STUDIES ON LACTOFERRIN GENE POLYMORPHISM IN DANGI CATTLE

THESIS

Submitted in partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE

IN

ANIMAL GENETICS AND BREEDING

BY

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(INDIA)

2023

Appendix-B

DECLARATION OF THE STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled **"STUDIES ON LACTOFERRIN GENE POLYMORPHISM IN DANGI CATTLE"** or part thereof has not been submitted for any of the other degree or diploma of any university, nor the data have been derived from any thesis or publications of any university or scientific organization. The sources of materials used, and all assistance received during the course of investigation have been duly acknowledged.

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Appendix-C (i)

DECLARATION OF ADVISORY COMMITTEE

Mr. DHOND ANKUSH RAMESHRAO has satisfactorily prosecuted his course of research for a period of not less than one semester and that the thesis entitled, **"STUDIES ON LACTOFERRIN GENE POLYMORPHISM IN DANGI CATTLE"** submitted by him is the result of research work is sufficient to warrant its presentation to the examination in subject of **ANIMAL GENETICS AND BREEDING** for the award of **MASTER OF VETERINARY SCIENCE (M.V.Sc.)** degree by the Maharashtra Animal and Fishery Sciences University, Nagpur.

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

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Appendix-C (ii)

CERTIFICATE

This is to certify that the thesis entitled, **“STUDIES ON LACTOFERRIN GENE POLYMORPHISM IN DANGI CATTLE”** submitted by **Mr DHOND ANKUSH RAMESHRAO** to the Maharashtra Animal and Fishery Sciences University, Nagpur, in partial fulfillment of the requirement for the degree of **MASTER OF VETERINARY SCIENCE (M.V.Sc.)** has been approved by the Student’s Advisory Committee after examination in collaboration with the External Examiner.

Name and Signature Of External Examiner	Signature and Seal Head of the Department	Advisor/Guide

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I place on record my apology and sincere thanks to the unmentioned personalities, who have played a role in this study and preparation of this manuscript.

Place:

Date: *(Dhond Ankush Rameshrao)*

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LIST OF ABBREVIATIONS

@	At the rate
%	Per cent
°C	Degree Celsius
µg	Micro gram
µl	Micro liter
A	Adenine
G	Guanine
bp	Base pair
C	Cytosine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EAR	East Antolian Red
gm	Gram
GVA	Gross Value Added
KCL	Potassium chloride
LTF	Lactoferrin
MY	Milk Yield
$MgCl_2$	Magnesium chloride
ml	Milliliter

mM	Mili molar
NaOH	Sodium hydroxide
Ng	Nano gram
Nm	Nano meter
No	Number
PCR	Polymerase chain reaction
pH	Power of hydrogen
Pmole	Pico moles
RFLP	Restricted Fragment Length Polymorphism
rpm	revolution per minute
SAR	South Anatolian Red
SCC	Somatic Cell Count
T	Thymine
TAE	Tris acetate EDTA
TE	Tris EDTA
UV	Ultra Violet
V	Volts

1. INTRODUCTION

Livestock is an essential part of agriculture and plays an important role in the improvement and expansion of the agricultural industry. The livestock sector contributes to the enhancement of human food and nutritional security by offering nutrient-rich food and food products. It also creates revenue jobs and supplies manure and draught power to the agriculture, improves fertility of lands and provide sources for native fuel such as dried dung, methane gas production from fermented dung from cattle and buffalo. Total livestock population of India is 536.76 million, of which 193.46 million cattle (35.98%) (indigenous cattle 142.11 million and crossbreed cattle 51.36 million), 109.85 million buffaloes (20.51%), 74.26 million sheep (13.86%), 148.88 million goats (27.79%), 0.25 million camels (0.04%), 9.06 million pigs (1.69%) and 0.34 million horses (0.06%). Whereas, the livestock population of Maharashtra state is 33.0 million, out of which 13.9 million cattle, 5.6 million buffaloes, 2.7 million sheep and 10.6 million goats (20th Livestock census 2019).

India stands first in milk production (209.9 million tons) contributing 24 per cent of global milk production (928.0 million tons) (Economic survey 2021-2022). The total milk production of India is contributed through buffaloes 45% by established breeds and nondescript buffaloes. Whereas, cattle contribute 51% of milk production, out of which 3% from exotic and 28 % crossbred cattle of total production. Indigenous milch cattle breeds and non-descript cattle contributed 20 % of the entire amount of milk production. Overall livestock sector contributed 30.87% of agricultural sector GVA and 6.17% of total GVA (Annual Report of Department of Animal Husbandry and Dairying, Government of India, 2021-2022).

Animal Husbandry and Dairying Department, Government of India is making continuous efforts to increase the contribution from native cattle and buffaloes by implementing different plans as Rastriya Gokul Mission etc., IT tools such as e-Gopala app etc. along with advanced selection and breeding tools. A genetic marker helps to explain quantitative differences and their use in animal husbandry by favourably linking alleles for quantitative features with knowledge about each gene's mechanism of action and how genes interact. DNA markers offer two potential future uses in animal selection: combining the best alleles of two or more breeds and choosing the best alleles within a species or lineage, DNA-based markers such as RFLPs and minisatellites, PCR-based markers such as microsatellites and SNPs, and other types of genetic markers are oftenly being used in animal breeding.

Several candidate genes for milk production have been published on the promising candidate genes that affected the milk production features in exotic cattle breeds, including, ABCG2 (ATP binding cassette), CSN1S1 (Casein alphas 1), CSN1S2 (Casein alphas 2), CSN2 (Casein beta), CSN3 (Casein kappa), DGAT1-(Diacylglycerol O-acyltransferase 1), GHR (Growth hormone receptor), LEP (Leptin), LGB (Lactoglobulin beta), PRL (Prolactin), STAT5A (Signal transducer and activator of transcription 5A), BoLA-DRB3 (Major histocompatibility complex, class II, DRB3), LTF (Lactoferrin) (Ogorevc *et al.*, 2009).

Bovine lactoferrin (LF) gene is one of the key candidate genes that affects milk production and serves as a key marker for healthy udders (Ogorevc *et al.*, 2009). As a "red protein from milk whey," the bovine lactoferrin gene was identified in 1939. On the q arm of chromosome number 22 (BTA 22q24), the bovine lactoferrin gene is found in cattle. Different species sizes range from 23 to 35 kbps. It has 16 introns, with exon-1 being the smallest at 82 base pairs, and 17 exons (exon-17). In the population of exotic and crossbred cattle, introns 4 and 6 were proved to be highly polymorphic and connected to features related to milk production and somatic cell count. However, a little relevant literature is available on native cattle breeds (Sharma *et al.*, 2015).

There are 53 well defined breeds of cattle and 20 breeds of buffalo, according to the ICAR-NBAGR. The Indian cattle breeds known for adaptability tough climatic condition, perform well on low-quality feed and fodder, be resistant to numerous topical diseases with high milk fat percentage. The Maharashtra state has seven cattle breeds viz. Deoni, Gaolao, Khillar, Red Kandhari, Kokan Kapilla, Kathani and Dangi. Dangi cattle is one of the draft purpose breed of Maharashtra. The native breeding tract of Dangi cattle is Dangas (former Bombay state known as Dangas) comprising the Nasik and Ahmednagar districts in the state of

Maharashtra, in Marathi Dang means mountains, and these animals living in the mountains are hence named as Dangi. Dangi cattle are medium-sized animals with deep bodies and generally of a ponderous build. They are usually of broken red and white or black and white colour, and both sexes usually have horns. The horns are short and thick with lateral pointing tips. Some animals are also available with inward-pointing horns or downward-pointing horn tips. They have a relatively small head with a slightly protruding forehead, and skin exudes an oily secretion, which protects them from heavy rain.

The Dangi cattle is known for its hardy nature and its excellent working qualities in heavy rainfall areas, rice fields, and hilly areas. These animals are extensively used for harrowing, ploughing, and other agricultural operations Average milk daily milk production of Dangi cattle ranges to 0.5 kg to 2.5 kg. However, there are few cows available with farmers with average milk production of 3-4 kg per day (Janmeda *et al*., 2013).

The polymorphism study of lactoferrin gene in Dangi cattle will exploit the genetic potential of Dangi cow milk traits associated with the lactoferrin gene, which will help in the selection and breeding of Dangi cattle. The lactoferrin gene polymorphism has several benefits which are related to lactation and a good indicator of udder health in the cattle breeds will help in further selection in Dangi cattle to improve the yield and the quality of the milk obtained.

Considering the importance of the Dangi cattle breed and lactoferrin gene polymorphism, the present work was undertaken with the following objectives:

1. To study the polymorphism of the bovine lactoferrin gene (Intron 6) in Dangi cattle.
2. To establish the association between bovine lactoferrin genotypes and milk traits in Dangi cattle, if any.

2. REVIEW OF LITERATURE

Molecular techniques allow the detection of variation or polymorphisms existing among individuals in the population for specific regions of DNA. These polymorphisms can be used to exploit the genetic potential of the selected population. A review of literature is an elemental part of the research, which provides the basis for findings, discussion, and interpretation. This chapter illuminates the research work related Bovine Lactoferrin gene in Dangi cattle and its association with milk production traits.

2.1 Bovine lactoferrin protein:

Lactoferrin (LTF) is a ferric ion ($Fe3+$) -binding 80- kDa glycoprotein found in secretory secondary granules of neutrophile blood cells (Cramer *et al.,* 1985). It is a bioactive glycoprotein and is found in most the mammalian secretions, including bovine, human, goat, and porcine. The amino acid sequences of the lactoferrin proteins are highly conserved in the species of higher mammals. It is thought to act as a general antibacterial protection molecule during inflammation and within glands and secretions of the mammary gland, especially in dairy cattle breeding. The level of protection depends on the concentration of LTF in milk. This varies with the different stages of lactation, being high in colostrum and lower by order of magnitude at full lactation (Tsuji *et al.,* 1990). Bovine milk is the most common source of commercially produced lactoferrin (0.1 mg/ml), and it contains about one-tenth of the lactoferrin found in other mammals, with concentrations ranging between 0.02 and 0.2 mg/ml (O'Halloran *et al.,* 2010).

2.2 Structure of bovine lactoferrin gene:

Goodman and Schanbacher, in the year 1991, described the detailed structure of the lactoferrin gene; consists of 17 exons and 16 introns that ranges from 82 bp to 225 bp. It has two lobes: N lobe and C lobe. N lobe has two regions N-I and N-II and C lobe has C-I and CII. N-I lobe consists of exon-2, exon-3 and exon-4, whereas N-II lobe is consisting of exon-9, exon-10, exon-11 and exon-12. C-I lobe comprises of exon-4, exon-5, exon-6, exon-7 and C-II lobe comprises of exon-12, exon-13, exon-14, and exon15. In between N lobe and the C lobe is the hinge region which consists of exon-9. The mRNA (2351 bases) codes for a 708 amino acids protein with a 19 amino acids signal peptide immediately preceding a sequence identical to N-terminal, 40 amino acids have been reported for bovine lactoferrin gene, The transferrin gene family of non-heme iron binding glycoproteins include lactoferrin, which is found in polymorphonuclear leukocytes, mucosal secretions, and epithelial secretions. The presence of multiple regulatory elements within the lactoferrin promoter contributes to differential gene expression and variable lactoferrin content in milk. Schwerin *et al.* (1994) gave the precise location of the bovine lactoferrin gene on the q arm of chromosome no. 22 (BTA 22q24) in cattle.

In bovines, it is about 34.5 kbp in which the promoter region consists of 1122 bp with GC-rich region and has a noncanonical TATA box, multiple stimulating protein (sp-1) and other putative binding sites for transcription factors, including nuclear factor-κB (NF- κB), activator protein 1 (AP1), signal transducer and activator of transcriptions 3 and 5 (STAT 3 and STAT 5) and steroid hormone receptors (Zheng *et al.*, 2005).

