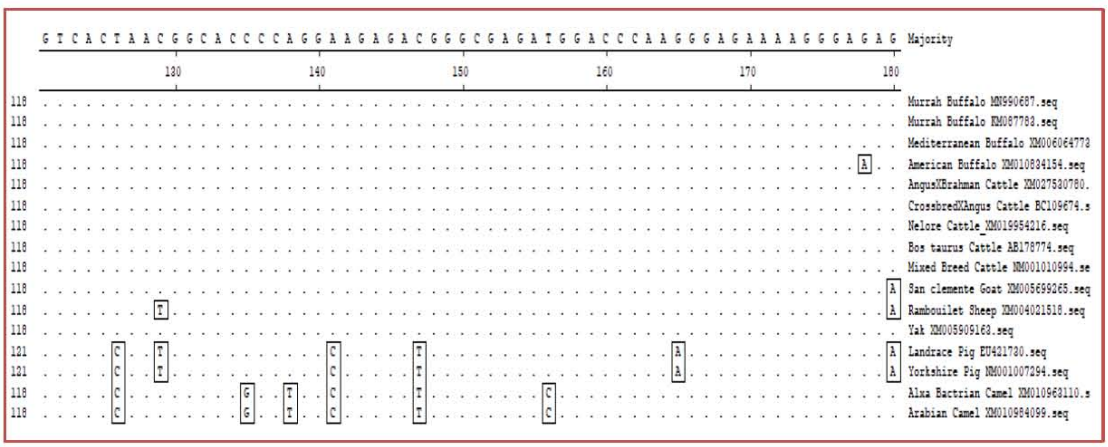
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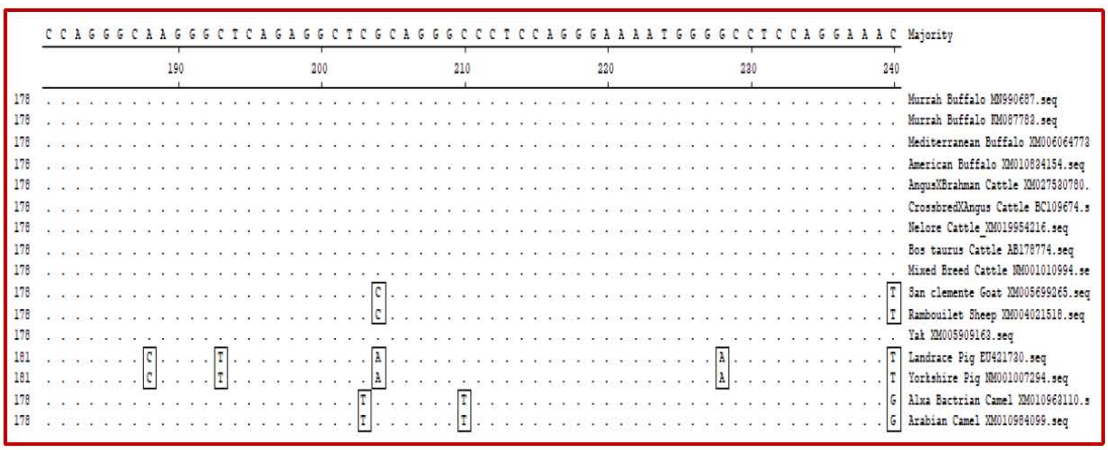
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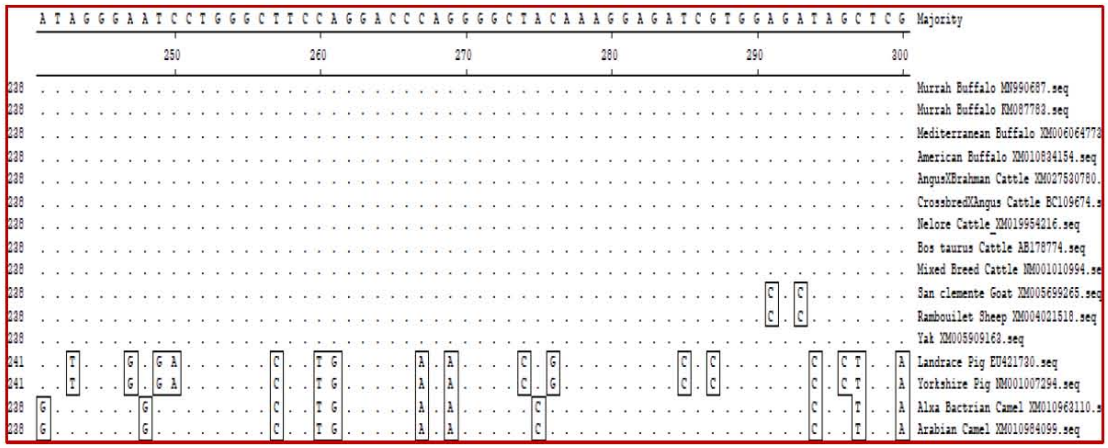
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Yak XM005909163.seq
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Yorkshire Pig NM001007294.seq
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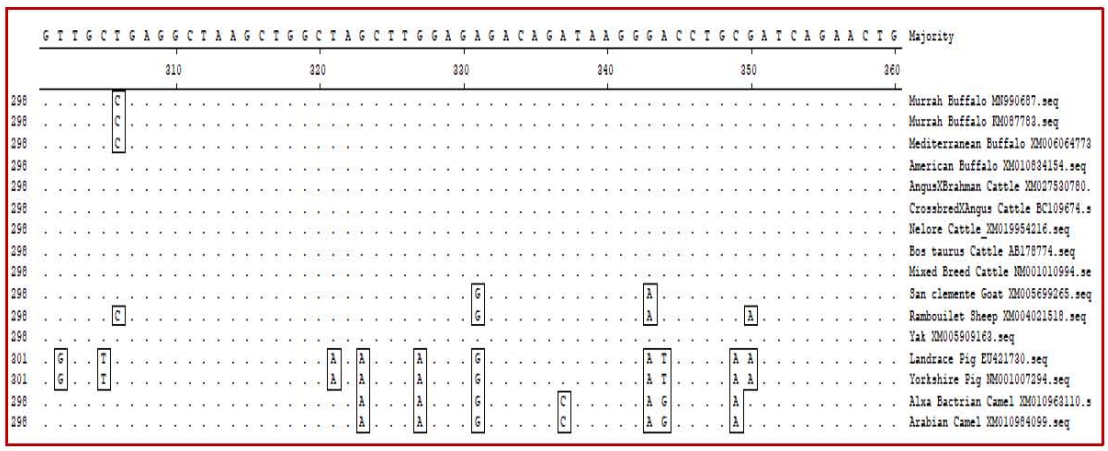
Arabian Camel XM010984099.seq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Majority
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330
340
350
360
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Murrah Buffalo KM087783.seq
Mediterranean Buffalo XM006064773
American Buffalo XM010834154.seq
AngusXBrahman Cattle XM027530780.
CrossbredXAngus Cattle BC109674.s
Nelore Cattle XM019954216.seq
Bos taurus Cattle AB178774.seq
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Rambouilet Sheep XM004021518.seq
Yak XM005909163.seq
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Alxa Bactrian Camel XM010963110.s
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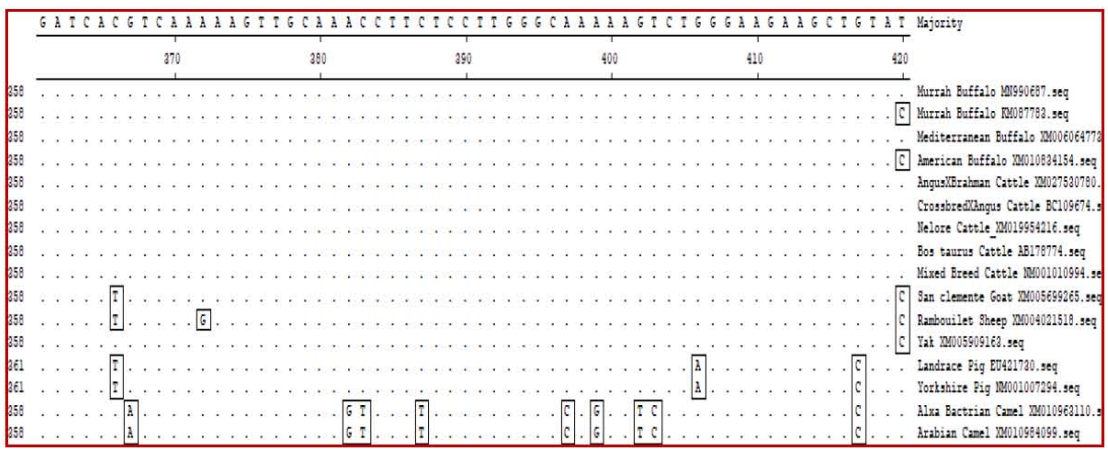
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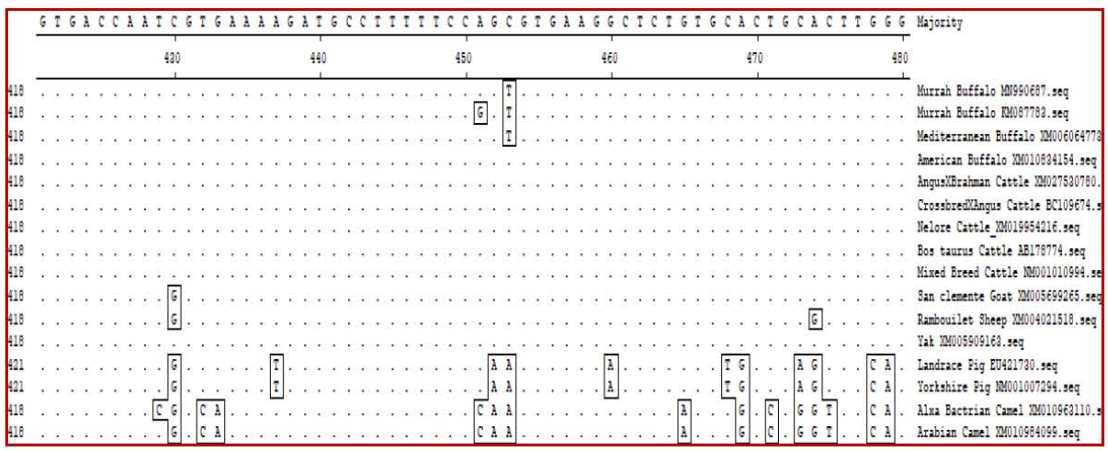
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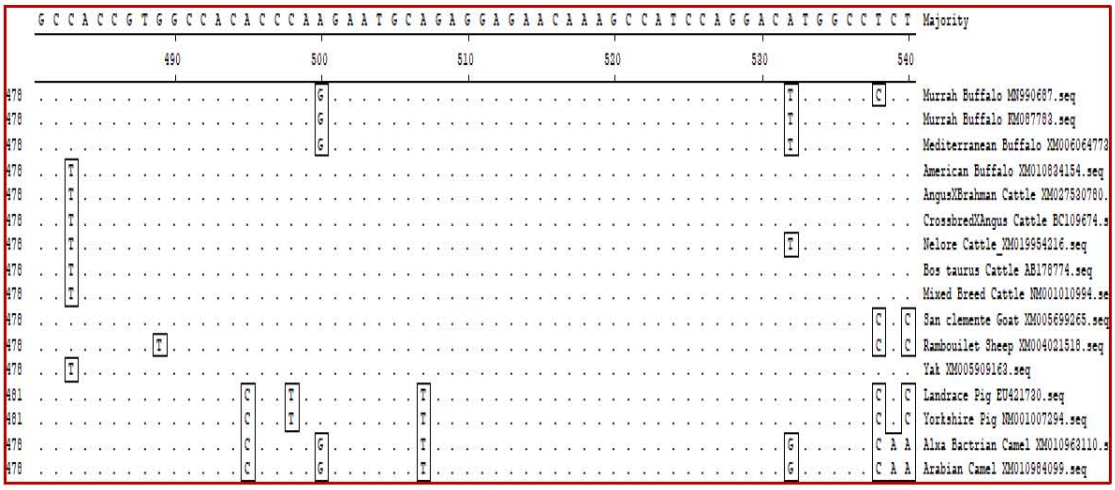
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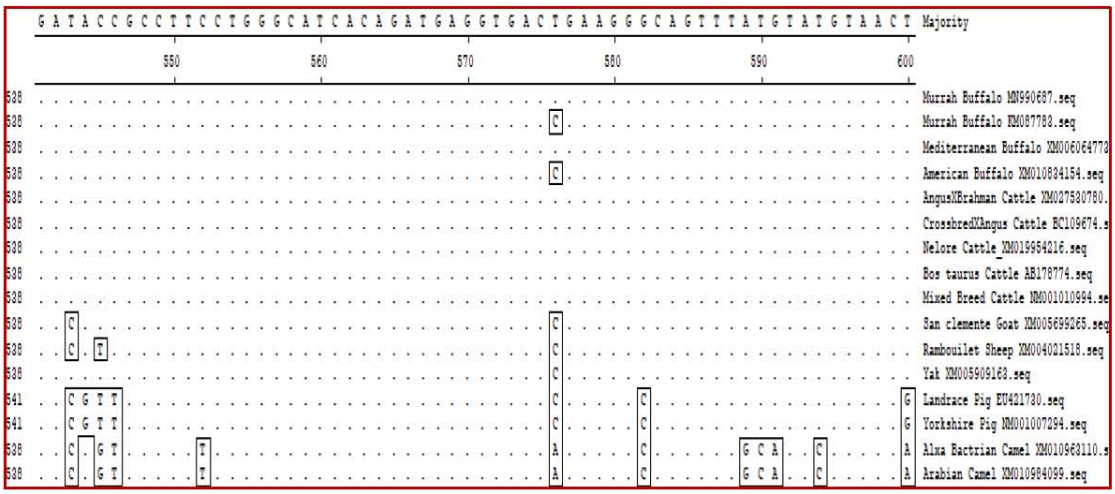
Majority
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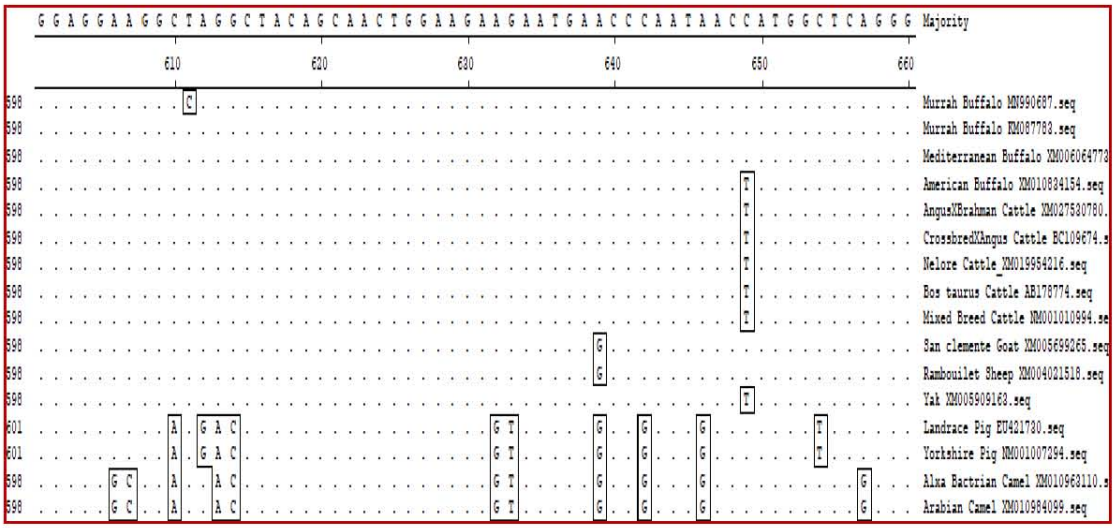
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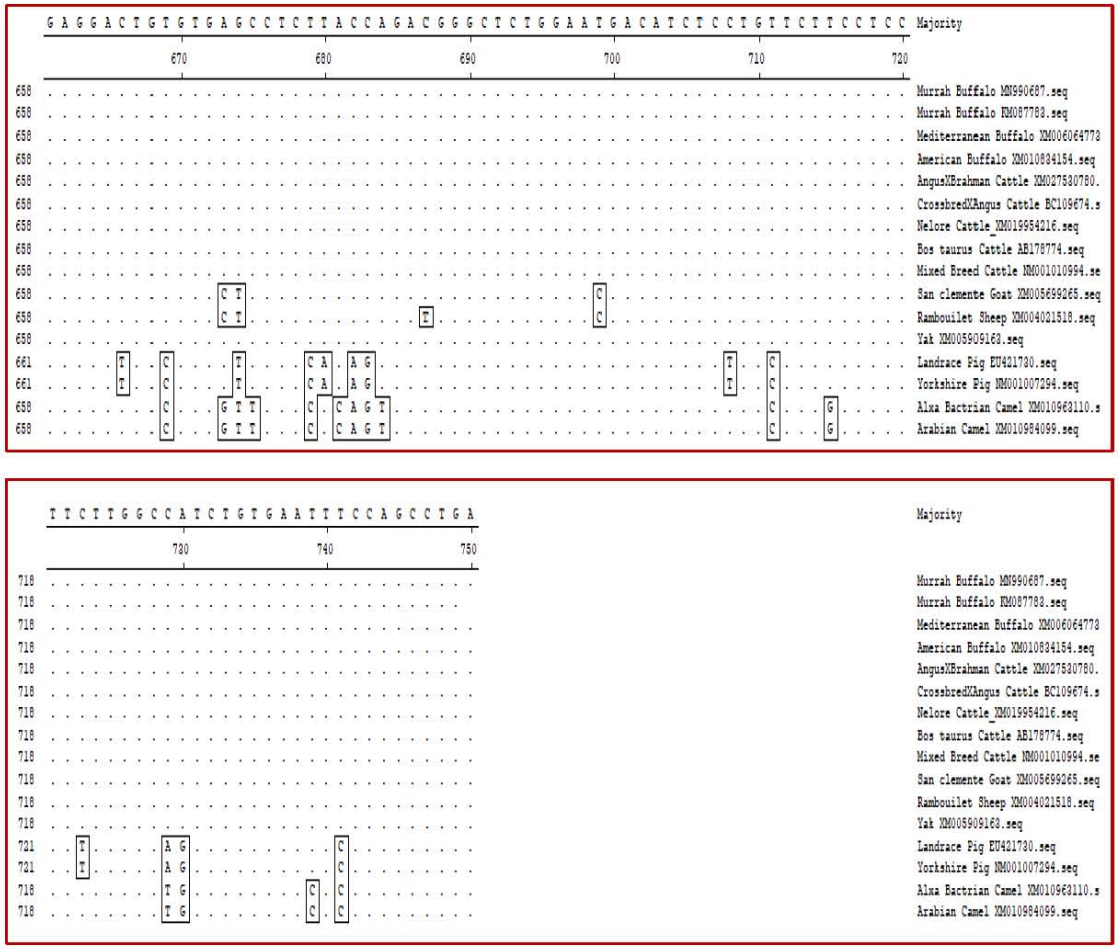

Fig. 4.9: Multiple sequence alignment of *MBL1* CDS of Murrah buffalo breed with other breeds of buffalo/cattle and other related species on the basis of CDS nucleotide sequences estimated using DNASTAR. Hide residue (....) that match the consensus exactly and Box residue that differ from the consensus. Majority: consensus.

4.1.11. Phylogenetic analysis

A phylogentic tree was constructed on the basis of deduced amino acid of *MBL1* CDS of Murrah buffalo and other related breeds/ species. The phylogenetic tree clearly indicates the splitting of the bovidae family into two clades: bovinae and caprinae subfamily showing a greater degree of variation leaving suidae and camelidae in two different clads (Fig. 4.11). Within the bovinae group, present studied Murrah buffalo *MBL1* sequence was to be most closely related to partial Murrah buffalo sequence followed by Mediterranean buffalo. However, American buffalo was to be most closely related to cattle breeds and yak. Caprinae group was more closely related to cattle and buffalo than suidae and camelidae group. In the phylogenetic tree, all buffalo breeds exhibited a close phylogenetic evolutionary relationship with cattle and yak.

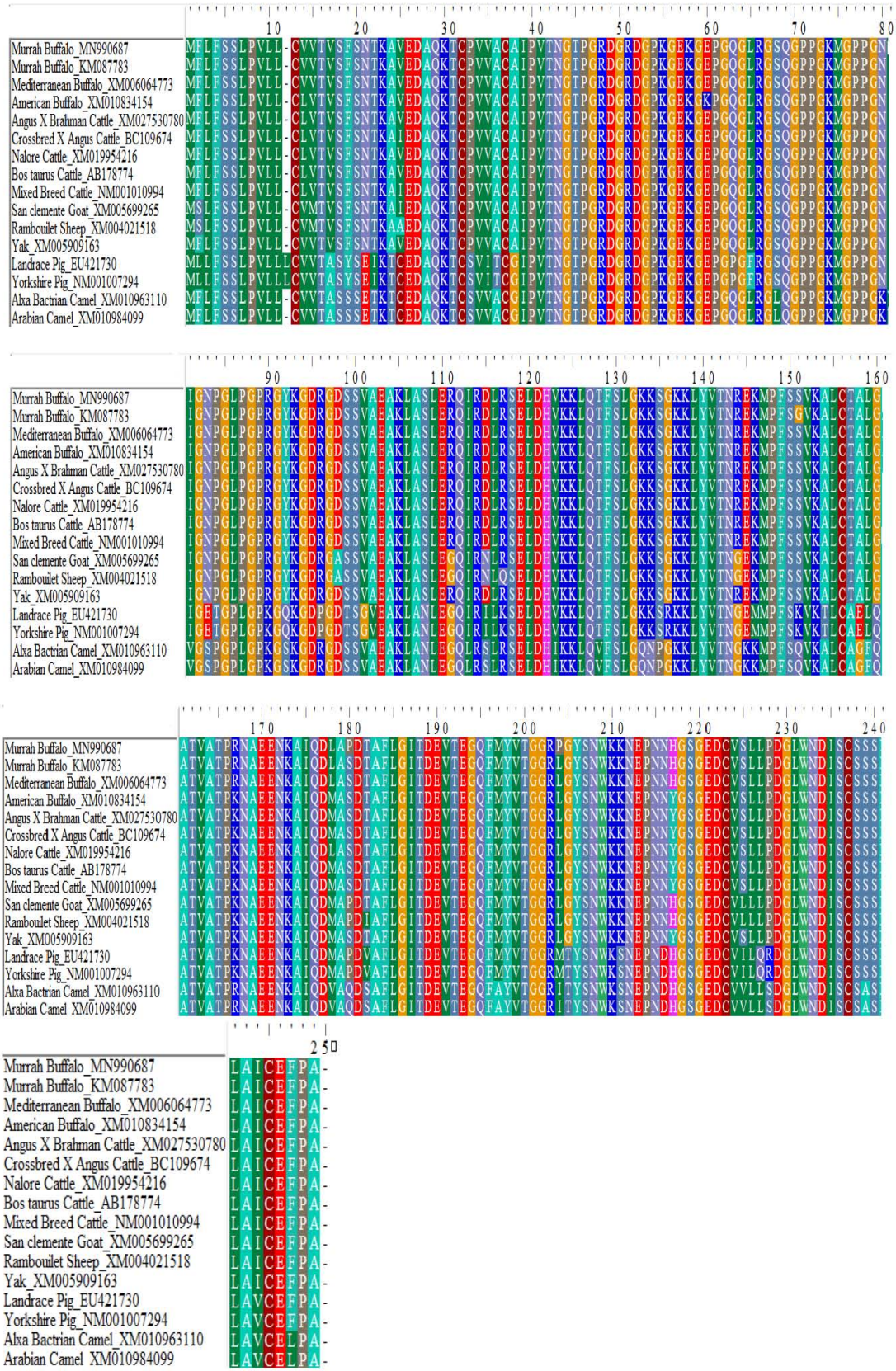

Fig. 4.10: Multiple sequence alignment of *MBL1* CDS of Murrah buffalo breed with other breeds of buffalo and other related species on the basis of CDS amino acid sequences estimated using BioEdit. Hide residue (....) that match the consensus exactly and box residue that differ from the consensus. Majority: Consensus.

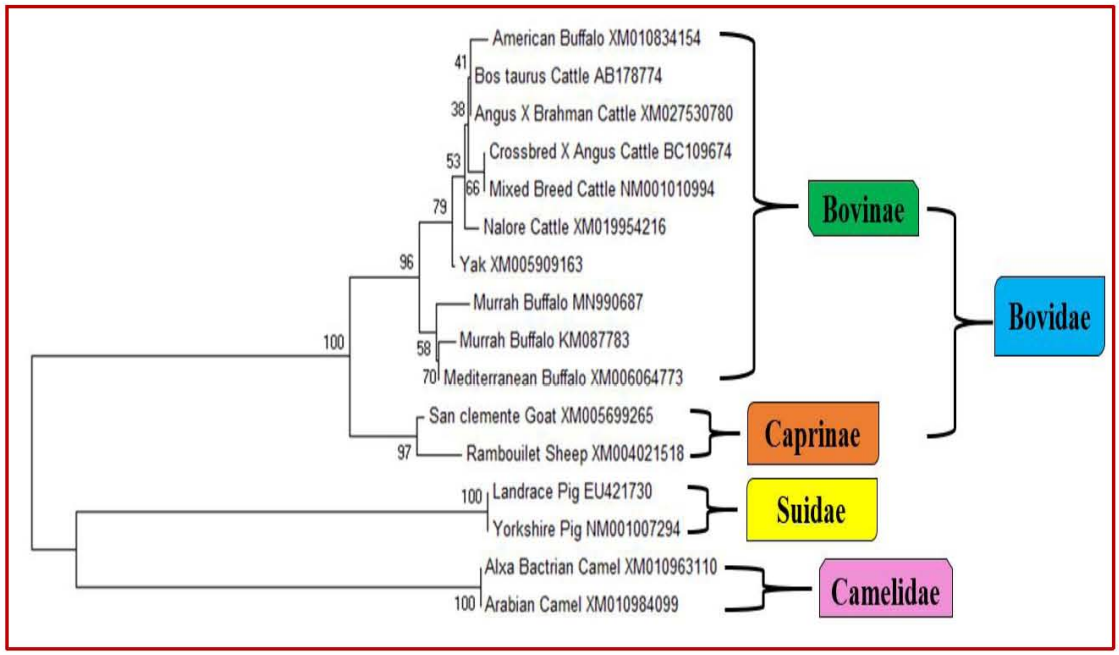

Fig. 4.11: Phylogenetic analysis based on deduced amino acids sequences of *MBL1* CDS for Murrah buffalo with other buffalo breeds and other related species using Neighbor-Joining method (Bootstrap test of phylogeny). The percentage of replicate trees (bootstrap value) in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

4.2 Somatic cell count

The lowest and highest somatic cell count and average cell size images generated from somatic cell counter LACTOSCAN are given in Fig. 4.12 to Fig. 4.15 for cattle and buffaloes irrespective of genotype of any polymorphism. The highest and lowest somatic cell count among cattle population were 1988.10^3 with 10.6 µm average cell size and 7.10^3 with 6.2 µm average cell size, respectively. The highest and lowest somatic cell count among buffalo population were 23.10^3 with average cell size 8.2 µm and 4.10^3 with average cell size 6.7 µm, respectively.

4.3 PCR-RFLP assay for polymorphism study

4.3.1 Isolation of genomic DNA

The quality of isolated genomic DNA of all the screened 150 samples (Hariana cattle = 50, Sahiwal cattle = 50 and Murrah buffaloes = 50) were checked by 0.7 % agarose gel electrophoresis. DNA was visualized under gel documentation system and revealed all the samples had single intact band without smearing near the well (Fig. 4.16).

The concentration of the DNA samples were estimated by using Nanodrop. The OD_{260}/OD_{280} ratio was ranged between 1.75 and 1.90 indicated purity of DNA without contamination of phenol and protein.

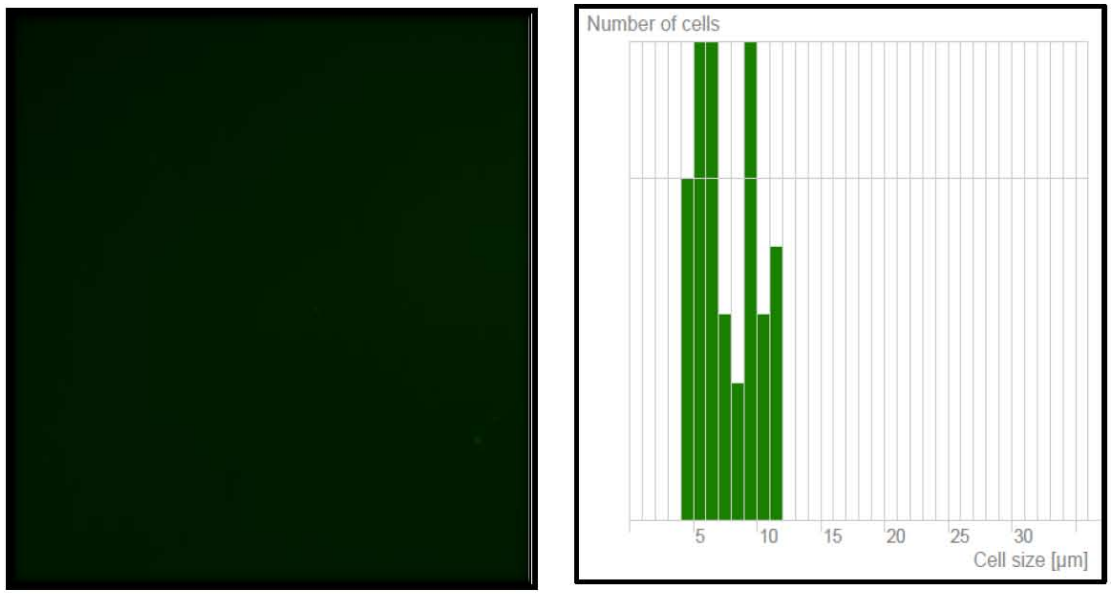

Fig. 4.12: Pictures from Lactoscan somatic cell counter A. Somatic cell count analysis image showing the lowest somatic cell count (7.103) among cattle population. B. Cell size histogram to show average cell size (6.2 µm) for the lowest somatic cell conunt among cattle population.

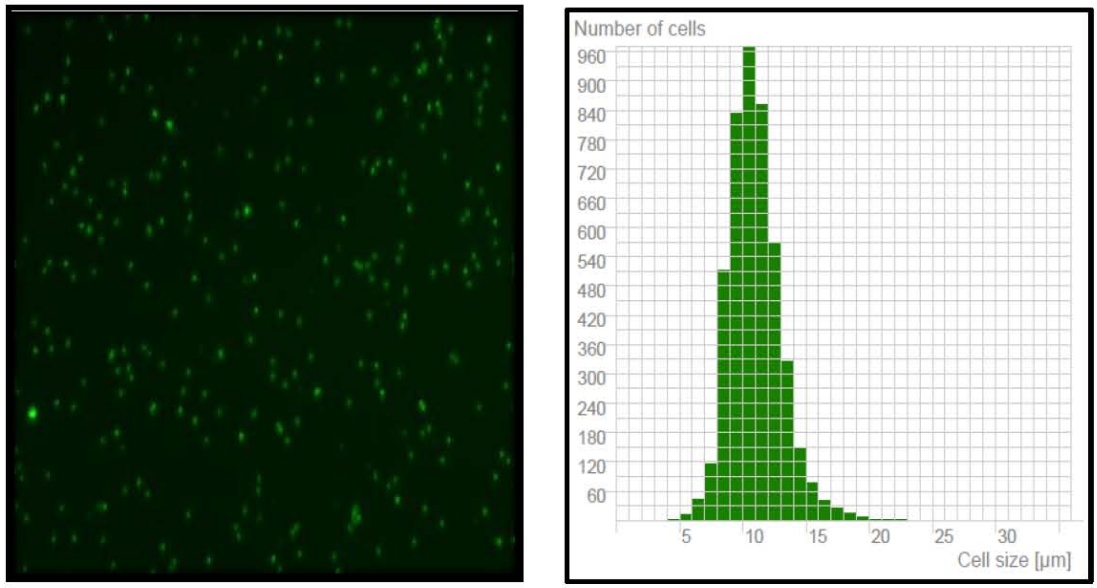

Fig. 4.13: Pictures from Lactoscan somatic cell counter A. Somatic cell count analysis image showing the highest somatic cell count (1988.103) among cattle population. B. Cell size histogram to show average cell size (10.6 µm) for the highest somatic cell conunt among cattle population.

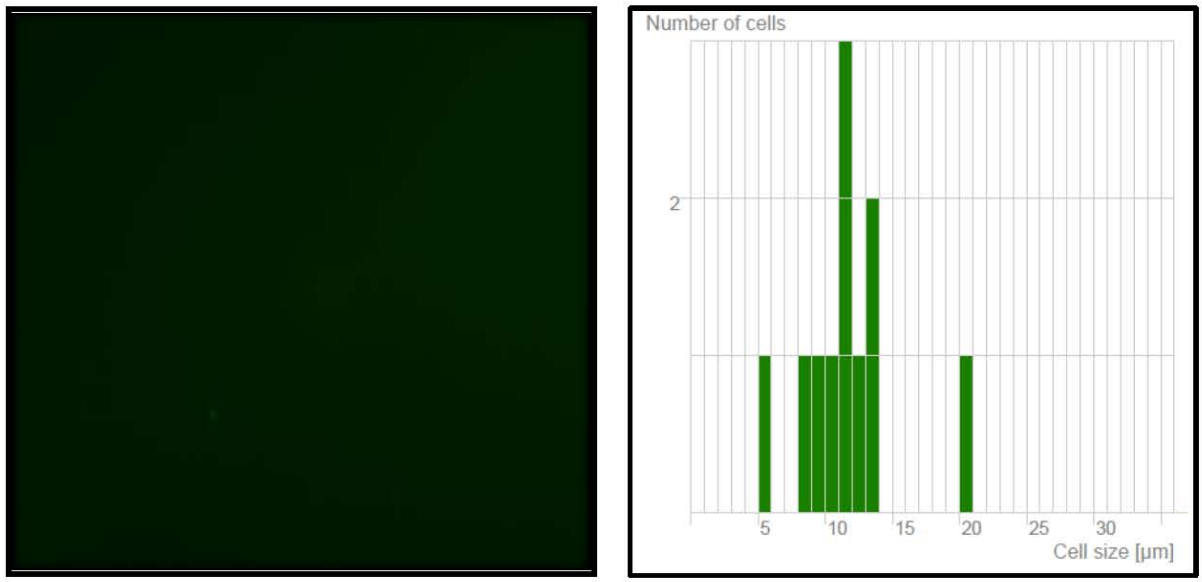

Fig. 4.14: Pictures from Lactoscan somatic cell counter A. Somatic cell count analysis image showing the lowest somatic cell count (4.103) among buffalo population. B. Cell size histogram to show average cell size (6.7 µm) for the lowest somatic cell conunt among buffalo population.

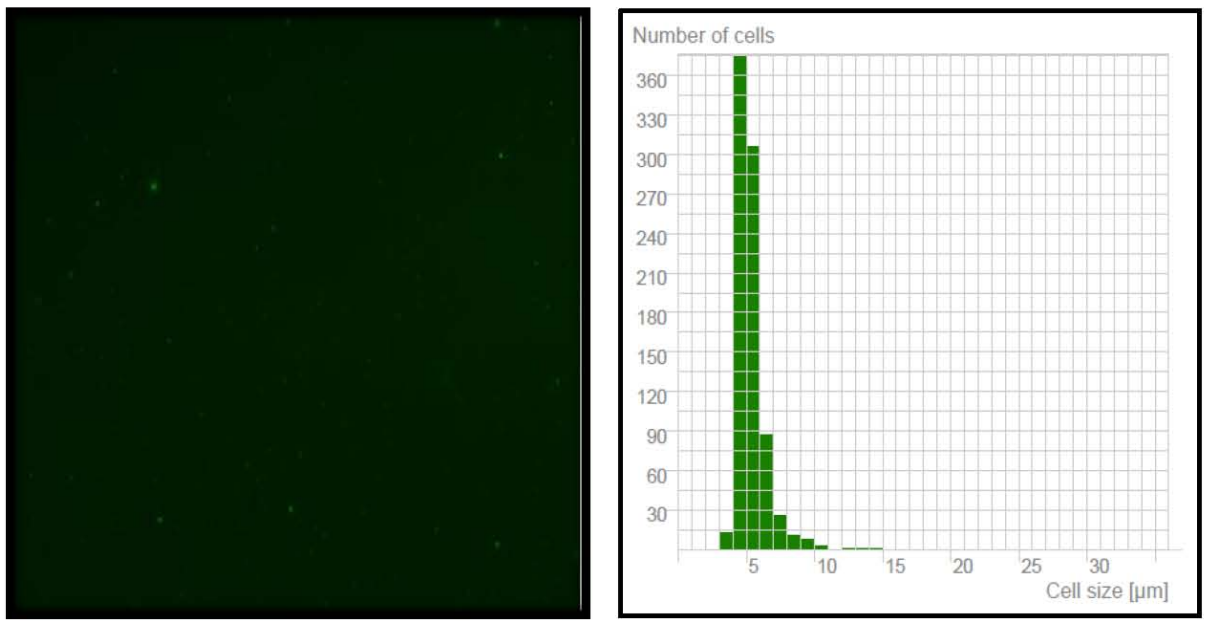

Fig. 4.15: Pictures from Lactoscan somatic cell counter A. Somatic cell count analysis image showing the highest somatic cell count (23.103) among buffalo population. B. Cell size histogram to show average cell size (8.2 µm) for the highest somatic cell conunt among buffalo population.

4.3.2 *Apa*I/intron I polymorphism study

4.3.2.1 Amplification of intron I region of *MBL1* gene

The PCR amplification of the *MBL1* intron I region produced 588 bp products in all the screened animals. The PCR products were resolved by performing 1.0% agarose gel electrophoresis and were documented using gel documentation system (Fig. 4.17).

4.3.2.2 *Apa*I/intron I-PCR-RFLP assay

For the screening of SNP g.855G>A in intron I region of *MBL1* gene, 588 bp amplified products were digested with *Apa*I restriction enzyme as per the protocol. The *Apa*I/intron I-PCR-RFLP assay revealed three types of genotypes *viz.* GG (band sizes 311 and 277 bp), GA (GG, 588, 311 and 277 bp) and AA (undigested; 588 bp) (Fig. 4.18). Out of 150 screened samples, most of the animals (n=122) were of GG genotype, then GA genotype (n= 25) and remaining were AA (wild) genotype (n=3). This revealed that the cattle and buffalo population used in the present study were polymorphic in nature with two types of alleles G and A.

