COMPARATIVE POLYMORPHIC ANALYSIS OF LYSOZYME GENE VARIANTS (LYZ) IN TWO BREEDS OF CATTLE

(*Holstein friesian&Bostaurus taurus*)

A DISSERTATION SUBMITTED TO ST. TERESA'S COLLEGE (AUTONOMOUS), ERNAKULAM IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF

DEGREE OF MASTER OF SCIENCE IN ZOOLOGY

SUBMITTED BY.
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CERTIFICATE

This is to certify that the dissertation entitled **"Comparative polymorphic analysis of lysozyme gene variants in two breeds of cattle. (*Holstein friesian* & *Bos taurus taurus*)"** is an authentic record of original project work carried out by Ameesha Grace MP (Reg No: SM23ZOO002), during the Academic year 2023-2025, under my guidance in partial fulfilment of the requirement of the Degree of Master of Science in Zoology from St. Teresa's College (Autonomous), Ernakulam.

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BONAFIDE CERTIFICATE

Certified that this thesis titled, **"Comparative polymorphic analysis of lysozyme gene (LYZ) gene variants in two breeds of cattle (*Holstein friesian* & *Jersey*)"** is a bonafide work of **AMEESHA GRACE, Reg no: SM23ZOO002**who carried out the Research under my supervision. Certified further, that to the best of my knowledge, the work reported herein does not form part of any other thesis or dissertation on the basis of which a degree or award was conferred on an occasion on this or any other candidate.

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I hereby declare that the dissertation entitled **"Comparative polymorphic analysis of lysozyme gene (LYZ) variants in two breeds of cattle. (*Holstein friesianand Bostaurus taurus)*"** submitted to St. Teresa's College (Autonomous),Ernakulam in partial fulfilment of the requirements for the award of the Degree of Master of Science in Zoology is a record of original research work done by me under the supervision and guidance of Dr.Reema Kuriakose , Associate Professor, Department of Zoology, St. Teresa's College (Autonomous),Ernakulam and to the best of my knowledge and belief, this project contains no material previously published or written by another person, except where due reference is made.

AMEESHA GRACE M P

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AMEESHA GRACE MP

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LIST OF ABBREVATIONS

1.	%	Percentage
2.	β	Beta
3.	°C	Degree Celsius
4.	Eg.	Example
5.	et al.	And other
6.	Etc.	And similar things
7.	Hrs	Hours
8.	Mins	Minutes
9.	g	Gram
10.	pH	Power of Hydrogen
11.	LYZ	Lysozyme gene
12.	PCR	Polymerase chain reaction
13.	Secs	Seconds
14.	ml	Millilitres
15.	UV	Ultraviolet
16.	EDTA	Ethylenediaminetetraacetic acid
17.	µl	Microlitres
18.	approx	Approximately
19.	HF	*Holstein friesian*
20.	Jersey	*Bostaurustaurus*
21,	NCBI	National centre for biotechnology information
22.	DNA	Deoxyribonucleic acid

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1. ABSTRACT

Lysozyme is an antimicrobial enzyme produced by animals that forms part of the innate immune system. It exerts antimicrobial activity against microorganismsespecially grampositive bacteria; by hydrolyzing 1,4 β linkages between N-acetyl muramic acid and N-acetyl glucosamine. Lysozyme is also a potential genetic marker of natural resistance process. The current study aims to identify the comparative polymorphic analysis of Lysozyme (LYZ) variants in different breeds of cattle. Blood samples were collected from two breeds of cattles ;*Holstein friesian*(HF) and *Bostaurustaurus*(Jersey). Primer was synthesised from the NCBI website and the FASTA sequence of the particular gene was obtained. Using QIAGEN KIT the DNA was isolated from each breed. The isolated DNA was amplified using Polymerase chain reaction. Then it was observed using agarose gel electrophoresis. To study the mutations the DNA was subjected for sequence analysis using Sanger's method. Then the reference sequence was compared with the mutation sequence to study the single nucleotide polymorphism in each breeds of cattle. It was found that there was six single nucleotide mutations, three in each breed used for the study. In *Holstein friesian* there were mutations in the 6353^{th}, 7062^{th} and7506^{th} positions. In *Bostaurustaurus* there were mutations in the 6383^{th}, 6514^{th}, and 7355^{th} positions. Single nucleotide polymorphism can affect the innate immunity of these two breeds of cattle. In case of these the mutations compromises the functionality of lysozyme, cattle would become more vulnerable to bacterial infections like mastitis, pneumonia, and enteric disease. It can also bring changes in innate and adaptive immunity becausethe gene for lysozyme plays a central role in innate immunity. Mutations in it would indirectly influence the effectiveness of other immune processes like macrophage activation and cytokine secretion.The presence of an inefficient lysozyme may disrupt the equilibrium of gut microbiota, which could result in gastrointestinal problems and systemic infections. Most importantly cattles with SNPs are susceptible for mastitis in the future.

Among the two breeds of cattle studied, *Holstein friesian* cattle generally have higher expression levels of lysozyme gene compared to *Bostaurustaurus*. Due to their higher lysozyme gene expression they show more active enzymatic defence mechanism. But more active mastitis resistance is found in Jersey cattle despite of comparatively low lysozyme gene expression but because of their greater natural resistance, including more robust immune system, and high fat and protein content in milk.We cannot declare that SNPs is the only reason for developing mastitis as it can also happen due to other reasons like bacterial infections and poor management.