2.3 Functions of bovine lactoferrin gene:

The main protein that binds iron is lactoferrin, which is present in both human and bovine milk as well as mammary secretions. It is well known for its function in providing iron to nursing infants as well as its contribution to the antibacterial defense of the maternal mammary gland and the gut of new-borns. Additionally, it influences the immune system's operations, the growth of the intestinal epithelium, granulocyte and lymphocyte mitogenesis. Other milk proteins are not subject to the same control as lactoferrin (Goodman and Schanbacher, 1991).

Teraguchi *et al.* (1995), observed a bacteriostatic effect against clostridia in the gut mice as part of a study at the Nutritional Science Laboratory. Even Sekine *et al.* (1997) advised application in chemotherapy for colon tumours in rats at Chemotherapy Division of The National Cancer Center Research Institute, Tokyo.

The primary function of LTF lies in its role in iron metabolism, including iron transport, storage, and chelation, but it is also thought to play a role in innate defense and exhibits a diverse range of biological activities, including antimicrobial activities, antiviral activities, antioxidant activities, immunomodulation, modulation of cell growth, and binding and inhibition of several bioactive compounds, such as lipopolysaccharide and glycosaminoglycan. The *in-vitro* activity of LTF also includes transcriptional activation of several genes. It is one of the important proteins that have anti-infective properties and can prevent or control mastitis in dairy cattle. Lactoferrin stimulates the immune system and serves as a natural antioxidant (Sharma *et al.,* 2015).

LTF exhibits strong antimicrobial activity against a broad spectrum of bacteria (gram-positive and negative), fungi, yeasts, viruses and parasites, also lactoferrin has potent antifungal action (Kirkpatrick *et al.,* 1971). The primary function of LTF lies in its role in iron metabolism. It is demonstrated that lactoferrin is involved not only in the transport of iron, zinc and copper, but also in the regulation of their intake, but it is also thought to play a role in innate defenses and exhibits a diverse range of biological activities (Shongwe *et al.,* 1992). It is a potent activator of immunological functions such as granulopoiesis, cytokine production, antibody synthesis, natural killer cell toxicity, lymphocyte proliferation and complement activation and production of interleukins (IL-1), (IL-2) and TNF (Kimber *et al.,* 2002). It is one of the important proteins that have anti-infective properties and can prevent or control mastitis in dairy cattle (Teng, 2002).

2.4 Lactoferrin content in milk and its association with milk traits:

Cheng *et al.* (2008) studied factors affecting the lactoferrin concentration in bovine milk at State Key Laboratory of Animal Nutrition, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, P. R. China. In which 198 Chinese Holstein cattle indicated that the mean concentration of LTF tended to increase across lactation and observed a growing trend in LTF concentration across the lactation of Holstein cows. Similarly, they reported in dairy Gir cows the highest LTF concentrations occurred in the ninth and tenth months of lactation and the lowest concentration in the third month.

Ujita *et al.* (2019) at Beef Cattle Research Center, Institute of Zootechnics, Brazil observed LTF concentration in 45 Gir cows in their first lactation, according to which the association between LTF and the milk components showed positive except for lactose %. But its magnitude was low with fat % and Total Solid %, and high magnitude with protein %. The high positive correlation of LF with protein % is probably due to the fact that LTF itself is a protein. Previous studies with LTF content in bovine milk estimated a positive and moderate genetic correlation (0.52, $P<0.01$), between Protein % and LTF, an expected value that might be explained by the quantitative trait loci (QTL) that affects protein production and its content in milk.

2.5 Concentration of lactoferrin in colostrum:

In contrast to other milk proteins, lactoferrin is regulated differently. Bovine lactoferrin (bLf) is a protein found in milk that is highest in colostrum (1-2 mg/ml) and lowest in milk (0.01-0.1 mg/ml). It also increases noticeably (20-100 mg/ml) during mammary involution when the secretion of milk and other milk proteins decreases, even while other milk proteins continue to be present in normal proportions after intramammary infection, lactoferrin is raised in milk (>2 mg/ml) for extended periods of time (Goodman and Schanbacher, 1991).

Lactoferrin concentration in milk range from 0.02 to 0.75 mg/mL, whereas lactoferrin concentration in colostrum range from 1.5 to 5 mg/mL (Hahn *et al.,* 1998).

Indyk and Filonzi (2005) discovered that the lactoferrin concentration in colostrum was 100 times higher than in milk. Cheng *et al.* (2008), observed a positive correlation between the volume of milk produced, and the stage of lactation with the lactoferrin concentration.

2.6 Bovine Lactoferrin gene polymorphism:

Seyfert and Kuhn (1994) at Research Institute for the Biology of Farm Animals, Germany, isolated and characterized the bovine lactoferrin gene in german black pied cattle and detected a polymorphic *Eco*RI restriction site in intron 6. PCR-RFLP was carried out in 301 bp fragment using *Eco*RI restriction and confirmed the polymorphic nature of this restriction site. The band pattern for genotypes was observed as AA, AB, and BB. Allele A does not contain an EcoRI restriction site, while allele B cut into 201 and 100 bp fragments.

Li *et al.* (2004) conducted a study to characterize the relevance between mastitis resistance and the variants of the lactoferrin gene by using PCR-SSCP, including five fragments within the 5′ region and all exons of the bovine lactoferrin gene they amplified and identified the nucleotide diversity. Mutations were observed in Lf5′-1, Lf5′-3, and Lf5′-5 in three different regions in the same individuals; exons 4, 8, 9, 11, 15, and intron 4. Further, they could not find the effects of all mutated loci on milk production traits, due to small sample size, high standard error, and imbalanced data.

Daly *et al.* (2006) analyzed the sequence of the promoter region in five different cattle breeds; Holstein Friesian, New Zealand Holstein, Montebéliard, Normande, and Norwegian Red at Food and Animal Biotechnology Centre, Moorepark Food Research Centre, Ireland, to determine the extent of polymorphic variation4, which exists in this region both within and across cattle breeds. They found different single nucleotide polymorphisms (SNPs) throughout this region. The most frequently encountered polymorphism found in all breeds was at position-28, which is immediately proximal to the TATA box of the promoter. Interestingly, most variable base position was -131, which included three types of heterozygotes including a single base deletion in one allele and three homozygote variants. Most of the nucleotide changes in these animals were within the 190 base pairs of the transcription start point, except for an insertion seen in three animals at position -478.

Kaminski *et al.* (2006) carried out a study and amplified the promoter region of LTF gene containing mutation (G/C) in position +32 has by PCR followed by genotyping by the SSCP and RFLP method in 358 Polish Holstein-Friesian cows at Department of Animal Genetics, University of Warmia and Mazury in Olsztyn, Poland, and reported the frequency of genotype 0.628,0.313. 0.059 for GG, GC, and CC. They observed CC cows show significantly higher ($P \leq 0.01$) protein content in milk in comparison with GG cows. However, other milk performance traits including SCC were also higher but at non-significant levels.

Sender *et al.* (2006) investigated association of the polymorphism of some genes with the occurrence of mastitis in cattle, at Institute of Genetics and Animal Breeding of the Polish Academy of Sciences, Poland. The purpose of this study was to investigate the association of the lactoferrin alleles and interaction of BoLA-DRB3 and lactoferrin alleles with the somatic cell count in cow milk. In which lactoferrin gene polymorphism was determined by the PCR-RFLP. The polymorphism of the lactoferrin gene was identified in blood samples collected from 125 cows. Udder health was determined by test - day milk somatic cell count. They observed that Lactoferrin genotype BB was significantly ($p<0.01$) associated with a decrease of the somatic cell count in cow milk which was good indicator of udder health, but could not find relationship between interaction of BB lactoferrin genotype and BoLA-DRB3 16 with somatic cell count due to the small sample.

Wojdak-Maksymiec *et al.* (2006) studied associations between bovine lactoferrin gene polymorphism and somatic cell count in milk at Department of Animal Genetics and Breeding, Agricultural University of Szczecin, Polandin 124 Polish Black-and-White dairy cows of various share of the Holstein-Friesian (HF) breed. Lactoferrin (LTF) gene polymorphism were obtained with PCR-RFLP method using *Eco*RI enzyme. Two alleles of LTF, A and B, were reported in the studied population. Their frequencies were 67.74% and 32.56%, respectively. They observed three genotypes: AA, BB and AB, of frequencies equal to 37.90%, 2.42% and 59.68%, respectively. They find that statistically significant associations exist between the somatic cell count (SCC) and LTF genotype, lactation month and parity. The highest somatic cell count was recorded in milk of the AB genotype, whereas the lowest in cows of the AA genotype.

Arnould *et al.* (2009) carried out genetic analysis of lactoferrin content in bovine milk at Animal and Microbial Biology Unit, and Animal Science Unit, Gembloux Agricultural University, Belgium. In which they studied bovine LF gene polymorphism and its effect on different economic traits as milk yield (MY), fat and protein percentages, and somatic cell score (SCS) using PCR amplification and sequencing of all exons. They observed three new polymorphisms in exon 2, exon 11, and intron 8. They did not observe significant association between polymorphism and different economic traits in studied population.

O'Halloran *et al.* (2010) investigated polymorphism in the bovine lactoferrin gene promoter region associated with reproductive performance and somatic cell count. In order to explore the relationship between single nucleotide polymorphisms of the lactoferrin promotor region and fertility and milk production traits, they performed DNA-PCR sequencing on 848 sire semen samples from an Irish dairy herd. They observed polymorphism in three SNPs; LF-586, LF-190, LF-28. Furthermore, they noted there was no association between the three SNPs and the yields of milk, fat, or protein. While the survival traits, calving interval showed improvement.

Sharifzadeh and Doosti (2011) carried out study on lactoferrin gene polymorphism in Iranian Holstein Cattle using PCR-RFLP technique at Department of Microbiology, Faculty of Veterinary Medicine, Islamic Azad University, Iran. They used the PCR-RFLP technique and *Eco*RI to analyze polymorphism in the 301 bp lactoferrin gene in 160 Iranian Holstein bulls. In the population under study, they discovered the two alleles 'A' and 'B', along with the genotypes AA, AB, and BB. The frequencies of the A and B alleles were, respectively, 67.74% and 32.26%, whereas those of the AA, AB, and BB were, respectively, 32.50%, 10%, and 57.50%. Furthermore, they noted that genotype AB was significantly associated with the highest SCC, whereas genotype AA was associated with the lowest somatic cell count ($P<0.01$).

Bahar *et al.* (2011) at Cell and Molecular Biology Laboratory, School of Agriculture, Food Science and Veterinary Medicine, Veterinary Science Centre, University College Dublin, Belfield, Ireland., conducted studies to identify, in silico, SNPs in the promoter region of the LTF gene that could affect transcription factor binding activity to investigate the effects of these SNPs in vitro by measuring promoter transcriptional activities of different bovine LTF promoter haplotypes and to investigate the genetic association between LTF promoter SNPs and milk lactoferrin concentration. Haplotypes were deduced from sequencing of the 2.2-kb bovine LTF promoter in 78 unrelated animals. In silico analysis of the 2.2-kb promoter revealed two major haplotypes (BtLTF_H1a and BtLTF_H2a) that differed at 10 SNP loci that affect transcription factors of both a constitutive and an inducible nature. The SNPs c.-28A>C, c.131T>C, c.-156A>G, c.-270T>C, c.-586C>T, c.-1702A>G, c.-1953G>A, c.-2047A>G, c.2077A>G, c.-2122C>T, c.-2140A>G and c.-2151G>A were associated (P< 0.001) with milk lactoferrin content in 372 Holstein-Friesian cows. In study 372 Holstein-Friesian cows were genotyped for 12 LTF polymorphisms and infection-free (SCC < 150 000 cells/ml) milk samples from these cows were assayed for lactoferrin concentration, this study support the concept that during the process of natural selection, variants in the promoter region of the LTF gene have evolved in dairy cows.