4.3.2.3 Calculation of *Apa*I/intron I allelic and genotypic frequency

The genotypic and allelic frequencies of *Apa*I/intron I were calculated and presented in Table 4.2. Among all the samples of cattle and buffalo, homozygote genotype GG was more frequent (81.33%) than heterozygote genotype GA (16.66%) and homozygote AA genotype (2.0%). The allele G was more frequent (0.90) than allele A (0.10). Genotype GG was highest (90.0%) in Murrah buffaloes than Hariana (80.0%) and Sahiwal cattle (74.0%). The genotype AA was equally (2.0%) present in Murrah buffaloes, Sahiwal and Hariana cattle whereas the occurrence of heterozygote genotype GA was lowest (8.0%) in Murrah buffalo than Sahiwal (24.0%) and Hariana cattle (18.0%). The allelic and genotypic frequencies from the screened animals were calculated as following:

$$\textbf{Allelic/gene frequency} = \frac{(2D + H)}{2N}$$

Allelic frequency f (G) = (2D+H)/2N

Allelic frequency f (G) = (2x122+25)/2x150 = 0.896

= 89.60%

Allelic frequency f (A) = (2R+H)/2N

Allelic frequency f (A) = (2x3+25)/2x150 = 0.10

= 10.30%

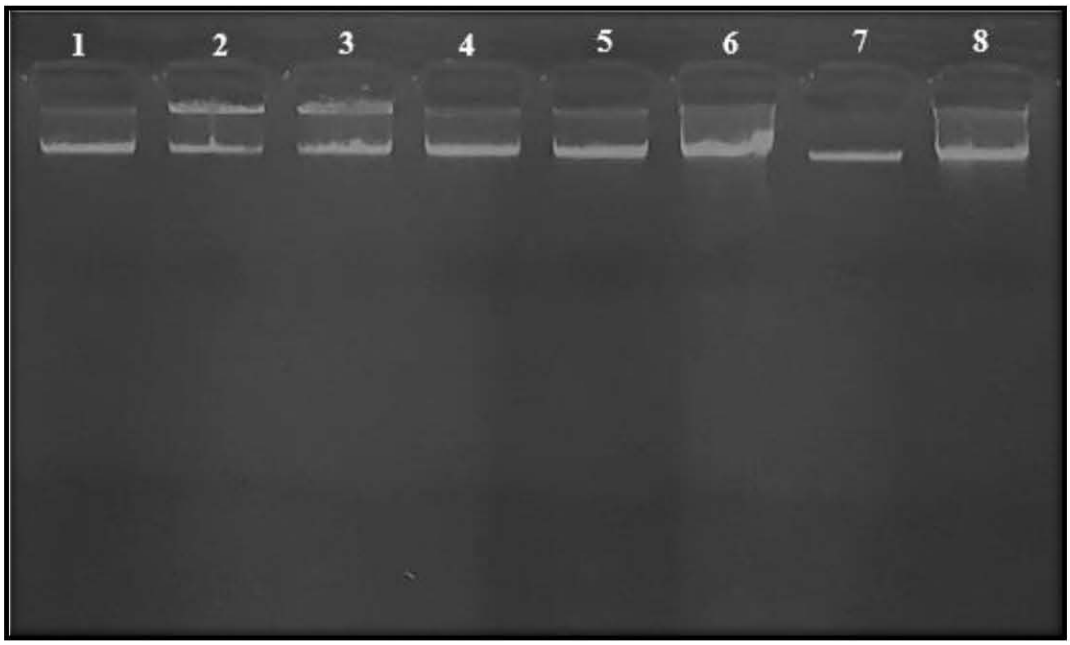

Fig. 4.16: Agarose (1%) gel electrophoresis showing single bands of DNA. Lane 1-3: DNA - bands of Hariana cattle, Lane 4-6: DNA bands of Sahiwal cattle and Lane 7-8: DNA bands of Murrah buffalo.

Fig. 4.17: Agarose (1.0%) gel electrophoresis showing amplification of intron 1 region (588 bp) of MBL1 gene of Hariana cattle (Lane 1-2), Sahiwal cattle (Lane 3-4) and Murrah buffalo (Lane 5-6), Lane M: Marker (100 bp DNA ladder).

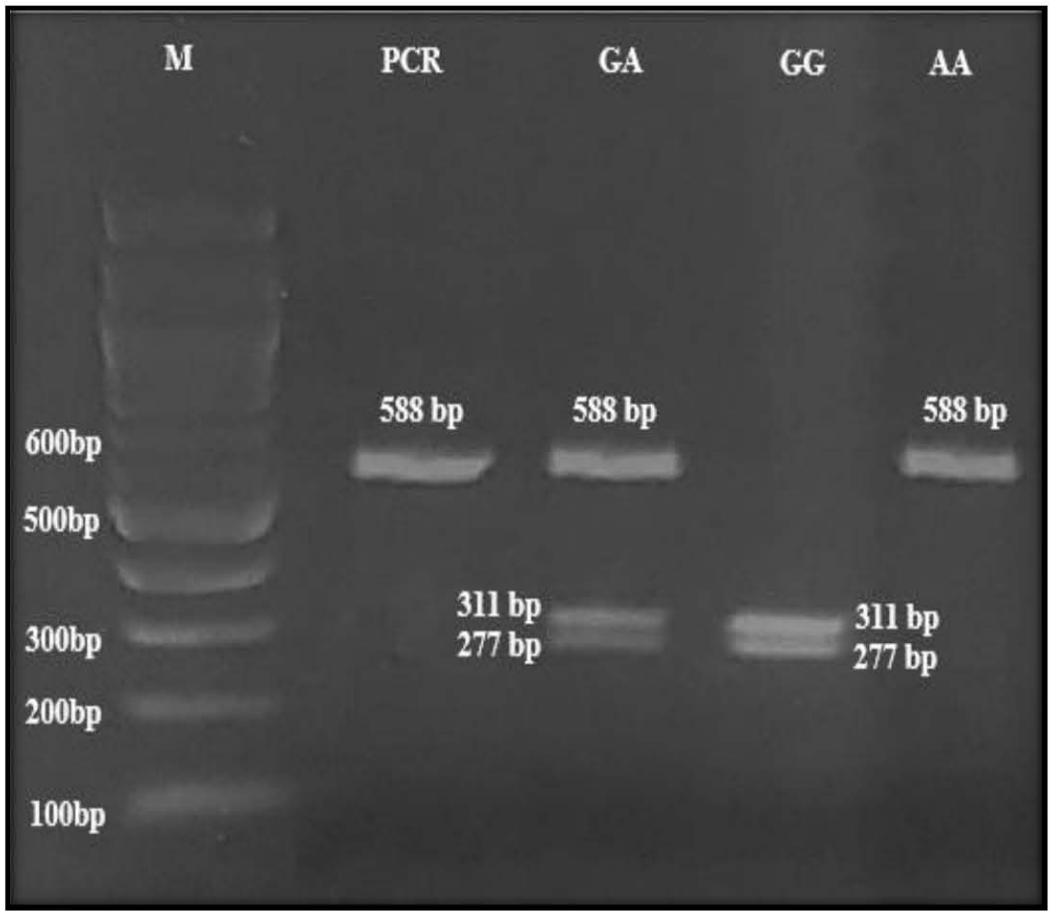

Fig. 4.18: RFLP products of *MBL1* intron I region digested with *Apa*I RE. Lane M: Marker (100 bp DNA ladder), Lane PCR: undigested PCR amplicon of 588 bp, Lane GA, GG & AA: RFLP products of size 588, 311 and 277 bp for genotype GA, 311 and 277 bp for genotype GG and 588 bp for genotype AA respectively.

$$\text{Genotypic frequency} = \frac{\text{Total no. of individual of particular genotype}}{\text{Total no. of individuals of all genotype}}$$

Genotypic frequency f (GG) = 122/150 = 0.813

= 81.30%

Genotypic frequency f (GA) = 5/150 = 0.033

= 3.30%

Genotypic frequency f (GG) = 3/150 = 0.02

= 2.0%

4.2.2.4 Calculation of χ^2 value for *ApaI*/ intron I

The χ^2 calculated value for *ApaI*/intron I were 0.32 and 0.004 for Hariana and Sahiwal cattle whereas it was 4.20 for Murrah buffalo (Table 4.2). The χ^2 table values were 3.841 and 6.635 at 5% and 1% level of significance, respectively for degree of freedom 1. These results revealed that $\chi^2_{(cal)} < \chi^2_{(tab)}$ at 5% level of significance for Hariana and Sahiwal while $\chi^2_{(cal)} < \chi^2_{(tab)}$ at 1% level of significance for Murrah buffalo hence selected population of cattle and buffalo was found in Hardy-Weinberg equilibrium.

Table 4.2: Genotypic and allelic frequencies of *Apa*I/intron I polymorphism in screened cattle and buffalo

Breed	Genotypic frequency (%)			Allelic frequency		Chi square
	GG	GA	AA	G	A	(χ2)
Hariana cattle (n=50)	80 (n=40)	18 (n=9)	2 (n=1)	0.89	0.11	0.32 *(P<0.05)*
Sahiwal cattle (n=50)	74 (n=37)	24 (n=12)	2 (n=1)	0.86	0.14	0.004 *(P<0.05)*
Total cattle (n=100)	77.00 (n=77)	21.00 (n=21)	2.00 (n=2)	0.88	0.12	0.23 *(P<0.05)*
Murrah buffalo (n=50)	90 (n=45)	8 (n=4)	2 (n=1)	0.94	0.06	4.2 *(P<0.01)*
Total (N=150)	81.33 (n=122)	16.66 (n=25)	2 (n=3)	0.90	0.10	1.5 *(P<0.05)*

Where; N= Sample size, n= Number of animals of particular breed and genotype.

4.2.2.5 Association studies of *ApaI*/intron I genotypes with milk production traits in Hariana cattle

The means with standard errors of mean (Mean± S.E.M.) for each trait related to each genotype for two lactations are given in Table 4.3. Only one AA genotype was available for association study so it was not included in association analysis.

4.2.2.5.1 Age at first calving (AFC)

AFC is defined as age of the animal at its first calving. The overall mean of AFC of 50 Hariana cattle was calculated as 2100.6±160.2 days. AFC of GG and GA genotypes were 2140.0 ±61.4 and 2060.0 ±259.0 days, respectively. There was no significant ($p>0.05$) difference for AFC between the two genotypes (Fig. 4.19).

4.2.2.5.2 Total milk yield (TMY)

TMY is defined as total milk produced during a lactation period of animal. The overall mean of TMY was calculated as 1495±208 liters and 1740±240 liters in first and second lactation, respectively. (Fig. 4.20). There was no significant ($p>0.05$) difference was observed for the TMY between these two genotypes in both the lactations.

4.2.2.5.3 Lactation Period (LP)

The number of days in animal is producing milk (period between calving date to dry date) is known as LP. The overall mean of LP was observed as in first and second lactation was 330.5±14.3 days and 320.5±15.0 days, respectively. (Fig. 4.21). There was no significant ($p>0.05$) difference was observed for the LP between these two genotypes in both the lactations.

4.2.2.5.4 Milk yield at 300 days (MY300)

Total milk produced in 300 days of lactation period is known as MY300. The overall mean of MY300 was calculated as 1344.0±136.5 liters and 1602.5±195.5 liters in first and second lactation, respectively. There was no significant ($P>0.05$) difference was observed for the MY300 between these two genotypes in both the lactations. (Fig. 4.22).

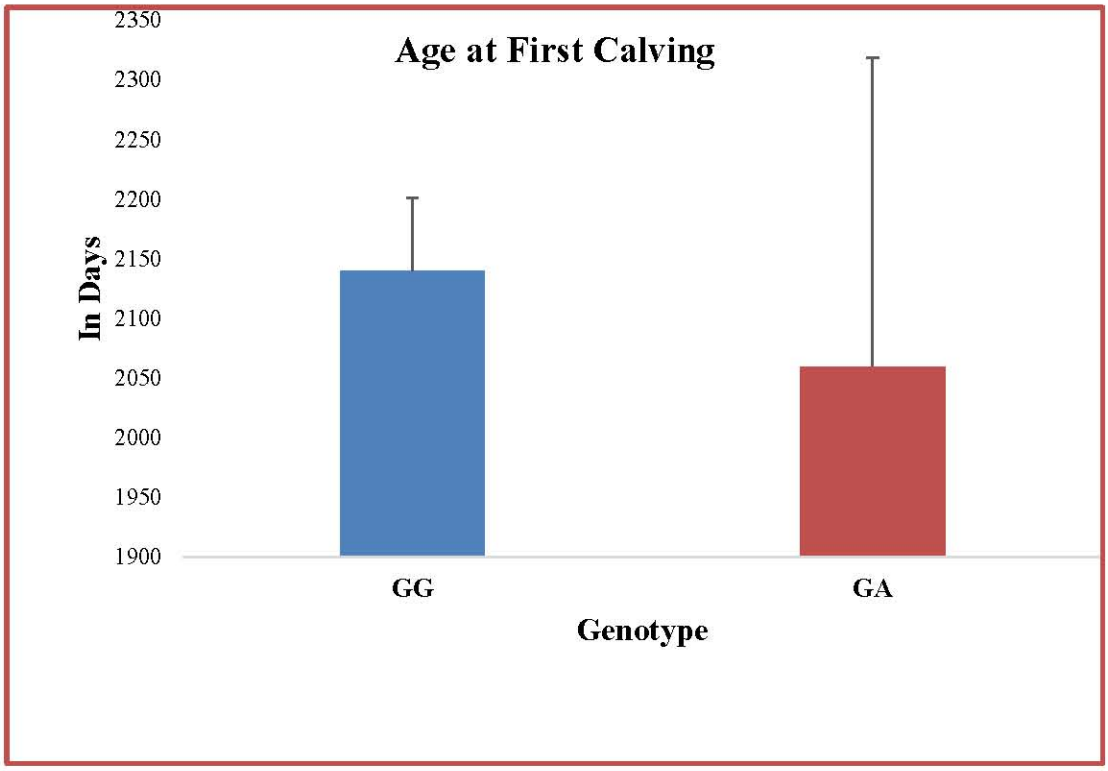

Fig. 4.19: Graphical representation of association study of *Apa*I/intron I genotypes with age at first calving in Hariana cattle.

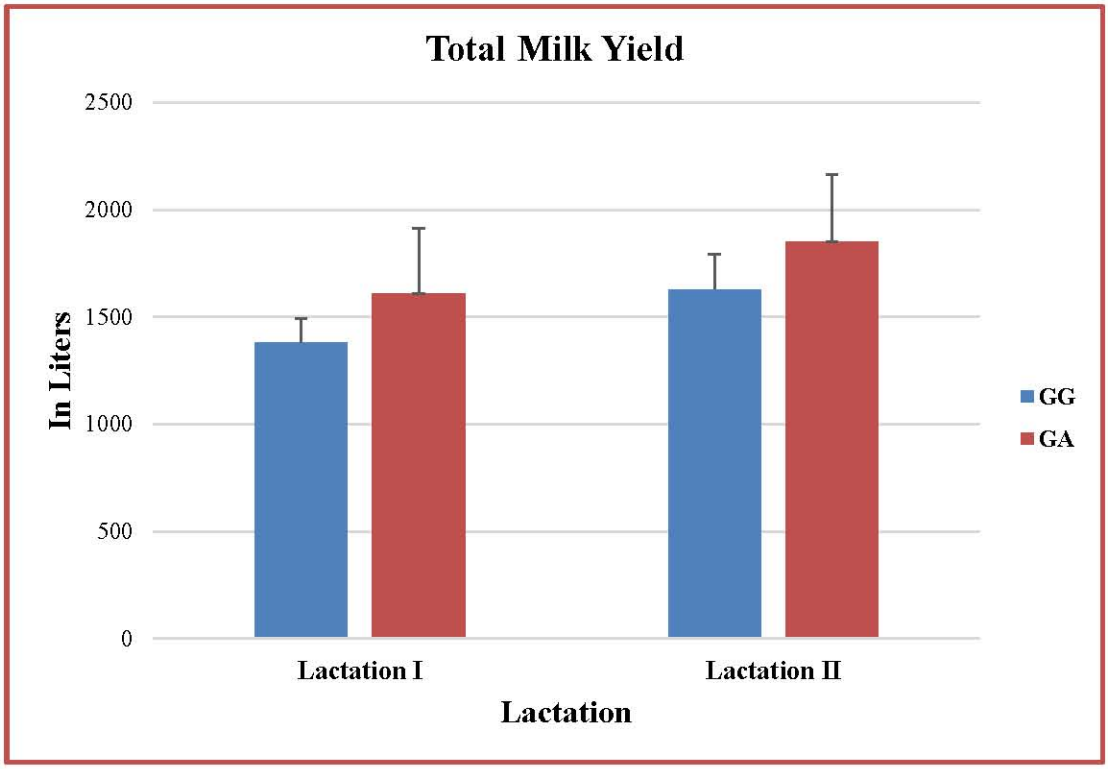

Fig. 4.20: Graphical representation of association study of *Apa*I/intron I genotypes with total milk yield for lactation I and lactation II in Hariana cattle.

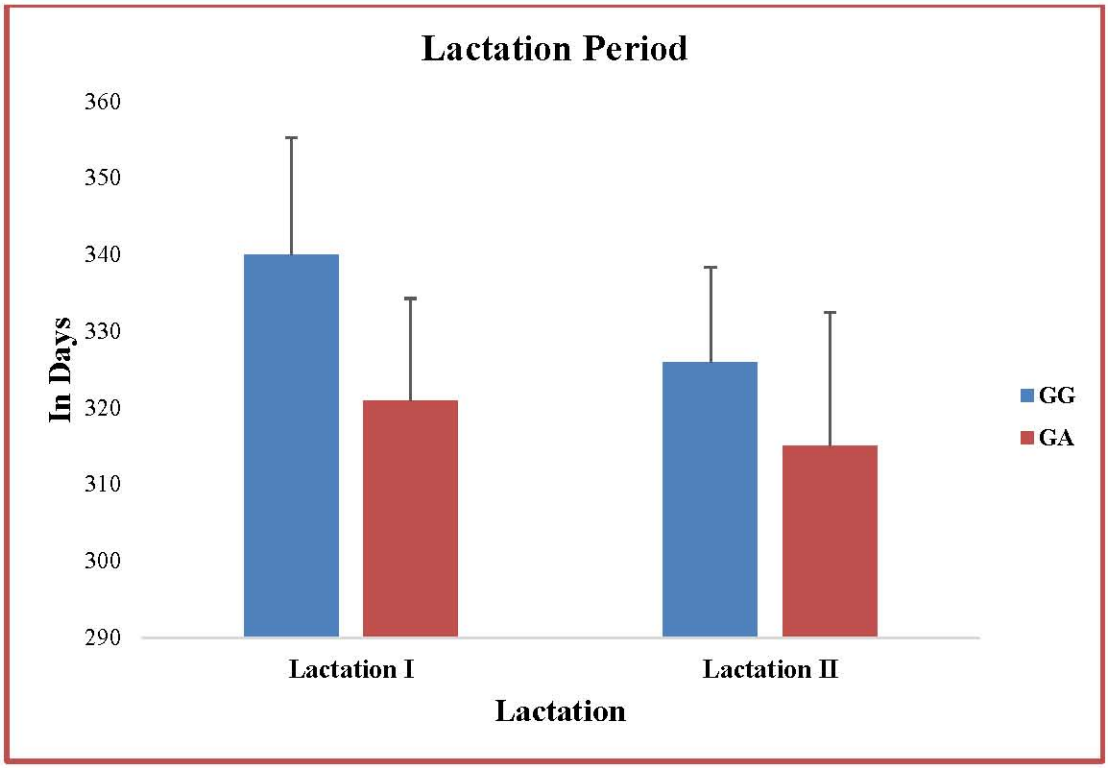

Fig. 4.21: Graphical representation of association study of *Apa*I/intron I genotypes with lactation period for lactation I and lactation II in Hariana cattle.

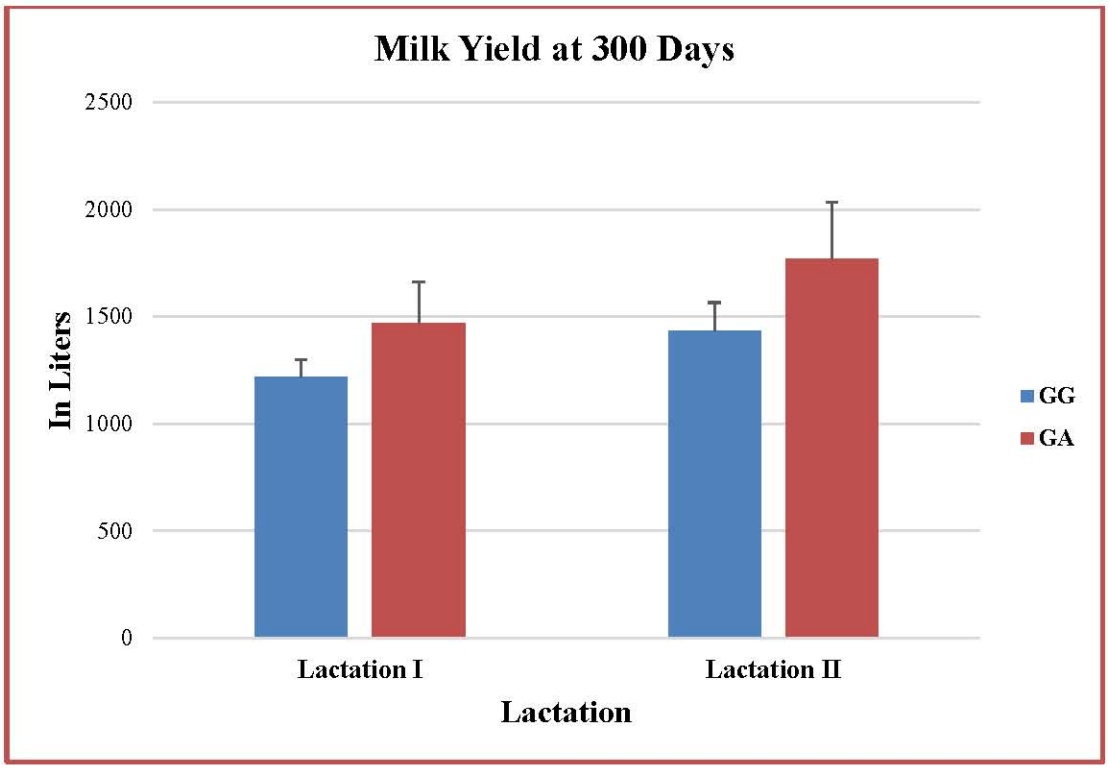

Fig. 4.22: Graphical representation of association study of *Apa*I/intron I genotypes with milk yield at 300 days for lactation I and lactation II in Hariana cattle.

Table 4.3: Association studies of *Apa*I/intron I genotypes with milk production traits in Hariana cattle

Lactation	Genotype	AFC (days)	TMY (liters)	LP (days)	MY300 (liters)
I (N=50)	GG (40)	2140.0 ±61.4 (40)	1380.0 ±114.0 (40)	340.0 ±15.3 (40)	1218.0 ±81.1 (40)
	GA (9)	2060.0 ±259.0 (9)	1610.0 ±302.0 (9)	321.0 ±13.3 (9)	1470.0 ±192.0 (9)
	AA (1)*	2270 (1)	1355 (1)	285 (1)	1355 (1)
II (N=44)	GG (36)	------	1630.0 ±164.0 (36)	326.0 ±12.4 (36)	1435.0 ±130.0 (36)
	GA (7)	------	1850.0 ±316.0 (7)	315.0 ±17.5 (7)	1770.0 ±261.0 (7)
	AA (1)*	------	1460 (1)	352 (1)	1240 (1)

Where AFC: Age at first calving; TMY: Total milk yield; LP: Lactation period and MY300: Milk yield upon 300 days. *Only one AA genotype was present in screened population so it has not included in association analysis.

4.2.2.6 Association studies of *ApaI*/intron I genotypes with milk production traits in Sahiwal cattle

The means with standard errors of mean (Mean± S.E.M.) for each trait related to each genotype for two lactations are given in Table 4.4. Only one AA genotype was available for association study so it was not included in association analysis.

4.2.2.6.1 Age at first calving (AFC)

The overall mean of AFC of 50 Sahiwal cattle was calculated as 1750.3±152.5 days. AFC of GG and GA genotypes were 1821.0 ±155.0 and 1800.0 ±150.0 days, respectively. There was no significant *(P>0.05)* difference between these two genotypes for AFC (Fig. 4.23) indicating that *Apa*I/intron I had no significant effect on AFC in Sahiwal cattle.

4.2.2.6.2 Total milk yield (TMY)

The overall mean of TMY was calculated as 1871.0±226.5 liters and 1897.5±231.0 liters in first and second lactation, respectively. There was no significant *(P>0.05)* difference was observed for the TMY between GG and GA genotypes in both the lactations. (Fig. 4.24).

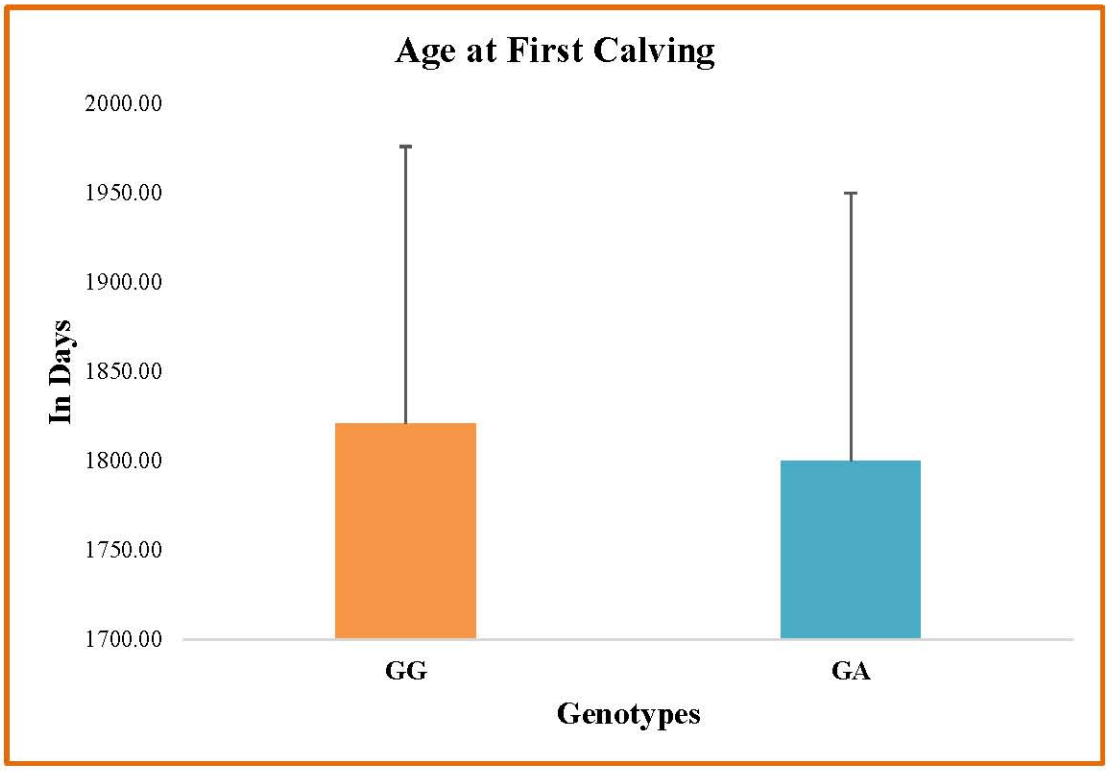

Fig. 4.23: Graphical representation of association study of *Apa*I/intron I genotypes with age at first calving in Sahiwal cattle.

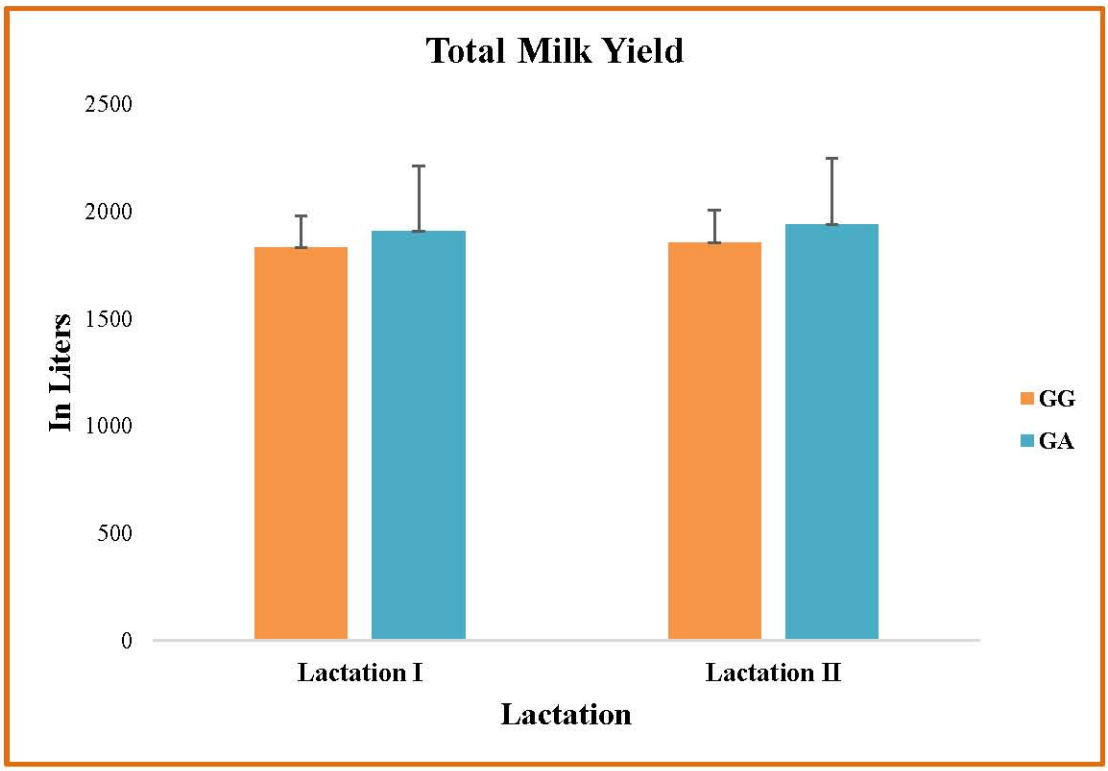

Fig. 4.24: Graphical representation of association study of *Apa*I/intron I genotypes with total milk yield for lactation I and lactation II in Sahiwal cattle.

4.2.2.6.3 Lactation Period (LP)

The overall mean of LP was observed as in first and second lactation was 357.0±33.3 days and 361.5±35.6 days for genotype GA and GG, respectively. (Fig. 4.25). There was no significant *(P>0.05)* difference was observed for the LP between the genotypes in both the lactations.

4.2.2.6.4 Milk yield at 300 days (MY300)

The overall mean of MY300 was calculated as 1502.5±89.5 liters and 1539.5±92.1 liters in first and second lactation, respectively. There was no significant *(P>0.05)* difference was observed for the MY300 between all the genotypes in both lactations. (Fig. 4.26).

Table 4.4: Association studies of *ApaI*/intron I genotypes with milk production traits in Sahiwal cattle

Lactation	Genotype	AFC (days)	TMY (liters)	LP (days)	MY300 (liters)
I (N=50)	GG (37)	1821.0 ±155.0 (37)	1832.0 ±149.0 (36)	376.0 ±21.2 (36)	1435.0 ±61.1 (23)
	GA (12)	1800.0 ±150.0 (24)	1910.0 ±304.0 (23)	338.0 ±45.5 (23)	1570.0 ±118.0 (23)
	AA (1)*	1750 (1)	2660 (1)	418 (1)	1910 (1)
II (N=46)	GG (34)	------	1855.0 ±153.0 (34)	372.0 ±20.2 (34)	1469.0 ±63.1 (34)
	GA (11)	------	1940.0 ±309.0 (11)	351.0 ±51.0 (11)	1609.0 ±121.0 (11)
	AA (1)*	------	2831 (1)	433 (1)	2100 (1)

Where AFC: Age at first calving; TMY: Total milk yield; LP: Lactation period and MY300: Milk yield upon 300 days. *Only one AA genotype was present in screened population so it has not included in association analysis.

4.2.2.7 Association studies of *ApaI*/intron I genotypes with milk production traits in Murrah buffalo

The means with standard errors (Mean± S.E.) for each trait related to each genotype for two lactations are given in Table 4.5.

4.2.2.7.1 Age at first calving (AFC)

The overall mean of AFC of 49 Murrah buffaloes was calculated as 1790.0±160.8 days. There was no significant difference ($P>0.05$) between the two genotypes for AFC (Fig. 4.27) indicating that *ApaI*/Intron I had no significant *(P>0.05)* effect on AFC in Murrah

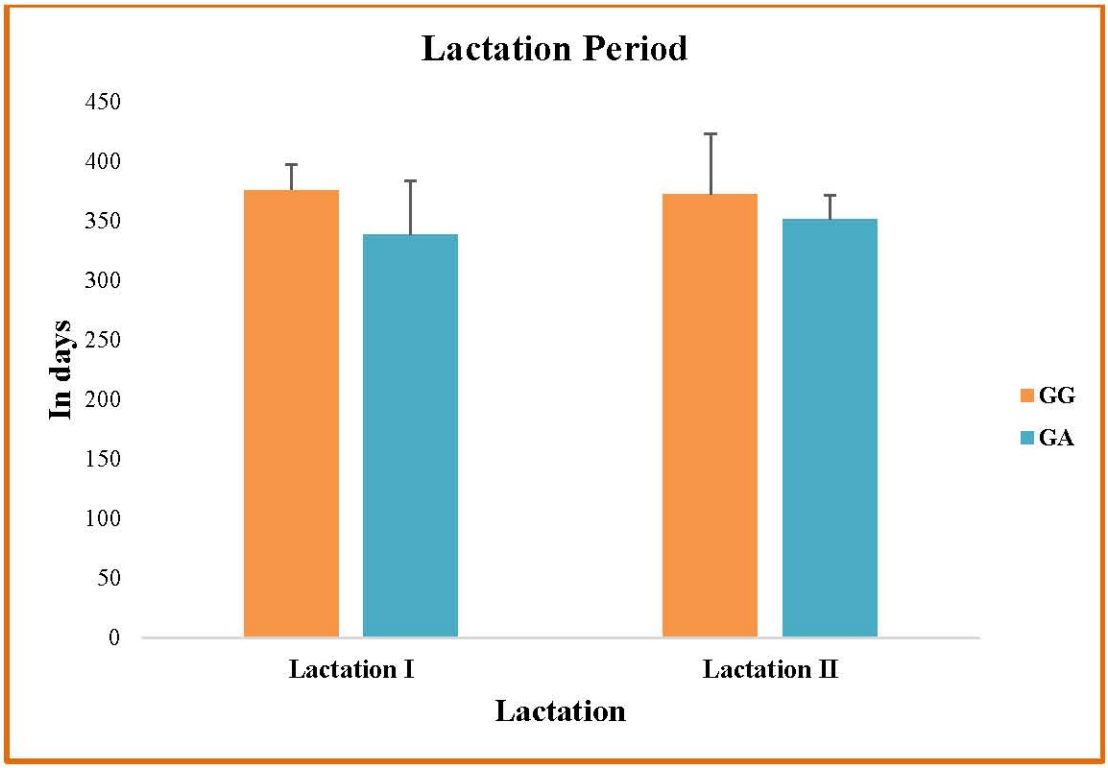

Fig. 4.25: Graphical representation of association study of *Apa*I/intron I genotypes with lactation period for lactation I and lactation II in Sahiwal cattle.

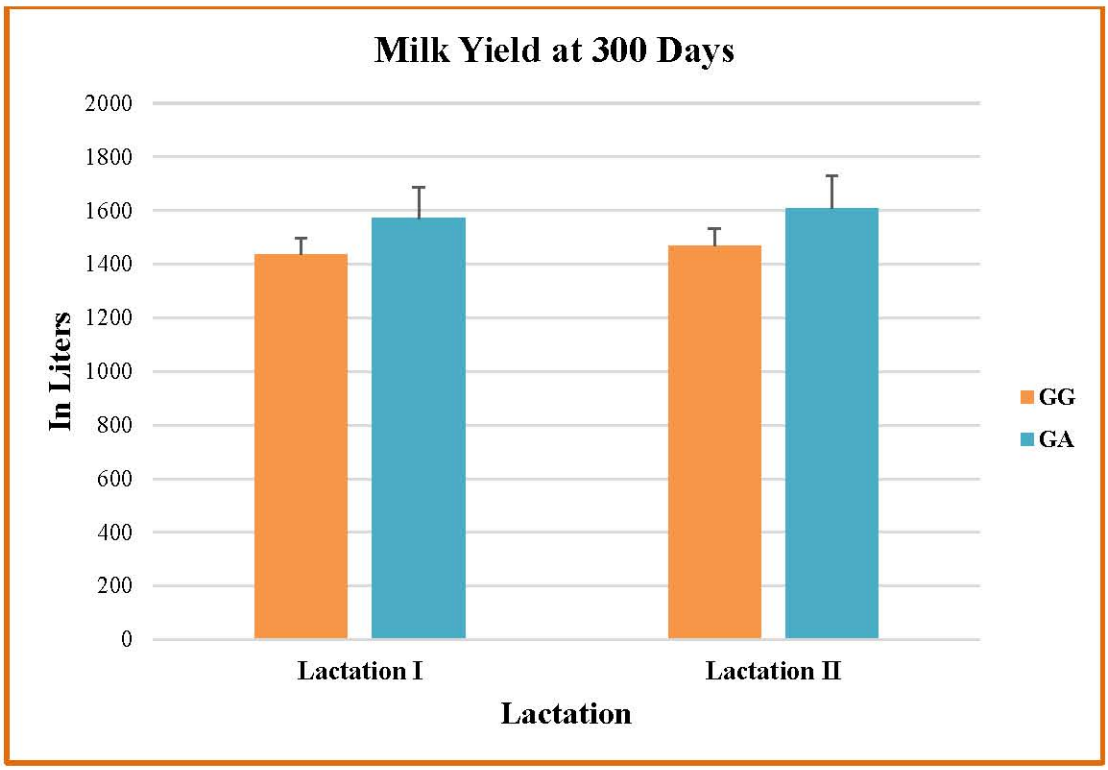

Fig. 4.26: Graphical representation of association study of *Apa*I/intron I genotypes with milk yield at 300 days for lactation I and lactation II in Sahiwal cattle.

buffaloes. AFC of GG and GA genotypes were 1960.0 ±105.6 and 1620.0 ±216.0 days, respectively.