Anggraeni *et al.* (2012) studied genetic polymorphism of the lactoferrin gene in HF cattle both Dairy and Beef Cattle at National Artificial Insemination and Embryo Transfer Stations at Research Institute for Animal Production, Indonesia, in 89 HF cattle. They observed only two genotypes AA (301 bp) and AB (301 bp, 201bp and 100bp) by *Eco*RI/PCR-RFLP of 301 bp fragment in both HF dairy and beef cattle. The frequencies of AA and AB were 0.50 and association was found between AA and AB genotype and Mastitis resistance.

Nanaei *et al.* (2012) studied lactoferrin gene polymorphism of Holstein cows in Isfahan province at College of Agriculture, Isfahan University of Technology, Isfahan, Iran. In which total 404 Holstein cows from five dairy herds in Isfahan province were used to investigate polymorphism of bovine lactoferrin (LTF) gene. They found two alleles A and B in population with frequency of 0.775 to 0.831, respectively. The occurrence of two genotypes AA and AB, with frequency of 0.606 and 0.394, respectively. Statistical analysis showed that there was no Hardy-Weinberg equilibrium between the observed and expected distribution of LTF genotypes. They suggested that LTF intron 6 polymorphisms might be associated with resistance to mastitis.

Zabolewicz *et al*. (2012) at Department of Animal Genetics, University of Warmia and Mazury in Olsztyn, Poland, conducted a study to find out whether a single SNP (A/C) located at position−28 (within the TATA box) influences the transcription complex in mammary epithelial cells and performance traits in dairy cattle. Four hundred fifty-nine Polish Holstein-Friesian bulls and 274 cows—the daughters of 6 bulls were screened by PCR-SSCP method. Cows were selected into two groups showing low (less than 105/ml) and a high (more than 106/ml) somatic cell count measured 10 times during the first lactation. Electrophoretic mobility shift assay (EMSA) showed that the LTF gene was expressed during lactation and that the A allele was bound by the nuclear proteins more efficiently. The C allele was significantly more frequent in cows with low number of SCC (P<0.0001). The effects of LTF genotype on the breeding value of bulls were estimated for 29 traits routinely evaluated in the national database. Significant negative effects of the AA genotype were found for 6 conformation traits and 3 fertility traits. The lack of an antagonistic correlation between allele C of LTF and milk performance traits highlights its benefit for breeding programs. In this the polymorphism which changes the consensus sequence of TATA box was investigated, also in addition to that, using an independent sample they assessed the effects of LTF genotypes on breeding values of Holstein-Friesian bulls, evaluated for 29 traits, including somatic cell score (SCS).

Wang *et al.* (2012) at College of Animal Science and Technology, inner Mongolia University for Nationality, China, tried to explore the association of polymorphisms of bovine lactoferrin gene with the susceptibility to mastitis and part milk trait. PCR-SSCP and DNA sequencing techniques were used to detect the polymorphisms of exon11 of 38 case cattle and 118 control cattle. The results analysis showed that the extremely significant differences in genotypic frequencies and allelic frequencies of exon11 were present in the treatment and control group (P<0.01), AA genotypic frequencies and A allelic frequencies were found highly significant in treatment group than the control group (P<0.01). The SCS of AA genotype individuals were significantly higher than AB and BB genotype (P<0.05), the milk protein of AA genotype individuals were extremely significantly higher than AB genotype (P<0.01) and significantly higher than BB genotype (P<0.05), the BB genotype individuals were significantly higher than AB genotype (P<0.05). These findings indicated a prospect that the A allelic of bLF-exon11 may have relevant to the susceptibility of mastitis in Simmental cattle.

Gursel *et al*. (2013) carried study on *Eco*RI polymorphism, intron 6 of the bovine lactoferrin gene in which 46 SAR and 46 EAR cattle were selected. Bovine LF gene was amplified by polymerase chain reaction (PCR) and the PCR products with digested by *Eco*RI restriction enzyme. The frequency of A allele was found higher than B allele in EAR cattle breed and the frequency of B allele was found higher than A allele in SAR cattle breed. They suggested that further studies should be conducted on the bovine LF gene in Bos taurus and Bos indicus cattle to understand the reason of high genotype frequency in SAR and EAR cattle breeds.

Rodriguez *et al.* (2013) explored the association of the intron 6 polymorphism of the bovine lactoferrin gene with milk production, fat and protein percentages, and somatic cell count in a population of Holstein cows. The information about 382 Holstein cows were collected from the Antioquia province. 1345 lactations records were taken for the association analysis with milk production and 293 lactations were taken for fat and protein percentages and for somatic cell count (SCC). The genotyping was performed using the PCR-RFLP technique and the conclusion that the LTF bovine gene is a good indicator of the susceptibility or the resistance to subclinical mastitis and is associated with the protein percentage in the milk.

Maletic *et al.* (2013) conducted study on analysis of lactoferrin gene polymorphism and its association to milk quality and mammary gland health in Holstein-Friesian cows, at University of Belgrade, Faculty of Veterinary Medicine, Serbia. In which they decided to monitor the distribution of LTF gene genotypes in 46 Holstein Friesian cows of different age. DNA was isolated from blood sample and the polymorphism of lactoferrin gene was determined by PCR- RFLP method using *Eco*RI. They reported that two allelic forms of the gene were found, and two genotype AA and AB were found in the ratio of 71.7% and 28.3%. Further, they reported that genotype AA and AB differ significantly for total milk production.

Hemati *et al.* (2014) carried out study on association between bovine lactoferrin gene variant and somatic cell count in milk based on *Eco*RI restriction site at Laboratory for Molecular Genetics and Animal Biotechnology, Faculty of Animal Science and Fisheries, Sari Agricultural Sciences and Natural Resources

University, Sari, Iran, in which effects of a lactoferrin gene polymorphism (intron 6) on milk somatic cell count (SCC) and subclinical mastitis was investigated in 121 Holstein dairy cattle. Two alleles of A and B and two genotypes of AA and AB were found in an EcoRI recognized single nucleotide polymorphism in intron 6 of lactoferrin gene with the frequencies of 85.12, 14.88, 70.25 and 29.75%, respectively. No homozygous BB cows were detected in the studied population. Marker-traits association analysis showed that *Eco*RI marker site in lactoferrin gene was significantly related to SCC ($P<0.01$) with AB as a desirable genotype. They suggested the selection of the AB genotypes for *Eco*RI recognized single nucleotide polymorphism might contribute to a reduction of SCC in Holstein dairy cattle.

Pawlik *et al.* (2014) investigated the association between bovine lactoferrin gene polymorphism and production traits and in Polish Holsteins. They found associations between four SNPs, localized in the 5’-flanking region (-926 and +32) and in exons 4 and 9 of the lactoferrin gene, and examined their dairy performance. Agarose gel electrophoresis revealed the following bands LF-926- AA (229bp), AG (229, 168 and 61bp) and GG- (168 and 61bp); LF+32- CC (264, 166 and 56bp), GC (430,264,166 and 56bp) GG (430 and 56bp); LFex4- AA (117 and 78bp), AG (195, 117 and 78bp) and GG (195 bp); LFex9- CC (159bp), CT (159, 103 and 56bp) and (TT: 103 and 56bp). The frequencies of AA, AG and GG are 0.01, 0.12 and 0.87. Genotypes CC, GC and GG were found in frequencies 0.04, 0.27 and 0.69 and genotypes AA, AG and GG were found in frequencies of 0.60, 0.37 and 0.03 respectively. Association was found between the genotypes and the milk traits but it was also found that the genotype by environment interaction may significantly change associations between genes and traits.

Bukhari *et al.* (2015) at Division of Animal Genetics and Breeding, Sher-E-Kashmir University of Agricultural Science and Technology-Jammu, R S Pura, India, conducted study in which total of 50 samples of blood and milk of Jersey crossbred cattle were collected from which genomic DNA was isolated and specific primer was used for the amplification of the promoter region of lactoferrin gene. The PCR product comprised of 115 bp. The PCR product was digested with *Taq*I restriction enzyme. The amplicon has no restriction site and all the 50 animals revealed only one type of genotype (HH) with respect to *Taq*I, PCR-RFLP. The same PCR products were subjected to SSCP, and single genotype was found to be present in all animals. Hence, promoter region of lactoferrin gene was found monomorphic in Jersey crossbred cattle under the present study.

Chopra *et al.* (2015) carried out study on lactoferrin gene promoter variants and their association with clinical and subclinical mastitis in indigenous and crossbred cattle at ICAR-National Dairy Research Institute, Karnal (Haryana), India. Sahiwal and Karan Fries 350 cows were genotyped for *Taq*I/PCR-RFLP analysis. The 115 bp PCR product was used to detect the polymorphism of the bovine lactoferrin gene promoter. According to their findings, Sahiwal and Karan Fries cattle had various levels of polymorphism in the lactoferrin gene promoter, which was found to be polymorphic. They found Sahiwal and Karan Fries cattle to be three different genotypes, GG, GH, and HH. In Sahiwal, the genotype frequencies for GG, GH, and HH were 0.23, 0.53, and 0.24, compared to 0.867, 0.506, and 0.406 in Karan fries. Additionally, they observed non-significant associations between mastitis incidence and association studies of SNPs linked with mastitis.

Mao *et al.* (2015) at College of Animal Science and Technology, Yangzhou University, Yangzhou, P. R. China, screened 20 Chinese Holstein cows. PCR amplification and sequencing of the bovine lactoferrin gene promoter region and used for SNP discovery in the region between nucleotide positions -461 to -132. Three SNPs (-270T>C, 190G>A and -156A>G) was observed in bovine lactoferrin, Chinese Holstein cows (n=866) were genotyped using Sequenom Mass ARRAY machine and the associations between SNPs or haplotype and milk somatic cell score (SCS) and production traits were analysed by the least-squares method in the GLM procedure of SAS. SNPs -270T>C and -156A>G showed close linkage disequilibrium (r(2)=0.76). The SNP -190G>A showed a significant association with SCS, and individuals with genotype GG had higher SCS than genotypes AG and AA. Associations were observed between the SNPs -270T>C and -190G>A with SCS and the milk composition.

Patel *et al.* (2015) at Animal Genetics Division, Indian Veterinary Research Institute, Izatnagar, India, conducted a study in which they isolated Genomic DNA from the blood of 100 crossbred cattle (HF, Jersey, Brown Swiss x Hariana cattle). Exon 4 and intron 6 regions of lactoferrin (Lf) gene were characterized by PCR-SSCP. Two alleles of exon 4, namely E and F showing two genotypes EE and EF and two alleles of intron 6, namely A and B showing two genotypes AA and AB were found in studied population. Cloning and sequencing results revealed that in exon 4, there was one SNP in allele E and two SNPs in allele F, which changed amino acid isoleucine to valine. In intron 6 there was one SNP in allele A and three SNPs in allele B. Least square analysis for SCC and FLMY revealed that genotype AA was significantly associated with low SCC, while genotype AB was significantly associated with high SCC. However, genotype EE was significantly associated with FLMY as compared to genotype EF.

Nanaei *et al.* (2016) at Department of Animal Sciences, College of Agriculture, Isfahan University of Technology, Iran, examined 404 Holstein dairy cow farms Iran. A 301 bp fragment of intron 6 for bovine LTF gene was amplified and the animals were genotyped using PCR-RFLP method. Two alleles of bovine LTF, A and B, were identified in the studied population. Frequency of A and B, were identified in population was 6.606 and 0.393, respectively. They found that AB cows had significantly higher ($P<0.05$) fat percentage and SCS in milk in comparison with AA genotype. Other traits did not show any significant difference.

El-Domany *et al.* (2019) at Department of Theriogenology, Faculty of Veterinary Medicine, Mansoura University, Egypt conducted a study to explore the genetic polymorphisms in LTF/*Eco*RI and TLR4/*Alu*I loci and their association with milk and reproductive performance in Holstein cattle. A randomly selected 800 Holstein dairy cows from two dairy farms (400 animals each) from Egypt were used. Based on the two farm records, association between LTF/*Eco*RI genotypes and milk performance traits (order of lactation, daily milk yield, days in milk, corrected milk at 305 day and dry period) was carried out. Meanwhile, exploring of TLR4/*Alu*I genotypes effect was done on data for reproductive performance (age at first freshening, calving interval, number of services per conception, ovarian rebound and days open). DNA was extracted from blood samples collected from Holstein dairy cows of the both farms and restriction analysis of 301-bp PCR products of LTF gene revealed two genotypes: AA genotype (301 bp) and AB genotype (301, 201 and 100 bp). Meanwhile, restriction analysis of 382-bp PCR products of TLR4 gene digested with *Alu*I yielded two alleles (A and B) and three genotypes (AA, AB and BB). The A allele was indicated by two bands at 300 and 82 bp, and the B allele resulted in three fragments of 160, 140 and 82 bp. There was a significant association ($p \leq 0.05$) between LTF genotypes and milk performance traits except for days in milk. The TLR4 genotypes had significant effects ($p \leq 0.05$) on age at first freshening, calving interval, number of services per conception, ovarian rebound and days open. Ordinal logistic regression statistical model also revealed that it is possible to calculate high reproductive performance traits and to predict favourable dairy cows based on LTF and TLR4 genotypes. Their research reveals the effectiveness of LTF/*Eco*RI and TLR4/*Alu*I loci as candidates for reproductive performance assessment in Holstein cattle.

Zainab Sabeeh Al-Allak (2020) investigated lactoferrin gene polymorphism in Iraqi native cattle using PCR-RFLP technique at Department of Animal Production, College of Agriculture University of Misan, Iraq. In order to acquire information on the polymorphism of the bovine lactoferrin (LTF) gene for a potential genetic marker, a total of 30 cows from three dairy herds in the province of Misan were used. Two alleles A, B with two genotypes AA, AB were reported in the examined population.

Sharma *et al.* (2020) conducted a study to find polymorphism in LF gene using PCR-RFLP in 50 Jersey crossbred cattle maintained at Central Cattle Breeding Farm, Jammu. PCR-RFLP was done using *Eco*RI and digestion of 301 bp product revealed three genotypes namely AA (301 bp), AB (301 bp, 201 bp and 100 bp), and BB (201 bp and 100 bp). Genotypic frequency of AA, AB and AB was 0.50, 0.10, and 0.40, respectively. Allelic frequencies for A and B alleles were 0.55 and 0.45, respectively.

Zinnatov *et al.* (2020) at Kazan State Academy of Veterinary Medicine, Russia conducted the tested milk productivity of cows with different LTF genotype in their pedigrees showed that the maximum milk yield was in cows of the Ayvengo line with the LTF^{AB} genotype. These cows were distinguished from other cows by their high protein content, and these animals had the lactoferrin gene. And the animals of the Ayvengo line differed in the level of fat in milk. The comparative PCR-RFLP analysis of the blood DNA samples of the first-calf Holstein cows showed the benefit virtually by all milk production indicators (milk yield, milk protein and fat), including by somatic cell content in milk of the Ayvengo and Sovereign line cows with the LTF^{AB} genotype. The Aydial line stock with the LTF^{AB} genotype is worthy of notice regarding the selection activity.

Khalil *et al.* (2021) at Department of Animal Production, Faculty of Agriculture at Moshtohor, Benah University, Egypt, conducted study on total 494 lactation records from 180 milking cows raised in three experimental herds in Egypt, for lactoferrin gene polymorphism using PCR-RFLP. They, investigated the association of lactoferrin genotypes with lactation traits, yields of milk, fat and protein in Friesian breed and local Baladi herd. A mixed model was used in analysing data of each herd separately. An amplified PCR product of the lactoferrin gene was performed at 800 bp and digested using *HinfI* restriction enzyme. Three-band patterns for three genotypes were obtained, involving AA with one band (800 bp), AB with three bands (800, 650, and 150 bp), and BB with two bands (650 and 150 bps). They found an association between the genotypes and the yields of milk fat and protein. The strong associations among genotypes of the lactoferrin gene and yields of milk, fat, and protein, was found that indicates the potential of the lactoferrin gene to select as a candidate gene for lactation traits in Frisian and Baladi cattle in Egypt. They found an association between genotypes of lactoferrin gene with milk fat and protein yields in three studied herds.

Kim *et al.* (2021) conducted genome-wide association to identify chromosomal loci and candidate genes with potential effects on milk production phenotypes in a Korean Holstein population. A total of eight significant quantitative trait locus regions were identified for milk yield (Bos taurus autosome (BTA) 7 and 14), adjusted 305-d fat yield (BTA 3, 5, and 14), adjusted 305-d protein yield (BTA 8), and somatic cell score (BTA 8 and 23) of milk production traits. Furthermore, they discovered three main candidate genes (diacylglycerol O-acyltransferase 1 (DGAT1), phosphodiesterase 4B (PDE4B), and anoctamin 2 (ANO2)) through bioinformatics analysis. The DGAT1 gene had an impact on the MY (milk yield) and FY (fat yield) phenotypes via being involved in glycolipid metabolism, fat digestion and absorption, metabolic pathways, and retinol metabolism pathways. However, no strong candidate genes were chosen for the PY (protein yield) and SCS (somatic cell score) traits, even though substantial regions were found, indicating the need for additional research. For the genetic selection of those phenotypes to increase milk production in Korean Holstein cattle, their findings were anticipated to provide crucial information.

Miyakawa *et al.* (2022) investigated clinical reports on the effects of bovine lactoferrin (bLF) in children using PubMed, Google Scholar, and additional hand searching, at food ingredients and technology institute, R&D Division, Morinaga milk industry, Kanagawa, Japan. They discussed main effects were protection from infection, modulation of iron metabolism, body growth, cerebral development, and regulation of the fecal microbiome. They found that bLF may contribute to the child health, particularly by suppressing or alleviating gastrointestinal and respiratory symptoms and improving the iron status of children with anemia or those at a high risk of anemia. The dose of bLF varies depending on the expected effect and target age, ranging from 35.8 to 1000 mg per day, and it may not necessarily have to be as high as that found in human breast milk. Subject characteristics, field conditions and quality of bLF may have led to inconsistent results. In addition, some beneficial effects of bLF were observed in the secondary analysis of the study. Further clinical evidence as the primary endpoint is needed to accurately describe the beneficial effects of bLF on the child health.

Maulana *et al.* (2022) conducted study at Biology Department, Faculty of Mathematics and Natural Science, Indonesia. The purpose of the study was to determine whether the volume of milk produced by Indonesian Friesian Holstein is correlated with LTF gene variation (IFH). In this investigation, fifty cows were sampled. Individual cow sample LTFs were genotyped utilizing the PCR-RFLP technique and *Eco*RI restriction enzyme. As a result, the genotypes AB and AA were discovered, with frequencies of 0.58 and 0.42, respectively. Comparing the AB genotype to the AA genotype, more milk is produced during lactation (3741 1520.29 kg vs. 3338 1317.57 kg), although there is no statistically significant difference ($p > 0.05$). The polymorphism of the LTF gene did not correlate with milk production in the population of dairy cattle investigated.

Pham *et al.* (2022) carried out study on association of lactoferrin and toll-like receptor 2 genotypes with mastitis and milk components in Vietnamese Holstein Cattle, at Key Laboratory of Animal Cell Technology, National Institute of Animal Sciences, Vietnam. They examined the association of polymorphisms in Lactoferrin (LTF) and Toll-like receptor 2 (TLR2) genes with mastitis score and milk component traits, association of TLR2 and LTF polymorphisms with subclinical mastitis and milk components in the Holstein breed raised in Vietnam. Among 192 samples, they identified 44 mastitis-positive samples (22.92%). The mastitis significantly reduced the fat and lactose components in milk ($p < 0.001$) but increased the protein concentration in milk. A total of 94 (49%) and 98 (51%) cows had AA and AB genotypes for the LTF gene at promotor region, respectively. No significant association was found between the LTF genotypes and the milk component traits or mastitis incidence ($p > 0.05$).

3. MATERIALS AND METHODS

The present research work was carried out at Genetic Investigation Laboratory (GIL), Department of Animal Genetics and Breeding and Department of Livestock Product Technology, Mumbai Veterinary College, Mumbai. The molecular laboratory work was performed at GIL, whereas, the forthright analysis of milk samples for milk fat, milk protein, SNF and lactose was attempted at the Department of Livestock Product Technology.

3.1 Selection of animals

The study comprised of 30 lactating Dangi cows from Cattle Breeding Farm and Dangi Cow Research Station, Igatpuri, District-Nashik. The lactating cows only in the first phase of lactation (3-4 months) were selected to ensure the availability of milk samples for the next 6 months of period for generation of sufficient data.

Collection of blood samples and milk samples:

The blood samples (05 ml) from each cow were collected aseptically in EDTA vacutainers with proper labels with animal ID, and transported to the Genetic Investigation Laboratory (GIL), Department of Animal Genetics and Breeding, Mumbai Veterinary College, Parel, Mumbai, by maintaining the cold chain. The samples were stored at 4°C until further DNA extraction.

A total 10 ml of milk samples were collected from each cow in milk collecting bottles fortnightly and transported to the laboratory in an ice pack to avoid spoiling of the milk samples. The milk samples were analyzed on the same day by using LACTOSCAN, at Department of Livestock Product Technology, Mumbai Veterinary College, Mumbai, and the data related to the milk composition of 30 lactating cows were generated for a period of 6 months.

3.2 Isolation of genomic DNA

The genomic DNA was isolated from the blood samples of 30 lactating Dangi cows using, Phenol: Chloroform: Isoamyl alcohol (P: C: I) by Sambrook and Russell (2006).

Stepwise DNA extraction protocol

1. The blood samples were removed from 4°C storage and thawed at room temperature, then made uniform by inverting them slowly.

2. 2 ml of blood was taken in 15 ml centrifuge tubes to which 4 ml of chilled RBC lysis buffer1X (double the amount of blood) was added and then mixed properly. These tubes were incubated for 10-15 minutes in ice-filled beakers before being centrifuged at 5,500 rpm for 15 minutes.

3. After centrifuging, there was a separation of the supernatant and sediment layer. The lysed RBCs were found in the supernatant those were discarded by inverting the centrifuge tubes.

4. The tubes were again filled with 4 ml of chilled RBC lysis buffer approximately and the pellet of WBCs was dispersed by tapping the tube bottom and centrifuged for 15 minutes at 5,500 rpm.

5. The process of discarding the supernatant, addition of RBC lysis buffer, dispersion of WBC pellet and then centrifuging was repeated till the pellet became white in colour, indicating that the RBCs had been fully lysed and discarded, leaving the pellet white.

6. Until the WBC pellet becomes white in colour, the step four and five were repeated.

7. 600 µl of DNA extraction buffer was added. The WBC pellet was then dispersed by tapping the tubes to disperse the WBC pellet in the extraction buffer and incubated in water bath for 30 minutes at 37°C

8. 40 µl of 10 per cent Sodium Dodecyl Sulphate (SDS) was added to the tubes and gently mixed by inverting the centrifuge tubes.

9. 2.5 microlitre proteinase K was added to the tubes and mixed gently end to end, and kept in the water bath at 50°C for 3 hours. Another 2.5 microlitres of proteinase K was added, and tubes were incubated at 50°C overnight in the water bath.

10. Next morning, the contents in the centrifuge tubes were transferred into sterile autoclaved 1.5 ml Eppendorf tubes. An equal amount of tris equilibrated phenol was added to the tubes and properly mixed for 15 minutes by inverting the tubes gently. The mixture was then centrifuged at 5,500 rpm for 15 minutes.