4.2.2.7.2 Total milk yield (TMY)

The overall mean of TMY was calculated as 1920.0±189.0 liters and 2045.0±141.5 liters in first and second lactation, respectively. However, TMY of GG genotype (2320.0 ±127.0 liters) was higher than GA genotype (1770.0 ±156.0 liters) in second lactation (Fig. 4.28). There was no significant *(P>0.05)* difference was observed for the TMY between all the genotypes with the lactations.

4.2.2.7.3 Lactation Period (LP)

The overall mean of LP was observed in first and second lactation was 369.0±40.3 and 333±37.3 days, respectively. There was no significant *(P>0.05)* difference was observed for the LP between all the genotypes in both lactations. LP of genotype GG was much higher (370.0 ±48.4) than genotype GA (296.0 ±26.3) in second lactation (Fig. 4.29).

4.2.2.7.4 Milk yield at 300 days (MY300)

The overall mean of MY300 was calculated as 1595.0±78.8 liters and 1815.0±67.0 liters in first and second lactation, respectively. There was no significant *(P>0.05)* difference was observed for the MY300 between the two genotypes in both lactations. (Fig. 4.30).

Table 4.5: Association studies of *Apa*I/intron I genotypes with milk production traits in Murrah buffalo

Lactation	Genotype	AFC (days)	TMY (liters)	LP (days)	MY300 (liters)
I (N=49)	GG (44)	1960.0 ±105.6 (42)	1850.0 ±195.0 (42)	363.0 ±51.8 (42)	1600.0 ±91.8 (42)
	GA (4)	1620.0 ±216.0 (3)	1990.0 ±183.0 (3)	375.0 ±28.8 (3)	1590.0 ± 65.7 (3)
	AA (1)*	1735 (1)	1840 (1)	260 (1)	1840 (1)
II (N=43)	GG (39)	-----	2320.0 ±127.0 (39)	370.0 ±48.4 (39)	1860.0 ±72.7 (39)
	GA (3)	-----	1770.0 ±156.0 (3)	296.0 ±26.3 (3)	1770.0 ±61.2 (3)
	AA (1)*	-----	2162 (1)	358 (1)	1765 (1)

Where AFC: Age at first calving; TMY: Total milk yield; LP: Lactation period and MY300: Milk yield upon 300 days. *Only one AA genotype was present in screened population so it has not included in association analysis.

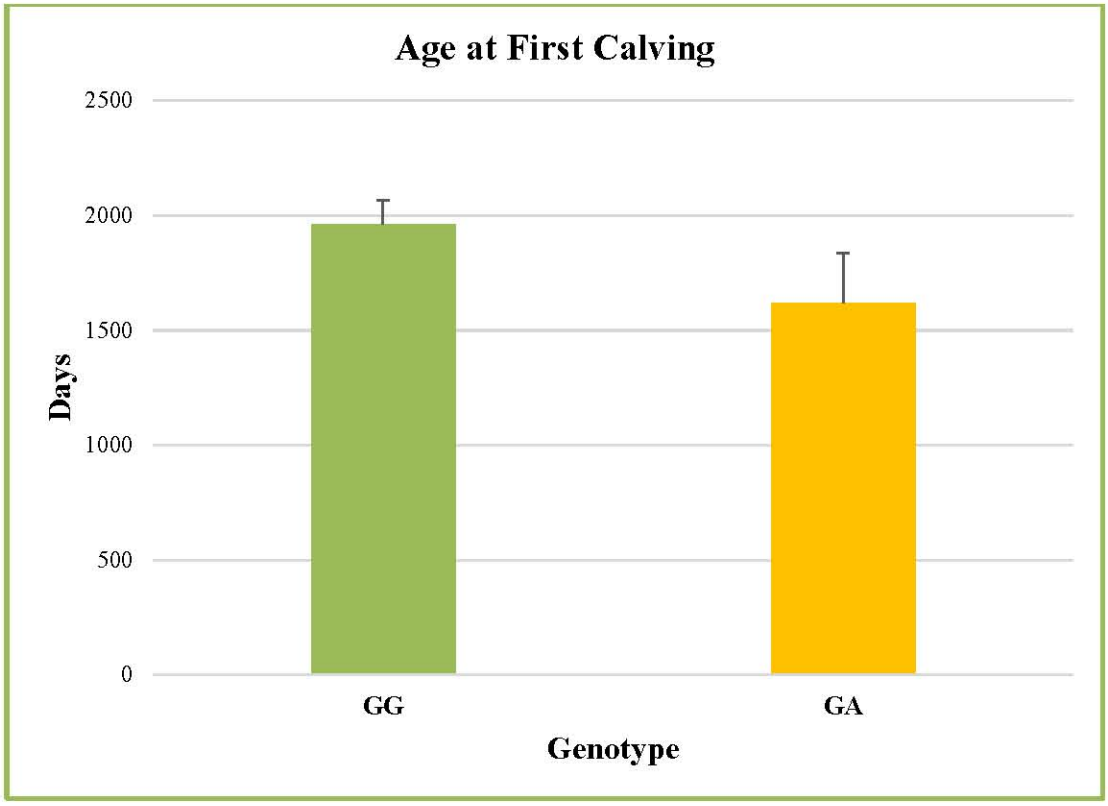

Fig. 4.27: Graphical representation of association study of *Apa*I/intron I genotypes with age at first calving in Murrah buffalo.

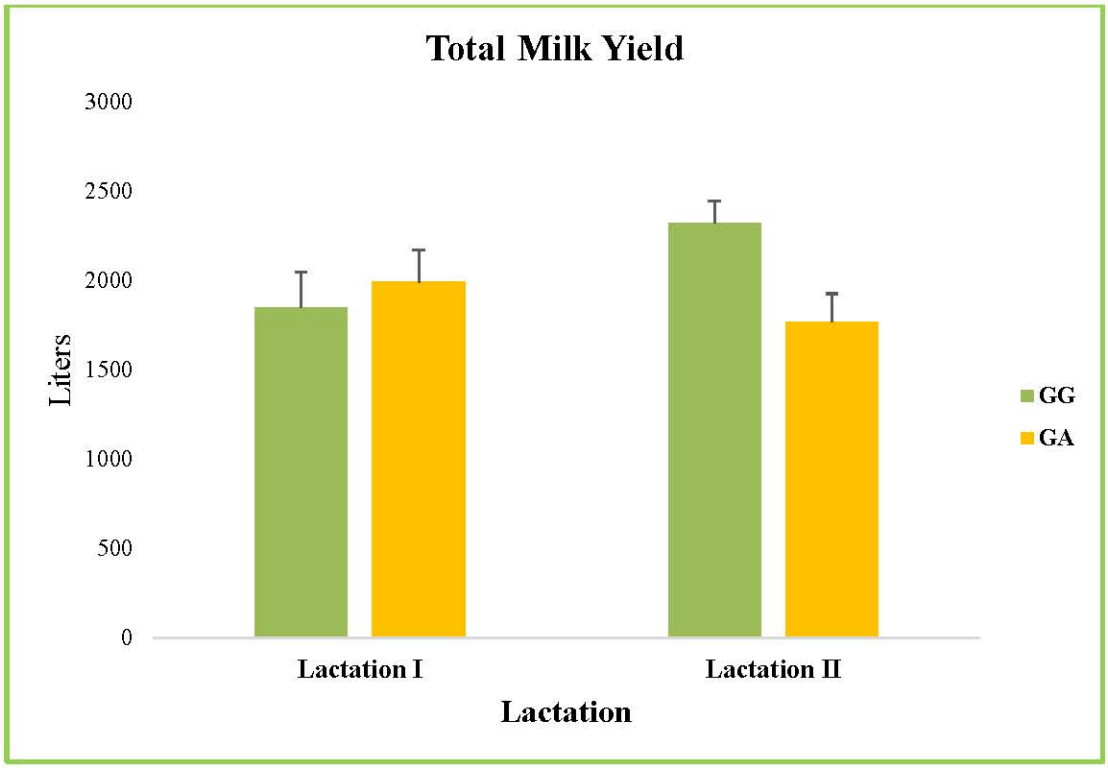

Fig. 4.28: Graphical representation of association study of *Apa*I/intron I genotypes with total milk yield for lactation I and lactation II in Murrah buffalo.

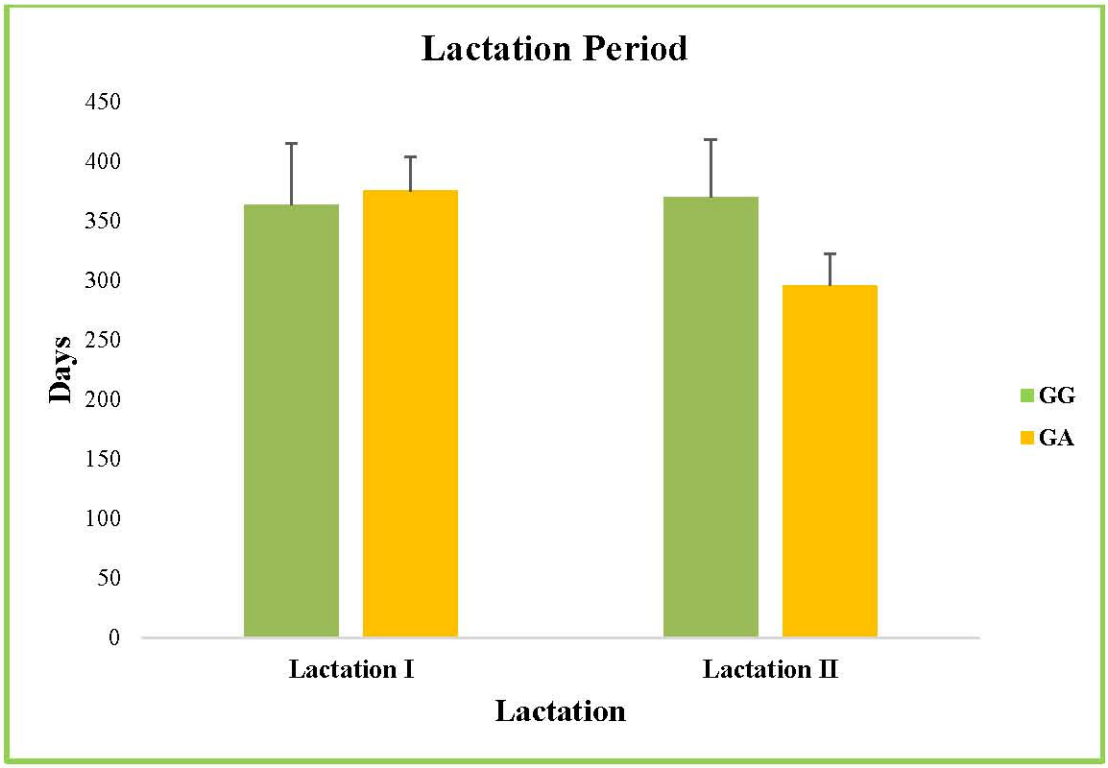

Fig. 4.29: Graphical representation of association study of *Apa*I/intron I genotypes with lactation period for lactation I and lactation II in Murrah buffalo.

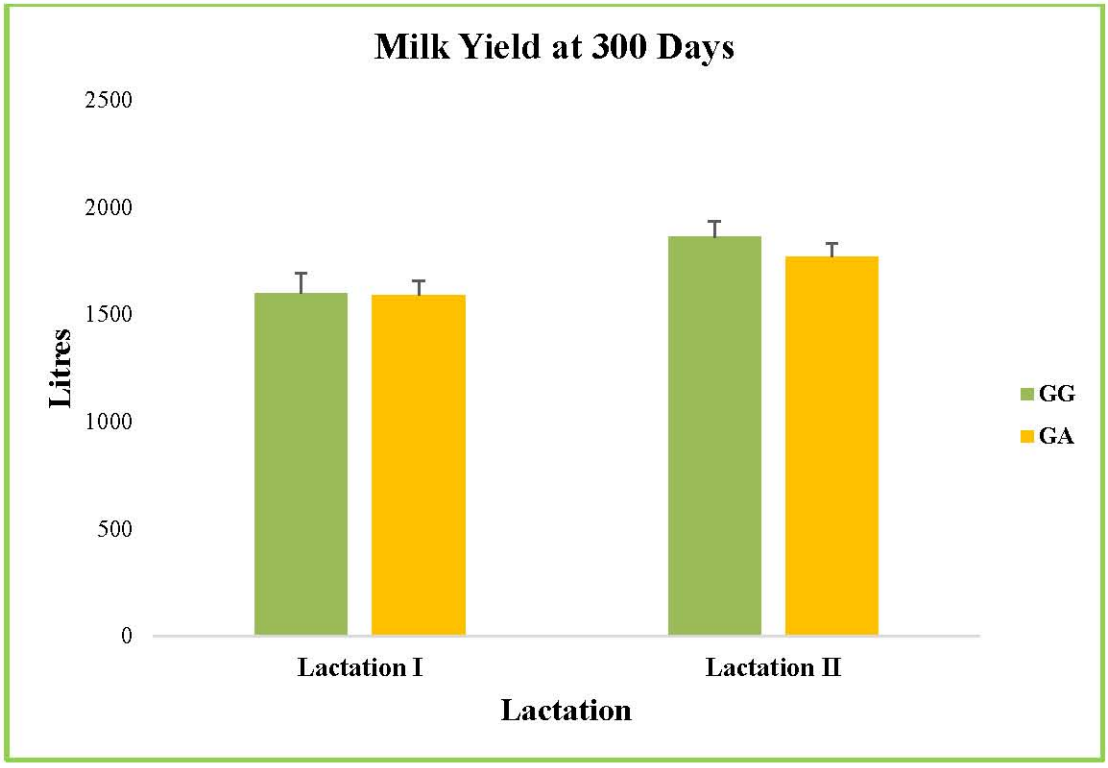

Fig. 4.30: Graphical representation of association study of *Apa*I/intron I genotypes with milk yield at 300 days for lactation I and lactation II in Murrah buffalo.

4.2.2.8 Association studies of *Apa*I/intron I genotypes with SCS in cattle (Hariana and Sahiwal)

The means with standard error of means (Mean± S.E.M.) for SCS related to each genotype are given in Table 4.6. Only one AA genotype was available in the screened population so it was not included in association analysis for any of the trait.

The overall mean of SCS was 652.0±229.5 and 943.5±515.0 for Hariana (n=48) and Sahiwal cattle (n=47), respectively. There was no significant difference was observed for SCS between two genotypes in both the cattle breeds (Fig. 4.31). However, genotype GA was higher (1140.0±668.0) than GG genotype (747.0±362.0) in Sahiwal cattle while genotype GG (675.0±223.0) and genotype GA (630.0±236.0) had very less difference in Hariana cattle.

4.2.2.9 Association studies of *Apa*I/intron I genotypes with somatic cell score in Murrah buffaloes

The means with standard errors of mean (Mean± S.E.M.) for SCS related to each genotype are given in Table 4.6. Only one AA genotype was available in the screened population so it was not included in association analysis for any of the trait.

The overall mean of SCS was 53.6±5.4 for 46 Murrah buffaloes. There was no significant difference was observed between two genotypes for SCS in Murrah buffaloes (Fig. 4.32).

Table 4.6: Association studies of *Apa*I/intron I genotypes with SCS in cattle (Hariana and Sahiwal) and Murrah buffalo

Genotype	Hariana cattle	Sahiwal cattle	Murrah buffalo
GG	675.0±223.0 (38)	747.0±362.0 (36)	51.0±2.9 (41)
GA	630.0±236.0 (9)	1140.0±668.0 (10)	56.2±7.8 (4)
AA*	90.3 (1)	554.0 (1)	57.2 (1)

* Only one AA genotype was present in screened population so it has not included in association analysis.

4.2.3 *Hae*III/exon 2 polymorphism study

4.2.3.1 Amplification of exon 2 region of *MBL1* gene

Amplification of exon 2 region of *MBL1* gene by PCR produced fragments of 401 bp with exon 2 region specific primers with optimized PCR conditions in all the screened

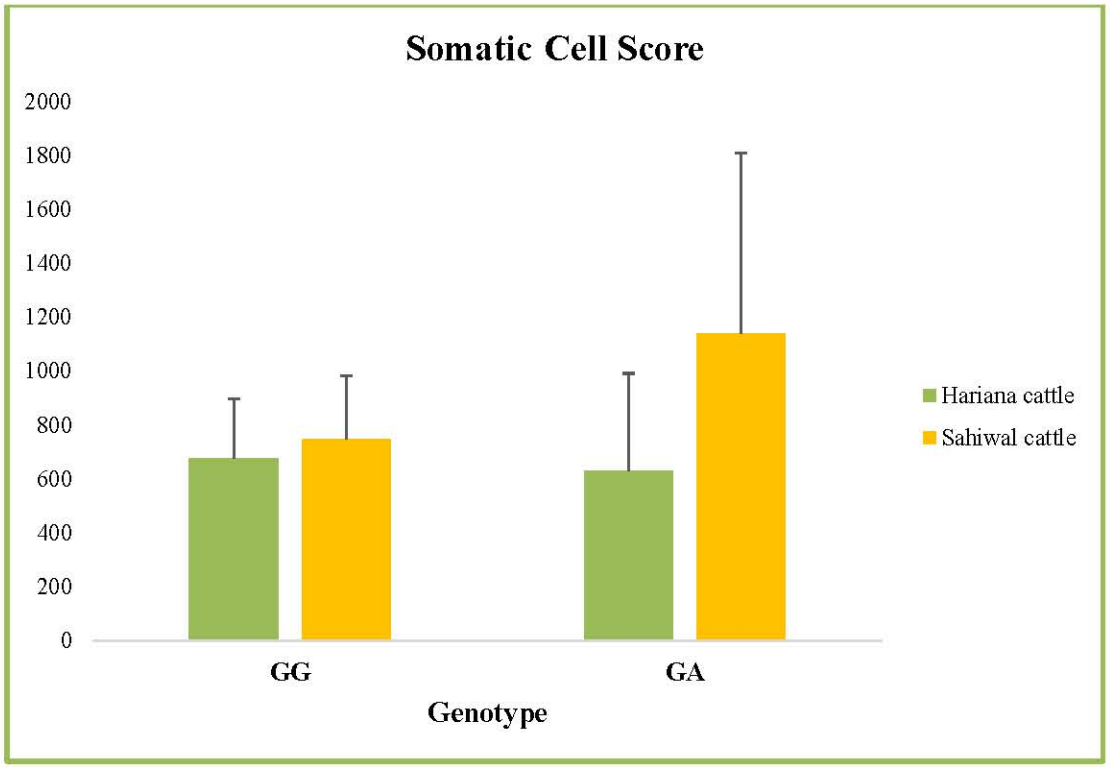

Fig. 4.31: Graphical representation of association study of *Apa*I/intron I genotypes with somatic cell score in cattle (Hariana and Sahiwal).

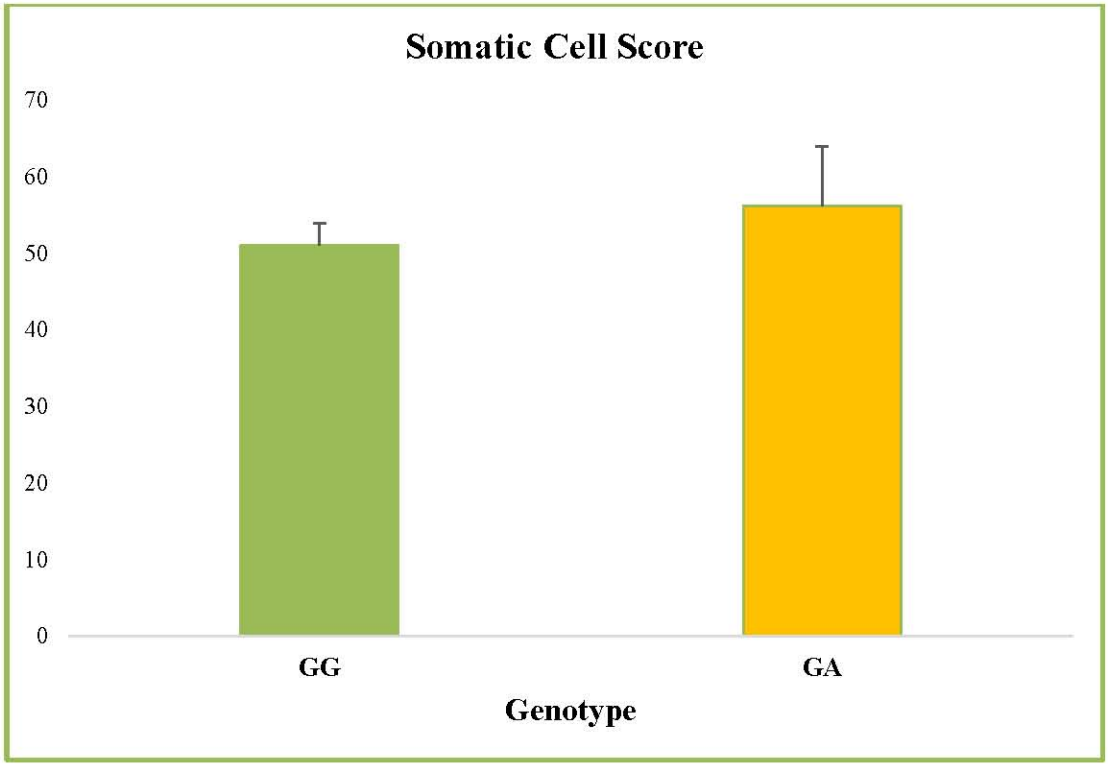

Fig. 4.32: Graphical representation of association study of *Apa*I/intron I genotypes with somatic cell score in Murrah buffaloe.

animals. PCR products were resolved on 1.0% agarose gel electrophoresis and were documented using gel documentation system (Fig. 4.33).

4.2.3.2 *Hae*III/ exon 2 PCR-RFLP assay

For screening of the SNP g.2686T>C in exon 2 region of *MBL1* gene, 401 bp amplified products were digested with *Hae*III restriction enzyme as per the protocol. The *Hae*III/exon 2 PCR-RFLP assay revealed banding pattern of 274 and 127 bp for genotype TT, 274, 184, 127 and 90 bp for TC genotype and 184, 127 and 90 bp for genotype CC (Fig. 4.34). Among all the animals (n=150), 22 were of heterozygote TC genotype while majority of animals (n=91) were of homozygote CC genotype and remaining 22 were of homozygote TT genotype. This revealed that the cattle and buffalo population used in the present study were polymorphic in nature with two types of alleles T and C.

4.2.3.3 Calculation of *Hae*III/exon 2 allelic and genotypic frequency

The genotypic and allelic frequencies of *Hae*III/exon 2 were calculated and presented in Table 4.7. Among all the animals homozygote genotype CC was more frequent (60.66%) than homozygote TT genotype (24.66%) and heterozygote genotype TC (14.66%). The allele C was more frequent (0.68) than allele T (0.32) among all cattle and buffaloes. Although, the cattle and buffalo populations had variation in pattern of genotype percentage such as genotype TT was highest (66.0%) in Murrah buffaloes while it was 34.0% in cattle population. Allelic frequency pattern was also varied in cattle and buffalo as allele C was much more frequent (0.85) in Murrah buffalo than cattle (0.60) and allele T was very less frequent (0.15) in buffalo than cattle (0.40).

The allelic and genotypic frequencies from the studied sample size of 150 (cattle=100 and buffalo=50) were calculated as following:

$$\textbf{Allelic/gene frequency} = \frac{(2D + H)}{2N}$$

Allelic frequency f (T) = (2D+H)/2N

Allelic frequency f (T) = (2x37+22)/2x150 = 0.32

= 32.0%

Allelic frequency f (C) = (2R+H)/2N

Allelic frequency f (C) = (2x91+22)/2x150 = 0.68

= 68.0%

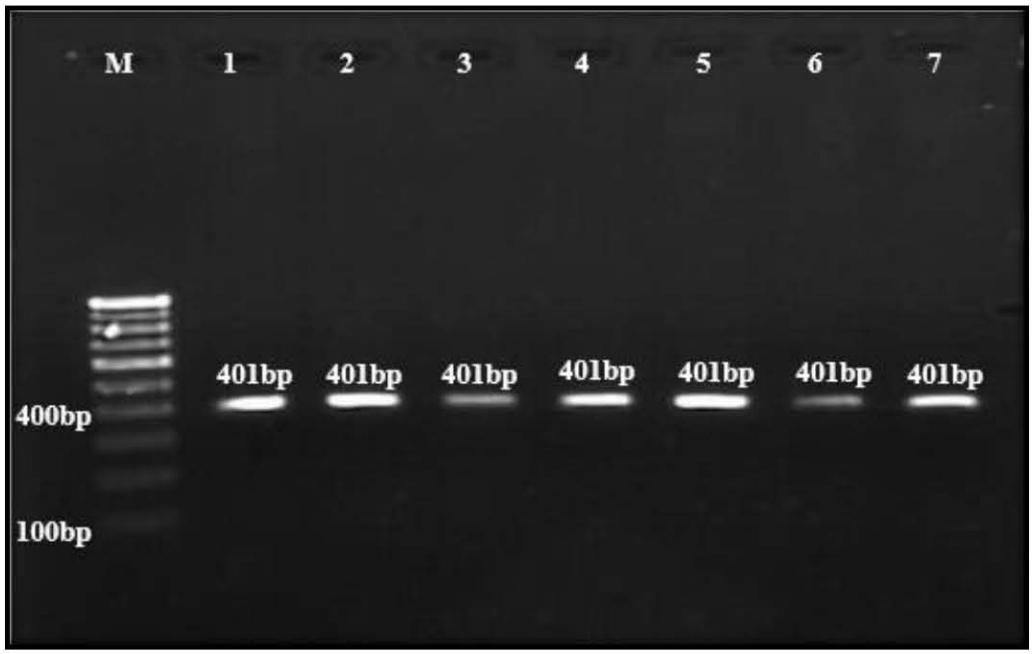

Fig. 4.33: Agarose (2.0%) gel electrophoresis showing amplification of exon 2 region (401 bp) of *MBL1* gene of Hariana cattle (Lane 1-2), Sahiwal cattle (Lane 3-4) and Murrah buffalo (Lane 5-7), Lane M: Marker (100 bp DNA ladder).

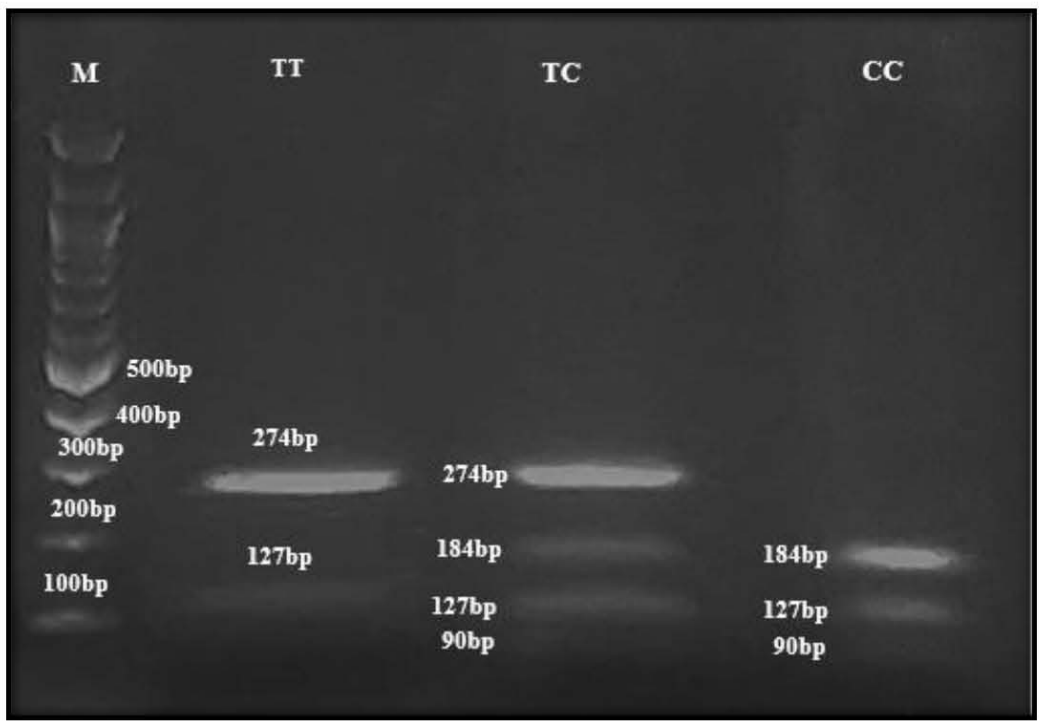

Fig. 4.34: RFLP products of exon 2 region of *MBL1* gene digested with *Hae*III RE. Lane M: Marker (100 bp DNA ladder), Lane TT, TC & CC: RFLP products of size 274, 127 bp for genotype TT, 274 and 184, 127 and 90 bp for genotype TC and 184, 127 and 90 bp for genotype CC respectively.

$$\text{Genotypic frequency} = \frac{\text{Total no. of individual of particular genotype}}{\text{Total no. of individuals of all genotype}}$$

Genotypic frequency f (TT) = 37/150 = 0.24

= 24.6%

Genotypic frequency f (TC) = 22/150 = 0.146

= 14.6%

Genotypic frequency f (CC) = 91/150 = 0.60

= 60.0%

4.2.3.4 Calculation of χ^2 value for *Hae*III/exon 2

The χ^2 calculated value for *Hae*III/exon 2 were 24.91 and 25.12 for Hariana and Sahiwal cattle whereas it was 3.89 for Murrah buffaloe (Table 4.7). The χ^2 table values were 3.841 and 6.635 at 5% and 1% level of significance, respectively for degree of freedom 1. These results revealed that $\chi^2_{(cal)} > \chi^2_{(tab)}$ at 5% and 1% level of significance for Hariana and Sahiwal while $\chi^2_{(cal)} < \chi^2_{(tab)}$ at 1% level of significance for Murrah buffaloe hence selected population of cattle was not in the Hardy-Weinberg equilibrium while the Murrah buffalo population was found in the equilibrium.

Table 4.7: Genotypic and allelic frequencies of *Hae*III/exon 2 genotypes in cattle and buffalo

Breed	Genotypic frequency (%)			Allelic frequency		Chi square (χ2)
	TT	TC	CC	T	C	
Hariana cattle (n=50)	32.00 (n=16)	14.00 (n=7)	54.00 (n=27)	0.39	0.61	24.91 *(P>0.05)*
Sahiwal cattle (n=50)	36.00 (n=18)	12.00 (n=6)	52.00 (n=26)	0.42	0.58	25.12 *(P>0.05)*
Total cattle (n=100)	34.00 (n=34)	13.00 (n=13)	53.00 (n=53)	0.40	0.60	54.53 *(P>0.05)*
Murrah Buffalo (n=50)	66.00 (n=3)	32.00 (n=9)	2.00 (n=38)	0.15	0.85	3.89 *(P<0.01)*
Total (N=150)	24.60 (n=37)	14.60 (n=22)	60.60 (n=91)	0.32	0.68	65.92 *(P>0.05)*

Where; N= Sample size, n= Number of animals of particular breed and genotype.

4.2.3.5 Association studies of *Hae*III/exon 2 genotypes with milk production traits in Hariana cattle

The means with standard errors of mean (Mean± S.E.M.) for each trait related to each genotype for two lactations in Hariana cattle are given in Table 4.8.

4.2.3.5.1 Age at first calving (AFC)

The overall mean of AFC for 49 Hariana cattle was calculated as 2220.0±133.6 days. AFC of TT, TC and CC genotypes were 1930.0±85.9, 2570.0±261.0 and 2160.0±53.9 days, respectively. There was a significant *(P<0.05)* difference for AFC among the genotypes TC and CC (Fig. 4.35).

4.2.3.5.2 Total milk yield (TMY)

The overall mean of TMY was calculated as 1343.0±138.0 liters and 1743.3±195.0 liters in first and second lactation, respectively. There was no significant *(P>0.05)* difference was observed for the TMY among all the genotypes in lactations I and II. However, TMY of TT genotype was numerically higher (1570.0±124.0 liters) than TC genotype (1250.0±154.0 liters) and CC genotype (1210.0±136.0) in first lactation but they were statistically same (Fig. 4.36).

4.2.3.5.3 Lactation Period (LP)

The overall mean of LP was observed as in first and second lactation was 336.0±16.4 and 321.0±17.97 days, respectively. There was no significant *(p>0.05)* difference was observed for the LP among all the genotypes in both lactations (Fig. 4.37).

4.2.3.5.4 Milk yield at 300 days (MY300)

The overall mean of MY300 was calculated as 1163.0±109.0 liters and 1573.3±154.6 liters in first and second lactation, respectively. There was no significant *(P>0.05)* difference was observed for the MY300 among all the genotypes in both the lactations (Fig. 4.38).

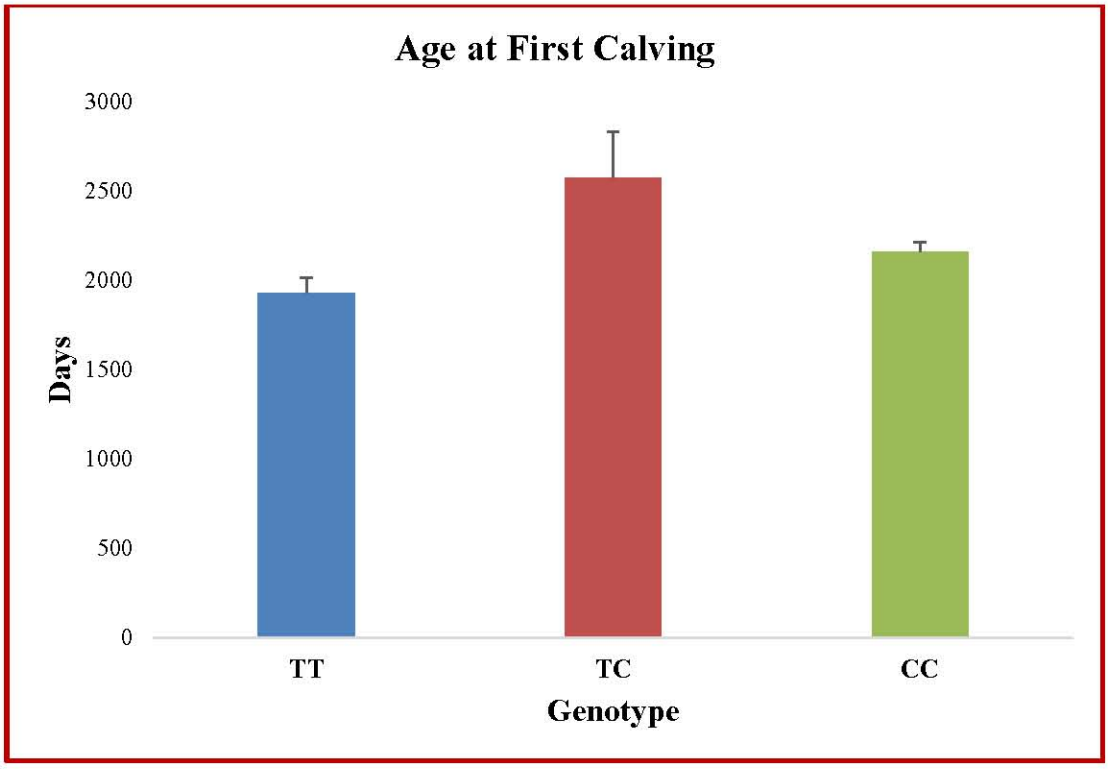

Fig. 4.35: Graphical representation of association study of *Hae*III/exon 2 genotypes with age at first calving in Hariana cattle.

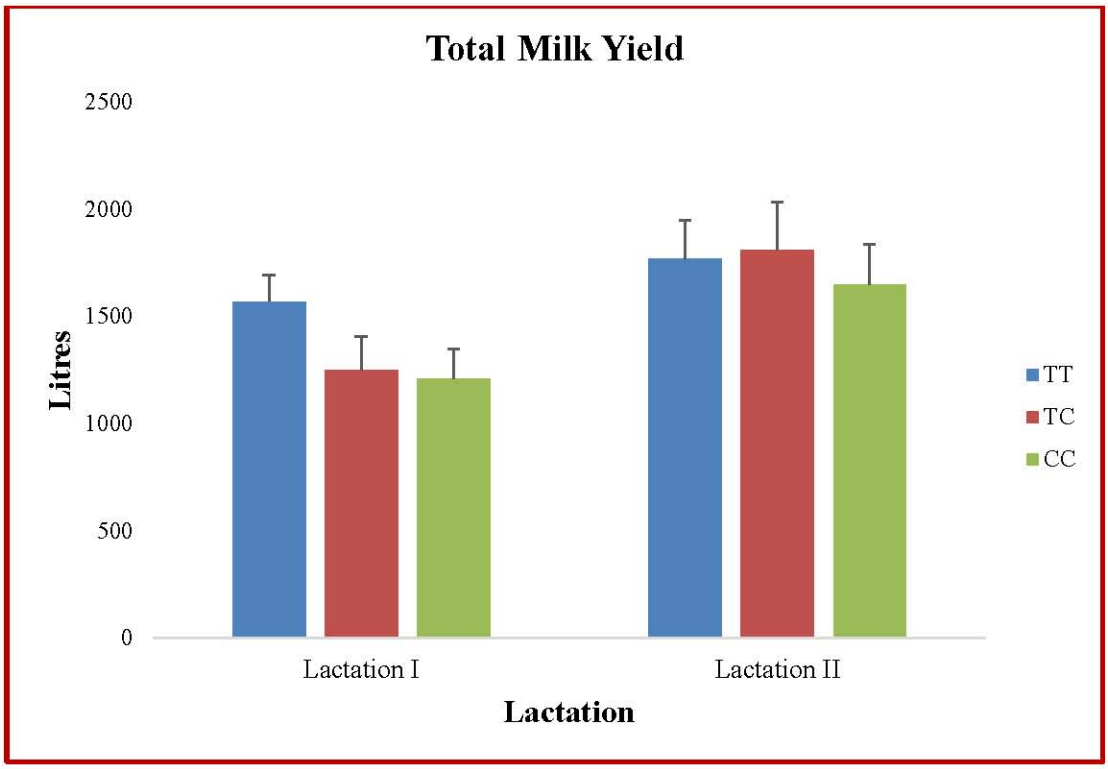

Fig. 4.36: Graphical representation of association study of *Hae*III/exon 2 genotypes with total milk yield for lactation I and lactation II in Hariana cattle.