11. The viscous upper aqueous phase containing DNA was transferred into different autoclaved eppendorf tubes.

12. The process was then repeated by adding equal amount of Phenol: Chloroform: Isoamyl alcohol (25:24:1) then with Chloroform: Isoamyl alcohol (24:1).

13. The 3 M sodium acetate at the rate of 100 µl/ 1 ml of the aqueous phase was added to the final aqueous phase and gently mixed.

14. The mixture was added with double the volume of chilled ethanol and mixed gently by inversion. These tubes were kept at room temperature for the precipitation of DNA.

15. Tubes were centrifuged at 5,500 rpm for 20 minutes at room temperature, and the supernatant was discarded by gentle inversion.

16. The DNA pellet obtained was given a wash by adding 500 µl of 70 percent ethanol and centrifugation at 5,500 rpm for 15 minutes at 4°C twice.

17. The tubes containing dried DNA pellet were then air-dried. After the complete evaporation of ethanol leaving the DNA pellet, 400 µl of autoclaved double distilled water was added and kept at 60°C for 2 hours in the water bath to inactivate DNAase or other enzymes and dissolution of DNA pellet.

18. The DNA in the microcentrifuge tubes was vortexed to dissolve and stored at 4°C for a week and finally stored at -20°C.

3.2 DNA quality and quantification:

The quantity and quality of DNA was checked using a spectrophotometer (NanoDrop ND-2000, Thermo, USA) and by agarose gel electrophoresis, respectively.

3.3.1 Spectrometry

Initially, 1 µl of ultra-pure water was used as a blank solution, followed by quantification and quality check of DNA samples. DNA samples with OD 260:280 ratio of 1.8 to 2.0 were diluted with nuclease-free water to obtain approximate final DNA concentration.

3.3.2 Agarose gel electrophoresis

The purity and integrity of the isolated genomic DNA samples were checked by running DNA with loading dye on 0.8 % agarose gel for 120 volts for 5 mins and then 90 volts for 40 mins.

3.4 PCR amplification of bovine lactoferrin gene

The present study comprised of intron 6 of bovine lactoferrin gene involved in milk production and fat percentage of milk.

3.4.1 Primers for bovine lactoferrin gene:

The intron 6 of the bovine lactoferrin gene was amplified by using the set of primers described by Seyfert and Kuhn (1994) (Table 3.1).

Table 3.1: Primer sequences for amplification of intron 6 of bovine lactoferrin Gene:

Sr. No	**Locus**	**Primer sequence (5´ 3´)**	**Base Pair**	**Author**
1	Intron 6	F 5’-GCC TCA TGA CAA CTC CCA CAC-3’	301 bp	Seyfert and Kuhn (1994)
		R 5’-CAG GTT GAC ACA TCG GTT GAC-3’		

3.4.2 PCR reaction for intron 6 of bovine lactoferrin gene:

The final reaction of volume 25 µl was used to optimize the PCR reaction for intron 6 of bovine lactoferrin gene. Commercially available PCR master mix, double distilled water, 0.5 µl of (10 pmole) forward primer, and 0.5 µl of (10 pmole) reverse primer for respective exons were aliquoted for a single reaction. One µl of genomic DNA (approx. 50-150 ng/ µl) was added in each PCR tube to make the final volume 25 µl (Table 3.2). A negative or no template control containing all the reaction components except the template DNA was used. Finally, the PCR tubes were kept in a pre-programmed thermal cycler (Mastercycler Epigradient S, Eppendorf, Germany).

Table 3.2 Composition of PCR reaction for amplification of intron 6 ofbovine lactoferrin gene:

PCR COMPONENTS	**VOLUME**	**CONCENTRATION (10 pmole/µl)**	**FINAL CONCENTRATION**
Nuclease free water	10.5 µl	-	
PCR Master Mix (SYMBIO,2X PCR Master Mix)	12.5 µl	1X	1X
Forward primer	0.5 µl	10 pmole	5 pmole
Reverse primer	0.5 µl	10 pmole	5 pmole
Template DNA	1.0 µl	50-150 ng/ µl	50-150 ng/ µl
Total	25 µl	-	

3.4.3 PCR Cycling conditions for intron 6 of bovine lactoferrin gene:

PCR protocols for amplification of the fragments of intron 6 of bovine lactoferrin gene were optimized. All the reactions were carried out in 0.2 ml thin-walled PCR tubes. PCR tubes containing the reaction mixture were tapped gently and quickly spun. The tubes were placed in a thermal cycler and subjected to PCR. The initial denaturation was at 95ºC for 5 minutes, and for the 34 cycles of denaturation at 94ºC for a minute, the annealing temperature was 62ºC for a minute, extension was at 72ºC for a minute, and the final extension at 72ºC for 10 minutes, was the cycling conditions (Table 3.2)

3.5 Agarose gel electrophoresis of PCR products of intron 6 of bovine lactoferrin gene:

PCR amplification of intron 6 of bovine lactoferrin gene was confirmed by running 5 µl of PCR product from each tube on 1.7 per cent agarose gel at 120 V for the first 10 minutes and next at 90 V for 45 minutes in 1X TAE buffer. Ethidium bromide (1X) was incorporated in 150 ml of agarose gel @ 10 µl (0.5 mg/ml of gel). A step DNA ladder (100 bp) was used as a molecular size marker. The amplified products were visualized under UV light and documented by a gel documentation system (UVP, UK).

3.6 Restriction digestion of intron 6 of bovine lactoferrin gene:

3.6.1 *Eco*RI restriction recognition sites:

The restriction endonuclease enzyme *Eco*RI recognizes the ssDNA sequence 5'-GAATTC'-3, and introduces a single-strand cut between the G and A nucleotides. This recognition site is a palindrome: the opposite strand also reads 5'-GAATTC'-3 and will be cut in the same manner. Digestion with *Eco*RI, therefore, cuts the DNA into a number of fragments with identical single-stranded ~~TTAA-5' tails or “sticky ends” at both ends.

3.6.2 Restriction digestion with *Eco*RI enzyme of PCR products:

Restriction enzyme *Eco*RI was used for performing PCR-RFLP studies on amplified fragment of intron 6 of bovine lactoferrin gene in the present study. The 10 µl of PCR products were digested using *Eco*RI enzyme in the final reaction volume of 20 µl, where 1 µl of *Eco*RI enzyme, 2 µl of RE-buffer, and 7 µl of nuclease-free water were added (Table 3.3). The final reaction mixture was then incubated at 37ºC for 18 hours in the hot water bath.

3.3 Composition of RFLP reaction for mix intron 6 of bovine lactoferrin gene using enzyme *Eco*RI:

S. No.	Reagent	Quantity
1	RE-enzyme-ECORI (10U/ µl)	1.00 µl
2	RE-buffer (10x buffer)	2.00 µl
3	PCR-product	10.00 µl
4	Nuclease free water	7.00 µl
Total reaction volume		20.00 µl

3.6.3 Agarose gel electrophoresis of RE digested products of intron 6 of bovine lactoferrin gene

After overnight restriction digestion at 37°C in the water bath, the PCR products were electrophoresed on 2.5 percent agarose gel containing Ethidium bromide @ 10 µl/100 ml at a constant voltage of 90 V for 40 minutes using 1X TAE buffer. A step DNA ladder (100 bp) was used as a molecular size marker. Thebands were visualized under UV light and documented by a gel documentation system. The band size was judged by comparing with a molecular size marker andrecorded.

3.7 Estimation of gene and genotypic frequencies:

The frequency of bovine lactoferrin gene and genotypes were estimated by standard procedure-

$$\text{Genotype frequency} = \frac{\text{Total no. of individuals of a particular genotype}}{\text{Total no. of individuals}}$$

$$\text{Gene frequency (Desired allele)} = \frac{D+1/2H}{N}$$

Where,

D= No. of desired homozygotes

H= No. of Heterozygotes, N= No. of Animals.

3.8 Association studies between genotypes and milk traits:

*Eco*RI-RFLP analysis of intron 6 of bovine lactoferrin was carried out. The milk samples were analyzed for milk the estimation of milk traits viz. milk fat, milk protein, SNF and lactose percentage. Association study was performed by applying two mean student's 't' test by William Gosset ("Statistical Methods" by G. W. Snedecor and W. G. Cochran,1980).

Test statistics for two mean student's 't' test is as follows:

$$\boxed{t = \frac{\bar{x}1 - \bar{x}2}{\sqrt{}(s2(\frac{1}{n1} + \frac{1}{n2}))}}$$

Where,

$\bar{x}$ 1 and $\bar{x}$ 2 = two sample means

n1 and n2 = two sample sizes

s = pooled or combined standard deviation of two samples.

4. RESULTS AND DISCUSSION

The present research work was carried out at Genetic Investigation Laboratory, Department of Animal Genetics and Breeding, and Department of Livestock Product Technology, Mumbai Veterinary College, Mumbai. The major objective of the work was to know the association between polymorphism of intron 6 of bovine lactoferrin gene and milk traits, especially milk components of purebred Dangi cows. The study involved 30 lactating Dangi cows in all, and the outcomes are presented and discussed under:

4.1 Isolation and quantification of genomic DNA

The genomic DNA was isolated from blood samples by using phenol-chloroform method described by Sambrook and Russell (2006). The concentration and purity of the DNA were examined using the Nanodrop Spectrophotometer. The DNA concentrations ranged between 46.6 ng/µl to 152.7 ng/µl, and the OD ratio of DNA between 1.8 to 2. The DNA quality was confirmed by using 0.8 % agarose gel electrophoresis. All the samples that were extracted showed a single, bright band of DNA, indicating that the DNA was of good quality for further research (Plate No.4.1).

4.2 PCR Amplification of intron 6 of bovine lactoferrin gene:

The annealing temperature was adjusted on the basis of Guanine and Cytosine (GC) content of the primer. For intron 6, several annealing temperatures were attempted to obtain a good quality band of 301 bp. The consistent results were obtained at 62°C. Similar results were recorded by Sharifzadeh and Doosti (2011) and Maulana *et al*. (2022). Seyfert and Kuhn (1994) used an annealing temperature of 60 to 70°C for 20 cycles and then touchdown to 60°C for 20 cycles. The annealing temperature was set at 60°C by Rodriguez *et al.* (2013) and Anggraeni *et al.* (2012). Whereas, Nanaei *et al.* (2012), Nanaei *et al.* (2016) and Lavanya K (2022) used 61°C annealing temperature to obtain a 301 bp band of bovine lactoferrin gene.

The intron 6 of the bovine lactoferrin gene was amplified using the appropriate pair of primers, and the amplified PCR product was visualized on 1.7%

agarose gel with a 100 bp ladder. On the agarose gel, the desired size of PCR product 301 bp (Plate No. 4.2), was observed. A similar 301 bp product was observed by Sender *et al.* (2006), Anggraeni *et al.* (2012), Rodriguez *et al*. (2013), El-Domany *et al.* (2019), Zinnatov *et al.* (2020) in Holstein Friesian cattle. Sharifzadeh and Doosti (2011), Nanaei *et al.* (2012), Patel *et al*. (2015) in crossbreed cows (HF, Jersey, Brown Swiss X Haryana), Nanaei *et al.* (2016) in Iranian Holstein Friesian cattle, Sharma *et al.* (2020) in Jersey crossbred cattle, Pham *et al*. (2022) in Vietnamese Holstein cows, Maulana *et al.* (2022) in Indonesian Friesian Holstein cows and Lavanya K (2022) HF crossbred cows.

Seyfert and Kuhn (1994) in German Black Pied cattle, Wojdak- Maksymiec *et al.* (2006) in Polish Black and White cattle, Gursel *et al*. (2013) in South Anatolian Red cattle and East Anatolian cattle, Lavanya K (2022) in purebred Gir cows.

However, there were a few reports on various bovine lactoferrin gene fragments, including those by Kaminski *et al*. (2006), who observed a 281 bp fragment in Polish HF cattle, Bukhari *et al.* (2015) studied a 115 bp fragment in Jersey crossbred cattle, Chopra *et al.* (2015) observed a 115 bp fragment in Karan fries, and Sahiwal cattle. Zainab *et al.* (2020) amplified 175 bp fragment in Iraqi native cattle, and Khalil *et al.* (2021) reported 800 bp in Baladi cattle.

4.3 PCR-RFLP analysis of intron 6 of bovine lactoferrin gene:

Different digestion time periods were tested to digest the 301 bp PCR product of intron 6, bovine lactoferrin gene at 37 °C with *Eco*RI restriction digesting enzyme; complete digestion was observed at 37 °C for 18 hours.

Maulana *et al.* (2022) 37 °C for 2 hours in Indonesian Friesian Holstein cows, Rodriguez *et al*. (2013) achieved complete digestion at 37 °C for 3 hours in Holstein cows, Sharifzadeh and Doosti (2011) for 4 hours in Iranian Holstein cattle, Sharma *et al.* (2020) for 6 hours in Jersey crossbred cattle, Nanaei *et al.* (2016) for 6 hours in Iranian Holstein cattle, Gursel *et al.* (2013) 37 °C for overnight in South Anatolian Red cattle (SAR), East Anatolian Red (EAR) cattle and Lavanya K (2022) 37 °C for overnight at purebred Gir and HF crossbred cows.

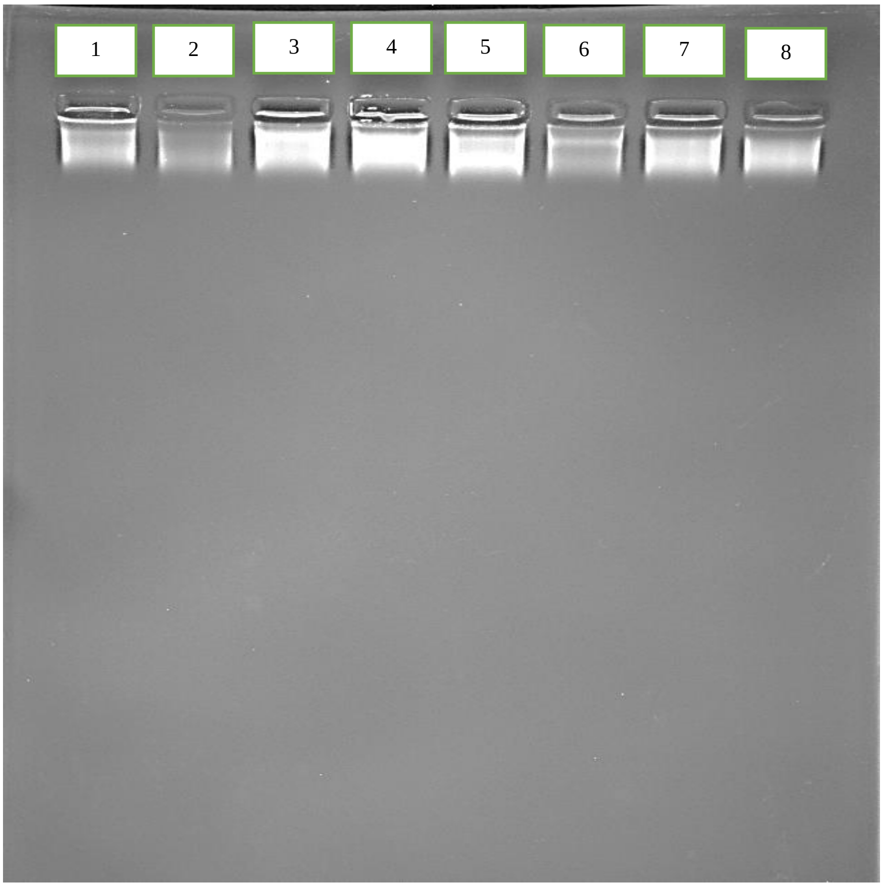

Plate 4.1 Genomic DNA Agarose gel (0.8 %) electrophoresis at 120V for 5 min and 90V for 40 min.

Sample no. 1-8.

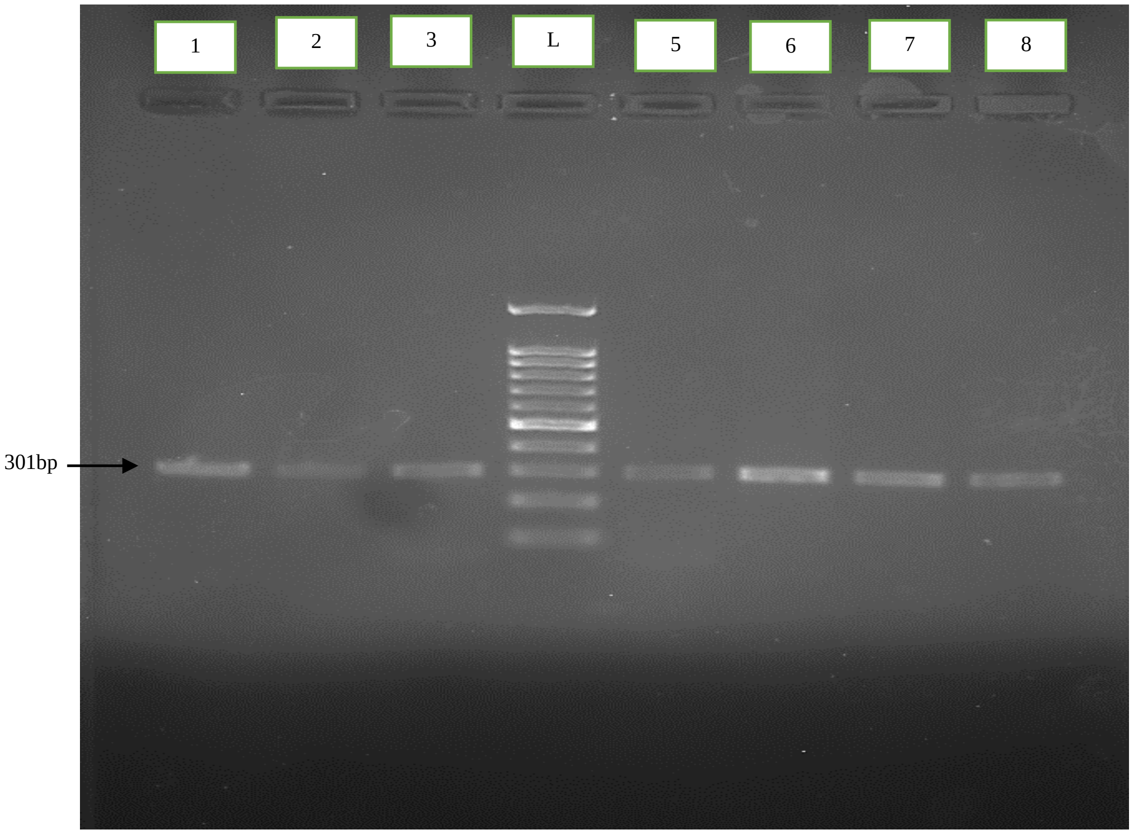

Plate 4.2 Agarose gel (1.7%) electrophoresis of PCR Amplification of intron 6 polymorphism of bovine lactoferrin gene at 120V for 10 min and 90V for 45 min.

Lane L-100 bp pair ladder

Lane- 1-3 and 5-8 PCR Amplificon of Intron 6 of Bovine Lactoferrin Gene (301 bp)

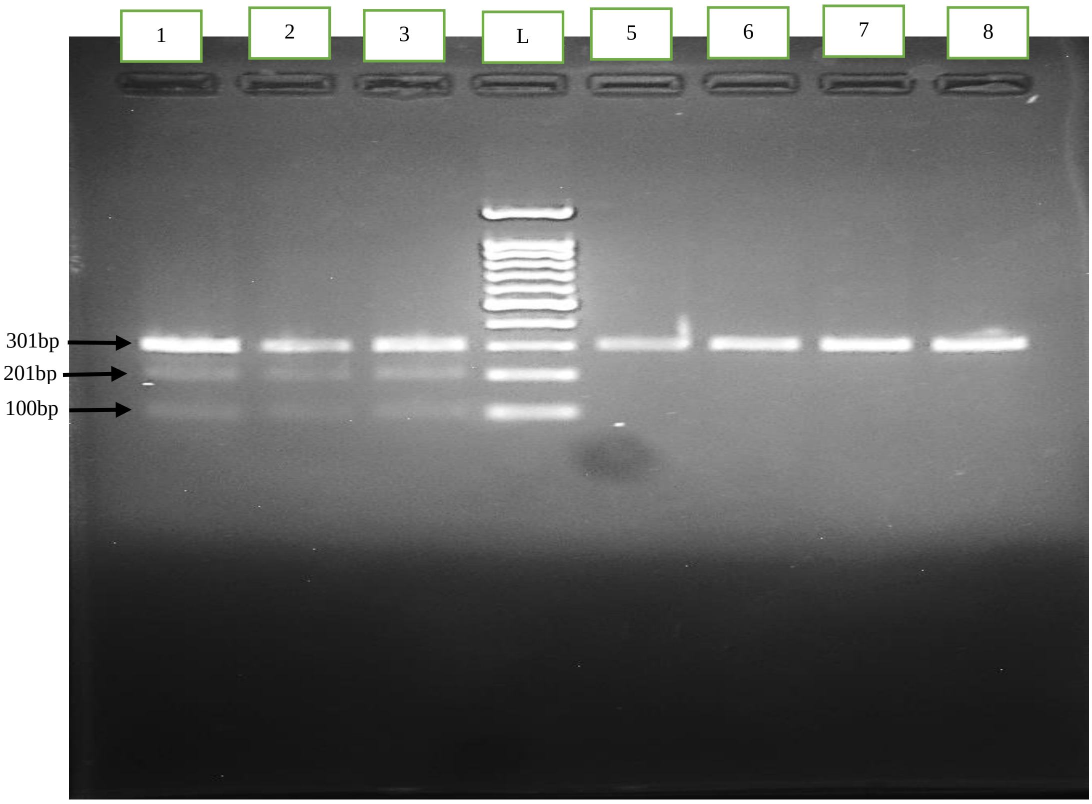

Plate 4.3 Agarose gel (2.5%) electrophoresis PCR-RFLP of intron 6 of bovine lactoferrin gene using *Eco*RI in Dangi cows at 90V for 40 min.

Lane L- 100 bp pair ladder,

Lane 1 to 3- AB genotype (301 bp, 201 bp and 100 bp)

Lane – 5,6,7 and 8 AA genotype (301 bp)

A 2.5% agarose gel was used to examine the digested PCR products (Plate No. 4.3). *Eco*RI/PCR-RFLP analysis restriction band patterns in Dangi cow revealed two patterns, one with a single band of size 301 bp and the other with three bands of size 301 bp, 201 bp, and 100 bp. The cows were classified into two genotypes; 27 cows with “AA" (301 bp) and 3 cows with "AB" (301 bp, 201 bp, and 100 bp) based on the observed RFLP band pattern. Only two genotypes were observed i.e., AA and AB, whereas the genotype "BB" was not observed in the Dangi cows under investigation.

Similar findings with two genotypes AA and AB were observed by Anggraeni *et al.* (2012), Maletic *et al.* (2013), El-Domany *et al.* (2019), Zinnatov *et al.* (2020) in Holstein Friesian cows. Nanaei *et al.* (2012), Patel *et al*. (2015) in Crossbred cattle (HF, Jersey, Brown Swiss X Hariana), Nanaei *et al.* (2016) in Iranian Holstein cattle, Zainab *et al.* (2020) in Iraqi native cattle, Pham *et al*. (2022) Vietnamese Holstein cows, Maulana *et al.* (2022) in Indonesian Friesian Holstein cows, Lavanya K (2022) in purebred Gir and HF crossbred cows. According to Anggraeni *et al.* (2012), the absence of the BB genotype may be influenced by the animal’s diverse genetic origins used in their research. Differences in genetic background throughout generations may be caused by different breeding objectives and selection criteria. The lack of the genotype BB may also be significantly influenced by geographic differences.