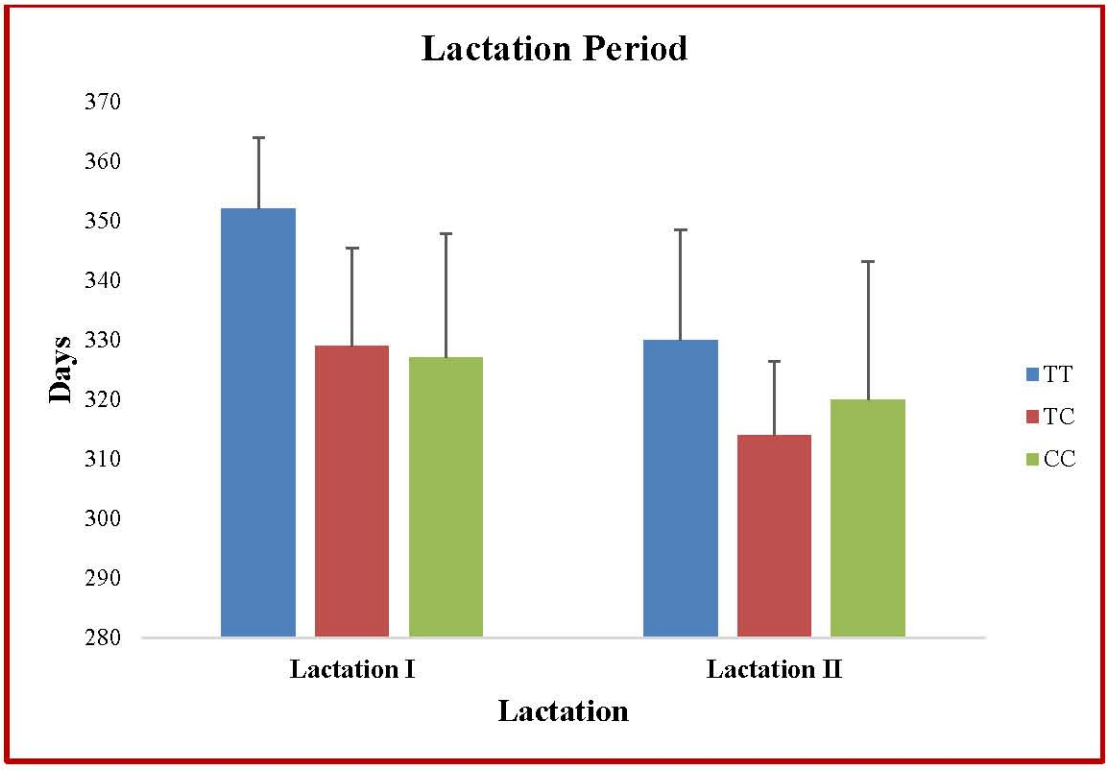

Fig. 4.37: Graphical representation of association study of *Hae*III/exon 2 genotypes with lactation period for lactation I and lactation II in Hariana cattle.

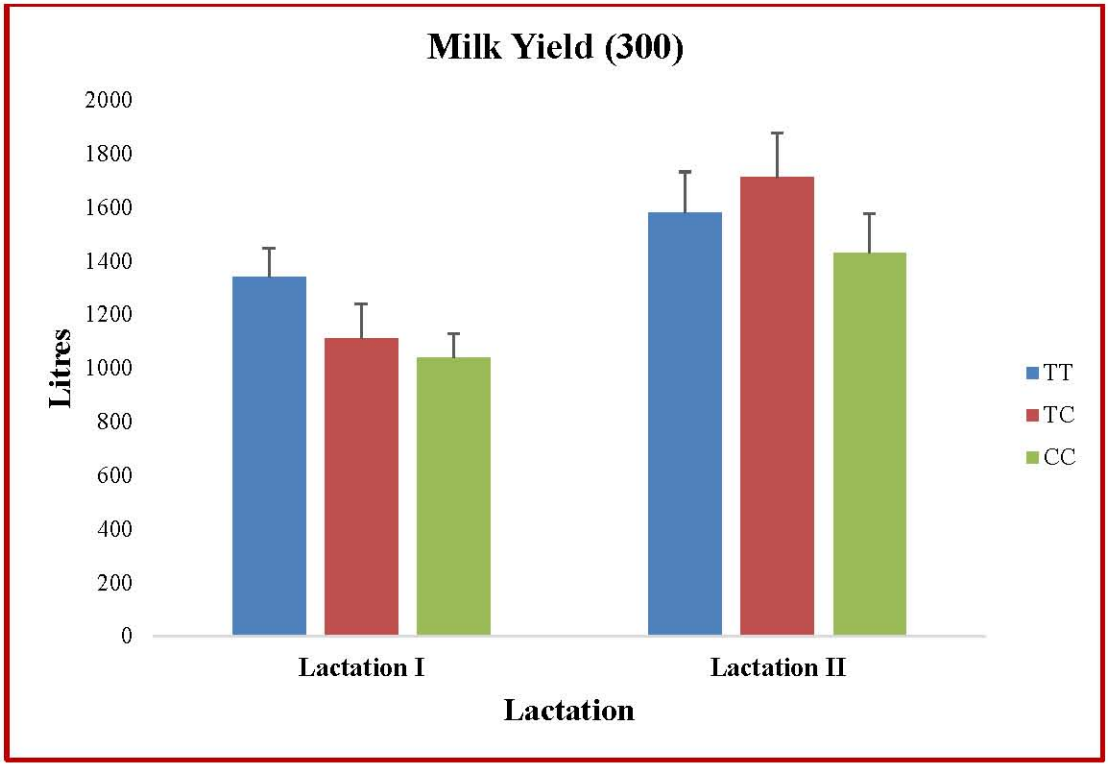

Fig. 4.38: Graphical representation of association study of *Hae*III/exon 2 genotypes with milk yield at 300 days for lactation I and lactation II in Hariana cattle.

Table 4.8: Association studies of *Hae*III/exon 2 genotypes with milk production traits in Hariana cattle

Lactation	Genotype	AFC (days)	TMY (liters)	LP (days)	MY300 (liters)
I (N=49)	TT (16)	1930.0±85.9[ab] (16)	1570.0±124.0 (13)	352.0±12.0 (13)	1340.0±106.0 (13)
	TC (6)	2570.0±261.0[b] (6)	1250.0±154.0 (5)	329.0±16.4 (5)	1109.0±131.2 (5)
	CC (27)	2160.0±53.9[a] (27)	1210.0±136.0 (23)	327.0±20.8 (23)	1040.0±89.8 (23)
II (N=36)	TT (12)	------	1770.0±177.0 (12)	330.0±18.4 (12)	1580.0±152.0 (12)
	TC (4)	------	1810.0±223.0 (4)	314.0±12.4 (4)	1710.0±167.0 (4)
	CC (20)	------	1650.0±185.0 (18)	320.0±23.1 (18)	1430.0±145.0 (18)

Where AFC: Age at first calving; TMY: Total milk yield; LP: Lactation period and MY300: Milk yield upon 300 days.

4.2.3.6 Association studies of *Hae*III/exon 2 genotypes with milk production traits in Sahiwal cattle

The means with standard errors of means (Mean± S.E.M.) for each trait related to each genotype for two lactations in Sahiwal cattle are given in Table 4.9.

4.2.3.6.1 Age at first calving (AFC)

The overall mean of AFC of 50 Sahiwal cattle was calculated as 1886.6±68.3 days. AFC of TT, TC and CC genotypes were 1770.0±76.0, 2140.0±18.8 and 1750.0±110.0 days, respectively. There was no significant *(P>0.05)* difference for AFC among all the genotypes (Fig. 4.39).

4.2.3.6.2 Total milk yield (TMY)

The overall mean of TMY was calculated as 1833.3±208.1 liters and 1926.0±152.4 liters in first and second lactation, respectively. There was no significant *(P>0.05)* difference was observed for the TMY among all the genotypes in both lactations (Fig. 4.40).

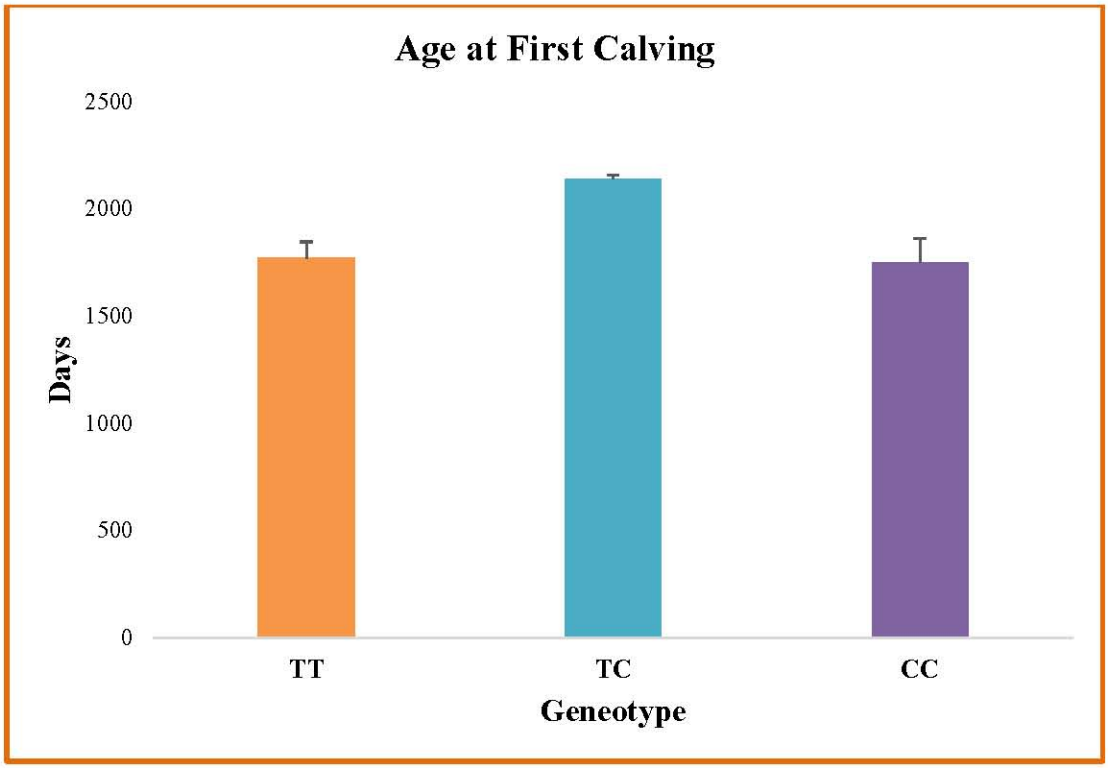

Fig. 4.39: Graphical representation of association study of *Hae*III/exon 2 genotypes with age at first calving in Sahiwal cattle.

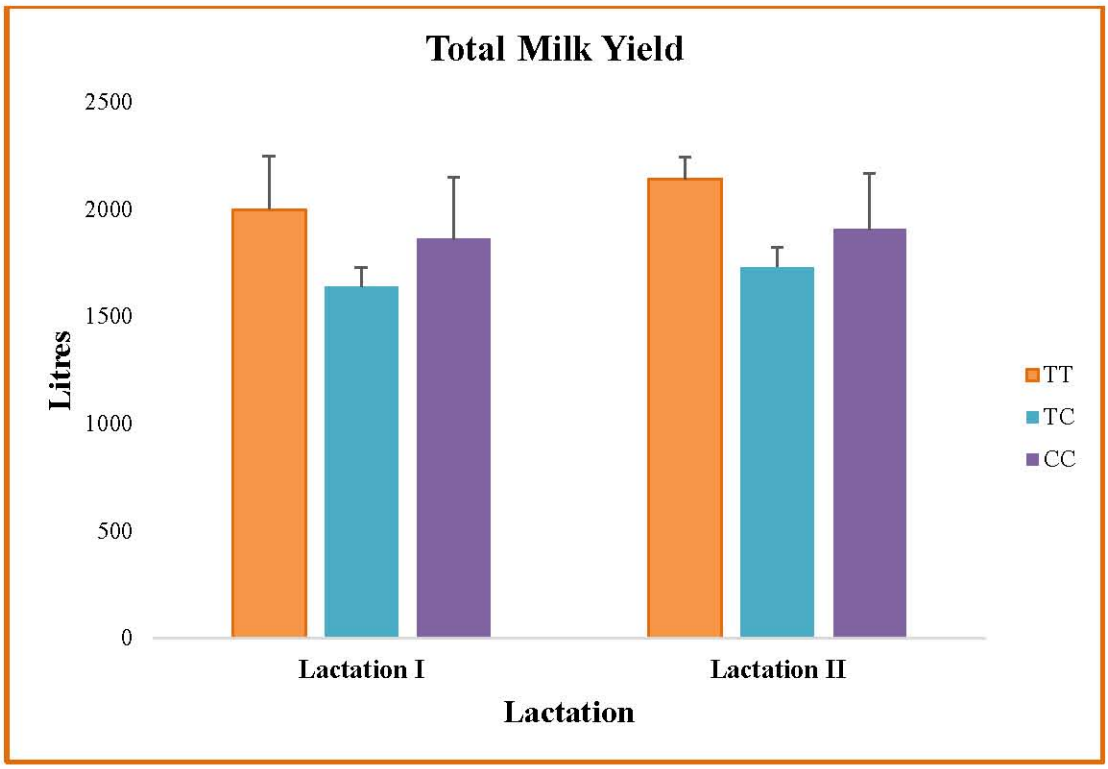

Fig. 4.40: Graphical representation of association study of *Hae*III/exon 2 genotypes with total milk yield for lactation I and lactation II in Sahiwal cattle.

4.2.3.6.3 Lactation Period (LP)

The overall mean of LP was observed as in first and second lactation was 337.3±26.2 and 347.0±25.7 days, respectively. There was no significant *(P>0.05)* difference was observed for the LP among all the genotypes in both lactations (Fig. 4.41).

4.2.3.6.4 Milk yield at 300 days (MY300)

The overall mean of MY300 was calculated as 1516.6±82.5 liters and 1569.0±79.5 liters in first and second lactation, respectively. There was no significant *(P>0.05)* difference was observed for the MY300 among all the genotypes in both lactations (Fig. 4.42).

Table 4.9: Association studies of *Hae*III/exon 2 genotypes with milk production traits in Sahiwal cattle

Lactation	Genotype	AFC (days)	TMY (liters)	LP (days)	MY300 (liters)
I (N=50)	TT (18)	1770.0±76.0 (18)	2000.0±246.0 (18)	396.0±23.2 (18)	1460.0±123.0 (18)
	TC (6)	2140.0±18.8 (6)	1640.0±89.3 (5)	299.0±36.9 (5)	1580.0±46.2 (5)
	CC (26)	1750.0±110.0 (26)	1860.0±289.0 (25)	317.0±18.6 (25)	1510.0±78.3 (25)
II (N=42)	TT (15)	------	2142.0±103.0 (15)	385.0±25.2 (15)	1541.0±109.0 (15)
	TC (4)	------	1731.0±92.3 (4)	325.0±32.8 (4)	1482.0±48.3 (4)
	CC (23)	------	1905.0±262.0 (23)	331.0±19.2 (23)	1685.0±81.2 (23)

Where AFC: Age at first calving; TMY: Total milk yield; LP: Lactation period and MY300: Milk yield upon 300 days.

4.2.3.7 Association studies of *Hae*III/exon 2 genotypes with milk production traits in Murrah buffalo

The means with standard errors of means (Mean± S.E.M.) for each trait related to each genotype for two lactations in Sahiwal cattle are given in Table 4.10.

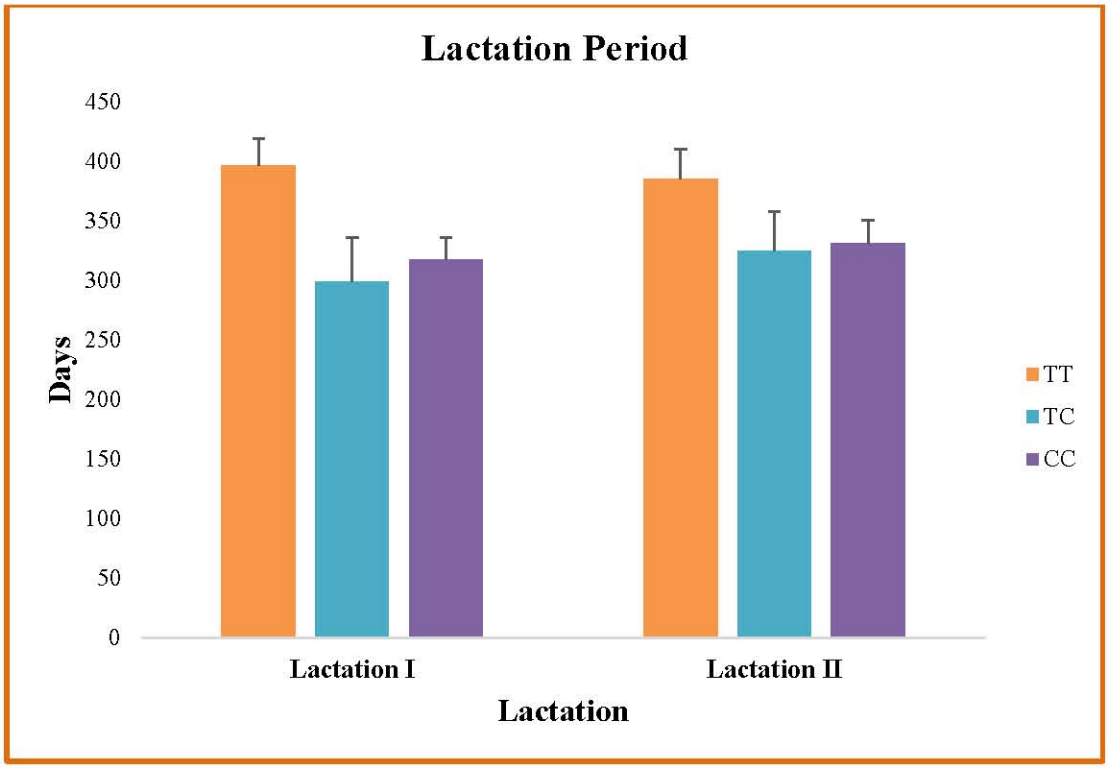

Fig. 4.41: Graphical representation of association study of *Hae*III/exon 2 genotypes with lactation period for lactation I and lactation II in Sahiwal cattle.

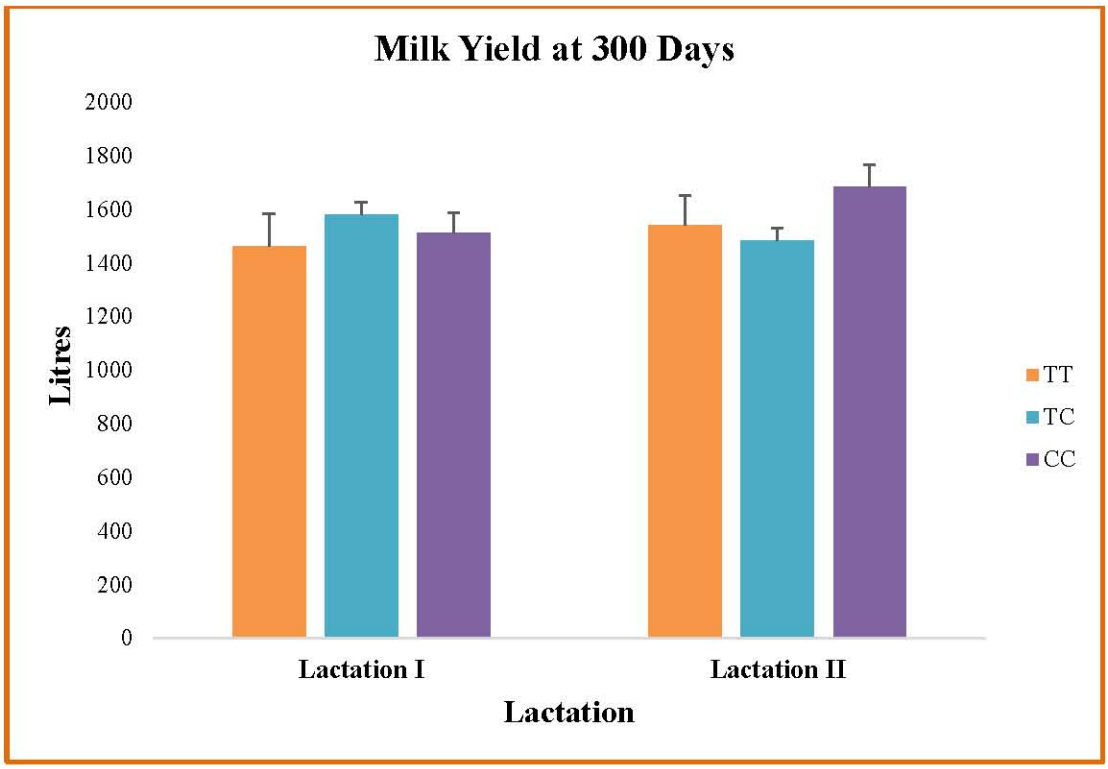

Fig. 4.42: Graphical representation of association study of *Hae*III/exon 2 genotypes with milk yield at 300 days for lactation I and lactation II in Sahiwal cattle.

4.2.3.7.1 Age at first calving (AFC)

The overall mean of AFC of 47 Murrah buffaloes was calculated as 1856.6±169.3 days. AFC of TT, TC and CC genotypes were 1760.0±226.0, 1860.0±169.0 and 1950.0±113.0 days, respectively. There was no significant difference ($P>0.05$) for AFC among all the genotypes (Fig. 4.43).

4.2.3.7.2 Total milk yield (TMY)

The overall mean of TMY was calculated as 1783.3±165.5 liters and 2546.6±240.0 liters in first and second lactation, respectively. There was no significant *(P>0.05)* difference was observed for the TMY among all the genotypes in both the lactations (Fig. 4.44).

4.2.3.7.3 Lactation Period (LP)

The overall mean of LP was observed as in first and second lactation was 342.0±22.5 and 400.0±36.4 days, respectively. There was no significant *(P>0.05)* difference was observed for the LP among all the genotypes in both the lactations (Fig. 4.45).

4.2.3.7.4 Milk yield at 300 days (MY300)

The overall mean of MY300 was calculated as 1580.0±133.8 liters and 1903.3±83.5 liters in first and second lactation, respectively. There was no significant *(P>0.05)* difference was observed for the MY300 among all the genotypes in both lactations (Fig. 4.46).

Table 4.10: Association studies of *Hae*III/exon 2 genotypes with milk production traits in Murrah buffalo

Lactation	Genotype	AFC (days)	TMY (liters)	LP (days)	MY300 (liters)
I (N=47)	TT (2)	1760.0±226.0 (2)	1400.0±194.0 (2)	271.0±10.5 (2)	1570.0±276.0 (2)
	TC (9)	1860.0±169.0 (9)	2010.0±204.0 (9)	392.0±39.9 (9)	1550.0±69.7 (9)
	CC (36)	1950.0±113.0 (36)	1940.0±98.6 (36)	363.0±17.1 (36)	1620.0±55.9 (36)
II (N=40)	TT (2)	------	2870.0±251.0 (2)	466.0±51.2 (2)	1850.0±87.3 (2)
	TC (7)	------	2580.0±339.0 (7)	371.0±36.8 (7)	2060.0±103.0 (7)
	CC (32)	------	2190.0±130.0 (32)	363.0±21.3 (32)	1800.0±60.3 (32)

Where AFC: Age at first calving; TMY: Total milk yield; LP: Lactation period and MY300: Milk yield upon 300 days.

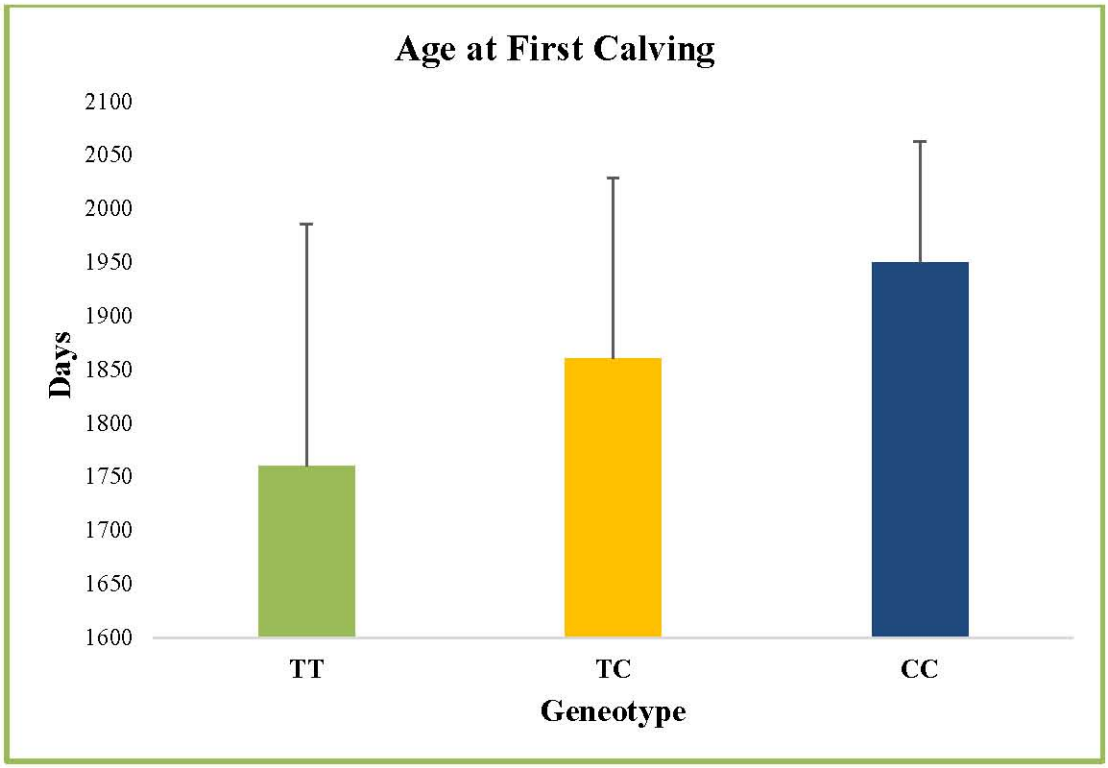

Fig. 4.43: Graphical representation of association study of *Hae*III/exon 2 genotypes with age at first calving in Murrah buffaloe.

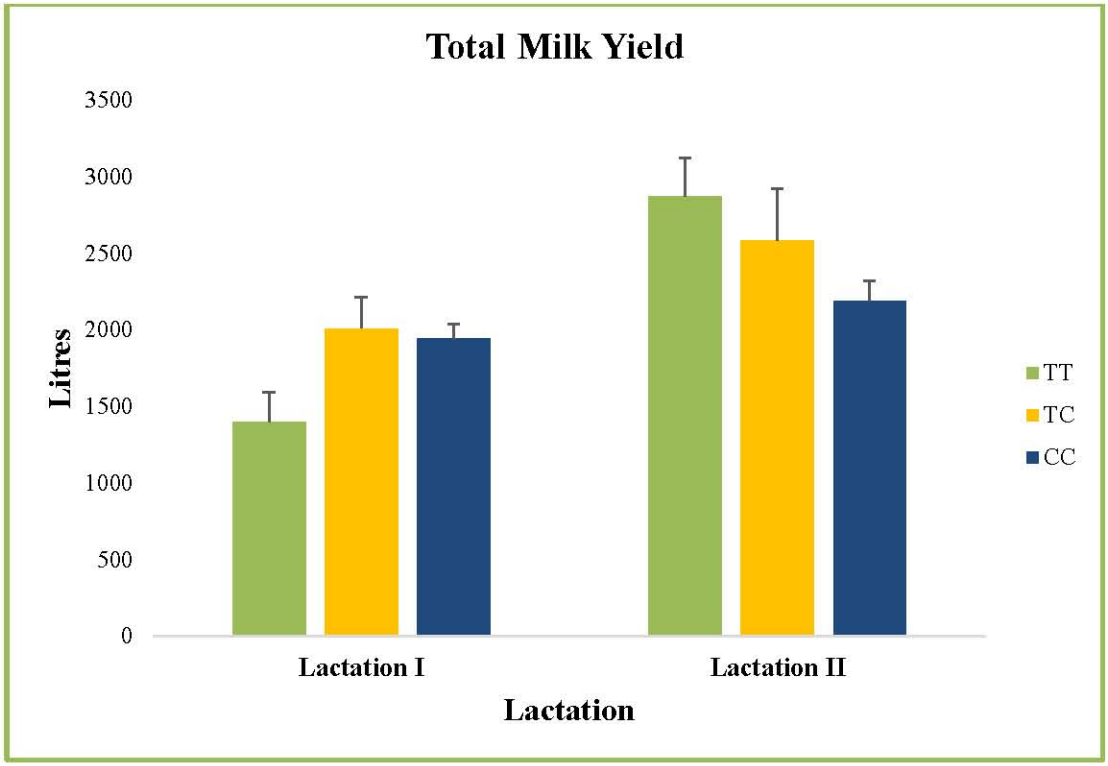

Fig. 4.44: Graphical representation of association study of *Hae*III/exon 2 genotypes with total milk yield for lactation I and lactation II in Murrah buffalo.

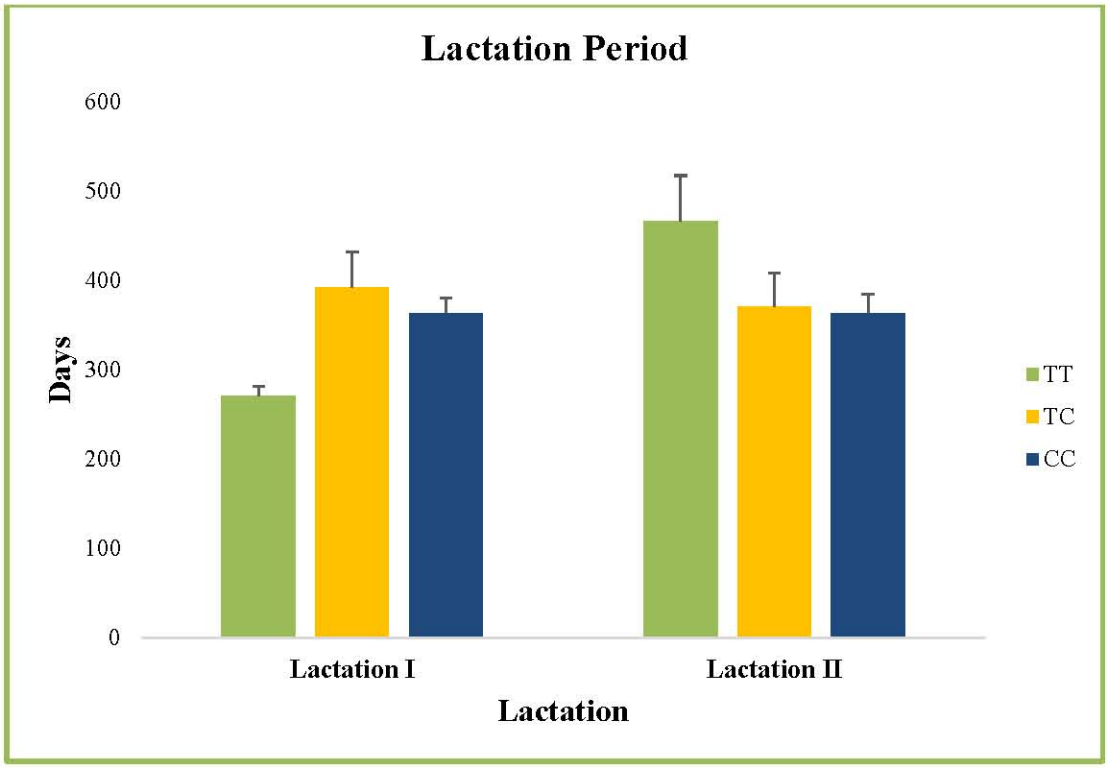

Fig. 4.45: Graphical representation of association study of *Hae*III/exon 2 genotypes with lactation period for lactation I and lactation II in Murrah buffalo.

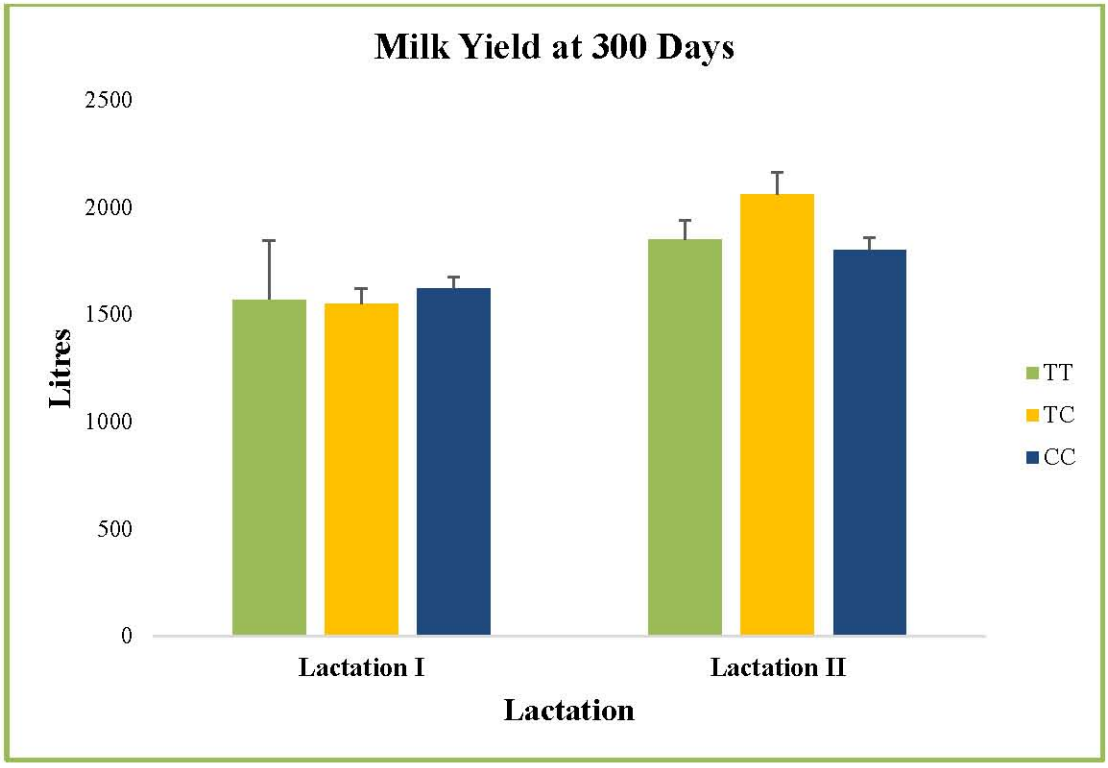

Fig. 4.46: Graphical representation of association study of *Hae*III/exon 2 genotypes with milk yield at 300 days for lactation I and lactation II in Murrah buffalo.

4.2.3.8 Association studies of *Hae*III/exon 2 genotypes with somatic cell score in cattle (Hariana and Sahiwal)

The means with standard errors of means (Mean± S.E.M.) for SCS related to each genotype are given in Table 4.11. The overall mean of SCS was 830.3±283.8 and 804.3±325.4 for Hariana (n=48) and Sahiwal cattle (n=47), respectively. There was a significant *(P<0.05)* difference for SCS between genotype TC (1480.0±505.0) and genotype CC (170.0±51.5) of Hariana cattle (Fig. 4.47). There was no significant difference was observed between two genotypes for SCS in Sahiwal cattle. However, genotype CC was higher (1190.0±648.0) than genotype TC (82.02±249.0) and genotype TT (401.0±79.6).

4.2.3.9 Association studies of *Hae*III/exon 2 genotypes with somatic cell score in Murrah buffaloes

The means with standard errors of mean (Mean± S.E.M.) for SCS related to each genotype are given in Table 4.11. Only one TT genotype was available in the screened population so it was not included in association analysis for SCS. The overall mean of somatic cell score was 51.27±5.0 for Murrah buffaloes (n=44). There was no significant *(P>0.05)* difference was observed between two genotypes for SCS in Murrah buffaloes (Fig. 4.48).

Table 4.11: Association studies of *Hae*III/exon 2 genotypes with SCS in cattle (Hariana and Sahiwal) and Murrah buffalo

Genotype	Hariana cattle	Sahiwal cattle	Murrah buffalo
TT	841.0±295.0^{ab} (15)	401.0±79.6 (17)	63.2 (1)
TC	1480.0±505.0^{b} (7)	822.0±249.0 (6)	50.7±6.8 (8)
CC	170.0±51.5^{a} (26)	1190.0±271.0 (24)	51.9±3.2 (35)

4.2.4 *Sty*I/exon 2 Polymorphism study

4.2.4.1 Amplification of exon 2 region of *MBL1* gene

For screening of the SNP g.2686T>C in exon 2 region of *MBL1* gene A 162 bp of amplified product of exon 2 region of *MBL1* gene was successfully produced by using specific primer pairs and optimized PCR conditions. PCR products were resolved on 1.0% agarose gel electrophoresis and were documented using gel documentation system (Fig. 4.49).

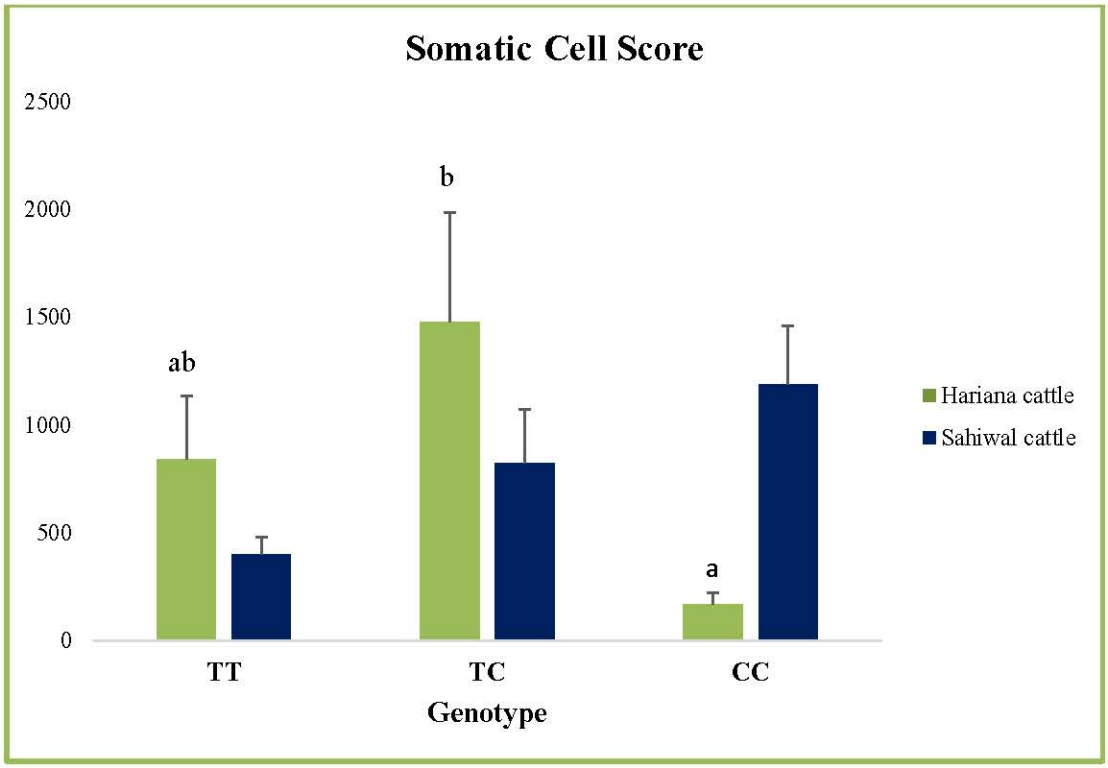

Fig. 4.47: Graphical representation of association study of *Hae*III/exon 2 genotypes with somatic cell score in cattle (Hariana and Sahiwal).