However, all three genotypes AA, AB, and BB were reported in various populations of cattle studied by Seyfert and Kuhn (1994) in German Black Pied cattle, Sender *et al.* (2006) in Holstein cows, Wojdak-Maksymiec *et al.* (2006) studied Polish Black-and-White dairy cows, Sharifzadeh and Doosti (2011) in Iranian Holstein cattle, Gursel *et al.* (2013) in South Anatolian Red (SAR) and East Anatolian Red (EAR) cattle, Rodriguez *et al*. (2013) in Holstein cows and Sharma *et al.* (2020) in 50 Jersey crossbred cattle.

4.4 Genotypic Frequencies in Dangi Cattle:

In the present study out of 30 Dangi cows only three cows were with two genotypes AA and AB remaining 27 cows were with AA genotype only, BB genotype is absent in studied population of Dangi Cattle. Genotypic frequencies of

AA, AB, and BB in 30 Dangi cows were 0.9, 0.1, and 0.0, respectively (Table 4.1). The genotypic frequencies reported by other workers in different cattle breeds as; Nanaei *et al*. (2016) and of AA and AB as 0.606 and 0.393 in Iranian Holstein cattle, respectively. In Holstein Friesian cattle by Hemati *et al.* (2014) reported that frequencies of AA and AB were 0.702 and 0.297, Maletic *et al.* (2013) reported frequencies of AA and AB were 0.717 and 0.283, Zinnatov *et al.* (2020) reported frequencies of AA and AB were 0.723 and 0.277. Patel *et al.* (2015) reported frequencies of AA and AB were 0.77 and 0.23 in crossbred cattle in HF cattle and Lavanya K (2022) reported frequencies of AA and AB were 0.667 and 0.333 in HF crossbred cow, 0.767 and 0.233 in purebred Gir cows, respectively.

However, all three genotypic frequencies were reported by Wojdak - Maksymiec *et al*. (2006), with the frequencies of AA, AB and BB were 0.379, 0.242 and 0.597 in Polish HF cows, Sharifzadeh and Doosti (2010) 0.325, 0.1 and 0.575 in Iranian HF cows, Rodriguez *et al.* (2013), 0.6, 0.36 and 0.04 in HF cows, Gursel *et al.* (2013) 0.239,0.456,0.304 in South Anatolian Red (SAR), 0.391,0.478,0.130 in East Anatolian Red (EAR) cattle and Sharma *et al*. (2020) 0.5, 0.1 and 0.4 in Jersey crossbred, respectively.

Table 4.1: Genotypic frequencies of Dangi cow for bovine lactoferrin gene

Breed	**Genotype**	**Genotypic frequency**
Dangi	AA	0.90
	AB	0.10
	BB	0.0

4.5 Gene Frequencies in Dangi Cattle:

The observed frequencies were 0.95 and 0.05 for ‘A’ and ‘B’ alleles, respectively in Dangi cows (Table 4.2). The current results indicated the predominance of ‘A’ allele in Dangi cattle population under study.

The allele frequency reported with A and B alleles by Seyfert and Kuhn (1994) 0.755 and 0.245 in German Black Pied cattle, Wojdak - Maksymiec *et al*.

(2006) 0.677 and 0.325 in Polish HF cows, Sharifzadeh and Doosti (2011) 0.674 and 0.326 in Iranian HF cows, Gursel *et al.* (2013) 0.467 and 0.532 in Anatolian Red cattle, 0.630 and 0.369 in East Anatolian Red (EAR) cattle. in HF cows by Maletic *et al.* (2013) 0.86 and 0.14, Rodriguez *et al.* (2013) 0.78 and 0.22, Hemati *et al*. (2014) 0.851 and 0.149, Zinnatov *et al.* (2020) 0.84 and 0.16. Patel *et al.* (2015) 0.88 and 0.12 in crossbred cows, Nanaei *et al.* (2016) 0.803 and 0.197 in Iranian HF cows, Sharma *et al*. (2020) 0.55 and 0.45 in Jersey crossbred and Lavanya K (2022) 0.834 and 0.166 in HF crossbred cows, 0.884 and 0.116 in purebred Gir cows.

Table 4.2: Gene frequencies of Dangi cow for bovine lactoferrin gene

Breed	**Allele**	
	A	**B**
Dangi	0.95	0.05

4.6 Estimated average of milk traits recorded

The average estimated milk traits in Dangi cows were; milk fat- 3.85±0.18, milk protein- 3.15±0.03, SNF- 8.55±0.10, and lactose percentage- 4.70±0.05. However, the average of Dangi cows with genotype AA were: milk fat- 3.861±0.188, milk protein- 3.139±0.036, SNF- 8.516±0.106 and lactose percentage- 4.686±0.057 and the average of Dangi cows with genotype AB were: milk fat- 3.704±0.534, milk protein- 3.250±0.086, SNF- 8.846±0.254 and lactose percentage- 4.818±0.159.

4.7 Association study between bovine lactoferrin gene genotypes and milk traits in Dangi cattle:

The association between the genotypes of the bovine lactoferrin gene AA and AB of intron 6 and milk traits viz., milk fat, milk protein, SNF, and lactose content, was established by applying two mean student t-test. The results were found to be non-significant for the milk fat, milk protein, SNF, and lactose content in Dangi cows under investigation. It indicated that there was no association between the AA and AB genotypes with milt traits viz. milk fat, milk protein, SNF and lactose percentage in the present study.

Table 4.3: Association between lactoferrin gene and milk fat percentage, milk protein percentage, SNF percentage and lactose percentage in Dangi cattle

Breed	Genotypes	n	Milk fat percentage (Mean±SE)	P value*
Dangi	AA	27	3.861±0.188	0.265
	AB	3	3.704±0.534	
	Genotypes	**n**	**Milk protein percentage (Mean±SE)**	-0.093
	AA	27	3.139±0.036	
	AB	3	3.250±0.086	
	Genotypes	**n**	**Milk SNF percentage (Mean±SE)**	-0.995
	AA	27	8.516±0.106	
	AB	3	8.846±0.254	
	Genotypes	**n**	**Milk lactose percentage (Mean±SE)**	-0.723
	AA	27	4.686±0.057	
	AB	3	4.818±0.159	

The present results are consistent with those of Maletic *et al.* (2013) in Holstein Friesian cows observed a non-significant association between polymorphism of intron 6 and milk fat percentage. Nanaei *et al.* (2016), also observed a similar type of association between the polymorphism and milk yield, milk fat % and milk protein %. Whereas, Zinnatov *et al.* (2020) found an association between the intron 6 polymorphism and milk production traits viz., milk yield, milk protein, milk fat, and SCCs in HF cows of the Ayvengo and Sovereign lines with the LTF^{AB} genotype. Lavanya K (2022) observed significant association between AB genotype and milk yield in Gir and HF crossbreed cows, no significant association with milk fat percentage.

However, research carried out on other regions of bovine lactoferrin gene by Khalil *et al.* (2021) observed three genotypes in the promoter region and found a significant association between the genotypes AA, AB and BB and milk fat %, and milk protein% in Friesian and Baladi cows. Further, they observed that cows with BB genotype had significant 180-day milk yield and milk fat yield, while cows with AA genotype had significantly higher in milk fat and protein yields.

The present study confirmed the polymorphic nature of intron-6 of bovine lactoferrin gene in Dangi cattle, with AA and AB genotypes in the population. The present findings may be useful in planning further breeding strategy of Dangi cattle. The association study showed non-significant relation between AA and AB genotypes with milk fat, milk protein, SNF and lactose percentage. It might be due to the smaller sample size (30) and out of 30 cows only 03 cows had polymorphism.

5. SUMMARY AND CONCLUSIONS

The present research work was carried out at the Genetic Investigation Laboratory, Department of Animal Genetics and Breeding, and Department of Livestock Product Management, Mumbai Veterinary College, Mumbai. The aim of the research was to identify the polymorphism in intron 6 of the bovine lactoferrin gene and determine whether there was any association between the polymorphism and the indicators of milk production, such as milk fat percentage, milk protein percentage, SNF percentage, and lactose percentage in Dangi cattle. Total 30 lactating Dangi cows from the Cattle Breeding Farm and Dangi Cow Research Station, Igatpuri, District Nashik and were tested for polymorphism and association study. The blood samples from 30 lactating cows for extraction of genomic DNA, while milk samples were collected fortnightly and processed at the Department of Livestock Product Technology, to estimate the milk fat percent, milk protein percent, SNF percent, and lactose percent.

The genomic DNA was isolated from the blood samples by using Phenol: Chloroform method. The range of DNA concentration ranged from 46.6 ng/µl to 152.7 ng/µl, and the OD ratio of samples ranged between 1.8 to 2. The DNA fragment of bovine lactoferrin gene intron 6 region was amplified successfully at an annealing temperature of 62^{o}C. Agarose gel (0.8 %) electrophoresis yielded the desired PCR product of 301 bp. Further, the PCR products were subjected to restriction digestion with *Eco*RI enzyme at 37^{o}C for 18 hours. EcoRI restriction digestion revealed two patterns; one band of size 301 bp and three bands of sizes 301 bp, 201 bp, and 100 bp. It confirms the polymorphic nature of the intron 6 bovine lactoferrin gene in Dangi cows. The genotypes of the cows were determined using the PCR-RFLP pattern as: AA (301 bp) and AB (301, 201, 100 bp), however, no existence of BB genotype was found in Dangi cattle population under investigation.

In 30 lactating Dangi cows, the AA (27 cows) and AB (03 cows) genotypic frequencies were 0.9 and 0.1, respectively. A and B allele gene frequencies in Dangi cows were 0.95 and 0.05, respectively. Overall, gene frequencies for alleles A and B supported the 'A' allele's dominance in the population of selected Dangi cows.

The average estimated milk traits in Dangi cows were; milk fat -3.85±0.18, milk protein - 3.15±0.03, SNF -8.55±0.10, and lactose percentage- 4.70±0.05. However, the average of Dangi cows with genotype AA were: milk fat-3.861±0.188, milk protein-3.139±0.036, SNF-8.516±0.106 and lactose percentage-4.686±0.057 and the average of Dangi cows with genotype AB were: milk fat-3.704±0.534, milk protein-3.250±0.086, SNF-8.846±0.254 and lactose percentage-4.818±0.159.

Association analysis revealed no statistically significant effect of the *Eco*RI polymorphism of the bovine lactoferrin gene's intron 6 on the milk fat and protein percentage as well as SNF and lactose percentage in Dangi cows.

On the basis of the results obtained following are the conclusions of the current research:

1. The intron 6 of the bovine lactoferrin gene was successfully amplified with the suitable primer at an annealing temperature of 62^{o}C, and the desired 301 bp PCR product was obtained in Dangi cow.
2. *Eco*RI/PCR-RFLP analysis of the bovine lactoferrin gene's intron 6, revealed two genotypes, AA and AB, and confirmed the polymorphic nature of the lactoferrin gene in Dangi cattle. However, the BB genotype was not found in the population of Dangi cows for the study.
3. The gene and genotypic frequencies of the lactoferrin gene indicated the predominance of ‘A’ alle in the Dangi cattle population.
4. The present study did not reveal a significant association between the genotypes (AA, AB) and milk traits, milk fat, milk protein, SNF, and lactose percentage in Dangi cattle.