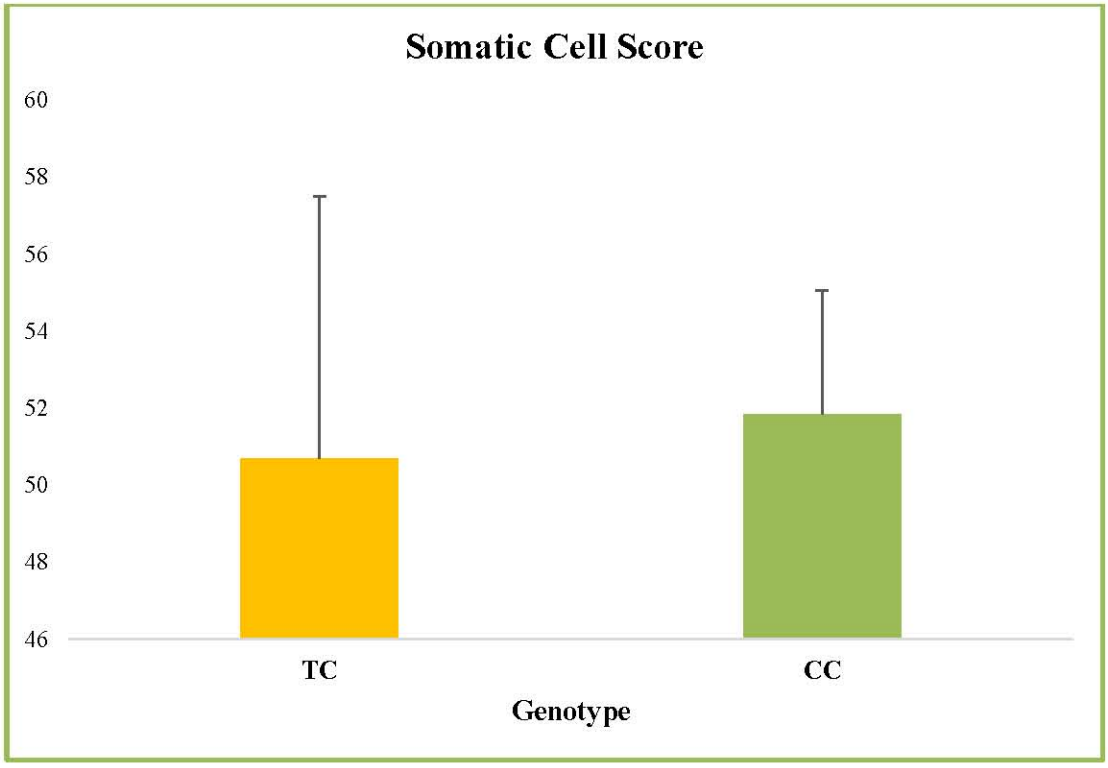

Fig. 4.48: Graphical representation of association study of *Hae*III/exon 2 genotypes with somatic cell score in Murrah buffalo.

4.2.4.2 *Sty*I/exon 2 PCR-RFLP assay

For the screening of SNP g.2651G>A in the exon 2 region of *MBL1* gene, 162 bp amplified products were digested with *Sty*I restriction enzyme as per the manufacturer's protocol. The *Sty*I/exon 2 PCR-RFLP assay revealed monomorphic pattern with only one genotype GG having undigested product of 162 bp (Fig. 4.50). This confirmed that all the screened cattle and buffalo used in the present study were monomorphic in nature with only GG (wild) genotype with G allele.

4.2.4.3 Calculation of *Sty*I/exon 2 allelic and genotypic frequency

The *Sty*I/exon 2 allelic and genotypic frequencies were calculated and presented in Table 4.12. The allelic and genotypic frequencies from the studied sample size of 150 (cattle=100 and buffalo=50) were calculated as following:

$$\textbf{Allelic/gene frequency} = \frac{(2D + H)}{2N}$$

Allelic frequency f (G) = (2D+H)/2N

Allelic frequency f (G) = (2x150+0)/2x150 = 1.00

= 100.00%

Allelic frequency f (A) = (2R+H)/2N

Allelic frequency f (A) = (2x0+0)/2x150 = 0.00

= 0.00%

$$\textbf{Genotypic frequency} = \frac{\text{Total no. of individual of particular genotype}}{\text{Total no. of individuals of all genotype}}$$

Genotypic frequency f (GG) = 150/150 = 1.00

= 100.00%

Genotypic frequency f (GA) = 0/150 = 0.00

= 0.00%

Genotypic frequency f (AA) = 0/150 = 0.00

= 0.00%

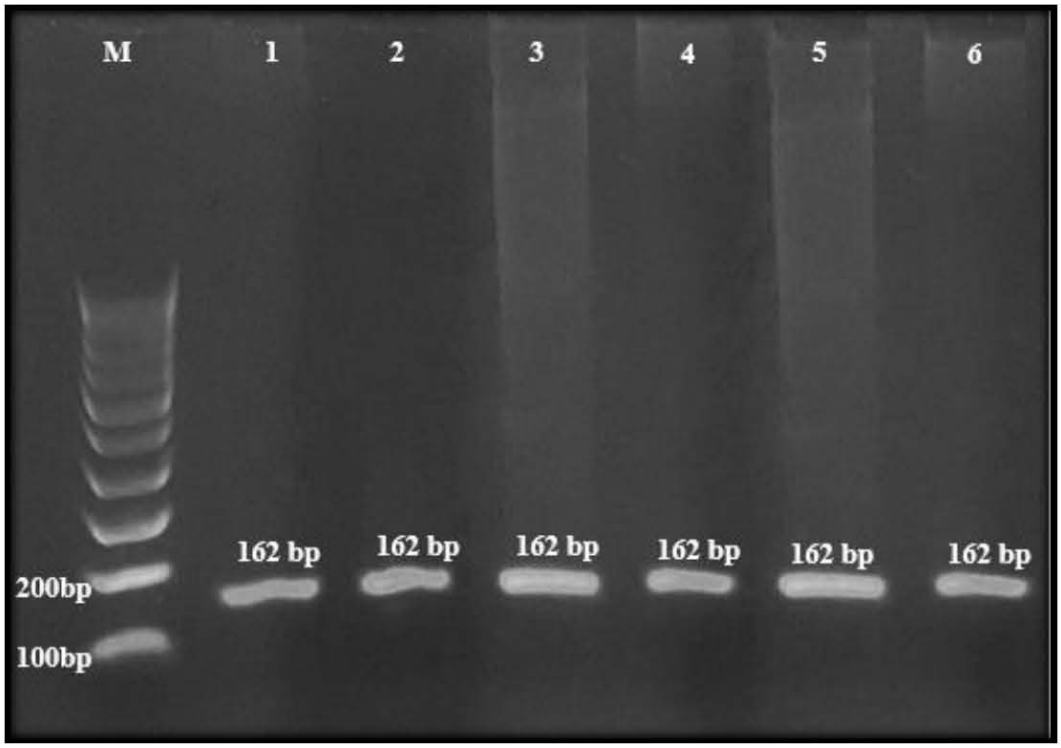

Fig. 4.49: Agarose (2.0%) gel electrophoresis showing amplification of exon 2 region (162 bp) of *MBL1* gene of Hariana cattle (Lane 1-2), Sahiwal cattle (Lane 3-4) and Murrah buffalo (Lane 5-6), Lane M: Marker (100 bp DNA ladder).

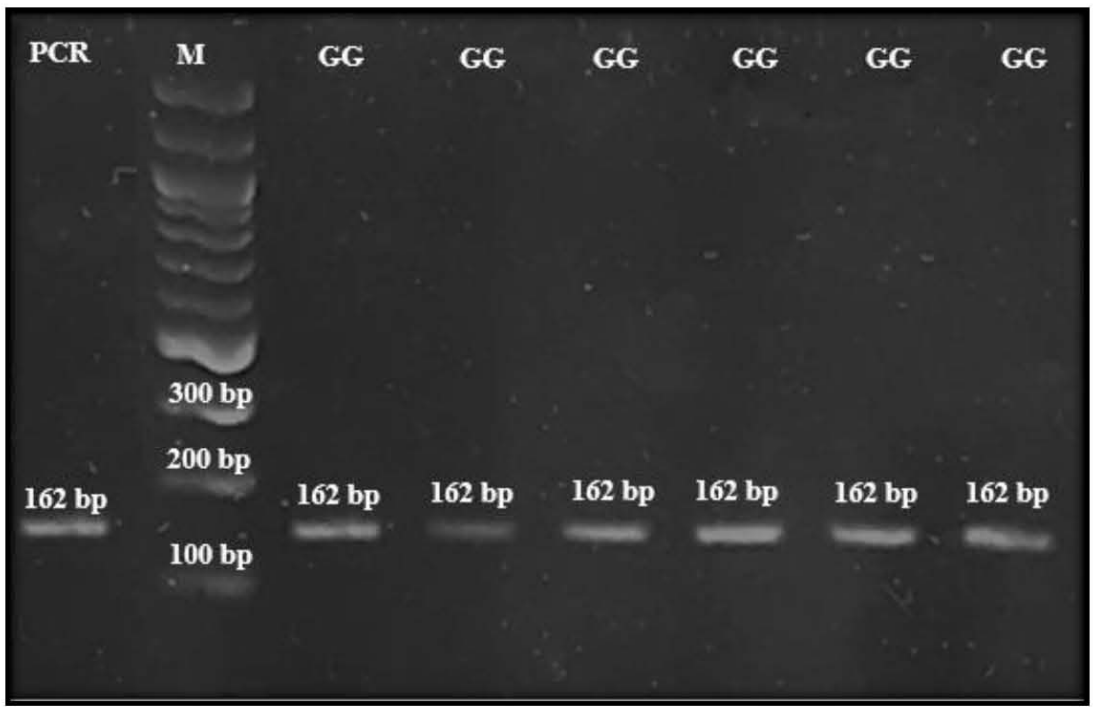

Fig. 4.50: RFLP products of exon 2 region of *MBL1* gene digested with *Sty*I RE showing monomorphic pattern. Lane PCR: Undigested PCR product (162 bp), M: Marker (100 bp DNA ladder), Lane 1-6: GG genotype (uncut band; 162 bp).

Table 4.12: Genotypic and allelic frequencies of *Sty*I/exon 2 in cattle and buffalo

Breed	Genotypic frequency (%)			Allelic frequency	
	GG	GA	AA	G	A
Hariana cattle (n=50)	100.00 (n=50)	0.00 (n=0)	0.00 (n=0)	1.00	0.00
Sahiwal cattle (n=50)	100.00 (n=50)	0.00 (n=0)	0.00 (n=0)	1.00	0.00
Total cattle (n=100)	100.00 (n=100)	0.00 (n=0)	0.00 (n=0)	1.00	0.00
Murrah buffalo (n=50)	100.00 (n=50)	0.00 (n=0)	0.00 (n=0)	1.00	0.00
Total (N=150)	100.00 (n=150)	0.00 (n=0)	0.00 (n=0)	1.00	0.00

Where; N= Sample size, n= Number of animals of particular breed and genotype.

4.2.4.3 Association studies of *Sty*I/exon 2 genotypes with milk production traits and somatic cell count

The association analysis of *Sty*I/exon2 genotypes with milk production traits and SCS was not performed due to monomorphic pattern in all the screened cattle and buffalo population.

CHAPTER-5

DISCUSSION

In the present study, two cattle breeds (Hariana and Sahiwal) and one buffalo breed (Murrah) of India, representing diverse production characteristics were included. In present study, complete coding sequence of *MBL1* gene was cloned and characterized in Indian buffalo breed Murrah. It would provide an opportunity to explore novel mutations and their exploitation to enhance milk production.

MBL gene is one of the most important components of the innate immune system (Neth *et al*., 2000). A number of variations have been found in both the coding and the non-coding regions of the *MBL* gene in human, sheep, porcine and cattle, of which several affect the assembly of *MBL*, thus leading to the innate immune dysfunctions (Madsen *et al*., 1998; Juul-Madsen *et al*., 2011a; Lillie *et al*., 2007; Liu *et al*., 2011; Zhao *et al*., 2011; Wang *et al*., 2011). Mutations of both *MBL1* and *MBL2* have been shown to vary the susceptibility of animals to various infections (Lillie *et al*., 2005).

5.1 Characterization of *MBL1* CDS

Previously *MBL1* gene has been isolated from rabbits (Kozutsumi *et al*., 1980; Kawai *et al*., 1998), humans (Kawasaki *et al*., 1983; Ezekowitz *et al*., 1988 and Taylor *et al*., 1989), rats (Oka *et al*., 1988), cattle (Holmskov *et al*., 1993; Kawai *et al*.,1997), chickens (Laursen *et al*., 1998a), mice (Sastry *et al*., 1995), rhesus monkey (Mogues *et al*., 1996), mouse (Liu *et al*., 2001), pigs (Agah *et al*., 2001; Lillie *et al*., 2006a; Juul-Madsen *et al*., 2006), horse (Podolsky *et al*., 2006), chimpanzees (AAX84948 and AAX84954), baboons (AAX84951 and AAX84957) and gorilla (AAX84949 and AAX84955). Two homologous forms of MBL, designated MBL-A and MBL-C, have been characterized by cDNA isolation from all these species except humans, pigs, cows and chickens (Kawai *et al*., 1997; Ezekowitz *et al*., 1988; Laursen *et al*., 1998a; Agah *et al*., 2001). The primary sequence of human *MBL* has been elucidated and the four domains have been characterized based on their homology with other lectins (Ezekowitz *et al*., 1988).

The bovine *MBL1* gene span over 5223 bp long and is located on BTA 28 (BBU4) (Gjerstorff *et al*., 2004b). It contains five exons and four introns, encoding a protein (MBL-A) of 248 aa as per *MBL1* gene and *MBL1* mRNA sequences available on NCBI with accession no. AC_000185.1 and NM_001010994.3, respectively. Complete coding sequence of the Murrah buffalo *MBL1* gene of present study was submitted in GenBank database with accession no. MN990687.

5.1.1 At nucleotide level

In the present study, a 951 bp cDNA sequence was amplified consisted a 158 bp (part of exon 1 and exon 2) long 5′-UTR followed by 747 bp CDS and a sort 46 bp 3′-UTR. A 747 bp CDS contained 57 bp analogous to a signal peptide of 19-aa residues, 690 bp analogous to the mature protein resulting a 248 aa protein. Similarly, a 747 bp CDS encoding a protein of 248 aa residues in bovine (*Bos taurus*) was reported by Kawai *et al*. (1997). However, they obtained 1409 bp cDNA that contained a short (15 bp) 5′-UTR, followed by 747 bp CDS (57 bp corresponding to a signal peptide of 19-aa residues and 690 bp corresponding to the mature protein) and remaining 647 bp long 3′-UTR. They reported that the bovine mannose binding protein (MBP) had high homology to human MBP which was much higher than to that of rat or mouse MBP-C or MBP-A. On comparison with *MBL1* mRNA sequence of *Bos taurus* (NM_001010994) available on NCBI, it showed 98.3% homology at nucleotide level.

On comparison with previously reported partial *MBL1* CDS sequence (Accession no. KM087783) of Murrah buffalo, the present study sequence exhibited 99.1% homology. The present study *MBL1* CDS was characterized by cloning and sequencing of *MBL* mRNA. On the contrary, the previous sequence was characterized by genomic DNA sequencing of *MBL1* gene. The last three nucleotides *viz.* TGA which codes for termination or stop codon were lacked a nucleotide ‘A’ in previously reported partial *MBL1* sequence of Murrah buffalo. The presence of a stop codon is generally a signal to terminate protein synthesis and this process constitutes the last essential stage of translation, as it ensures the formation of full-sized proteins. Additionally, this partial sequence was lacking 5′-UTR and 3′-UTR, however, the present study sequence of Murrah buffalo *MBL1* gene had 158 bp of 5′-UTR and 46 bp of 3′-UTR. In such a way, the sequence of present study fulfilled this gap and characterized complete CDS of *MBL1* gene in Murrah buffalo.

Multiple nucleotide sequence comparison of *MBL1* CDS sequences revealed that Murrah buffalo *MBL1* sequence of present study was more similar to that of exotic Mediterranean buffalo predicted sequence (99.6%) than partial *MBL1* CDS sequence of Murrah buffalo (99.1%) and exotic American buffalo (*Bison bison*) (98.1%). It was more (98.3-98.5 %) similar to exotic cattle breeds than American buffalo (98.1%). No report is availableon the sequence similarity at nucleotide level among cattle breeds or between cattle and buffalo breeds. However, three bovine MBPs have been reported till date. One of these was eluted as a peak of 600 kDa on gel chromatography and was shown to consist of 45-kDa polypeptide subunits on SDS-PAGE performed under reducing conditions (Kawasaki *et al*., 1985). The other bovine MBP was found to have a 28-kDa polypeptide chain who’s N-terminal aa sequence showed reasonable homology to previously reported MBPs (Holmskov

et al., 1993). The third one was found to have a 33-kDa bovine MBP and showed an amino acid sequence which was almost the same as that of the 28-kDa MBP (Kawai *et al*., 1997). The *MBL1* CDS of present study showed 98.4% similarity with its wild relative *Bos mutus* (yak) and 96.1% and 95.2% similarity with sequences of *Capra hircus* (goat) and *Ovis aries* (sheep), respectively indicating a high degree of sequence conservation. On the other hand, it showed less similarity with pig breeds (Landrace pig and Yorkshire pig) (83.5%) and camel breeds (Alxa Bactrian camel and Arabian camel) (83.5-83.7%). In case of similarity with porcine CDS sequence, Lillie *et al*. (2006a) reported 83.4% identity between porcine and bovine *MBL1 CDS* sequences which is similar to the present findings.

The 951 bp *MBL1* cDNA of present study contained a 149 bp (1-149 bp) segment as exon 1 that was transcribed but untranslated. This type of non-coding sequence was referred as exon 0 or exon 1 in previously characterized *MBL1* gene of several species. Similarly, *Bos taurus* sequence (NM_001010994), Liu *et al*. (2011) and Lillie *et al*. (2006a) also reported 149 bases in the non-coding region of first exon in the bovine and porcine *MBL1* genes, respectively. Wang *et al*. (2011) and Liu *et al*. (2011) also stated the non-coding exon in their study on China Holstein cattle and represented it as exon 1. Such an exon has also been found in the mouse (Laursen and Nielsen 2000), pig (Lillie *et al*., 2007a) and murine (Sastry *et al*., 1995) *MBL1* genes as well as in the rat and mouse *MBL2* genes (Laursen and Nielsen 2000). Naito *et al*. (1999) also reported an alternative exon (exon 0) 1.0 kb upstream of the *MBL2* exon 1 in humans and stated that the non-coding exon 0 was present before the putative start of exon 1 that was similar to the present finding in which the non-coding exon 1 was present before the start of exon 2. In human *MBL2* gene, transcription may initiate either at exon 1 or at an additional, non-coding 1.0 kb upstream located, exon 0 (Sastry *et al*., 1989, Taylor *et al*., 1989 and Naito *et al*., 1999). Heitzeneder *et al*. (2012) assumed that 10–15% of MBL protein in serum derives from exon '0' transcription.

In Murrah buffalo *MBL1* cDNA exon 1 started from nucleotide position 1 to 149 (non-coding), exon 2 from 150-339 (190 bp long), exon 3 from 340-456 (117 bp long), exon 4 from 457-531 (75 bp long) and incomplete exon 5 from 532-951 nucleotides (420 bp long), however, only 347 bp of exon 5 were the parts of CDS. At the start of exon 2 (150-339), which contains the translation start site (ATG) at the position 159-161, there are 9 non-coding nucleotides of 5′ UTR. The CDS region was located from 159-905 (747 bp) nucleotide position in 951 bp cDNA sequence. Similarly, Lillie *et al*. (2006a) characterized bovine and porcine *MBL1* gene in their study and obtained a 934 bp cDNA in bovine (*Bos taurus*) that included a full coding sequence (747 bp) of bovine MBL-A and parts of the 5′ and 3′ UTRs.

For porcine, they obtained a 875 bp cDNA that contained 42 bp of 5′-UTR, a 750 bp segment encoding an *MBL* CDS and 83 bp of 3′ UTR in Yorkshire cross pigs.

Murrah buffalo *MBL1* CDS exhibited that exon 2 encoded the signal peptide (1-19 aa), a cysteine rich N-terminus region (20-42 aa) and a part of collagenous region (43-60), exon 3 encoded the remainder of the collagenous region (61-99 aa), exon 4 encoded an α-helical coiled-coil structure known as 'neck region' (100-124 aa) and exon 5 encoded the CRD (125-248 aa), which adopts a globular configuration. Similar findings were reported in *MBL1* CDS of bovine (NM_001010994.3 and AY861663), porcine *MBL1* CDS (Agah *et al*., 2001; Lillie *et al*., 2006a) and human *MBL2* gene (Weis *et al*., 1992; Madsen *et al*., 1995 and Wallis *et al*., 2004).

5.1.2 At amino acid level

The deduced amino acid sequence of the CDS of Murrah buffalo *MBL1* was found to have conserved sequences required for specific MBL functions such as two cysteine residues in the short N-terminal domain, a GEKGEP sequence in the collagen domain, a PGKXGP sequence (PGKMGP) at the C-terminal end of the collagen like domain and a Glu–Pro–Asn (EPN) motif in their CRD. The collagen-like domain consisted of numerous Gly-X-Y motifs and one Gly-X-Gly (a characteristic of MBL) which leads to the formation of a triple helix structure as reported previously. There was an interruption in the collagen Gly-X–Y triple helix that introduces a kink needed for the sertiform structure of MBLs. This finding was consistent with the kink observed in this domain in all other MBL proteins (Wallis *et al*., 2004). Similar conserved sequences were reported in deduced amino acid sequences of previously characterized bovine *MBL1* gene (Kawai *et al*., 1997; Lillie *et al*., 2006a) and porcine *MBL1* gene (Agah *et al*., 2001;Lillie *et al*., 2006a).

At least two cysteine residues are needed in N-terminal region for the stabilization of trimers in higher order multimers (McCormack *et al*., 1999; Ohashi *et al*., 2004; Jensen *et al*., 2005). In the collagen domain, a GEKGEP sequence has been associated as a site for interaction with the C1q receptor C1qRp in MBL of other species (Arora *et al*., 2001). A PGKXGP sequence at the C-terminal end of the collagen like domain is part of the putative MASP-binding motif (Wallis *et al*., 2004) which suggests the Murrah buffalo MBLA protein has the potential to activate the lectin-complement pathway. It was stated that collagenous lectins with mannose-sugar-type binding specificity have a Glu–Pro–Asn (EPN) motif in their CRD, whereas those with galactose-sugar-type specificity, have Gln–Pro–Asp (Weis *et al*., 1992; Ogasawara and Voelker, 1995), according to this Murrah buffalo MBL-A was predicted to have mannose-type sugar specificity similar to other mammalian MBLs. These previous

findings about MBLs indicates that Murrah buffalo MBL1 of present study is a true mannose-binding lectin.

The deduced amino acid sequence from Murrah *MBL1* CDS was compared with that of other buffalo and cattle breeds and other related species available in the GenBank database. On comparison of MBL1 amino acid sequences, it was revealed that Murrah buffalo MBL1 sequence of present study was equally similar (98.8%) to another partial Murrah buffalo and exotic Mediterranean buffalo. It was least similar to exotic American buffalo (96.8%) among buffalo breeds. Among all the cattle breeds, amino acid sequence of Murrah buffalo MBL1 showed more similarity with Nellore cattle (*Bos indicus*, Brazilian origin) (97.2%). It showed 97.6% similarity with its wild relative *Bos mutus* (yak), 95.2% and 94.4% similarity with sequences of goat and sheep, respectively. A high relative homology was observed in the sequences of both nucleotide and deduced amino acid sequences across analysed large and small ruminant species. No report is available regarding the sequence similarity at amino acid level among different cattle breeds or between cattle and buffalo breeds. However, Holmskov *et al*. (1993) and Kawai *et al*. (1997) isolated bovine MBPs and reported sequence similarity results of alignment with human, mouse and rat. Holmskov *et al*. (1993) compared the sequence of the first 26 N-terminal amino acid residues of the 28 kDa lectin with the N-terminal amino acid sequences of rat MBP-A, rat MBP-C (Drickamer *et al*., 1986), human MBP (Ezekowitz *et al*., 1988; Taylor *et al*., 1989), and cDNA derived sequences of mouse MBP-A and MBP-C (Sastry *et al*., 1991). The highest degree of sequence similarity was found with human MBP and rat MBP-C. This was especially pronounced around the cysteine residues which were probably involved in the interchain attachments.

Kawai *et al*. (1997) purified a 33-kDa bovine MBP and aligned it with rat, human, and mouse (Drickamer *et al*., 1986; Taylor *et al*., 1989; Sastry *et al*., 1991) which showed that bovine MBP was the most homologous to human MBP and revealed that bovine MBP (249 aa) has 65% aa identity with human MBP (248 aa) when two gaps are allowed in the alignment. Mouse (244 aa) and rat MBP-C (244 aa) have similar identity (58%) to bovine MBP. Moreover, Lillie *et al*. (2006a) showed 79.9, 77.1, 67.2 and 69.9% homology with rhesus monkey (L43912/CO583176), human (AF019382), rat (NM_012599) and mouse (NM_010775) respectively. Agah *et al*. (2001) isloated porcine liver *MBL* cDNA and exhibited 64.9% identity with human MBL and 50.2% and 56.7% identity with rat A and C MBL, respectively.

The deduced amino acid sequence of Murrah buffalo *MBL1* showed less similarity with pig breeds (79.9%) and camel breeds (81.9%), respectively. Almost similar homology (80.7%) was observed between bovine and porcine MBl-A by Lillie *et al*. (2006a). Podolsky

et al. (2006) characterized a horse MBL (eMBL/equine MBL) and reported that the deduced amino acid sequence of eMBL was 63% similar to bovine MBL1.

5.2 Identification of single nucleotide polymorphism (SNPs)

The *MBL1* sequences were aligned and compared with previously published *MBL1* sequences for inspection of polymorphic sites. The *MBL1* CDS of Murrah buffalo contained three unique nucleotide substitutions, a G→A synonymous mutation at nucleotide position 87 which did not resulted any amino acid substitution (Q29Q) and was located in the N-terminal region of the protein encoded by exon 2. Similarly, Cosenza *et al*. (2012) detected two synonymous mutations (g.993G→A) and g.1092C→T in first coding exon of sheep MBL and none of these mutations was found to be associated with any effect. Yuan *et al*. (2013) identified a synonymous mutation T→C at position 2569 and Wang *et al*. (2011) and Liu *et al*. (2011) identified a T→C mutation with synonymous substitution (GCT (Ala) → GCC (Ala) in N-terminal region of *Bos taurus MBL1*. These synonymous SNPs did not found to be associated with any structural dysfunction or any other trait. However, several non-synonymous mutations in first coding exon of the human *MBL* gene were frequently described as being associated with MBL plasma concentration, reduced ligand-binding capacity and failure to activate complement system (Larsen *et al*., 2004). Moreover, SNPs in N-terminal region of *MBL1* gene have been reported to be associated with low serum level of MBL1 and milk production traits. Wang *et al*. (2011); Liu *et al*. (2011) and Yuan *et al*. (2013) detected a non-synonymous mutation G→A resulted in (GTT (Val) → ATT (Ile) substitution at amino acid position 24 in N-terminal region of the bovine MBL1 gene and found to be significantly correlated with milk SCS and MBL-A serum levels suggesting it might be useful as a marker for selection of dairy mastitis resistance in cattle. Juul-Madsen *et al*. (2011a) identified a SNP A77G in N-terminal domain of porcine MBL-A which resulted in substitution of glycine for glutamic acid at amino acid 26, although it did not disturb the assembling of the MBL subunits into the basic trimer structure but they indicated that G allele of SNP A77G may also contribute to a lower concentration of MBL-A in serum in a haplotype combination. Another non-synonymous SNP A112G resulting in a conservative change of amino acids from asparagine to aspartic acid was detected by Bergman *et al*. (2014) in the same region and was unlikely to affect the three-dimensional structure of the molecule.

Among the two unique non-synonymous mutations in Murrah buffalo *MBL1* CDS, one T→C substitution at nucleotide position 611 was found which resulted in amino acid substitution of Leucine 204 Proline (L204P) and the another one was found at nucleotide position 538, a T→C transition which lead to amino acid substitution (non-synonymous) of Serine 180 Proline (S180P). These both non-synonymous mutations were located in the

carbohydrate recognition domain (CRD) of amino acid sequence. Apart from these unique nucleotide changes found in present *MBL1* CDS of Murrah buffalo, a non-synonymous SNP was found in CRD of previously reported partial CDS of Murrah buffalo that was 451A→G transition causing a non-synonymous mutation with amino acid substitution of AGC (serine) to GGC (glycine) at amino acid position 151. Comparable mutation to leucine residue was identified by Juul-Madsen *et al*. (2006) in porcine *MBL1* gene at nucleotide position 698 in CRD which resulted in substitution of Leucine 233 Serine (L233S) and suggested it may affect the binding properties of CRD. Lillie *et al*. (2006b) identified a synonymous C→T SNP in porcine *MBL1* gene at position 687 (codon 219-aspartic acid) in CRD. Phatsara *et al*. (2007) reported two synonymous SNPs found at the positions G579A and G645A of porcine *MBL2* cDNA which were located at codons 193 (Lys) and 215 (Val) in CRD of predicted amino acid sequence. They suggested, *MBL* gene as functional and positional candidate gene for *in vivo* complement activity mediated *via* the lectin pathway. Two synonymous SNPs (C3233T and C3344T) were detected by Juul-Madsen *et al*. (2011a) in CRD of MBL1 amino acid sequence, however, no association was observed between these SNPs and any trait. Fraser *et al*. (2018) identified a non-synonymous mutation Cys237Tyr in the same region of MBL-A protein of horse and predicted to be damaging. Whether the CRD mutations found in present study has similar effects on the structure of buffalo MBL-A is unknown, but is of particular interest as evidence suggests that CRD specific mutations have been associated with its binding properties and complement activation.

Additionally, American buffalo had a unique G→A nucleotide change at nucleotide position 178 which resulted in amino acid substitution (non-synonymous) of Glutamic acid 60 Lysine (E60K) and falls in the collagen like domain (CLD) of MBL1. The mutation occurs in the CLD of MBL-C was predicted to have a significant impact on protein structure and function as well as on higher order oligomerization through disruption of the Gly-X-Y collagen-like repeats (Sumiya *et al*., 1991). Lillie *et al*. (2006b) and Juul-Madsen *et al*. (2011a) identified one non-synonymous SNP at position 271 (G→T) of porcine *MBL1* which was predicted to be functionally important because it substituted cysteine for glycine (wild-type) in the 16th glycine-X-Y triplet (Gly91Cys) of the CLD. This triplet has been suggested to be involved in the initiation of the helical formation of the CLD, and the mutation may inhibit the assembly of MBL subunits into the basic trimer structure, thus reducing the amount of functional MBL in circulation (Wallis, 2002). Lillie *et al*. (2006b) also reported a C→T SNP at position 273 (codon 91) which corresponded to a silent nucleotide difference. Bergman *et al*. (2012) reported a SNP T871C which substituted a phenylalanine to leucine at amino acid 65 represented the X-position in G-X-Y motif 7 in CLD of pig MBL1. Fraser at

el. (2018) detected a SNP resulting in Gly82Ala substitution in CLD of horse MBL-A with unknown impact but assumed that it might be associated with infectious diseases.

In the CLD of *MBL2* gene, SNPs g.1197 C>A and g.1198 G>A changed proline to glutamine, whereas SNP g.1207 T>C was identified as a synonymous mutation. Correlation analysis showed that the g.1197 C>A marker was significantly correlated to SCS in Chinese cattle (Zhao *et al*., 2012). In humans, three SNPs have been identified in exon 1 at codons 52 (Arg → Cys), 54 (Gly → Asp) and 57 (Gly → Glu) located in the collagen domain (Sumiya *et al*., 1991; Lipscombe *et al*., 1992; Madsen *et al*., 1994), showed association with many innate immunological factors (Holmskov *et al*., 2003). Furthermore, the point mutations in exon 1 of the human *MBL* gene were frequently described as being associated with MBL plasma concentration and failure to activate complement (Larsen *et al*., 2004). Wang *et al*. (2012) detected three SNPs in *MBL2* gene of Chinese Holstein cattle, g.234 C>A and g.235 G > A (codon 42) resulted in Pro to Gln change at the 1st Gly-X-Y repeat of the CLD, while the SNP g.244 T > C (codon 45) was identified as a synonymous mutation (Asn > Asn) at the 2th Gly-X-Y repeat of the CLD. The SNP marker (g.234 C>A) was significantly correlated with SCS.

Several complement component gene variants have been reported to associate with resistance to different bacteria, viruses and parasites, such as *S. aureus* (*MBL2* in mice; Shi *et al*., 2004); *Streptococcus suis* and porcine circovirus 2 (*MBL2* in pigs; Lillie *et al*., 2007) and *Brucella abortus* (*MBL2* in water buffalos; Capparelli *et al*., 2008) Analysis of bovine *MBL1* SNPs may contribute to the finding of mastitis resistance-related genetic traits as bacteria are the most common causes of mastitis.

5.3 Phylogenetic analysis

The phylogenetic analysis of deduced amino acid of *MBL1* gene showed the closest genetic relationship with partial sequence of another Murrah buffalo than the exotic ones in which the bovidae family divided into two clades: bovinae and caprinae subfamily showing a greater degree of variation leaving suidae and camelidae in two different clads. Within the bovinae group, present studied Murrah buffalo *MBL1* sequence was to be most closely related to partial Murrah buffalo sequence followed by Mediterranean buffalo. However, American buffalo was to be most closely related to cattle breeds and yak. Caprinae group was more closely related to cattle and buffaloes than suidae and camelidae group. In the phylogenetic tree, all buffalo breeds exhibited a close phylogenetic evolutionary relationship with cattle and yak.

On the contrary, Lillie *et al*. (2006a) and Phatsara *et al*. (2007) reported a phylogenetic analysis of the predicted amino acid sequences of known mammalian MBL-A genes which revealed three distinct branches and observed porcine and bovine MBL-A came off together as a separate branch, with rodents and primate MBLs forming the other two branches. It might be because they aligned with MBL1 of non-related species other than bovine. Their branching pattern was consistent with that found for MBL-C by Fujita *et al*. (2004) using just the amino acid sequences of the CRD.

5.4 PCR-RFLP assay for polymorphism study

5.4.1 *Apa*I/intron I PCR-RFLP assay

In the present study, screened Sahiwal and Hariana cattle and Murrah buffalo population were found polymorphic for *Apa*I/intron I PCR-RFLP assay. It revealed three types of genotype *viz.* genotype GG with band sizes 311 and 277 bp; genotype GA with band sizes 588, 311 and 277 bp and wild type genotype AA with band size 588 bp. The present findings were similar to the reports of Liu *et al*. (2010) and Wang *et al*. (2011) in Chinese cattle breeds. Yuan *et al*. (2013) and Aksel *et al*. (2019) also reported an allelic variant with G→A mutation at location c.1252G>A in intron I region of bovine *MBL1* gene in Chinese cattle breeds and Turkey and Europian cattle breeds, respectively. Furthermore, Juul-Madsen *et al*. (2011a) identified six SNPs (G193C, G216T, C488T, T1046C, C1102T and S1103G) in intron I region of Landrace and Duroc breeds of pig. Marklund *et al*. (2000) and Phatsara *et al*. (2007) also reported a SNP within intron I of *MBL1* gene at position 328. However, intron sequences do not code for amino acids/proteins, they have been proved to play vital regulating parts in gene expression and its regulation (Nott *et al*., 2003), mRNA transcription and splicing (Zan *et al*., 2007). Zhai *et al*. (2019) also identified several intronic mutations in Hu Sheep *MBL* gene, g.288T>A in intron I, g.1091 T>C, g.1096A>C, g.1770G>C in intron II, g.2297C>T, g.2331G>A in intron III. Their result showed that intron I and II genotype differences in *MBL* serum levels were significant ($P<0.05$), which indicated that *MBL* gene can be used as candidate gene for disease resistance. Whether the identified mutation of g.855G>A affects gene expression and its regulation is unknown.

5.4.1.1 Genotype frequency

Genetic polymorphism of *Apa*I/intron I region of *MBL1* gene had been observed in different indigenous and exotic cattle breeds by other authors that are presented in Table 5.1.

Table 5.1: Genotypic frequencies of *Apa*I/intron I region of *MBL1* in different cattle breeds

Breed	Genotypic frequency			Reference
	GG (%)	GA (%)	AA (%)	
▪ Chinese Holstein cattle (CH)	79.50	19.10	1.00	Liu *et al*. (2010)
▪ Chinese Holstein cattle (CH)	76.00	23.00	1.00	Wang *et al*. (2011)
▪ Luxi Yellow cattle (LY)	1.00	0.00	0.00	
▪ Bohai Black cattle (BB)	1.00	0.00	0.00	
▪ Vrindavani cattle (Mastitis positive)	49.00	51.00	0.00	Asaf *et al*. (2014b)
▪ Vrindavani cattle (Mastitis negative)	46.00	54.00	0.00	
▪ **Sahiwal cattle**	**74.00**	**24.00**	**2.00**	**In present study**
▪ **Hariana cattle**	**80.00**	**18.00**	**2.00**	**In present study**
• **Murrah buffalo**	**90.00**	**8.00**	**2.00**	**In present study**

In the present study, the frequency of GG genotype in Sahiwal and Hariana cattle and Murrah buffalo was 74.0%, 80.0% and 90.0%, respectively. The value of GG genotype Sahiwal and Hariana cattle was in accordance with the findings of Liu *et al*. (2010) and Wang *et al*. (2011) in Chinese Holstein (CH) cattle (79.50% and 76.0%, respectively) while in contrast with Asaf *et al*. (2014b) which might be due to breed difference. The value of GG genotype in Murrah buffalo was not in agreement with the reports of Liu *et al*. (2010) and Wang *et al*. (2011) as well as with the GG genotype value of Sahiwal and Hariana cattle of present study. The value of GG genotype in cattle (Sahiwal and Hariana) and Murrah buffalo breed were in contrast with the findings of Wang *et al*. (2011) in Luxi Yellow (LY) cattle (1.0%) and Bohai Black (BB) cattle (1.0%) breeds of China and Asaf *et al*. (2014b) in Vrindavani cattle (49.0%).

The value of GA genotype of Sahiwal and Hariana cattle and Murrah buffalo in present study was observed as 24.0%, 18.0.0% and 8.0%, respectively. The values of GA genotype in Sahiwal cattle was similar to the findings of Wang *et al*. (2011) in CH cattle (23.0%) whereas in contrast with the reports of Asaf *et al*. (2014b) in Vrindavani cattle. Additionally, it was slightly differed with the findings of Liu *et al*. (2010) in CH cattle (19.0%).The genotypic value of GA genotype of Hariana cattle in presence study was in accordance with Liu *et al*. (2010) in CH cattle (19.10) while it was slightly differed with the genotypic frequency value (23.0%) reported by Wang *et al*. (2011) and in contrast with the

value (51.0%) reported by Asaf *et al*. (2014b). The genotypic value of GA genotype of Murrah buffalo in presence study was not supported by any reports; this may be because of the difference between cattle and buffalo. No findings are available for g.855G>A SNP in buffaloes. The value of GA genotype in cattle (Sahiwal and Hariana) and buffalo breeds were in contrast with the findings of Wang *et al*. (2011) in LY cattle (0%) and BB cattle (0%) breeds of China.

In present study, the value of AA genotype was equal (2.0%) for Sahiwal and Hariana cattle and Murrah buffalo, respectively which is slightly differed from the findings of Liu *et al*. (2010) and Wang *et al*. (2011) in CH cattle (1.0%) while it was in contrast with the findings in Luxi Yellow (0%) and Bohai Black cattle (0%) reported by Wang *et al*. (2011) and Asaf *et al*. (2014b) in Vrindavani cattle.

5.4.1.2 Allelic frequency

In the present study allelic frequency of allele G in Sahiwal and Hariana cattle and Murrah buffalo was higher than that of A allele. These findings of Sahiwal and Hariana cattle was in concurrence with the repo.rts of Liu *et al*. (2010) in CH cattle and Wang *et al*. (2011) in CH, LY and BB cattle breeds of China and Asaf *et al*. (2014b) in Vrindavani cattle. Although, the frequency of allele A was lower in Vrindavani cattle reported by Asaf *et al*. (2014b) which might be due the difference in breeds. In case of Murrah buffalo, allelic frequency of allele G was much higher (0.94) than allele A (0.06) which was differed from Sahiwal and Hariana cattle of present study as well as reported studies (Table 5.2).

Table 5.2: Allelic frequencies *Apa*I/intron I region of MBL1 in different cattle breeds

Breed	Allelic frequency		Reference
	G	A	
• Chinese Holstein cattle	0.89	0.10	Liu *et al*. (2010)
▪ Chinese Holstein cattle	0.87	0.13	Wang *et al*. (2011)
▪ Luxi Yellow cattle	1.0	0.0	
▪ Bohai Black cattle	1.0	0.0	
▪ Vrindavani cattle (Mastitis positive)	0.74	0.25	Asaf *et al*. (2014b)
▪ Vrindavani cattle (Mastitis negative)	0.72	0.27	
• **Sahiwal cattle**	**0.86**	**0.14**	**Present study**
• **Hariana cattle**	**0.89**	**0.11**	**Present study**
• **Murrah buffalo**	**0.94**	**0.06**	**Present study**

5.4.1.3 χ^2 -test for *Apa*I/ intron I in screened population

In the current study, results of χ^2 -test indicated that there were significant differences in genotypic and allelic distributions of g.855G>A for Hariana and Sahiwal cattle and Murrah buffalo breed. The screened population of cattle and buffalo was in Hardy-Weinberg equilibrium (HWE). These finding were in agreement with the findings of Liu *et al*. (2010) in CH cattle and Wang *et al*. (2011) in CH, LY and BB cattle breeds of China.

5.4.1.4 Association studies of *Apa*I/intron I polymorphism with milk production traits

In present study, there was no significant difference observed between *Apa*I/intron I genotypes and milk production traits including AFC, TMY, LP and MY300 for both the lactations in Sahiwal and Hariana cattle and Murrah buffalo. However, TMY of genotype GG was much higher (2320.0 ±127.0 liters) than genotype GA (1770.0 ±156.0 liters) and LP of genotype GG was much higher (370.0 ±48.4 days) than genotype GA (296.0 ±26.3 days) in second lactation of Murrah buffalo. Liu *et al*. (2010) and Wang *et al*. (2011) also reported no significant difference between genotypes of *Apa*I/intron I and milk production traits in Chinese breeds of cattle.

5.4.1.5 Association studies of *Apa*I/intron I genotypes with SCS

There was no significant difference was observed for SCS between two genotypes in both the cattle breeds and buffalo breed. However, genotype GA was much higher than GG genotype in Sahiwal cattle. Similarly, Liu *et al*. (2010) and Wang *et al*. (2011) also reported insignificant difference between genotypes of *Apa*I/intron I and SCS in Chinese cattle breeds.

5.4.2 *Hae*III/exon 2 PCR-RFLP assay

In the present study, screened Sahiwal and Hariana cattle and Murrah buffalo population were found polymorphic for *Hae*III/exon 2 PCR-RFLP assay which was performed to screen a synonymous SNP (g.2686T>C). It revealed banding pattern of 274 and 127 bp for genotype TT, 274, 184, 127 and 90 bp for genotype TC and 184, 127 and 90 bp for genotype CC. The genotypic frequency pattern in the present study was different from the reports of Liu at al. (2010) in CH cattle as well as Wang *et al*. (2011) and Liu *et al*. (2011) in CH, LY and BB cattle. However, it was slightly similar to Kamaldeep *et al*. (2017a) and (2017b) in Sahiwal cattle and these variations in the frequency of different genotypes might be due to the geographical distribution, population size and selection history. Moreover, the genotypic pattern of Murrah buffalo for g.2686T>C SNP was in totally opposite with the finding of Kamaldeep *et al*. (2019) who reported a monomorphic pattern for this SNP in Murrah buffalo. Yuan *et al*. (2013) and Aksel *et al*. (2019) reported a comparable silent SNP

c.2569T>C with no association with milk SCS in bovine *MBL1* gene. Zhao *et al*., (2012) reported a similar synonymous SNP g.1207 T>C (codon 45) in *MBL2* gene of CH cows with no significant association with milk production traits or SCS, however, a significant association existed between the combined genotypes of 4 SNPs (g.1164 G>A, g.1197 C>A, g.1198 G>A and g.1207 T>C) and milk production traits and SCS, suggesting that one SNP may be influenced by others. Similarly, Lillie *et al*. (2006b) reported a synonymous C→T SNPs at position 273 in codon 91 of porcine MBL-A.

5.4.2.1 Genotype frequency

Genetic polymorphism of *Hae*III/exon 2 region of *MBL1* gene had been observed in different indigenous and exotic cattle breeds by other authors that are presented in Table 5.3.

Table 5.3: Genotypic frequencies of *Hae*III/exon 2 region of *MBL1* in different cattle breeds

Breed	Genotypic frequency			Reference
	TT (%)	TC (%)	CC (%)	
▪ Chinese Holstein cattle	16.70	57.30	26.00	Liu *et al*. (2010)
▪ Chinese Holstein cattle	14.00	58.00	28.00	Wang *et al*. (2011)
▪ Luxi Yellow cattle	0.00	53.00	47.00	
▪ Bohai Black cattle	19.00	37.00	44.00	
▪ China Holstein cattle	12.79	75.48	11.73	Liu *et al*. (2011)
▪ Luxi Yellow cattle	0.00	54.55	45.45	
▪ Bohai Black cattle	20.83	29.17	50.00	
▪ Sahiwal cattle	39.20	42.90	17.90	Kamaldeep *et al*. (2017a)
▪ Sahiwal cattle	37.00	46.00	17.00	Kamaldeep *et al*. (2017b)
▪ **Sahiwal cattle**	**36.00**	**12.00**	**52.00**	**In present study**
▪ **Hariana cattle**	**32.00**	**14.00**	**54.00**	**In present study**
• **Murrah buffalo**	**66.00**	**32.00**	**2.00**	**In present study**

In current study, the frequency of TT genotype in Sahiwal and Hariana cattle and Murrah buffalo was 36.0%, 32.0% and 66.0%, respectively. The value of TT genotype in Sahiwal and Hariana cattle was in concurrence with the findings of Kamaldeep *et al*. (2017a, 2017b) in Sahiwal cattle (39.2% and 32.0%) while in contrast with the reports of Liu *et al*.

(2010) in CH cattle (16.7%), Wang *et al*. (2011) and Liu *et al*., (2011) in China Holstein (14.0% and 12.79%), LY (0%) and BB cattle (19.0% and 20.83%). Amusingly, the present study results indicated that TT genotype was much more frequently present in Murrah buffaloes than TT genotype in Sahiwal and Hariana cattle of present study as well as other breeds of above reported studies.

The genotypic value of TC genotype of Sahiwal and Hariana cattle and Murrah buffalo in present study was observed to be 12.0%, 14.0.0% and 32.0%, respectively. The frequency of TC genotype in Sahiwal and Hariana cattle was lower from other genotypes which was in contradictory of findings of Liu et al. (2010) in CH cattle, Wang *et al*. (2011) and Liu *et al*. (2011) in CH, LY and BB cattle, Kamaldeep *et al*. (2017a) and (2017b) in Sahiwal cattle. However, in case of Murrah buffalo, the value of TC genotype was in contrast to the frequency of TC genotype of Sahiwal and Hariana cattle of presence study, Liu *et al*. (2010), Wang *et al*. (2011), Liu *et al*., (2011) in CH cattle and Kamaldeep *et al*. (2017a) and 2017 (b) in Sahiwal cattle whereas similar to the findings of Wang *et al*. (2011) in BB cattle (37%) and Liu *et al*. (2011) in BB cattle (29.17).

The value of CC genotype for Sahiwal and Hariana cattle and Murrah buffalo in present study were 52.0%, 54.0% and 2.0%, respectively. The frequency of CC genotype in Sahiwal and Hariana cattle was in agreement with that of LY cattle (47.0%) and BB cattle (44.0%) by Wang *et al*. (2011) and LY cattle (45.45%) BB cattle (50.0%) by Liu *et al*. (2011) while in contrast with CH cattle (26.0, 28.0 and 11.73%) by Liu *et al*. (2010), Wang *et al*. (2011) and Liu *et al*. (2011), respectively and Sahiwal cattle (17.0%) by Kamaldeep *et al*. (2017). Captivatingly, the presence of CC genotype in Murrah buffaloes was very less and was in opposite of Sahiwal and Hariana cattle of present study as well as other cattle breeds used in reported studies by above mentioned researchers. Such variations in frequency of g.2686T>C genotypes might be due to the variations in number of samples tested and breeds involved.

5.4.2.2 Allelic frequency

In the current study, allele C was more frequently present than allele T in Sahiwal and Hariana cattle as well as in Murrah buffalo breed. The findings of Sahiwal and Hariana cattle were in accordance with the reports of Liu *et al*. (2010) in CH cattle, Wang *et al*. (2011) and Liu *et al*. (2011) in CH and BB cattle and Kamaldeep *et al*. (2017a) and (2017b) in Sahiwal cattle. Amusingly, the results revealed huge variations in frequency of T and C alleles in cattle and buffalo population as the value of allele C was very less and value of allele T was much high in Murrah buffaloes than Sahiwal and Hairana cattle of present study as well as

other cattle breeds tested by Liu *et al*. (2010), Wang *et al*. (2011), Liu *et al*. (2011) and Kamaldeep *et al*. (2017a) and (2017b), however, somewhat similar results were reported by Wang *et al*. (2011) and Liu *et al*. (2011) in LY cattle.

Table 5.4: Allelic frequencies *Hae*III/exon 2 region of *MBL1* in different cattle breed

Breed	Allelic frequency		Reference
	T	C	
• Chinese Holstein cattle	0.45	0.54	Liu *et al*. (2010)
▪ Chinese Holstein cattle	0.43	0.57	Wang *et al*. (2011)
▪ Luxi Yellow cattle	0.26	0.74	
▪ Bohai Black cattle	0.37	0.63	
▪ Chinese Holstein cattle	0.50	0.49	Liu *et al*. (2011)
▪ Luxi Yellow cattle	0.27	0.72	
▪ Bohai Black cattle	0.35	0.64	
▪ Sahiwal cattle	0.39	0.60	Kamaldeep *et al*. (2017a)
▪ Sahiwal cattle	0.40	0.60	Kamaldeep *et al*. (2017b)
• **Sahiwal cattle**	**0.42**	**0.58**	**Present study**
• **Hariana cattle**	**0.39**	**0.61**	**Present study**
• **Murrah buffalo**	**0.15**	**0.85**	**Present study**

5.4.2.3 χ^2 -test for *Hae*III/exon 2 in screened population

The results of χ^2 –test done in present study revealed that there was no significant difference in genotypic and allelic distribution of g.2686T>C genotypes for tested cattle population (Sahiwal and Hariana) whereas a significant difference was observed in genotypic and allelic distribution for Murrah buffalo population. Hence, the tested population of Sahiwal and Hariana was not in the Hardy-Weinberg equilibrium while the Murrah buffalo population was found in the equilibrium. In case of cattle, similar findings were reported by Liu *et al*. (2010) in CH cattle, Wang *et al*. (2011) and Liu *et al*. (2011) in LY and CH cattle while, in contrary, BB cattle by Wang *et al*. (2011) and Liu *et al*. (2011) and Sahiwal cattle by Kamaldeep *et al*. (2017a, 2017b) were found in HW equilibirium which was in agreement with the results for Murrah buffalo of present study.

In present study, Murrah buffalo population was found in HW equilibrium for both *Apa*I/intron I and *Hae*III/exon2 assays however, the cattle population (Sahiwal and Hariana) was found in HWE in case of *Apa*I/intron I only while it did not meet equilibrium in case of

*Hae*III/exon2 in spite of same animals used in both the assays, it might be because the animals used in study belonged to a single closed herd.

5.4.2.4 Association studies of *Hae*III/exon 2 polymorphism with milk production traits

The association study of g.2686T>C genotypes revealed no significant association between any of the genotypes and milk production traits (TMY, LP and MY300) for both the lactations in cattle (Sahiwal and Hariana) and Murrah buffaloes and these findings were in agreement with the reports of Liu *et al*. (2010), Wang *et al*. (2011), Liu *et al*. (2011) and Kamladeep *et al*. (2017b) who found no association between any of the genotype and milk production traits. However, in case of AFC in Hariana cattle, a significant difference ($P<0.05$) was observed in which TC genotype was significantly higher (2570.0±261.0 days) than CC genotype (2160.0±53.9 days) which was in contrast with the finding of Kamladeep *et al*. (2017b) in Sahiwal cattle.

However, Wang *et al*. (2011) and Liu *et al*. (2011) interestingly, found a significant difference between combined genotypes of the three SNPs (g.855G>A, g.2651G>A and g.2686T>C) and milk production traits, suggesting that the genotype of one SNP may be influenced by other SNPs. Therefore, the analysis of haplotype combination for marker assisted selection will be more valuable than the study of a single SNP which would be in consistent with the conclusion drawn in human SNP and inheritance studies by Fallin *et al*. (2001).

5.4.2.5 Association studies of *Hae*III/exon 2 genotypes with SCS

In present study, there was a significant difference ($P<0.05$) for SCS between genotype TC (1480.0±505.0) and genotype CC (170.0±51.5) of Hariana cattle, however no significant difference was observed between any of the genotypes and SCS in case of Sahiwal cattle and Murrah buffalo. Similar to the findings of Sahiwal cattle and Murrah buffalo, Liu *et al*. (2010), Wang *et al*. (2011), Liu *et al*. (2011) and Kamaldeep *et al*. (2017a) did not found any significant difference between g.2686T>C genotypes and SCS.

5.4.3 *Sty*I/exon 2-PCR-RFLP assay

In the present study, screened cattle (Sahiwal and Hariana) and Murrah buffalo population were found monomorphic for *Sty*I/exon 2 PCR-RFLP assay performed to screen g.2651G>A SNP. It revealed only one type of banding pattern (GG genotype) which was of 162 bp in all screened samples of cattle (Sahiwal and Hariana) and Murrah buffaloes. PCR-RFLP was used to screen g.2651G>A in present study, however, Wang *et al*. 2011 genotyped this SNP by CRS–PCR with primer pair containing a nucleotide mismatch specifically the

second base A was replaced by C from the 3′ end of reverse primer, which created a new *Sty*I restriction site. Similarly, Liu *et al*. (2011) used CRS-PCR to screen this SNP. On the other hand, Asaf *et al*. (2014a) used allele specific PCR to screen g.2651G>A in which he used a set of three primers *viz.* forward, wild reverse and mutant reverse.

5.4.3.1 Genotypic frequency

In present investigation, the GA and AA genotype were absent (0.0) and the genotypic frequency of GG genotype was 100% in all the screened Hariana and Sahiwal cattle and Murrah buffaloes. These findings were in contrast with the results of Wang *et al*. (2011), Liu *et al*. (2011) in CH cattle, LY and BB cattle and Asaf *et al*. (2014a) in Vrindavani crossbred cattle and genotypic frequency of GG genotype was ranged from 30% to 57% in different cattle breeds as presented in Table 5.5.

Table 5.5: Genotypic frequencies of *Sty*I/exon 2 region of *MBL1* gene in different cattle breeds

Breed	Genotypic frequency			Reference
	GG (%)	GA (%)	AA (%)	
▪ Chinese Holstein cattle	30.00	55.00	15.00	Wang *et al*. (2011)
▪ Luxi Yellow cattle	55.00	39.00	5.00	
▪ Bohai Black cattle	52.00	48.00	0.00	
▪ Chinese Holstein cattle	30.00	62.00	8.00	Liu *et al*. (2011)
▪ Luxi Yellow cattle	57.00	38.00	5.00	
▪ Bohai Black cattle	50.00	50.00	0.00	
▪ Vrindavani cattle (Mastitis positive)	46.00	31.00	23.00	Asaf *et al*. (2014a)
▪ Vrindavani cattle (Mastitis negative)	48.00	42.00	10.00	
▪ **Sahiwal cattle**	**100.00**	**0.00**	**0.00**	**In present study**
▪ **Hariana cattle**	**100.00**	**0.00**	**0.00**	**In present study**
• **Murrah buffalo**	**100.00**	**0.00**	**0.00**	**In present study**

+

5.4.3.2 Allelic frequency

In the present work, the allelic frequency of allele G and A was observed as 1.0 and 0.0, respectively in all the screened animals and these findings were in contrast with the reports of Wang *et al*. (2011), Liu *et al*. (2011) and Asaf *et al*. (2004a) in which the frequency

distribution of G allele ranged from 0.58 to 0.76, whereas C allele ranged from 0.23 to 0.42 in different cattle breeds as shown in Table 5.6. However, in all the reported studies allele G was more frequent than allele A.

Table 5.6: Allelic frequencies *Sty*I/exon 2 region of *MBL1* gene in different cattle breed

Breed	Allelic frequency		Reference
	G	A	
▪ Chinese Holstein cattle	0.58	0.42	Wang *et al*. (2011)
▪ Luxi Yellow cattle	0.75	0.25	
▪ Bohai Black cattle	0.76	0.24	
▪ Chinese Holstein cattle	0.61	0.38	Liu *et al*. (2011)
▪ Luxi Yellow cattle	0.76	0.23	
▪ Bohai Black cattle	0.75	0.25	
▪ Vrindavani cattle (Mastitis positive)	0.62	0.38	Asaf *et al*. (2014a)
▪ Vrindavani cattle (Mastitis negative)	0.69	0.31	
• **Sahiwal cattle**	**1.0**	**0.0**	**Present study**
• **Hariana cattle**	**1.0**	**0.0**	**Present study**
• **Murrah buffalo**	**1.0**	**0.0**	**Present study**

5.4.3.3 χ^2-test for *Sty*I/exon 2 in screened population

The χ^2 –test could not performed as only GG genotype was found in all the screened animals for g.2651G>A SNP by *Sty*I/exon 2 assay. Wang *et al*. (2011) and Liu *et al*. (2011) reported that g.2651G>A SNP did not meet the HW equilibrium, however, LY and BB cattle population were in HW equilibrium. Asaf *et al*. (2014a) found Vrindavani crossbred cattle population in equilibrium as there was no significant difference between observed and expected frequencies of genotypes.

5.4.3.4 Association studies of *Sty*I/exon 2 polymorphism with milk production traits

In the present study, only GG genotype was found in all the screened animals for g.2651G>A SNP by *Sty*I/exon 2 assay, so association analysis could not be performed with milk production traits. However, several authors including Wang *et al*. (2011), Liu *et al*. (2011) and Asaf *et al*. (2014a) found polymorphic pattern for this SNP and studied the association of g.2651G>A variant with milk production traits in dairy cattle. Wang *et al*. (2011) used CRS-PCR to identify g.2651G>A and found no significant association between g.2651G>A and milk production traits (305d milk yield, fat content and protein content).

However, 305 d milk yield and milk protein content were significantly different in different combinations of haplotypes of three SNPs (g.855G>A, g.2686T>C and g.2561G>A) suggesting that the genotype of one SNP may be influenced by other SNPs. Liu *et al.* (2011) also used CRS-PCR to screen g.2561G>A and found no association with milk production traits. Although, g.2651G>A SNP affected the serum MBL-A concentrations and the serum CH50 values suggesting *MBL1* may be regarded as a functional and positional candidate gene to complement CH50 activity, similar to the manner in which it did in pigs (Phatsara *et al.*, 2007). Asaf *et al*. (2014a) investigated SNP g.2651G>A in Vrindavani crossbred (Holstein Friesian/ Brownswiss/ Jersey × Hariana) cattle (mastitis positive and mastitis negative) by allele specific PCR and found non-significant association with the clinical mastitis, suggesting further studies have to be undertaken in large population to validate the impact of g.2651G>A and the host response to mastitis.

5.4.3.5 Association studies of *Sty*I/exon 2 genotypes with SCS

In the current study, only GG genotype was found in all the screened animals for g.2651G>A SNP by *Sty*I/exon 2 assay, so association analysis could not be performed with SCS. However, Wang *et al*. (2011) observed a significant association between g.2651G>A genotypes and SCS, suggesting a possible role of this SNP in the host response against mastitis. On the other hand, Liu *et al*. (2011) and Asaf *et al*. (2014a) reported no significant association between g.2651G>A and SCS and concluded that differences in results may be due to the small number of tested samples in their study, however, in a haplotype combination, Liu *et al*. (2011) indicated that g.2651G>A can be useful in dairy cattle mastitis resistance breeding.

CHAPTER- 6

SUMMARY AND CONCLUSIONS

MBL (mannose-binding lectin) is primarily a liver-derived collagen-like serum protein and one of the important components of innate immunity. It recognizes patterns and binds sugar structures on micro-organisms (bacteria, viruses, fungi, parasites) as well as on dying host cells leading to opsonization and phagocytosis, and activation of the complement pathway resulting in lysis of the pathogen. It activates complement system in an antibody in C1q independent manner and requires MBL-associated serine proteases (MASP-1, MASP-2 and MASP-3). MBL belongs to a family of proteins called the collectins, which possess both collagenous regions and lectin domains. Most mammals have two *MBL* genes, *MBL1* and *MBL2*, which encode the MBL-A and MBL-C proteins, respectively. A number of mutations have been found in both the coding as well as the non-coding regions of the *MBL1* gene in various species, of which several variations affected the assembly of *MBL1*, thus lead to a low level of plasmic MBL and innate immune dysfunctions. *MBL1* contributed to bacterial infection resistance and was proposed as an indirect molecular marker of milk production traits to control mastitis and to improve dairy mastitis resistance traits in cattle. *MBL1* gene has 5 exons in its structure. The complete coding sequence of *MBL1* gene has not been characterized yet in buffalo breeds. Moreover, the polymorphic studies of *MBL1* gene have not been reported in Hariana breed and Murrah buffalo breed (excluding a g.2686T>C study). In the present study, complete CDS of *MBL1* gene was cloned and characterized in Murrah buffalo breed. *Apa*I/PCR-RFLP in intron I region and *Hae*III/PCR-RFLP and *Sty*I/PCR-RFLP in exon 2 region of *MBL1* gene were also performed in cattle breeds (Sahiwal and Hariana) and Murrah buffalo breed to detect the polymorphism and its association with milk production traits and somatic cell score (SCS).

For characterization study, a liver tissue sample from adult female buffalo of Murrah breed (n=2) was collected from slaughter house. The total RNA was isolated using GeneJET RNA purification kit. The first strand cDNA was synthesized using Revert Aid First strand cDNA Synthesis Kit. *MBL1* CDS region was amplified using suitable primers which were designed and commercially synthesized. On amplification, a band at 951 bp was observed and confirmed having *MBL1* CDS region. Cloning of *MBL1* PCR product of 951 bp was done using PUREGENE –Quick clone PCR cloning kit.

Amplified PCR products were cloned into pTZ57/RT cloning vector and then transformed into *E.coli* DH5α strain. Recombinant clones were identified from the

transformed bacterial colonies using blue and white colony selection. White colonies were transferred to LB-ampicillin broth media and incubated overnight. Next day morning, plasmid purification was carried out using GeneJET plasmid mini prep kit. Sequencing was done commercially from Eurofins genomics India Pvt Ltd, Bangalore using a plasmid having gene insert. Obtained sequences were submitted to NCBI GenBank database with accession no. MN990687. The nucleotides and deduced amino acid sequences of *MBL1* CDS of Murrah buffalo breed was aligned with Indian/exotic breeds of buffalo and cattle as well as with other species available in the GenBank database using ClustalW method of MegAlign program of Lasergene software (DNASTAR, USA).

The 951 bp cDNA of *MBL1* gene contained a long 158 bp 5′-untranslated region (5′-UTR) followed by 57 bp analogous to a signal peptide of 19-aa residues, 690 bp analogous to the mature protein and a sort 46 bp 3′-untranslated region (3′UTR). It consisted five exons including 149 bp long first non-coding exon 1 (1-149) that was transcribed but untranslated, 190 bp long exon 2 (150-339), 117 bp long exon 3 (340-456), 75 bp long exon 4 (457-531) and 420 bp long incomplete exon 5 but only 347 bps of exon 5 were the part of CDS while the remaining nucleotides were noncoding (part of 3′ UTR).

The complete CDS of buffalo *MBL1* consisted of 747 base pairs and encodes a protein of 248 amino acids. Among the total base count, it contains adenine (A) =194 (25.97%), guanine (G) =216 (28.92%), thymine (T) =152 (20.35%) and cytosine (C) =185 (24.77%). The *MBL1* CDS sequence had, A+T =346 (46.32%) and C+G =401 (53.68%). Among the 248 amino acids, 29 were strongly basic (+) amino acids (K and R), 27 were strongly acidic (-) amino acids (D and E), 72 were hydrophobic amino acids (A, I, L, F, W and V) and 64 were polar amino acids (N, C, Q, S, T and Y).

At nucleotide level, *MBL1* CDS of Murrah buffalo was more similar to that of exotic Mediterranean buffalo (99.6%) than another (partial CDS) Murrah buffalo (99.1%) and American buffalo (98.1%) sequence. Studied Indian buffalo breed showed more similarity with exotic cattle breeds (98.3-98.5%) and yak (98.4%) than exotic American buffalo (98.1%). It was 96.1% similar to goat, 95.2% similar to sheep and 83.5-83.7% similar to other breeds including Landrace pig and Yorkshire pig (83.5%) and Alxa Bactrian camel (83.5%) and Arabian camel (83.7%). At amino acid level, Murrah buffalo *MBL1* CDS was 98.8% similar to another Murrah buffalo and Mediterranean buffalo while 96.8% similar to American buffalo. Indian buffalo breed revealed 96.8-97.2% similarity with exotic cattle breeds, 97.6% similarity with yak, 95.2% similarity with goat, 94.4% similarity with sheep and 79.9-81.9% with other species. It was least (79.9%) homologues to pig breeds (Landrace and Yorkshire pig) followed by both camel breeds (81.9%).

On comparison with buffalo and cattle breeds, the *MBL1* CDS of Murrah buffalo contained three unique nucleotide change *viz*. a synonymous nucleotide change at position 87G→A with no change in amino acid (Q29Q) and two non-synonymous nucleotide changes at position 538T→C and 611T→C which resulted in amino acid substitution of Serine to Proline (S180P) and Leucine to Proline (L204P), respectively. A non-synonymous nucleotide change at position 451A→G was observed in previously reported partial Murrah buffalo sequence, which caused a non-synonymous mutation with amino acid substitution of serine to glycine (S151G) and one non-synonymous nucleotide change 178G→A, which caused an amino acid substitution Glutamic acid to Lysine (E60K) was observed in American buffalo. Additionally, two synonymous nucleotide changes (420T→C and 576G→C) were observed in American buffalo as well as previously reported partial Murrah buffalo *MBL1* CDS. All the cattle breeds contained a synonymous nucleotide change 103G→A transition with no change in amino acid. A phylogenetic tree was constructed including Indian/exotic buffalo breeds, exotic cattle breeds and other related species. Cattle and buffalo formed a separate subgroup (bovinae) than sheep and goat which were in caprinae subgroup. Furthermore, in bovinae subgroup, Murrah buffalo was more closely related to another Murrah buffalo and Mediterranean buffalo. In the phylogenetic tree, all buffalo breeds exhibited a close phylogenetic evolutionary relationship with cattle and yak.

For polymorphism study, genomic DNA was isolated from venous blood of 100 cattle (50 Sahiwal and 50 Hariana cattle) and 50 Murrah buffaloes, maintained at LFC, DUVASU, Mathura. Amplification of SNPs *viz.* g.855G>A) in intron I region, g.2686T>C and g.2561G>A in exon 2 regions of *MBL1* was carried out using already published primers. Restriction digestion was carried out using *Apa*I, *Hae*III and *Sty*I enzymes using manufacturer instructions. Association analysis of milk production traits and SCS in relation to g.855G>A/ g.2686T>C genotypes was done using SPSS (version 16.0) software.

Amplification of *MBL1* intron I region revealed 588 bp PCR product. *Apa*I/intron I PCR-RFLP assay revealed three types of genotypes *viz.* GG (311 and 277 bp), GA (588, 311 and 277 bp) and AA (undigested; 588bp) which confirmed the polymorphic pattern. GG genotype was more frequent (81.3%) in all screened animals followed by GA (16.6%) and AA (2.0%). The allelic frequency of G allele (0.89) was more than that of allele A (0.10). Although, genotype GG was highest (90.0%) in Murrah buffaloes than Hariana (80.0%) and Sahiwal cattle (74.0%). The genotype AA was equally (2.0%) present in Murrah buffaloes and Sahiwal and Hariana cattle whereas the occurrence of genotype GA was lowest (8.0%) in Murrah buffaloes than Sahiwal (24.0%) and Hariana cattle (18.0%). The χ^2 calculated value for *ApaI*/intron I were 0.32 and 0.004 for Hariana and Sahiwal cattle which was less than χ^2 table value (3.841 for df 1) at 5% level of significance, it revealed screened cattle population

was found in Hardy-Weinberg equilibrium (HWE). On the other hand, χ^2 value was 4.20 for Murrah buffaloes which was less than χ^2 table value (6.635 for df1) at 1% level of significance, it revealed Murrah buffalo population was found in HWE. Association study of *Apa*I/intron I genotypes of Sahiwal and Hariana cattle as well as Murrah buffalo showed no significant difference with milk production traits and SCS.

For the *Hae*III/exon 2 PCR-RFLP assay, a 401 bp PCR product was produced upon amplification. *Hae*III/exon 2 PCR-RFLP assay confirmed the polymorphic pattern by showing three types of genotype *viz.* TT (274 and 127 bp), TC (274, 184, 127 and 90 bp) and CC (184, 127 and 90 bp). The CC genotype was more frequent (60.6%) than TT (24.6%) and TC (14.6%) in total screened animals. Frequency of C allele was higher (0.68) than T allele (0.32). Although, the cattle and buffalo populations had variation in pattern of genotype percentage such as genotype TT was highest (66.0%) in Murrah buffaloes while it was 34.0% in cattle population. Additionally, genotype CC was lowest (2.0%) in Murrah buffaloes while it was highest (53.0%) in cattle population. Allele C was highly frequent (0.59) in cattle while Murrah buffalo had allele T as highly frequent (0.85). The χ^2 calculated value for *Hae*III/exon 2 were 24.91 and 25.12 for Hariana and Sahiwal cattle which was higher than χ^2 table value (3.841 and 6.635 for df 1) at 5% and 1% level of significance, it revealed screened cattle population was not in HWE. While, χ^2 value was 3.89 for Murrah buffaloes which was less than χ^2 table value (6.635 for df1) at 1% level of significance, it revealed Murrah buffalo population was found in HWE. Association study of *Hae*III/exon 2 genotypes with milk production traits showed a significant *(P<0.05)* difference for age at first calving (AFC) in Hariana cattle only, where TC genotype was significantly higher (2570.0±261.0 days) than CC genotype (2160.0±53.9 days). Association study of *Hae*III/exon 2 genotypes with SCS showed a signification *(P<0.05)* difference for SCS in Hariana cattle only, where genotype TC was significantly higher (1480.0±505.0) than genotype CC (170.0±51.5).

*Sty*I/exon 2 PCR-RFLP assay revealed monomorphic pattern with only single undigested band of 162 bp. This revealed that the screened animal used in the present study were monomorphic with GG genotype with only allele G. Therefore, association analysis of this SNP could not performed with milk production traits and SCS.

CONCLUSION

- Complete coding sequence (CDS) of *MBL1* gene in Murrah buffalo was characterized and submitted to NCBI GenBank database with Accession number *viz.* MN990687.
- It consisted five exons including 149 bp long first non-coding exon 1 (1-149) that was transcribed but untranslated, 190 bp long exon 2 (150-339), 117 bp long exon 3 (340-

456), 75 bp long exon 4 (457-531) and 420 bp long incomplete exon 5 but only 347 bps of exon 5 were the part of CDS while the remaining nucleotides were untranslated (part of 3′ UTR).

- Multiple sequence alignment of *MBL1* CDS nucleotide sequence with Indian/exotic buffalo breeds and exotic cattle breeds as well as other species at nucleotide level revealed Murrah buffalo complete CDS was more similar to that of exotic Mediterranean buffalo (99.6%) than another (partial CDS) Murrah buffalo (99.1%) and American buffalo (98.1%) sequence.
- Studied Murrah buffalo sequence showed (98.3-98.5%) similarity with exotic cattle breeds, 98.4% similar to yak, 96.1% similar to goat, 95.2% similar to sheep and 83.5-83.7% similar to other breeds including Landrace pig and Yorkshire pig (83.5%) and Alxa Bactrian camel (83.5%) and Arabian camel (83.7%) at nucleotide level.
- At amino acid level, *MBL1* CDS of Murrah buffalo breed was 98.8% to another (partial) Murrah buffalo and Mediterranean buffalo while 96.8% similar to American buffalo. Indian buffalo breed revealed 96.8-97.2% similarity with exotic cattle breeds, 97.6% similarity with yak, 95.2% similarity with goat, 94.4% similarity with sheep and 79.9-81.9% with other species including camel breeds (81.9%) and pig breeds (79.9%).
- A total of eight SNPs were found in MBL1 CDS sequence including three SNPs in present study Murrah buffalo, one SNP in previously reported Murrah buffalo, three SNPs in American buffalo and one SNP in all cattle breeds. Out of eight, four synonymous and four non-synonymous nucleotide changes were identified which encoded three amino acid changes at the position 60E→K in American buffalo, 151G→S (previous Murrah buffalo CDS), 180P→S and 204P→L (present study Murrah buffalo CDS) in amino acid sequence of *MBL*, respectively.
- Phylogenetic analysis clearly indicated that Murrah buffalo was more closely related to another Murrah buffalo followed by Mediterranean buffalo breed and exhibited a close phylogenetic evolutionary relationship with cattle and yak.
- PCR-RFLP method detected polymorphic pattern for *Apa*I/intron I and *Hae*III/exon 2 while monomorphic pattern for *Sty*I/exon 2 in screened cattle breeds (Sahiwal and Hariana) and Murrah buffalo breed.
- *Apa*I/intron I PCR-RFLP assay revealed three types of genotypes namely GG, GA and AA. Among themGG genotype was more frequent (81.3%) than GA (16.6%) and AA (2.0%) and frequency of G allele was higher (0.89) than A allele (0.10) in all the screened animals.
- Association study of *Apa*I/intron I genotypes with milk production traits and somatic cell score (SCS) revealed no significant difference was observed in screened animals.

- *Hae*III/exon 2 PCR-RFLP assay revealed three types of genotypes namely CC, CT and TT. Among them, CC genotype was more frequent (60.6%) than TT (24.6%) and TC (14.6%) and frequency of C allele was higher (0.68) than T allele (0.32) in all the screened animals.
- Association study of observed *Hae*III/exon 2 genotypes with milk production traits and SCS revealed significant difference ($P<0.05$) with age at first calving (AFC) and SCS in Hariana cattle only.
- *Sty*I/exon 2 PCR-RFLP assay revealed monomorphic pattern and generated only GG genotypes (162 bp; 100%) with G allele (1.0). Therefore, association analysis could not performed with milk production traits and SCS.