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APPENDIX – I

List of chemicals used and their preparation

I. 0.5M EDTA Solution

EDTA Disodium Salt	186.1 gm
Dissolved in double distilled water	800 ml

Adjust the pH 8.0 using NaOH

Finally the volume was adjusted to 1000ml using double distilled water

II. 1M Tris Buffer

Tris Base	121.1 gm
Distilled Water	800 ml

Adjust pH 8.0 using Concentrated HCl

Adjust Volume up to 1000 ml using double distilled water.

III. RBC Lysis Buffer

Ammonium Chloride (NH4Cl)	8.3 gm
Potassium Bicarbonate	1.0 gm
0.5 M EDTA (pH 8.0)	299 µl

Double Distilled water up to 1000 ml. Autoclaved and stored at room temperature.

IV. DNA Extraction Buffer

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

Autoclaved in batches of 100 ml and stored at room temperature.

V. 10% Sodium Dodecyl Sulphate (SDS)

Sodium Dodecyl Sulphate	10 gm
Double Distilled water up to	100 ml

Adjust pH 7.2 using concentrated HCl

Heat in water bath till 60ºC to dissolve and then stored at room temperature.

VI. 3 M Sodium Acetate

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

Adjust pH 5.2 using Glacial Acetic Acid.

Autoclaved and stored at room temperature.

VII. 70% Ethanol

Ethanol	70 ml
Double distilled water	30 ml

Mixed thoroughly and stored in amber colored bottle as 4ºC

VIII. Tris Equilibrated Phenol

108952 SRL Chemicals

IX. Phenol:Chloroform:Isoamyl alcohol (25:24:1)

1624296 SRL Chemicals

X. Chloroform:Isoamylalcohol (24:1)

0381C015 SRL Chemicals

XI. Proteinase K

36331 SRL Chemicals

XII. Ethidium Bromide

Ethidium Bromide	10 mg

Add 1 ml distilled water, dissolve and store at 4ºC

XIII. 10X TAE

Tris base powder	48.4 gm
Glacial Acetic Acid	11.2 ml
0.5 M EDTA	20 ml

Dissolve in 800 ml deionized water and adjust pH 8.3 using NaOH. Finally, volume made 1000 ml using double distilled water.

XIV. Agarose Gel 2 per cent

Agarose powder	1 gm
1XTAE	50 ml

Solution is heated till the agarose powder is completely dissolved. 5 µl EtBr is added (1µl/10 ml solution). The solution poured into a tray with a comb attached and left to solidify for about half hour.

APPENDIX- II

Equipment’s used in the Present study

S. No	Name of Equipment’s
01	Refrigerated Centrifuge (Thermoscientific, USA)
02	Weighing balance (Sartorius, Germany)
03	Micro centrifuge (Minispin, Eppendorf, Germany)
04	Micropipettes (Eppendorf, Germany)
05	Spectrophotometer (NanoDrop ND-2000, Thermo, USA)
06	Gel electrophoresis apparatus (Technosource, India)
07	Power pack for gel electrophoresis (Biorad, USA)
08	Mastercycler nexus gradiet (Eppendorf, Germany)
09	Gel documentation system (UVP, UK)
10	Horizontal and Vertical laminar flow
11	pH Meter (LMPH-10, Labman Scientific Instruments)
12	Water bath with Circulator (Neolab Instruments)

THESIS ABSTRACT

1	Title of the thesis (In Capital letters)	**STUDIES ON LACTOFERRIN GENE POLYMORPHISM IN DANGI CATTLE**
2	Full name of student	**DHOND ANKUSH RAMESHRAO**
3	Name and address of Major Advisor	**Dr. M. P. SAWANE** Professor, Department of Animal Genetics and Breeding, Mumbai Veterinary College, Mumbai-400012.
4	Degree to be awarded	**M.V.Sc.**
5	Year of award of degree	2023
6	Major subject	Animal Genetics and Breeding
7	Total number of pages in the thesis	
8	Number of words in the abstract	340
9	Signature of Student	
10	Signature, Name and address of forwarding authority (HOD/SH)	.
11	Name and Signature of the Associate Dean	

ABSTRACT

The present study was conducted at the Genetic Investigation Laboratory, Department of Animal Genetics and Breeding and Department of Livestock Product Technology, Mumbai Veterinary College, Mumbai. The major objective of the present study was to identify the polymorphism of intron 6 bovine lactoferrin gene and its association with milk traits, viz., milk fat percentage, milk protein percentage, SNF, and lactose content. Total of 30 lactating Dangi cows were selected from Cattle Breeding Farm and Dangi Cow Research Station, Igatpuri, Dist. Nashik for the present research work. The milk samples from each cow were collected fortnightly and processed for the estimation of milk traits, and simultaneously blood samples were collected for the extraction of Genomic DNA. The genomic DNA was extracted from blood samples and subjected to quantification and quality testing. All the DNA samples were found to be of good quality with OD ratio 1.8 to 2. PCR amplification of intron 6 of the bovine lactoferrin gene was performed with a suitable primer. The amplified PCR product of size 301 bp was obtained at an annealing temperature of 62ºC. Restriction digestion of the PCR product with *Eco*RI enzyme at 37°C for 18 hours produced two band patterns in Dangi cows. On the basis of *Eco*RI-RFLP analysis pattern, the cows were genotyped as AA (301 bp) and AB (301, 200,101 bp). The BB genotype was found to be absent in the studied population of Dangi cattle. The genotypic frequencies of AA and AB in 30 Dangi cows were 0.9 and 0.1, respectively, and allelic frequencies for A and B were 0.95 and 0.05, respectively, in Dangi cows. Association studies revealed no significant association between the genotypes of intron 6 of bovine lactoferrin gene AA, AB, milk fat, milk protein, SNF, and lactose percentage.

The present study confirms the polymorphic nature of intron 6 of the bovine lactoferrin gene in Dangi cattle with no significant association between milk traits under study. The non-significant association may be due to the small sample size, but the findings will be useful in further genetic improvement programs in Dangi cattle.

प्रबंध सारांश

1	प्रबंधाचे शीर्षक (मोठ्या अक्षरे मध्ये):	डांगी गुरांमध्ये लॅक्टोफेरिन जनुक बहुरूपी पॉलीमॉर्फिझमवर अभ्यास
2	विद्याथ्याचे पूर्ण नाव:	धोंड अंकुश रमेशराव
3	प्रमुक मार्गदर्शकाचे नाव व पत्ता:	डॉ. म. प. सवाणे प्राध्यापक पशुअनुवंशिक व पैदास शास्त्र विभाग, मुंबई पशुवैद्यकीय महाविद्यालय, मुंबई
4	पदवी दिली जाईल:	एम. व्ही. एस. सी.
5	पदवी पुरस्काराचे वर्ष:	२०२३
6	मुख्य विषय:	पशुअनुवंशिक व पैदास शास्त्र विभाग
7	प्रबंधातिल पानाची ऐकूण संख्या:	
8	सारांशाचे ऐकूण:	२९४
9	विद्यार्थीयाची सही:	
10	प्रबंध पाठवणाऱ्या अधिकाऱ्याचे संपूर्ण नाव व पत्ता:	.
11	सहयोगी अधिष्ठता, मुंबई पशुवैद्यकीय महाविद्यालय, मुंबई	

सारांश

सदर संशोधन कार्य पशु अनुवंशिक अन्वेषण प्रयोगशाळा, पशुअनुवंश शास्त्र व पैदास विभाग आणि पशुधन उत्पादन तंत्रज्ञान विभाग, मुंबई पशुवैद्यकीय महाविद्यालय, मुंबई येथे करण्यात आला. संशोधन कार्र्याचे प्रमुख उदिष्ट इंट्रॉन 6 बोवाइन लैक्टोफेरिन जनुकाचे बहुरूपता ओळखणे आणि त्याचा दुधाच्या गुणधर्मांशी संबंध तपासणे (उदा., दुधातील स्निग्धांश टक्केवारी, दुधाची प्रथिने टक्केवारी, एसएनएफ आणि लैक्टोज टक्केवारी). सध्याच्या संशोधन कार्यासाठी डांगी गाय संशोधन केंद्र, इगतपुरी, जिल्हा नाशिक येथून एकूण 30 दुधातील डांगी गायींची निवड करण्यात आली. प्रत्येक गायीच्या दुधाचे नमुने पंधरवड्याने गोळा करून त्यांचे पृथकरण केले, व जीनोमिक (अनुवंशिक रचना) डीएनए काढण्यासाठी रक्ताचे नमुने गोळा केले गेले. जीनोमिक डीएनए रक्ताच्या नमुन्यांमधून वेगळे काढले गेले आणि त्याचे प्रमाण आणि गुणवत्ता चाचणी केली गेली. सर्व डीएनए नमुने OD गुणोत्तर 1.8 ते 2 सह चांगल्या दर्जाचे असल्याचे आढळले. बोवाइन लॅक्टोफेरिन जनुकाच्या इंट्रोन 6 चे पीसीआर प्रवर्धन योग्य प्राइमरसह केले गेले असता 301 bp आकाराचे प्रवर्धित पीसीआर उत्पादन 62°C च्या एनीलिंग तापमानात प्राप्त झाले. 18 तासांसाठी 37 डिग्री सेल्सिअस तापमानावर *Eco*RI प्रतिबंधित विघटनांद्वारे डांगी गायींमध्ये दोन प्रकारचे खंडनमुने तयार झाले. EcoRI-RFLP विश्लेषण नमुन्या आधारे, गायींना AA (301 bp) आणि AB (301, 200,101 bp) जीनोटाइप असे वर्गीकरण केले गेले. सदर अभ्यासलेल्या डांगी गायींमधे BB जीनोटाइप अनुपस्थित असल्याचे आढळून आले. 30 डांगी गायींमध्ये AA आणि AB ची जीनोटाइपिक वारांवरता अनुक्रमे 0.9 आणि 0.1 होती, आणि A आणि B साठी 0.95 आणि 0.05, डांगी गायींमध्ये ॲलेलिक वारांवरता आढळून आली. सदर संशोधनात बोवाइन लैक्टोफेरिन जनुक AA, AB, जीनोटाइप आणि दुधातील स्निग्धांश टक्केवारी, दुधाची प्रथिने टक्केवारी, एसएनएफ आणि लैक्टोज टक्केवारी या मध्ये कोणताही महत्त्वाचा परस्पर संबंध आढळला नाही.

सदर संशोधनाद्वारे डांगी गोवंशातील बोवाइन लॅक्टोफेरिन जनुकाच्या इंट्रॉन 6 च्या बहुरूपी स्वरूपाची पुष्टी मिळते आणि अभ्यासाधीन दुधाच्या वैशिष्ट्यांमध्ये कोणताही महत्त्वाचा संबंध नाही. सदर शोध कार्य हे केवळ ३० गायीवर करण्यात आल्यामुळे परस्पर संबंध प्रस्थापित झालेले आढळले नाही, परंतु डांगी गुरांमधील पुढील अनुवांशिक सुधारणा कार्यक्रमांसाठी सदर निष्कर्ष उपयुक्त ठरतील.

VITA

The author Mr. Dhond Ankush Rameshrao was born on Jan 1st, 1997 at Latur District of Maharashtra State.

He has completed his Secondary School Certificate (SCC) examination from Lal Bahadur Shastri Vidyalaya, Udgir and subsequently completed Higher Secondary Certification from Jaikranti Junior College, Latur.

After completion of school and intermediate college education he entered the portals of College of Veterinary and Animal Sciences, Parbhani as a student of 2015 batch and obtained his Bachelor of Veterinary Sciences & Animal Husbandry (B. V. Sc. & A.H.) degree from Maharashtra Animal and Fishery Sciences University, Nagpur in 2020.

Being interested, he joined the joined the Department of Animal Genetics and Breeding at Mumbai Veterinary College in March 2021 for pursuing his M.V.Sc. and successfully completed his research titled “Studies on lactoferrin gene polymorphism in Dangi Cattle” as a part his research work.