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ABSTRACT

Mannan-binding lectin (MBL), a collagen like pattern recognizing serum protein, is one of the important components of innate immunity. It provides first line of defence by its ability to bind the bacterial surface through its carbohydrate recognition domain and activates the complement pathway leading to lysis of bacteria independent of antibody. Most mammals have two *MBL* genes, *MBL1* and *MBL2*, which encode the MBL-A and MBL-C proteins, respectively. A number of mutations have been found in both the coding as well as the non-coding regions of the *MBL1* gene in various species, of which several variations affected the assembly of *MBL1*, thus lead to a low level of plasmic MBL and innate immune dysfunctions. *MBL1* possibly contributed to bacterial infection resistance and was proposed as an indirect molecular marker of milk production traits to control mastitis and to improve dairy mastitis resistance traits in cattle. Hence, sequence analysis is important to locate mutations in nucleotide and amino acid sequence of *MBL1* gene. In the present study, complete CDS of *MBL1* gene was cloned and characterized in Murrah buffalo breed and DNA polymorphisms were also investigated in Indian cattle (Sahiwal and Hariana) and buffalo (Murrah) breed. Multiple sequence alignment revealed that the complete CDS of Murrah buffalo was 98.1-99.6% similar to buffalo breeds and 98.3-98.5% similar to cattle breeds at nucleotide level. It was 96.8-98.8% similar to buffalo breeds and 96.8-97.2% similar to cattle breeds at amino acid level. Two unique non-synonymous mutations were found in *MBL1* CDS of Murrah buffalo at position 538 (T→C) that caused an amino acid substitution at 180 (Serine→Proline) and 611 (T→C) that caused an amino acid substitution at position 204 (Leucine→Proline). Phylogenetic analysis revealed that Murrah buffalo was more closely related to another Murrah buffalo followed by Mediterranean buffalo breed and exhibited a close phylogenetic evolutionary relationship with cattle and yak. DNA polymorphism study was also conducted in a total 150 animals (50 Sahiwal, 50 Hariana cattle and 50 Murrah buffalo) maintained at LFC, DUVASU, Mathura (UP) by using PCR-RFLP assay. Amplification of intron I (g.855G>A), exon 2 (g.2686T>C) and exon 2 (g.2651G>A) regions of *MBL1* gene revealed 588 bp, 401 bp and 162 bp PCR products, respectively. *Apa*I/intron I PCR-RFLP assay generated three genotypes *viz.* GG (311 and 277 bp), GA (588, 311 and 277 bp) and AA (undigested; 588bp), where GG genotype was more frequent (81.3%) than GA (16.6%) and AA (2.0%) and frequency of G allele was higher (0.89) than A allele (0.10) in all the screened animals. Chi square (χ^2) analysis revealed that all the screened cattle and buffalo population was in Hardy-Weinberg equilibrium (HWE). No significant difference was observed among genotypes on performing association study with milk production traits and somatic cell score (SCS). *Hae*III/exon 2 PCR-RFLP assay revealed three genotypes *viz.* TT (274 and 127 bp), TC (274, 184, 127 and 90 bp) and CC (184, 127 and 90 bp), where CC genotype was more frequent (60.6%) than TT (24.6%) and TC (14.6%) and frequency of C allele was higher (0.68) than T allele (0.32) in all the careened animals. χ^2-test showed that cattle population was not found in HWE whereas Murrah buffalo population was found in the HWE. Association study of observed genotypes with milk production traits and SCS revealed significant *($P<0.05$)* difference with age at first calving and SCS in Hariana cattle only where, CC genotype had lower SCS (170±84) than other genotypes. *Sty*I/exon 2 PCR-RFLP assay revealed monomorphic pattern with only undigested 162 bp band (GG genotype). Therefore, association analysis could not perform with milk production traits and SCS.

Key words: Murrah, Sahiwal, Hariana, *MBL* gene, cloning, characterization, polymorphism, PCR-RFLP.

BIBLIOGRAPHY

20th Livestock Census, Govt of India (2019). All India Report, Ministry Of Agriculture Department Of Animal Husbandry, Dairying and Fisheries, http://dadf.gov.in/sites/default/filess/20th%20Livestock%20census2019%20All%20India%20Report.pdf

Agah, A.; Montalto M.C.; Young, K. and Stahl G.L. (2001). Isolation, cloning and functional characterization of porcine mannose-binding lectin. Immunology **102**: 338–343.

Aksel, E.G.; Arslan, K.; Özdemir, F. and Akyüz, B. (2019). Investigation of *MBL-1* gene polymorphism in some cattle breeds raised in Turkey. Mediterranean Agricultural Sciences. **32(1)**: 25-30.

Ali, Y.M.; Lynch, N.J.; Haleem, K.S.; Fujita, T.; Endo, Y.; Hansen, S.; et al. (2012). The lectin pathway of complement activation is a critical component of the innate immune response to pneumococcal infection. PLoS Pathog. **8**: e1002793.

Anders, E.M.; Hartley, C.A. and Jackson, D.C. (1993). Bovine and mouse serum β inhibitors of influenza A viruses are mannose-binding lectins. Proc. Natl. Acad. Sci. U.S.A. **87**: 4485–4489.

Arora, M.; Munoz, E. and Tenner, A.J. (2001). Identification of a site on mannan-binding lectin critical for enhancement of phagocytosis. Journal of Biological Chemistry. **276:** 43087–94.

Asaf, V.N.M.; Amod, K.; Mnjit, P.; Prashant, D.; Gaur, G.K. and Bharat, B. (2014a). Association of single nucleotide polymorphism in the MBL1 gene with mastitis in Vrindavani crossbred cattle. Indian Journal of Animal Sciences. **84(6)**: 703–705.

Asaf, V.N.M.; Bhushan, B.; Panigrahi, M.; Dewangan, P.; Kumar, A.; Kumar, P. and Gaur, G.K. (2014b). Association study of genetic variants at single nucleotide polymorphism rs109231409 of mannose-binding lectins 1 gene with mastitis susceptibility in Vrindavani crossbred cattle. Veterinary World. **7(10)**: 807-810.

Awale, M.M.; Dudhatra, G.B.; Kumar, A.; Chauhan, B.N.; Kamani, D.R.; Modi, C.M.; Patel, H.B. and Mody, S.K. (2012). Bovine mastitis: A threat to the economy. Sci. Rep.**1**: 295.

BAHFS (2019). Basic Animal Husbandry & Fisheries Statistics report, Ministry Of Agriculture Department Of Animal Husbandry, Dairying and Fisheries, http://dadf.gov.in/sites/default/filess/BAHS%20%28Basic%20Animal%20Husbandry%20Statistics-2019%29.pdf

Bansal, B.K. and Gupta, D.K. (2014). Subclinical mastitis in machine milked dairy farms in Punjab: Prevalence, distribution of bacteria and current antibiogram. Vet. World. **7(5)**: 291-294.

Beltrame, M.H.; Catarino, S.J.; Goeldner, I.; Boldt, A.B.W. and de Messias-Reason, I.J. (2015). The Lectin Pathway of Complement and Rheumatic Heart Disease. Frontiers in Paediatrics. **2**: 148.

Bergman, I.M.; Edman, K.; van As, P.; Huisman, A. and Juul-Madsen, H.R. (2014). A two-nucleotide deletion renders the mannose-binding lectin 2 (MBL2) gene nonfunctional in Danish Landrace and Duroc pigs. Immunogenetics. **66**: 171–184.

Bergman, I.M.; Sandholm, K.; Ekdahl, K.N.; Okumura, N.; Uenishi, H.; Guldbrandtsen, B.; Essler, S.E.; Knoll, A.; Heegaard, P.M.H.; Edfors, I. and Juul-Madsen, H.R. (2012). MBL1 genotypes in wild boar populations from Sweden, Austria, the Czech Republic, and Japan. International Journal of Immunogenetics. **40**: 131–139.

Blom, AM.; Villoutreix, B.O. and Dahlbäck, B. (2004). Complement inhibitor C4b-binding protein-friend or foe in the innate immune system. Molecular Immunology. **40**:1333–46.

Bouwman, L.H.; Roep, B.O. and Roos, A. (2006). Mannose-binding lectin: clinical implications for infection, transplantation, and autoimmunity. Hum Immunol. **67(4-5)**: 247-56.

Bramley, A. (1996). Current Concepts of Bovine Mastitis. National Mastitis Council, Madison, WI.

Brien, B.; Fitzpatrick, C.; Meaney, W.J. and Joyce, P. (1999). Relationship between somatic cell count and neutrophils in milk. Ir. J. Agric. Food Res. **38**: 288-296.

Capparelli, R.; Parlato, M.; Amoroso, M.G.; Roperto, S.; Marabelli, R.; Roperto, F. and Iannelli, D. (2008). Mannose-binding lectin haplotypes influence Brucella abortus infection in the water buffalo (Bubalus bubalis). Immunogenetics **60**: 157–165.

Carlen, E.; Strandberg, E. and Roth, A. (2004). Genetic parameters for clinical mastitis, somatic cell score, and production in the first three lactations of Swedish Holstein cows. J Dairy Sci. **87**:3062–3070

Chaka, W.; Verheul, A.F.; Vaishnav, V.V.; Cherniak, R.; Scharringa, J.; Verhoef, J.; Snippe, H. and Hoepelman, A.I. (1997). Induction of TNF-alpha in human peripheral blood mononuclear cells by the mannoprotein of Cryptococcus neoformans involves human mannose binding protein. Journal of Immunology. **159**: 6.

Chen, C. B. and Wallis, R. (2001). Stoichiometry of complexes between mannose-binding protein and its associated serine proteases. Defining functional units for complement activation. Journal of Biological Chemistry. **276**: 25894–25902

Chen, M.; Daha, M.R. and Kallenberg, C.G. (2010). The complement system in systemic autoimmune disease. Journal of Autoimmunity. **34**: J276–286.

Colley, K .J. and Baenziger, J .U. (1987). Identification of the post translational modifications of the core-specific lectin. The core-specific lectin contains hydroxyproline, hydroxylysine, and glucosylgalactosylhydroxylysine residues. Journal of Biological Chemistry. **262**: 10290–10295.

Cosenza, G.; Pauciullo, A.; Mancusi, A.; D'Avino, A.; Colimoro, L.; Di Berardino, D. and Ramunno, L. (2012). Identification of single nucleotide polymorphisms in the Nicastrese goat and Sardinia sheep mannose-binding lectin. Biochem Genet. **50**: 73–83.

Crouch, E.; Persson, A.; Chang, D. and Heuser, J. (1994). Molecular structure of pulmonary surfactant protein D (SP-D). Journal of Biological Chemistry. 269: 17311–17319.

Dahl, M.R.; Thiel, S.; Matsushita, M.; Fujita, T.; Willis, A.C.; Christensen, T.; Vorup-Jensen, T. and Jensenius, J.C. (2001). MASP-3 and its association with distinct complexes of the mannan-binding lectin complement activation pathway. Immunity. **15**: 127–135.

Davies, J.; Neth, O.; Alton, E.; Klein, N. and Turner, M. (2000). Differential binding of mannose-binding lectin to respiratory pathogens in cystic fibrosis. Lancet. **355**: 9218.

Degn, S.E.; Hansen, A.G.; Steffensen, R.; Jacobsen, C.; Jensenius, J.C. and Thiel, S. (2009). MAp44, a human protein associated with pattern recognition molecules of the complement system and regulating the lectin pathway of complement activation. Journal of Immunology. **183**: 7371–7378.

Degn, S.E.; Jensen, L.; Gál, P.; Dobó, J.; Holmvad, S.H.; Jensenius, J.C. and Thiel, S. (2010). Biological variations of MASP-3 and MAp44, two splice products of the MASP1 gene involved in regulation of the complement system. Journal of Immunological Methods. **361**: 37–50.

Degn, S.E.; Jensen, L.; Hansen, A.G.; Duman, D.; Tekin, M.; Jensenius, J.C. and Thiel, S. (2012). Mannan- binding lectin-associated serine protease (MASP)-1 is crucial for lectin path- way activation in human serum, whereas neither MASP-1 nor MASP-3 is required for alternative pathway function. Journal of Immunology. **189**: 3957–3969.

Degn, S.E.; Jensenius, J.C. and Thiel, S. (2011a). Disease-causing mutations in genes of the complement system. American Journal of Human Genetics. **88**: 689–705.

Degn, S.E.; Thiel, S.; Nielsen, O.; Hansen, A.G.; Steffensen, R. and Jensenius, J.C. (2011b). MAp19, the alternative splice product of the MASP2 gene. Journal of Immunological Methods. **373**: 89–101.

Dommett, R.M.; Klein, N. and Turner, M.W. (2006). Mannose-binding lectin in innate immunity: past, present and future. Tissue Antigens. **68(3)**: 193-209.

Drickamer, K. (1992). Engineering galactose-binding activity into a C-type mannose-binding protein. Nature. **360**: 6400.

Drickamer, K.; Dordal, M.S. and Reynolds, L. (1986). Mannose-binding proteins isolated from rat liver contain carbohydrate-recognition domains linked to collagenous tails. Journal of Biological Chemistry. **261**: 6878–6887.

Dunkelberger, J.R. and Song, W.C. (2010). Complement and its role in innate and adaptive immune responses. Cell Research. **20**: 34–50.

Eisen, D.P. and Minchinton, R.M. (2003). Impact of mannose-binding lectin on susceptibility to infectious diseases. Clin. Infect. Dis. **37**: 1496–1505.

Ezekowitz, R.A.; Day, L.E. and Herman, G.A. (1988). A human mannose binding protein is an acute-phase reactant that shares sequence homology with other vertebrate lectins. Journal of Experimental Medicine. **167**: 3.

Ezekowitz, R.A.; Kuhlman, M.; Groopman, J.E. and Byrn, R.A. (1989). A human serum mannose-binding protein inhibits in vitro infection by the human immunodeficiency virus. Journal of Experimental Medicine. **169**: 1.

Falconer, D.S. and Mackay, T.F.C. (1996). An Introduction to quantitative genetics. 4th ed., Songman Limited. Esser, England.

Fallin, D.; Cohen, A.; Essioux, L.; Chumakov, I.; Blumenfeld, M;, Cohen, D. and Schork, N. J. (2001). Genetic analysis of case/control data using estimated haplotype frequencies: application to APOE locus variation and Alzheimer's disease. Genome Research. **11:** 143-151.

Fishelson, Z.; Pangburn, M. and Muller-Eberhard, H. (1984). Characterization of the initial C3 convertase of the alternative pathway of human complement. Journal of Immunology. **132**: 1430–1434.

Fosbrink, M.; Niculescu, F. and Rus, H. (2005). The role of c5b-9 terminal complement com-plex in activation of the cell cycle and transcription. Immunol Res**. 31**: 37–46.

Frakking, F.N.; Brouwer, N.; Zweers, D.; Merkus, M.P.; Kuijpers, T.W.; Offringa, M. and Dolman, K.M. (2006). High prevalence of mannose binding lectin (MBL) deficiency in premature neonates. Clinical and Experimental Immunology. **145**: 1.

Fraser, D.A. and Tenner AJ. (2010). Innate immune proteins C1q and mannan-binding lectin enhance clearance of atherogenic lipoproteins by human monocytes and macrophages. Journal of Immunology. **185**: 7.

Fraser, R. S.; Lumsden, J. S. and Lillie, B. N. (2018). Identification of polymorphisms in the bovine collagenous lectins and their association with infectious diseases in cattle. Immunogenetics. **70**: 533–546.

Frederiksen, P.D.; Thiel, S.; Jensen, L.; Hansen, A.G.; Matthiesen, F. and Jensenius, J.C. (2006). Quantification of mannan-binding lectin. J Immunol Methods. **315(1-2)**: 49-60.

Fujita T. (2002). Evolution of the lectin complement pathway and its role in innate immunity. Nature Reviews Immunology. **2**: 346–353.

Fujita, T.; Endo, Y. and Nonaka, M. (2004). Primitive complement system--recognition and activation. Molecular Immunology. **41(2-3)**: 103-111.

Gabolde, M.; Guilloud-Bataille, M.; Feingold, J. and Besmond, C. (1999). Association of variant alleles of mannose binding lectin with severity of pulmonary disease in cystic fibrosis: cohort study. BMJ. **319**: 7218.

Gagnon, E.; Duclos, S.; Rondeau, C.; Chevet, E.; Cameron, P.H.; Steele-Mortimer, O.; Paiement, J.; Bergeron, J.J. and Desjardins, M. (2002). Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. Cell. **110**: 1.

Gallagher Jr, D.S.; Ryan, A.M.; Liou, L.S.; Sastry, K.N. and Womack, J.E. (1993). Somatic cell mapping of conglutinin (CGN1) to cattle syntenic group U29 and fluorescence in situ localization to Chromosome28. Mammalian Genome. **4**: 716–9.

Garred, P.; Larsen, F.; Seyfarth, J.; Fujita, R. and Madsen, H.O. (2006). Mannose-binding lectin and its genetic variants. Genes and Immunity. **7**: 85–94.

Ghiran, I.; Barbashov, S.F.; Klickstein, L.B.; Tas, S.W.; Jensenius, J.C. and Nicholson-Weller, A. (2000). Complement receptor 1/CD35 is areceptor for mannan-binding lectin. Journal of Experimental Medicine. **192**: 12.

Gjerstorff, M.; Hansen, S.; Jensen, B.; Dueholm, B.; Horn, P.; Bendixen, C. and Holmskov, U. (2004b). The gene encoding bovine SP-A, SP-D, MBL-A, conglutinin, CL–43 and CL–46 form a distinct collectin locus on *Bos taurus* chromosome 28 (BTA28) at position q.1.8–1.9. Animal Genetics. **35**: 333–37.

Gjerstorff, M.; Hansen, S.; Madsen, J.; Bendixen, C. and Holmskov, U. (2004a). Genomic and molecular characterization of bovine surfactant protein D (SP-D). Molecular Immunology. **41**: 369–376.

Groeneveld, T.W.; Oroszlán, M.; Owens, R.T.; Faber-Krol, M.C.; Bakker, A.C.; Arlaud, G.J.; McQuillan, D.J.; Kishore, U.; Daha, M.R. and Roos, A. (2005). Interactions of the extracellular matrix proteoglycansdecorin and biglycan with C1q and collectins. Journal of Immunology. **175**: 7.

Gulla, K.C.; Gupta, K. and Hajela, K. (2009). Functional estimation of mannose binding lectin associated serine protease (MBL-MASPs) in human serum. Indian Journal of Medical Research. **130**: 428–432.

Gupta, K.; Gupta, R.K. and Krishnan, H. (2006). Disease associations of mannose-binding lectin & potential of replacement therapy. Indian Journal of Medical Research. **127**: 431-440.

Håkansson, K .; Lim, N .K.; Hoppe, H.J. and Reid, K .B.M. (1999). Crystal structure of the trimeric a-helical coiled-coil and the three lectin domains of human lung surfactant protein D. Structure. Folding and Design. **7**: 255–264.

Håkansson, K. and Reid K. B.M. (2000). Collectin structure: A review. Protein Science. 9: 1607–1617.

Hakomori, S. (2001). Tumor-associated carbohydrate antigens defining tumor malignancy: basis for development of anti-cancer vaccines. Adv Exp Med Biol. **491**: 369-402.

Hamann, J. (2002). Milk quality and udder health in relation to modern milking. In: Recent Developments and Perspectives in Bovine Medicine. XXII World Buiatrics Congress, Hannover, August, **18-23**: p334- 345.

Hamvas, R.M.; Johnson, M.; Vlieger, A.M.; Ling, C.; Sherriff, A.; Wade, A.; Klein, N.J.; Turner, M.W. and Webster, A.D. (2005). Role for mannose binding lectin in the prevention of Mycoplasma infection. Infection and Immunity. **73**: 8.

Hansen, S. and Holmskov, U. (2002). Lung surfactant protein D (SP-D) and the molecular diverted descendants: conglutinin, CL-43 andCL-46. Immunobiology. **205**: 498–517.

Hansen, S. and Holmskov, U. (2003). Functional aspects of SP-D and the derived bovine collectins: conglutinin, CL-43 and CL-46. Immunobiology 205: In press.

Hansen, S.; Holm, D.; Moeller, V.; Vitved, L.; Bendixen, C.; Reid, K.B.M.; Skjoedt, K. and Holmskov, U. (2002). CL-46, a novel collectin highly expressed in bovine thymus and liver. Journal of Immunology. **169**: 5726–5734.

Hart, M.L.; Saifuddin, M.; Uemura, K.; Bremer, E.G.; Hooker, B.; Kawasaki, T. and Spear GT. (2002). High mannose glycans and sialic acid on gp120 regulate binding of mannose-binding lectin (MBL) to HIV type 1. AIDS Res Hum Retroviruses. **18**: 17.

Hartshorn, K.L.; Sastry, K.; White, M.R.; Anders, E.M.; Super, M.; Ezekowitz, R.A. and Tauber, A.I. (1993). Human mannose-binding protein functions as an opsonin for influenza A viruses. J Clin Invest. **91**: 4.

He, J.; Wiesmann, C. and Campagne M.V.L. (2008). A role of macrophage com-plement receptor CRIg in immune clearance and inflammation. Molecular Immunology. **45**: 4041–4047.

Heggelund, L.; Mollnes, T.E.; Espevik, T.; Müller, F.; Kristiansen, K.I.; Aukrust, P. and Frøland, S.S. (2005). Modulatory effect of mannose binding lectin on cytokine responses: possible roles in HIV infection. Eur J Clin Invest. **35**: 12.

Heitzeneder, S.; Seidel, M.; Förster-Waldl, E. and Heitger, A. (2012). Mannan-binding lectin deficiency - Good news, bad news, doesn't matter. Clinical Immunology. **143**: 1.

Héja, D.; Harmat, V.; Fodor, K.; Wilmanns, M.; Dobó, J.; Kékesi, K.A.; Závodszky, P.; Gál, P. and Gábor, P. (2012). Mono- specific inhibitors show that both mannan-binding lectin-associated serine protease-1 (MASP-1) and 2 are essential for lectin pathway activation and reveal structural plasticity of MASP-2. Journal of Biological Chemistry. **287**: 20290–20300.

Hoffmann, J.A.; Kafatos, F.C.; Janeway, C.A. and Ezekowitz, R.A.B. (1999). Phylogenetic perspectives in innate immunity. Science. **284**: 1313–1318.

Holmskov, U.; Holt, P.; Reid, K.B.; Willis, A.C.; Teisner, B. and J.C. Jensenius. (1993). Purification and characterization of bovine mannan-binding protein. Glycobiology. **3:** 147–153.

Holmskov, U.; Jensenius, J.C.; Tornoe, I. and Lovendahl, P. (1998). The plasma levels of coglutinin are heritable in cattle and low levels predispose to infection. Immunology **93**: 431–436.

Holmskov, U.; Laursen, S.B.; Malhotra, R.; Wiedemann, H.; Timpl, R.; Stuart, G.R.;Tornoe, I.; Madsen, P.S.; Reid, K.B. and Jensenius, J.C. (1995). Comparative study of the structural and functional properties of a bovine plasma C-type lectin, collectin-43, with other collectins. Biochem J. **305**: 889–896.

Holmskov, U.; Thiel, S. and Jensenius, J.C. (2003). Collectins and ficolins: humoral lectins of the innate immune defense. Annu. Rev. Immunol. **21**: 547–578.

Huang, J.; Wang, H.; Wang, C.; Li, J.; Li, Q.; Hou, M. and Zhong, J. (2010). Single nucleotide polymorphisms, haplotypes and combined genotypes of lactoferrin gene and their associations with mastitis in Chinese Holstein cattle. Mol Bio Rep. **37(1)**: 477-483.

Hughes, A.L. (2007). Evolution of the lung surfactant proteins in birds and mammals. Immunogenetics **59**: 565–572.

Iobst, S.T.; Wormald, M.R.; Weis, W.I.; Dwek, R.A. and Drickamer, K. (1994). Binding of sugar ligands to Ca2+-dependent animal lectins. I. Analysis of mannose binding by site-directed mutagenesis and NMR. Journal of Biochemistry. **269**: 15505–15511.

Ip, W.K.; Chan, K.H.; Law, H.K.; Tso, G.H.; Kong, E.K.; Wong, W.H.; To, Y.F.; Yung, R.W.; Chow, E.Y.; Au, K.L.; Chan, E.Y.; Lim, W.; Jensenius, J.C.; Turner, M.W.; Peiris, J.S. and Lau, Y.L. (2005). Mannose-binding lectin in severe acute respiratory syndrome coronavirus infection. Journal of Infectious Diseases. 191: 10.

Ip, W.K.; Takahashi, K.; Ezekowitz, R.A. and Stuart, L.M. (2009). Mannose binding lectin and innate immunity. Immunol Rev. **230**: 1.

Jack, D.L.; Jarvis, G.A.; Booth, C.L.; Turner, M.W. and Klein, N.J. (2001). Mannose-binding lectin accelerates complement activation and increases serum killing of Neisseria meningitidis serogroup C. Journal of Infectious Diseases. **184**: 7.

Janeway, C.A. (1989). Approaching the asymptote Evolution and revolution in immunology. Cold Spring Harbor Symp. Quant. Biol. **54**: 1–13.

Jensen, P.H.; Weilguny, D.; Matthiesen, F.; McGuire, K.A.; Shi. L. and Hojrup, P. (2005). Characterization of the oligomer structure of recombinant human mannan-binding lectin. Journal of Biological Chemistry. **280:** 11043–51.

Jensenius, J.C.; Jensen, P.H.; McGuire, K.; Larsen, J.L. and Thiel, S. (2003).Recombinant mannan-binding lectin (MBL) for therapy. Biochemical Society Transactions. **31(Pt 4)**: 763-767.

Ji, X.; Gewurz, H. and Spear, G.T. (2005). Mannose binding lectin (MBL) and HIV. Molecular Immunology. **42**: 2.

Juul-Madsen, H.R.; Kjaerup, R.M.; Toft, C.; Henryon, M.; Heegaard, P.M.H.; Berg, P. and Dalgaard, T.S. (2011a). Structural gene variants in the porcine mannose-binding lectin 1 (MBL1) gene are associated with low serum MBL-A concentration. Immunogenetics **63**: 309–317.

Juul-Madsen, H.R.; Krogh-Meibom, T.; Henryon, M.; Palaniyar, N.; Heegaard, P.M.H.; Purup, S.; et al. (2006). Identification and characterization of porcine mannan-binding lectin A (pMBL-A) and determination of serum concentration heritability. Immunogenetics. **58**: 129.

Juul-Madsen, H.R.; Norup, L.R.; Handberg, K.J. and Jorgensen, P.H. (2007). Mannan-binding lectin (MBL) serum concentration in relation to propagation of infectious bronchitis virus (IBV) in chickens. Viral Immunol. **20**: 562–570.

Juul-Madsen, H.R.; Norup, L.R.; Jørgensen, P.H.; Handberg, K.J.; Wattrang, E. and Dalgaard, T.S. (2011b). Crosstalk between innate and adaptive immune responses to infectious bronchitis virus after vaccination and challenge of chickens varying in serum mannose-binding lectin concentrations. Vaccine **29**: 9499–9507.

Kahn, S.J.; Wleklinski, M.; Ezekowitz, R.A.; Coder, D.; Aruffo, A. and Farr, A.(1996). The major surface glycoprotein of Trypanosoma cruziamastigotes are ligands of the human serum mannose-binding protein. Infection and Immunity. **64**: 7.

Kamaldeep, D.; Magotra, A.; Pander, B.L.; Manjeet, M.; Malik, B.S. and Dalal, D.S. (2017a). Identification of point mutation in Exon 2 of MBL1 gene and its relationship with Somatic Cell Score (SCS) in Sahiwal cattle. Indian Journal of Animal Research. **53(2)**: 200-203.

Kamaldeep, D.; Mgotra, A.; Pander, B.L.; Malik, A.; Garg, A.S.; Dalal, D.S. and Malik, B.S. (2017b). Association of G.2686T>C mutation of MBL1 gene with reproduction traits in Sahiwal cattle. International Journal of Agricultural Science and Research. **7(6)**:159-164.

Kamaldeep, D.; Pander, B.L.; Dalal, D.S.; Magotra, A.; Mittal, D.; Singh, M.; Malik, A. and Garg, A. (2019). Characterization and validation of point mutation in MBL1 gene and its relationship with mastitis in Murrah buffalo (*Bubalus bubalis)*. Biology. **38(3)**: 451-457.

Kawai, T.; Suzuki, Y.; Eda, S.; Ohtani, K.; Kase, T.; Fujinaga, Y.; Sakamoto, T.; Kurimura, T. and Wakamiya, N. (1997). Cloning and characterization of a cDNA encoding bovine mannan-binding protein. Gene. **186**: 161–165.

Kawai, T.; Suzuki, Y.; Eda, S.; Ohtani, K.; Kase, T.; Sakamoto, T.; Uemura, H. and Wakamiya, N. (1998) Molecular and biological characterization of rabbit mannan-binding protein. Glycobiology. **8**: 237–244.

Kawasaki, N.; Kawasaki, T. and Yamashina, I. (1985). Mannan-Binding Protein and Conglutinin in Bovine Serum. The Journal of Biochemistry. **98**: 1309-1320.

Kawasaki, N.; Kawasaki,T. and Yamashina, I. (1983). Isolation and characterization of a mannan-binding protein from human serum. Journal of Biochemistry. **94**: 937–947.

Kawasaki, T.; Etoh, R. and Yamashina, I. (1978). Isolation and characterization of mannan-binding protein from rabbit liver. Biochem. Biophys. Res. Commun. **81**: 1018–24.

Kelly, P.; Jack, D.L.; Naeem, A.; Mandanda, B.; Pollok, R.C.; Klein, N.J.; Turner, M.W. and Farthing, M.J. (2000). Mannose-binding lectin is a component of innate mucosal defense against Cryptosporidiumparvum in AIDS. Gastroenterology. **119**: 5.

Kilpatrick, D.C. (2002). Mannan-binding lectin: clinical significance and applications. Biochimica et Biophysica Acta. **1572(2-3)**: 401–413.

Kjaer, T.R.; Thiel, S. and Andersen G.R. (2013). Toward a structure-based comprehension of the lectin pathway of complement. Molecular Immunology. **56**: 413–422.

Kjaerup, R.M.; Dalgaard, T.S.; Norup, L.R.; Goto, R.M.; Miller, M.M.; Sørensen, P. and Juul-Madsen, H.R. (2014). Transcription efficiency of different chicken mannose-binding lectin promoter alleles. Immunogenetics. **66**: 737–742.

Kjaerup, R.M.; Norup, L.R.; Skjodt, K.; Dalgaard, T.S. and Juul-Madsen, H.R. (2013). Chicken mannose-binding lectin (MBL) gene variants with influence on MBL serum concentrations. Immunogenetics. **65**: 461–471.

Klabunde, J.; Uhlemann, A.C.; Tebo, A.E.; Kimmel, J.; Schwarz, R.T.; Kremsner, P.G. and Kun, J.F. (2002). Recognition of plasmodium falciparum proteins by mannan-binding lectin, a component of the human innate immune system. Parasitology Research. **88**: 2.

Koivula, M.; Mantysaari, E.A.; Negussie, E. and Serenius, T. (2005). Genetic and phenotypic relationships among milk yield and somatic cell count before and after clinical mastitis. J Dairy Sci. **88**: 827–833

Kozutsumi, Y.; Kawasaki, T. and Yamashina, I. (1980). Isolation and characterization of a mannan-binding protein from rabbit serum. Biochemical and Biophysical Research Communications. **95**: 658–664.

Krarup, A.; Sørensen, U.B.; Matsushita, M.; Jensenius, J.C. and Thiel, S. (2005). Effect of capsulation of opportunistic pathogenic bacteria on binding of the pattern recognition molecules mannan-binding lectin, Lficolin and H-ficolin. Infection and Immunity. **73**: 2.

Kristensen, M.; Thiel, S.; Sjöholm, A.; Matsushita, M. and Jensenius, J.C. (2007). Cooperation between MASP-1 and MASP-2 in the generation of C3 convertase through the MBL pathway. Int Immunol. **19**: 141–149.

Kuhlman, M.; Joiner, K. and Ezekowitz, R.A. (1989). The human mannose binding protein functions as an opsonin. Journal of Experimental Medicine. **169**: 5.

Kurata, H.; Cheng, H.M.; Kozutsumi, Y.; Yokota, Y. and Kawasaki, T. (1993). Role of the collagen-like domain of the human serum mannan bindingprotein in the activation of complement and the secretion of this lectin. Biochemical Biophysical Research Communications. **191**: 3.

Kwok, J.Y.;, Augst, R.M.; Yu, D.Y. and Singh, K.K. (2012). Sensitive CSFELISAs for the detection of MBL, MASP-2 and functional MBL/MASP-2. Journal of Neuroscience Methods. **209**: 1.

Larsen, F.; Madsen, H.O.; Sim, R.B.; Koch, C. and Garred, P. (2004). Disease associated mutations in human mannose-binding lectin compromise oligomerization and activity of the final protein. J Biol Chem. **279**: 21302–21311.

Lau, Y.L.; Chan, S.Y.; Turner, M.W.; Fong, J. and Karlberg, J. (1995). Mannose-binding protein in preterm infants: developmental profile and clinical significance. Clinical and Experimental Immunology. **102**: 3.

Laursen, S. B. and O. L. Nielsen. (2000). Mannan-binding lectin(MBL) in chickens: Molecular and functional aspects. Developmental and Comparative Immunology. **24**: 85–101.

Laursen, S.B.; Dalgaard, T.S.; Thiel, S.; Lim, B.L.; Jensen, T.V.; Juul-Madsen, H.R.; Takahashi, A.; Hamana, T.; Kawakami, M. and Jensenius, J.C. (1998a). Cloning and sequencing of a cDNA encoding chicken mannan-binding lectin (MBL) and comparison with mammalian analogues. Immunology. **93**: 421–430.

Laursen, S.B.; Hedemand, J.E.; Nielsen, O.L.; Thiel, S.; Koch, C. and Jensenius, J.C. (1998b). Serum levels, ontogeny and heritability of chicken mannan-binding lectin (MBL). Immunology. **94**: 587–593.

Leitner, G.; Shoshani, E.; Krifucks, O.; Chaffer, M. and Saran, A. (2000). Milk leukocyte population patterns in bovine udder infection of different etiology. J. Vet. Med. B. Infect. Dis. Vet. Public Health. **47(8)**: 581-589.

Lesavre, P.H.; Hugli, T.E.; Esser, A.F.; Muller-Eberhard, H. and Müller-Eberhard, H.J. (1979). The alternative pathway C3/C5 convertase: chemical basis of factor B activation. Journal of Immunology. **123**: 529–534.

Lillie, B.N.; Brooks, A.S.; Keirstead, N.D. and Hayes, M.A. (2005). Comparative genetics and innate immune functions of collagenous lectins in animals. Veterinary Immunology and Immunopathology. **108**: 97–110.

Lillie, B.N.; Hammermueller, J.D.; Macinnes, J.I.; Jacques, M. and Hayes, M.A. (2006a). Porcine mannan-binding lectin A binds to Actino bacillussuis and Haemophilus parasuis. Developmental and Comparative Immunology. **30**: 954–965.

Lillie, B.N.; Keirstead, D.N.; Brooks, S.A.; Squires, E.J.; Friendship, M.R. and Hayes, A.M. (2007b). Mannan binding lectin polymorphisms associated with common infectious diseases of pigs. The FASEB Journal. **21(6).**

Lillie, B.N.; Keirstead, N.D.; Squires, E.J. and Hayes, M.A. (2007a). Gene polymorphisms associated with reduced hepatic expression of porcine mannan-binding lectin C. Dev Comp Immunol **31**: 830–846.

Lillie, B.N.; Keirstead, N.D.; Squires, E.J.; Hayes, M.A. (2006b). Single nucleotide polymorphisms in porcine mannan-binding lectin A. Immunogenetics **58**: 983–993.

Lipscombe, R.J.; Sumiya, M.; Hill, A.V.; Lau, Y.L.; Levinsky, R.J.; Summerfield, J.A. and Turner, M.W. (1992). High frequencies in African and non- African populations of independent mutations in the mannose binding protein gene. Human Molecular Genetics. **1**: 709−715

Lipscombe, R.J.; Sumiya, M.; Summerfield, J.A. and Turner, M.W. (1995). Distinct physicochemical characteristics of human mannose binding protein expressed by individuals of differing genotype. Immunology. **85**: 4.

Liu, H.; Jensen, L.; Hansen, S.; Petersen, S.V.; Takahashi, K.; Ezekowitz, A.B.; Hansen, F.D.; Jensenius, J.C. and Thiel, S. (2001). Characterization and quantification of mouse mannan-binding lectins (MBL-A and MBL-C) and study of acute phase responses. Scandinavian Journal of Immunology. **53**: 489–497.

Liu, J.; Ju, Z.; Li, Q.; Huang, J.; Li, R.; Li, J.; Ma, L.; Zhong, J. and Wang, C. (2011). Mannose-binding lectin haplotypes influence serum MBL-A concentration, complement activity, and milk production traits in Chinese Holstein cattle. Immunogenetics **63**: 727–742.

Liu, M.; Ju, Z.H.; Li, Q.L.; Wang, H.M.; Huang, J.M.; Li, J.B.; Zhong, J.F. and Wang, C.F. (2010). Polymorphisms of MBL1 Gene Intron 1 and Exon 2 and Its Association with Mastitis and Milk Quality in Chinese Holstein Cattle. Scientia Agricultura Sinica. **43(11)**: 2363-2371.

Lu, J.; Laursen, S.B.; Thiel, S.; Jensenius, J.C. and Reid, K.B. (1993). The cDNA cloning of conglutinin and identification of the liver as the site of synthesis of conglutinin in members of the Bovidae. Biochemical Journal **292**: 157–162.

Lu, J.H .; Thiel, S.; Wiedemann, H.; Timpl, R. and Reid, K.B.M. (1990). Binding of the pentamer/hexamer forms of mannan binding protein to zymosan activates the proenzyme C1r2C1s2 complex, of the classical pathway of complement, without involvement of C1q. Journal of Immunology. **144(6)**: 2287–2294.

Lund, M.S.; Jensen, J. and Petersen, P.H. (1999). Estimation of genetic and phenotypic parameters for clinical mastitis, somatic cell production deviance, and protein yield in dairy cattle using Gibbs sampling. J Dairy Sci. **82**: 1045–1051

Ma, Y.; Uemura, K.; Oka, S.; Kozutsumi, Y.; Kawasaki, N. and Kawasaki, T. (1999). Antitumor activity of mannan-binding protein in vivo as revealed by a virus expression system: mannan-binding protein dependent cell-mediated cytotoxicity. Proceedings of the National Academy of Sciences of the United States of America. **96**: 2.

Madsen, H.O.; Garred, P.; Kurtzhals, J.A.; Lamm, L.U.; Ryder, L.P.; Thiel, S. and Svejgaard, A. (1994). A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. Immunogenetics **40**: 37–44.

Madsen, H.O.; Satz, M.L.; Hogh. B.; Svejgaard, A.; Garred, P. (1998). Different molecular events result in low protein level of mannan-binding lectin in population from southeast Africa and South America. Journal of Immunology. **161**: 3169-75.

Madsen, H.O; Garred, P.; Thiel, S.; Kurtzhals, J.A.; Lamm, L.U.; Ryder, L.P. and Svejgaard, A. (1995). Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. The journal of Immunology. **155:** 3013-3020.

Malhotra, R. and Sim, R.B. (1995). Collectins and viral infections. Trends in Microbiology. **3**: 240–244.

Malhotra, R.; Thiel, S.; Reid, K.B. and Sim, R.B. (1990). Human leukocyte C1q receptor binds other soluble proteins with collagen domains. Journal of Experimental Medicine. **172**: 3.

Marklund, L.; Shi, X. and Tuggle, C.K. (2000). Rapid communication: mapping of the Mannose-Binding Lectin 2 (MBL2) gene to pig chromosome 14. Journal of Animal Science. **78**: 2992−2993

Matsushita, M. and Fujita, T. (1992). Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. J. Exp. Med. **176**: 1497–502.

Mayer, S.; Raulf, M.K. and Lepenies, B. (2017). C-type lectins: their network and roles in pathogen recognition and immunity. Histochemistry and Cell Biology. **147(2)**: 223–237.

McCormack, F.X.; Damodarasamy, M. and Elhalwagi, B.M. (1999). Deletion mapping of N-terminal domains of surfactant protein A. The N-terminal segment is required for phospholipid

aggregation and specific inhibition of surfactant secretion. Journal of Biological Chemistry. **274:**3173–81.

Miller, M.E.; Seals, J.; Kaye, R. and Levitsky, L.C. (1968). A familial, plasma associated defect of phagocytosis: new cause of recurrent bacterial infection. Lancet **2**: 60–63.

Minchinton, R.M.; Dean, M.M.; Clark, T.R.; Heatley, S. and Mullighan, C.G. (2002). Analysis of the relationship between mannose-binding lectin (MBL) genotype, MBL levels and function in an Australian blood donor population. Scandinavian Journal of Immunology. **56**: 6.

Mogues, T.; Ota, T.; Tauber, A.I. and Sastry, K.N. (1996). Characterization of two mannose-binding protein cDNAs from rhesus monkey (Macacamulatta): structure and evolutionary implications. Glycobiology. **6**: 543–550.

Naito, H.; Ikeda, A.; Hasegawa, K.; Oka, S.; Uemura, K.; Kawasaki, N. and Kawasaki, T. (1999). Characterization of human serum mannan binding protein promoter. Journal of Biochemistry. **126**: 6.

Nakagawa, T.; Kawasaki, N.; Ma, Y.; Uemura, K. and Kawasaki, T. (2003). Antitumor activity of mannan-binding protein. Methods in Enzymology. **363**: 26-33

Nakamura, N.; Nonaka, M.; Ma, B.Y.; Matsumoto, S.; Kawasaki, N.; Asano, S. and Kawasaki T. (2009). Characterization of the interaction between serum mannan-binding protein and nucleic acid ligands. Journal of Leukocyte Biology. **86(3)**: 737–748.

NBAGR (2020). National Bureau of Animal Genetic Resources. http://14.139.252.116/agris/breed.aspx

Neth, O.; Jack, D.L.; Dodds, A.W.; Holzel, H.; Klein, N.J. and Turner, M.W. (2000). Mannose-binding lectin binds to a range of clinically relevant microorganisms and promotes complement deposition. Infection and Immunity. **68**: 688-93.

Ng, K. K.; D rickamer, K. and Weis, W.I. (1996). Structural analysis of monosaccharide recognition by rat liver mannose-binding protein. Journal of Biological Chemistry. 271: 663 –674.

Nielsen, B.B.; Kastrup, J.S.; Rasmussen, H.; Holtet, T.L.; Graversen, J.H.; Etzerodt, M.; Thogersen, H.C. and Larsen, I.K. (1997). Crystal structure of tetranectin, a trimeric plasminogen-binding protein with an alpha-helical coiled coil. FEBS Letters. **412**: 388–396.

Nonaka, M.; Ma, B.Y.; Ohtani, M.; Yamamoto, A.; Murata, M.; Totani, K.; Ito, Y.; Miwa, K.; Nogami, W.; Kawasaki, N. and Kawasaki, T. (2007). Subcellular localization and physiological significance of intracellular mannan-binding protein. Journal of Biochemistry. **282**: 24.

Norup, L.R.; Dalgaard, T.S.; Friggens, N.C.; Sørensen, P. and Juul-Madsen, H.R. (2009). Influence of chicken serum mannose-binding lectin levels on the immune response towards Escherichia coli. Poultry Science. **88**: 543-553.

Nott, A.; Meislin, S.H. and Moore, M.J. (2003). A quantitative analysis of intron effects on mammalian gene expression. RNA. **9(5)**: 607−617.

Ogasawara, Y. and Voelker, D.R. (1995). Altered carbohydrate recognition specificity engineered into surfactant protein D reveals different binding mechanisms for phosphatidylinositol and glucosylceramide. Journal of Biological Chemistry. **270**: 14725–14732.

Ogden, C.A.; deCathelineau, A.; Hoffmann, P.R.; Bratton, D.; Ghebrehiwet, B.; Fadok, V.A. and Henson, P.M. (2001). C1q and mannose binding lectin engagement of cell surface

calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. Journal of Experimental Medicine. **194**: 6.

Ohashi, T. and Erickson, H.P. (2004). The disulfide bonding pattern in ficolin multimers. Journal of Biological Chemistry. **279:** 6534–9.

Oka, S.; Ikeda, K.; Kawasaki, T. and Yamashina, I.(1988). Isolation and characterization of two distinct mannan-binding proteins from rat serum. Arch. Biochem. Biophys. **260**: 257–266.

Palaniyar, N.; Nadesalingam, J.; Clark, H.; Shih, M.J.; Dodds, A.W. and Reid, K.B. (2004). Nucleic acid is a novel ligand for innate immune pattern recognition collectins surfactant proteins A and D and mannose-binding lectin. Journal of Biological Chemistry. **279**: 32728–32736.

Park, K.H.; Kurokawa, K.; Zheng, L.; Jung, D.J.; Tateishi, K.; Jin, J.O.; Ha, N.C.; Kang, H.J.; Matsushita M.; Kwak, J.Y.; Takahashi, K. and Lee, B.L. (2010). Human serum mannose-binding lectin senses wall teichoicacid Glycopolymer of Staphylococcus aureus, which is restricted in infancy. Journal of Biological Chemistry. **285**: 35.

Phaneuf, L.R.; Lillie, B.N.; Hayes, M.A. and Turner, P.V. (2007). Binding of mouse mannan-binding lectins to different bacterial pathogens of mice. Veterinary Immunology and Immunopathology. **118**: 129–133.

Phatsara, C.; Jennen, D.G.; Ponsuksili, S.; Murani, E.; Tesfaye, D.; Schellander, K. and Wimmers, K. (2007). Molecular genetic analysis of porcine mannose-binding lectin genes, MBL1 and MBL2, and their association with complement activity. Int J Immunogenet **34**: 55–63.

Philipsson, J.; Ral, G. and Berglund, B. (1995). Somatic-cell count as a selection criterion for mastitis resistance in dairy-cattle. Livestock Production Science. **41**: 195–200**.**

Pillai, S.R.; Kunze, E.; Sordillo, L.M. and Jayarao, B.M. (2001). Application of differential inflammatory cell count as a tool to monitor udder health. Journal of Dairy Science. **84**: 1413–1420.

Podolsky, M.J.; Lasker, A.; Flaminio, M.J.B.; Gowda,L.D.; Alan, B.; Ezekowitz, R.A. and Takahashi, K. (2006). Characterization of an equine mannose-binding lectin and its roles in disease. Biochemical and Biophysical Research Communications. **343**: 928–936.

Polotsky, V.Y.; Belisle, J.T.; Mikusova, K.; Ezekowitz, R.A. and Joiner, K.A. (1997). Interaction of human mannose-binding protein with Mycobacterium avium. Journal of Infectious Diseases. **175**: 5.

Rakhshandehroo, M.; Stienstra, R.; de Wit, N.J.; Bragt, M.C.; Haluzik, M.; Mensink, R.P.; Müller, M. and Kersten, S. (2012). Plasma mannose-binding lectin is stimulated by PPARα in humans. American Journal of Physiology: Endocrinology and Metabolism. **302**: 5.

Reichhardt, M.P.; Loimaranta, V.; Thiel, S.; Finne, J.; Meri, S. and Jarva, H. (2012). The salivary scavenger and agglutinin binds MBL and regulates the lectin pathway of complement in solution and on surfaces. Frontiers in Immunology. **3**: 205.

Ricklin, D.; Hajishengallis, G.; Yang, K. and Lambris, J.D. (2010). Complement: a key system for immune surveillance and homeostasis. Nature Immunology. **11**: 785–797.

Riis AL, Hansen TK, Thiel S, Gravholt CH, Gjedde S, GormsenLC, Jørgensen JO, Weeke J, Møller N (2005). Thyroid hormone increases mannan-binding lectin levels. European Journal of Endocrinology. **153**: 5.

Rupp, R. and Boichard, D. (1999). Genetic parameters for clinical mastitis, somatic cell score, production, udder type trait and milking ease. J. Dairy Sci. **82**: 2198-2204.

Russell, C.D.; Widdison, S.; Leigh, J.A. and Coffey, T.J. (2012). Identification of single nucleotide polymorphisms in the bovine toll-like receptor 1 gene and association with health traits in cattle. Vet. Re. **43**: 17.

Saevarsdottir, S.; Oskarsson, O.O.; Aspelund, T.; Eiriksdottir, G.; Vikingsdottir, T.; Gudnason, V. and Valdimarsson, H. (2005). Mannan binding lectin as an adjunct to risk assessment for myocardial infarction in individuals with enhanced risk. Journal of Experimental Medicine. **201**: 1.

Saevarsdottir, S.; Vikingsdottir, T.; Vikingsson, A.; Manfredsdottir, V.; Geirsson, A.J. and Valdimarsson, H. (2001). Low mannose binding lectin predicts poor prognosis in patients with early rheumatoid arthritis. A prospective study. Journal of Rheumatology. **28**: 4.

Saifuddin, M.; Hart, M.L.; Gewurz, H.; Zhang, Y. and Spear, G.T. (2000). Interaction of mannose-binding lectin with primary isolates of human immunodeficiency virus type 1. J Gen Virol, **81**: Pt 4.

Sallenbach, S.; Thiel, S.; Aebi, C.; Otth, M.; Bigler, S.; Jensenius, J.C.; Schlapbach, L.J. and Ammann, R.A. (2011). Serum concentrations of lectin-pathway components in healthy neonates, children and adults: mannan-binding lectin (MBL), M-, L- and H-ficolin, and MBL associated serine protease-2 (MASP-2). Paediatrics Allergy and Immunology. **22**: 4.

Sambrook, J. and Russel, D.W. (2001). Molecular cloning: A laboratory manual cold spring harbor tab. Press, New York, pp: 6.4-6.11.

Santos, I.K.; Costa, C.H.; Krieger, H.; Feitosa, M.F.; Zurakowski, D.; Fardin, B.; Gomes, R.B.; Weiner, D.L.; Harn, D.A.; Ezekowitz, R.A. and Epstein, J.E. (2001). Mannan-binding lectin enhances susceptibility to visceralleishmaniasis. Infect Immun. **69**: 8.

Sastry, K.; Herman, G.A.; Day, L.; Deignan, E.; Bruns, G.; Morton, C.C. and Ezekowitz, R.A. (1989). The human mannose-binding protein gene. Exon structure reveals its evolutionary relationship to a human pulmonary surfactant gene and localization to chromosome 10. Journal of Experimental Medicine. **170**: 1175–1189.

Sastry, K.; Zahedi, K.; Lelias, J.M.; Whitehead, A.S. and Ezekowitz, R.A.B. (1991). Molecular characterization of the mouse mannose-binding proteins. The mannose-binding protein A but not C is an acute phase reactant. Journal of Immunology. **147**: 692–697.

Sastry, R.; Wang, J.S.; Brown, D.C.; Ezekowitz, R.A.; Tauber, A.I. and Sastry, K.N. (1995). Characterization of murine mannose-binding protein genes Mbl1 and Mbl2 reveals features common to other collectin genes. Mammalian Genome. **6**: 103–110.

Schou, T.W.; Permin, A.; Christensen, J.P.; Cu, H.P. and Juul-Madsen, H.R. (2010). Mannan-binding lectin (MBL) in two chicken breeds and the correlation with experimental Pasteurella multocida infection. Comp Immunol Microbiol Infect Dis **33**: 183–195.

Seyfarth, J.; Garred, P. and Madsen, H.O. (2005). The 'involution' of mannose binding lectin. Human Mol Genet. **14**: 2859–2869.

Seyfarth, J.; Garred, P. and Madsen, H.O. (2006). Extra-hepatic transcription of the human mannose-binding lectin gene (mbl2) and the MBL-associated serine protease 1-3 genes. Molecular Immunology. **43**: 7.

Sharma, B.S.; Leyva, I.; Schenkel, F. and Karrow, N.A. (2006). Association of toll-like receptor 4 polymorphisms with somatic cell score and lactation persistency in Holstein bulls. J Dairy Sci. **89**: 3626–3635.

Sharma, N.; Singh, N.K. and Bhadwal, M.S. (2011). Relationship of somatic cell count and mastitis: An overview. Asian Aust. J. Anim. Sci. **24(3)**: 429-438.

Sheriff, S.; Chang, C.Y. and Ezekowitz, R.A. (1994). Human mannose- binding protein carbohydrate recognition domain trimerizes through a triple alpha-helical coiled-coil. Nature Structural & Molecular Biology. **1**: 11.

Shi, L.; Takahashi, K.; Dundee, J.; Shahroor-Karni, S.; Thiel, S.; Jensenius, J.C.; Gad, F.; Hamblin, M.R.; Sastry, K.N. and Ezekowitz, R.A. (2004). Mannose-binding lectin-deficient mice are susceptible to infection with Staphylococcus aureus. J Exp Med **199**: 1379–1390.

Skjoedt, M-O.; Hummelshoj, T.; Palarasah, Y.; Honore, C.; Koch, C.; Skjodt, K.; et al. (2010). A novel mannose-binding lectin/ficolin-associated protein is highly expressed in heart and skeletal muscle tissues and inhibits complement activation. Journal of Biological Chemistry. **285**: 8234–8243.

Snedecor, G.W. and Cochran, W.G. (1989). Statistical methods, 8th Ed, Iowa state univ press.

Sørensen, C.M.; Hansen, T.K.; Steffensen, R.; Jensenius, J.C. and Thiel, S. (2006). Hormonal regulation of mannan-binding lectin synthesis in hepatocytes. Clinical and Experimental Immunology. **145**: 1.

Steffensen, R.; Thiel, S.; Varming, K.; Jersild, C. and Jensenius, J.C. (2000).Detection of structural gene mutations and promoter polymorphisms in the mannan-binding lectin (MBL) gene by polymerase chain reaction with sequence-specific primers. J Immunol Methods. **241**: 1-2.

Stover, C.M.; Thiel, S.; Thelen, M.; Lynch, N.J.; Vorup-Jensen, T.; Jensenius, J.C.; et al. (1999). Two constituents of the initiation complex of the mannan-binding lectin activation pathway of complement are encoded by a single structural gene. Journal of Immunology. **162**: 3481–3490.

Sumiya, M.; Super, M.; Tabona, P.; Levinsky, R.J.; Arai, T.; Turner, M.W. and Summerfield, J.A. (1991). Molecular basis of opsonic defect inimmuno deficient children. Lancet **337**: 1569–1570.

Super, M.; Gillies, S.D.; Foley, S.; Sastry, K.; Schweinle, J.E.; Silverman, V.J. and Ezekowitz, R.A. (1992). Distinct and overlapping functions of allelic forms of human mannose binding protein. Nature Genetics. **2**: 1.

Super, M.; Thiel, S.; Lu, J.; Levinsky, R.J. and Turner, M.W. (1989). Association of low levels of mannan-binding protein with a common defect of opsonisation. Lancet. **2**: 8674.

Swanson, A.F.; Ezekowitz, R.A.; Lee, A. and Kuo, C.C. (1998). Human mannose-binding protein inhibits infection of HeLa cells by Chlamydia trachomatis. Infection and Immunity **66**: 4.

Takahashi, K.; Gordon, J.; Liu, H.; Sastry, K.N.; Epstein, J.E.; Motwani,M.; Laursen, I.; Thiel, S.; Jensenius, J.C.; Carroll, M. et al. (2002). Lack of mannose-binding lectin-A enhances survival in a mouse model of acute septic peritonitis. Microbes and Infection. **4**: 773–784.

Takahashi, K.; Shi, L.; Gowda, L.D. and Ezekowitz, R.A. (2005). Relative roles of complement factor 3 and mannose-binding lectin in host defense against infection. Infect. Immun. **73(12)**: 8188–8193.

Takahashi, M.; Endo, Y.; Fujita, T. and Matsushita, M. (1999). A truncated form of mannose-binding lectin-associated serine protease (MASP)-2 expressed by alternative polyadenylation is a component of the lectin complement pathway. International Immunology. **11**: 859–863.

Tal-Stein, R.; Fontanesi, L.; Dolezal, M.; Scotti, E.; Bagnato, A.; Russo,V.; Canavesi, F.; Friedmann, A.; Soller, M. and Lipkin, E. (2010). A genome scan for quantitative trait loci affecting milk somatic cells core in Israeli and Italian Holstein cows by means of selective DNA pooling with single- and multiple-marker mapping. J Dairy Sci. **93**: 4913–4927

Taylor, M.E.; Brickell, P.M.; Craig, R.K. and Summerfield, J.A. (1989). Structure and evolutionary origin of the gene encoding a human serum mannose-binding protein. Journal of Biochemistry. **262**: 763–771.

Teillet, F.; Gaboriaud, C.; Lacroix, M.; Martin, L.; Arlaud, G.J. and Thielens, N.M. (2008). Crystal structure of the CUB1-EGF-CUB2 domain of human MASP-1/3 and identification of its interaction sites with mannan-binding lectin and ficolins. Journal of Biological Chemistry. **283**: 37.

Tenner, A.J.; Robinson, S.L. and Ezekowitz, R.A. (1995). Mannose binding protein (MBP) enhances mononuclear phagocyte function via areceptor that contains the 126,000 M(r) component of the C1qreceptor. Immunity. **3**: 4.

Thiel, S. and Gadjeva, M. (2009). Humoral pattern recognition molecules: mannan-binding lectin and ficolins. Adv Exp Med Biol. **653**: 58-73.

Thiel, S.; Holmskov, U.; Hviid, L.; Laursen, S.B. and Jensenius, J.C. (1992).The concentration of the C-type lectin, mannan-binding protein, inhuman plasma increases during an acute phase response. Clinical and Experimental Immunology. **90**: 1.

Thiel, S.; Jensen, L.; Degn, S.E.; Nielsen, H.J.; Gál, P.; Dobó, J. and Jensenius, J.C. (2012). Mannan-binding lectin (MBL)-associated serine protease-1(MASP-1), a serine protease associated with humoral pattern recognition molecules: normal and acute-phase levels in serum and stoichiometry of lectin pathway components. Clinical and Experimental Immunology. **169**: 38-48.

Thiel, S.; Vorup-Jensen, T.; Stover, C.M.; Schwaeble, W.; Laursen, S.B. et al. (1997). A second serine protease associated with mannan-binding lectin that activates complement. Nature **386**: 506–510.

Townsend, R.; Read, R.C.; Turner, M.W.; Klein, N.J. and Jack, D.L. (2001). Differential recognition of obligate anaerobic bacteria by human mannose-binding lectin. Clinical and Experimental Immunology. **124**: 2.

Turner, M.W. (1996). Mannose-binding lectin: the pluripotent molecule of the innate immune system. Immunology Today. **17:** 11.

Turner, M.W. and Hamvas, R.M.J. (2000). Mannose-binding lectin: structure, function, genetics and disease associations. Rev. Immunogenet. **2**: 305-322.

Turner, M.W.; Mowbray, J.F. and Roberton, D.R. (1981). A study of C3b deposition on yeast surfaces by sera of known opsonic potential. Clin Exp Immunol, **46**: 2.

Valdimarsson, H.; Stefansson, M.; Vikingsdottir, T.; Arason, G.J.; Koch, C.; Thiel, S.; *et al.* (1998). Reconstitution of opsonizing activity by infusion of mannan-binding lectin (MBL) to MBL-deficient humans. Scandinavian Journal of Immunology. **48**: 124-126.

Van de Watering, J.K.; Van Golde, L.M.G. and Batenburg, J.J. (2004). Collectins: Players of the innate immune system. Eur. J. Biochem. **271**: 1229-1249.

Van Emmerik, L.C.; Kuijper, E.J.; Fijen, C.A.; Dankert, J. and Thiel, S. (1994). Binding of mannan-binding protein to various bacterial pathogens of meningitis. Clinical and Experimental Immunology. **97**: 3.

Voss,T.; Melchers,K.; Scheirle,G. and Scha¨fer,K .P. (1991). Structural comparison of recombinant pulmonary surfactant protein SP-A derived from two human coding sequences: implications for the chain composition of natural human SP-A. American Journal of Respiratory Cell Molecular Biology. **4**: 88–94.

Wallis, R. (2002). Dominant effects of mutations in the collagenous domain of mannose-binding protein. Journal of Immunology. **168**: 4553−4558

Wallis, R.; Shaw, J.M.; Uitdehaag, J.; Chen, C.B.; Torgersen, D. and Drickamer, K. (2004). Localization of the serine protease-binding sites in the collagen-like domain of mannose-binding protein: indirect effects of naturally occurring mutations on protease binding and activation. Journal of Biological Chemistry **279**: 14.

Walport, M.J. (2001). Complement. First of two parts. New Engl Journal Med. **344**: 1058–66.

Wang, C.; Liu, M.; Li, Q.; Ju, Z.; Huang, J.; Li, J.; Wang, H. and Zhong, J. (2011). Three novel single-nucleotide polymorphisms of MBL1 gene in Chinese native cattle and their associations with milk performance traits. Vet Immunol Immunopathol. **139**: 229–236.

Wang, X.; Ju, Z.; Huang, J.; Hou, M.; Zhou, L.; Qi, C.; Zhang, Y.; Gao, Q.; Pan, Q.; Li, G.; Zhong, J. and Wang, C. (2012). The relationship between the variants of the bovineMBL2 gene and milk production traits, mastitis, serum MBL-C levels and complement activity. Veterinary Immunology and Immunopathology. **148**: 311–319.

Wang, X.; Xu, S.; Gao, Z.; Li, J.; Ren, H. and Luoren, Z. (2008). Cloning and SNP screening of the TLR4 gene and the association between its polymorphism and somatic cell score in dairy cattle. South African Journal of Animal Science. **38(2)**: 101-109.

Weis, W.I. and Drickamer, K. (1994). Trimeric structure of a C-type mannose-binding protein. Structure. **2**: 1227–1240.

Weis, W.I.; Taylor, M.E. and Drickamer, K. (1998). The C-type lectin superfamily in the immune system. Immunological Reviews. **163**: 19–34.

Weis,W.I.; Crichlow, G.V.; Murthy, H.M.; Hendrickson, W.A. and Drickamer, K. (1991). Physical characterization and crystallization of the carbohydrate-recognition domain of a mannose-binding protein from rat. Journal of Biological Chemistry. **266**: 20678–20686.

Weis,W.I.; Drickamer, K. and Hendrickson,W.A. (1992). Structure of a C-type mannose-binding protein complexed with an oligosaccharide. Nature. **360**: 127 –134.

White, R.A.; Dowler, L.L.; Adkison, L.R.; Ezekowitz, R.A. and Sastry, K.N. (1994). The murine mannose-binding protein genes (Mbl 1and Mbl 2) localize to chromosomes 14 and 19. Mammalian Genome. **5**: 807–809.

Wild, J.; Robinson, D. and Winchester, B. (1983). Isolation of mannose binding proteins from human and rat liver. Biochemical Journal. **210**: 1.

Williams, J.L.; Eggen, A.; Ferretti, L.; et al. (2002). A bovine whole genome radiation hybrid panel and outline map. Mammalian Genome. **13**: 469–74.

Worthely, D.L.; Bardy, P.G. and Mullighan, C.G. (2005). Mannose-binding lectin: biology and clinical implications. Intern Med J. **35(9)**: 548-55.

Yadav, A.K.; Singh, J. and Yadav, S.K. (2017). Characteristic features of registered Indigenous Buffalo Breeds of India: A Review. Int. Journal Pure App. Biosci. **5(4):** 825-83.

Ying, H.; Ji, X.; Hart, M.L.; Gupta, K.; Saifuddin, M.; Zariffard, M.R. and Spear, G.T. (2004). Interaction of mannose-binding lectin with HIV type 1 is sufficient for virus opsonization but not neutralization. AIDS Res Hum Retroviruses. **20**: 3.

Yokota, Y.; Arai, T. and Kawasaki, T. (1995). Oligomeric structures required for complement activation of serum mannan-binding proteins. Journal of Biochemistry.**117:** 2.

Yoshizaki, T.; Ohtani, K.; Motomura, W.; Jang, S.J.; Mori, K.; Kitamoto, N.; Yoshida, I.; Suzuki, Y. and Wakamiya, N. (2012). Comparison of human blood concentrations of collectin kidney 1 and mannan-binding lectin. Journal of Biochemistry. **151:** 1.

Ytting, H.; Christensen, I.J.; Thiel, S.; Jensenius, J.C.; Svendsen, M.N.; Nielsen, L; Lottenburger, T. and Nielsen, H.J. (2007). Biological variation in circulating levels of mannan-binding lectin (MBL) and MBL-associated serine protease-2 and the influence of age, gender and physical exercise. Scandinavian Journal of Immunology. **66**: 458–464.

Yuan, Z.; Chu, G.; Dan, Y.; Li, J.; Zhang, L.; Gao, X.; Gao, H.; Xu, S. and Liu, Z. (2012). BRCA1: a new candidate gene for bovine mastitis and its association analysis between single nucleotide polymorphisms and milk somatic cell score. Mol Biol Rep. **39**: 6625–6631.

Yuan, Z.; Li, J.; Li, J.; Gao, X. and Xu, S. (2013). SNPs identification and its correlation analysis with milk somatic cell score in bovine MBL1 gene. Mol Biol Rep. **40**: 7-12.

Yuan, Z.R.; Li, J.; Liu, L.; Zhang, L.P.; Zhang, L.M.; Chen, C.; Chen, X.J.; Gao, X..; Li, J.Y.; Chen, J.B.; Gao, H.J. and Xu, S.Z. (2011). Single nucleotide polymorphism of CACNA2D1 gene and its association with milk somatic cell score in cattle. Mol Biol Rep. **38**: 5179–5183.

Zan, L.S.; Zhang, J.L. and Liu, X.W. (2007). Association study on AGPAT6 intron 3 polymorphism and milk performance of dairy cattle. Scientia Agricultura Sinica. **40**: 1498−1503.

Zhai, M.; Mou, J.; Zhu, M.; Liang, Y.; Wang, M.; Zhao, Z. and Zhang, H. (2019). Polymorphisms of *MBL* gene introns and their association with *MBL* serum levels in Hu Sheep. Kafkas Univ Vet Fak Derg. **25(3)**: 349-356.

Zhang, C.; Wang, Y.; Chen, H.; Gu, C. and Fang, X. (2009). SLC11A1 gene polymorphisms are not associated to somatic cell score and milk yield in Chinese Holstein. Veterinary Immunology and Immunopathology . **127(3-4)**: 389-292.

Zhao, F.; Zhao, Z.; Yan, G.; Wang, D.; Ban, Q.; Yu, P.; Zhang, W. and Luo, Y. (2011). Polymorphisms in mannose-binding lectin (MBL) gene and their association with MBL protein levels in serum in the Hu sheep. Vet Immunol Immunopathol **140**: 297–302.

Zhao, Z.L.; Wang, C.F.; Li, Q.L.; Ju, Z.H.; Huang, J.M.; Li, J.B.; Zhong, J.F. and Zhang, J.B. (2012). Novel SNPs of the mannan-binding lectin 2 gene and their association with production traits in Chinese Holsteins. Genetics and Molecular Research. **11**: 3744–3754.

A. REAGENTS FOR DNA ISOLATION

1. 2.7% EDTA solution (pH-8.0)

EDTA disodium salt	2.7 gm
Double distilled water up to	100 ml

Adjust pH 8.0 using NaOH pellets

Sterilize by autoclaving and store at room temperature

2. 0.5 M EDTA solution (pH 8.0)

EDTA disodium salt	186.1 gm
Double distilled water up to	1000 ml

Adjust pH 8.0 using NaOH pellets

(Approximate 20g NaOH) because EDTA dissolves only at pH 8.0.

Autoclave and store at room temperature.

Functions of EDTA – 1.Chelates Mg^{++} ions.

2. Protects from nucleases

3. Makes plasma membrane more fragile

3. RBC lysis buffer (1X)

Ammonium chloride (NH_4Cl)	8.3 gm
Potassium bicarbonate ($KHCO_3$)	1.0 gm
0.5 M EDTA (pH 8.0)	299 µl
Double distilled water up to	1000 ml

Autoclave and store at room temperature.

4. 5 M NaCl solution

Sodium chloride (NaCl)	29.22 gm
Double distilled water up to	100 ml

Autoclave and store at room temperature.

5. DNA extraction buffer

1 M Tris buffer (pH 8.0)	5 ml
5 M NaCl	40 ml
0. 5 M EDTA buffer (pH 8.0)	2 ml
Double distilled water up to	500 ml

Autoclave in batches of 100 ml and store at room temperature

6. 10% Sodium dodecyl sulfate (SDS) (*also called sodium lauryl sulfate)*

SDS (sodium dodecyl sulfate)	10 gm
Autoclaved double distilled water up to	100 ml

(Adjust pH 7.2 using Conc. HCl). Heat in water bath at 60°C to dissolve and then store at room temperature.

Caution: Wear a mask when weighing SDS and wipe down the weighing area and balance after use because the fine crystals of SDS disperse easily. There is no need to sterilize 10% SDS.

Functions of SDS 1. Helps in cell membrane lysis,
2. Acts as a catalyst.

7. 3 M sodium acetate

Sodium acetate (Anhydrous)	24.6 gm
Double distilled water up to	100 ml

(Adjust pH 5.5 using glacial acetic acid)

Autoclave in batches of 20 ml

Function of 3M sodium acetate- 1. Precipitates DNA

8. Equilibration of phenol Tris saturated

Most batches of commercial liquefied phenol are clear and colourless and can be used in molecular cloning without redistillation. Occasionally batches of liquefied phenol are pink and yellow, and these should be rejected and returned to the manufacturer. Crystalline phenol is not recommended because it must be redistilled at 160°C to remove oxidation products such as quinones that cause the breakdown of phosphodiester bonds or cause cross linking of RNA and DNA.

***Caution*:** Phenol is highly corrosive and can cause severe burns. Wear gloves, protective clothing and safety glasses when handling phenol. All manipulations should be carried out in a chemical hood. Any areas that come in contact with phenol should be rinsed with large volume of water and washed with soap and water.

Before use, phenol must be equilibrated to a pH > 7.8, because DNA will partition into the organic phase at acid pH.

1. 100 g of phenol i.e. crystalline phenol was melted at 65°C and distilled (which takes around ½ - 1 hr). Liquefied phenol should be stored at –20°C.As needed remove phenol from the freezer and then melt at 68°C
2. 8-hydroxyquinolone to a final concentration of 0.1% was added. It is an antioxidant, partial inhibitor of RNase and a weak chelator of metal ions. In addition, its yellow color provides convenient way to identify the organic phase.

3. Equal volume of 0.5 M Tris (pH 8.0) (at room temperature) was added, stirred for ½ hr and kept overnight.
4. Next day supernatant was removed; 0.1 M Tris (pH 8.0) was added stirred for ½ hr and placed back in the refrigerator.
5. In the evening, the supernatant was removed; 0.1 M Tris pH 8.0 was added stirred for ½ hr and placed back in the refrigerator overnight.
6. Another saturation with 0.1 M Tris is given in the morning and the pH tested after decanting some amount of supernatant Tris. The pH of phenolic phase can be checked with indicator paper and should be 8.0.If it is not 8.0, repeat the above steps till pH is obtained.
7. After the phenol is equilibrated and the final aqueous phase (supernatant) has been removed add 0.1 volume of 0.1 M Tris base (pH 8.0) (10 ml for 100ml phenol) containing 0.2% β-mercaptoethanol. The phenol solution may be stored in this form under 100 mM Tris base (pH 8.0) in a light tight bottle at 4°C for period up to 1 month.

Note: Phenol prepared with 8-hydroxyquinolone can be stored in brown bottle for 1 month

Functions of phenol

1. **RNA with polyA tail is dissolved in alkaline phenol.**
2. Inhibits RNase
3. Weakly chelates metal ions

Functions of 8- hydrohxyquinolone

1. Prevents oxidation of phenol
2. Yellow colour provides convenient way to identify the organic phase

9. Chloroform-isoamyl alcohol preparation (24:1)

Chloroform	24 ml
Isoamylalcohol	1 ml

Mix thoroughly and store in amber coloured bottle at 4^{O}C.

10. Phenol-chloroform-isoamyl alcohol preparation (25:24:1)

Tris -saturated phenol	25 ml
Chloroform isoamyl alcohol (24:1)	25 ml

Mix thoroughly and store in amber coloured bottle at 4^{O}C.

11. 70% ethanol

Ethanol	70 ml
Autoclaved double distilled water	30 ml

Mix thoroughly and store in amber coloured bottle at 4^{O}C.

12. 1 M Tris (pH 8.0)

(a) Using Tris HCl

Tris HCl 157.6 g

Distilled water up to 1000 ml. Adjust pH to 8.0 with NaOH pellets.

Autoclave in 100 ml batches.

(b) Using Tris Base.

Dissolve 121.1 g of Tris Base in 800 ml of DDW. Adjust the pH to desired value by adding concentrated HCl. Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 litre with DDW. Dispense into aliquots and sterilize by autoclaving.

pH – HCl

8.0 – 42 ml

7.6 – 60 ml

7.4 – 70 ml

If the 1 M solution has a yellow colour, discard it and obtain better quality Tris. The pH of Tris solutions is temperature-dependent and decreases approximately 0.03 pH units for each 1°C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9 and 8.6 at 5°C, 25°C and 37°C, respectively.

13. 1X TE buffer- pH 8.0 for DNA

1M Tris	250 µl
0.5 M EDTA (pH 8.0)	50 µl
Double distilled water up to	25 ml

Autoclaved and stored at 4^{o}C

14. PBS, pH 7.4

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
H_2O	800ml

Adjust the pH to 7.4 with HCl .Add H_2O to 1L. Dispense the solution into aliquots and stabilize them by autoclaving for 20 minutes at 15 lbs/sq inch, Store at room temperature.

15. β-mercaptoethanol (BME)

Usually obtained as a 14.4M solution. Store in dark bottles.

B. REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

1. Ethidium bromide (10 mg/ml)

Ethidium bromide	10 mg
Autoclaved distilled water upto	1 ml

Wrap in aluminium foil (Photosensitive) and store in dark place at room Temperature.

2. 6 X Gel loading Dye

a) Bromophenol blue	0.25%
Xylene cyanol	0.25%
Sucrose in water	40%

Mix and store at 4^{o}C

b) Bromophenol blue	0.25%
Sucrose in water	40% (w/v)

Mix and store at 4^{o}C

c) Xylene cyanol FF	0.25%
Bromophenol blue.	0.25%
Glycerol in water	30%

Store at 4^{0}C

These gel loading buffers serve three purposes: They increase the density of the sample ensuring that the DNA drops evenly into the well; they add colour to the sample, thereby simplifying the loading process; and they contain dyes that, in an electric field, move toward the anode at predictable rates. Bromophenol blue migrates through agarose gels approximately 2.2-fold faster than xylene cyanol FF, independent of the agarose concentration. Bromophenol blue migrates through agarose gels run in 0.5 x TBE at approximately the same rate as linear double-stranded DNA 300 bp in length, whereas xylene cyanol FF migrates at approximately the same rate as linear double stranded DNA 4 kb in length. These relationships are not significantly affected by the concentration of agarose in the gel over the range of 0.5% to 1.4%.

3. 5 X TBE

Tris base	54 gm
Boric acid	27.5 gm
0.5 M EDTA (pH 8.0)	20 ml
Autoclaved distilled water upto	1000 ml

Autoclave and store at room temperatures.

4. 50 X Stock TAE

Tris base	242 gm
Glacial Acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Mix Tris with Stir bar to dissolve in about 600 ml of double distilled water. Add the EDTA & Acetic acid and make the final volume up to 1 liter by adding the double distilled water. Store at room temperature.

C. MEDIA AND REAGENTS FOR GENE CLONING

1. LB (Luria Bertani) media (1000 ml)

To make the medium all the components were dissolved in 800 ml of water pH adjusted to desired leve (pH 7) and final volume made to 1000 ml with water. For LB-agar medium, LB broth was supplemented with bacteriological agar at 1.5 % level. Both the LB broth and LB-agar were autoclaved at 15 psi of stream for 15 minutes, before use. Composition of LB media is given below:

Bacto Tryptone	10 gm
Bacto Yeast extract	5 gm
NaCl	5 gm
Agar	15 gm
Distilled water to make	1000 ml
pH adjusted to	7.0

2. Ampicillin stock solution (50 mg/mL)

Ampicillin sodium salt	2.5 gm
Deionized water	50 ml

Filter sterilize and store in aliquots at -20 ºC.

3. X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) stock solution (20 mg/ml)

X-Gal	200 mg
N, N-dimethylformamide	10 ml

Store at -20 ºC in the dark. 40 µl was used per plate.

4. IPTG (isopropyl-D-thiogalactopyranoside) stock solution

IPTG	1.2 gm
Deionized water	50 ml

Filter sterilized, aliquoted and stored at 4 ºC. 40 µl was used per plate.

CV OF STUDENT

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ACADEMIC QUALIFICATIONS:

Degree	University/ Board	Year of Passing	Percentage of marks	Subjects
Masters (M.Sc.)	IMS, Ghaziabad, CCS University Meerut, U.P.	2012	78.0	Biotechnology
Graduation (B.Sc.)	B.I.M.R. College, Jiwaji University, Gwalior, M.P.	2010	62.5	Biotechnology, Chemistry, Botany and Foundation Course
Intermediate	U.P. Board	2007	66.6	Physics, Chemistry, Biology, English, Hindi, Sports and Physical Education
High School	U.P. Board	2005	59.6	Hindi, English, Mathematics, Science, Social Studies and Drawing

Extra-curricular activities:

- Served as DICE committee member in XXVIII Annual Convention and International Conference on "Challenges and Opportunities in Animal Heath at the face of Globalization and Climate change" held at DUVASU, Mathura by IAVMI.
- Participated in Hindi Essay writing and Painting competition of "National Productivity Week" held in CIRG, Farah, Mathura.

Publications: 3 abstracts

- 1 abstract in compendium of XIV Biennial National Conference of Association of Public Health Veterinarians (APHV) held in January, 2020 at C.V.Sc, DUVASU, Mathura (U.P.).
- 2 abstracts in compendium of XVII National Symposium on "Enhancement of Farmer's Income through Management of Animal Genetic Resources" organized at COVSc & AH, Mhow on 10-11 February, 2020.

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I, **Manali Baghel**, Enrollment No. B-1385/14 undertake that I give copy right to DUVASU, Mathura of my thesis entitled "**Molecular characterization of *mannose binding lectin gene1 (MBL1)* in *Bubalus bubalis* & its variability in cattle and buffalo genome**".

I also undertake that patent, if any, arising out of research work conducted during the programme shall be filled by me only with due permission of the competent authority of DUVASU, Mathura (UP).

Manali

Signature of Student