2.INTRODUCTION

The immune system protects the body from pathogens and includes defence mechanisms against the majority of microorganisms in addition to a targeted and highly precise reaction to a specific invader. There are two types of immune responses: innate, which is non-specific, and adaptive, which is extremely particular. The body always defends itself against a disease in a similar way thanks to the innate reaction, which is frequently our first line of protection against anything alien. The reticuloendothelial system, the skin barrier, tears, saliva, different cytokines, complement proteins, lysozyme, bacterial flora, and a variety of cells, such as neutrophils, basophils, eosinophils, monocytes, macrophages, NK cells, epithelial cells, endothelial cells, red blood cells, and platelets, are examples of these natural mechanisms.

Lysozyme, an anti-bacterial enzyme, is mostly found in the body fluids, various tissues and secretions of animals and humans, and confers immunity against a wide range of bacterial species. As a vital component of the innate immune system, lysozyme has potent antibacterial properties that combat viral, fungus, and bacterial infections. It fortifies the immune system, works as a natural antibiotic, and increases the effectiveness of other medicines in addition to providing infection prevention. Lysozyme damages or kills bacteria by lysing their cell wall peptidoglycan, rupturing bacterial membranes, and activating autolytic enzymes within the bacterial cell wall. It does this by acting as both a lytic enzyme and a tiny cationic protein. Lysozyme breaks down the peptidoglycan in the bacterial cell wall by targeting, hydrolysing, and dissolving the mucopolysaccharide component of the peptidoglycan. In a similar vein, chitin's glycosidic linkages can be broken by this enzyme. One component of the mammary gland's non-specific defensive mechanism is the enzyme lysozyme. Despite its extremely low concentration in milk, it is a regular component that affects the udder's defence against infectious agents and general health. As a result, an overview is provided of the variables affecting the amount of lysozyme in cow's milk, its importance for the bactericidal properties of milk, how it affects mastitis and thus may be used in diagnostic procedures, and the therapeutic applications of lysozyme-rich milk.

The lysozyme gene in cows serves as a vital tool in improving their immune defence mechanism, especially against bacterial infections. Lysozyme is an enzyme that degrades the peptidoglycan layer of the bacterial cell wall, thus serving as a natural antibacterial factor. In cows, this gene is particularly vital in defending the udder against infection caused by mastitis, a prevalent and economically significant disease in the dairy sector. Breeds with increased expression of lysozyme are more resistant to such infections, enhancing herd health and milk yield. The function of lysozyme in maintaining the health of the gut by regulating the population of harmful bacteria also enhances digestion and nutrient uptake. Efficient lysozyme gene variants may therefore improve resistance to diseases, lower veterinary expenses, and enhance productivity in cattle rearing.

Although the lysozyme gene is important in the immune protection of cattle through the degradation of bacterial cell walls, there are some disadvantages that can be experienced as a result of genetic mutations or variations in the gene. Single nucleotide polymorphisms (SNPs) in the lysozyme gene have the potential to decrease the enzymatic activity of the

gene, thus compromising the animal's resistance to bacterial infections such as mastitis. Bovine breeds with compromised lysozyme activity are likely to show increased susceptibility to such infections, affecting their health and productivity in terms of milk production. Additionally, lysozyme gene mutations can hinder proper immune function, increasing the vulnerability of cattle to environmental pathogens and stress factors. Genetic variations in certain instances can also influence growth rates and metabolic energy efficiency, further decreasing animal productivity in commercial agricultural systems. Thus, though the lysozyme gene is crucial to immunity, impaired function of this gene can lead to serious risks to cattle health and economic efficiency.

The breeds of cattle included in this study are *Holstein friesian* and *Bostaurustaurus*.

Originating on Jersey, one of the Channel Islands, the Jersey breed of small short-horned dairy cattle is believed to have descended from French cattle. The Jersey is typically of a fawn or cream colour, although darker shades are also common. In the late 18th century, regulations were enacted to prohibit the importation of cattle into Jersey, except for immediate slaughter. By the early 19th century, the indigenous breed was officially recognized as pure. Initially introduced into England in large numbers, with one of the earliest herds established in 1811, Jersey cattle have since been distributed worldwide. The first recorded exportation of registered Jerseys to the United States occurred in 1850. Known for their adaptability to various conditions, Jersey cattle are sought after for their remarkably rich butterfat content in milk. Consequently, these animals are highly valued for crossbreeding with native stock to improve butterfat percentages. They play a significant role in regions where butter is a primary product, such as in New Zealand.

Originating in the northern provinces of North Holland and West Friesland of the Netherlands, the *Holstein-friesian*is recognized as the largest and most popular dairy breed, known for its exceptional milk production capabilities. Historically, the breed exhibited both black and white or red and white coloring, but the black and white characteristics became predominant after 1750 due to selective breeding practices. Despite this, the gene for red coloring persists within the breed. Grazing in the fertile polders of North Holland led to the selection of cows with high genetic merit for milk production. Notably, several Holstein-Friesian cows have achieved remarkable milk yield of over 27,000 kg in a 305-day lactation period within the last three decades.

Considered by many experts as the paramount breed of cattle globally, the Holstein–Friesian breed is highly specialized in the conversion of feed into protein for human consumption. These bovines are distinguished by their distinctive black-and-white color pattern, with well-defined spots that can vary in proportions. Despite the presence of the red allele in small frequencies, Holstein cattle are renowned for their exceptional milk production, averaging between 25 to 35 kg per day. Although some breeds may exhibit higher percentages of fat, protein, and solids, the overall milk quantity of the Holstein remains unparalleled. Moreover, these cattle are notably large, weighing between 600 to 800 kilograms.

Comparative polymorphism analysis of Lysozyme gene (LYZ) variants in two breeds of Cattles is the focus of this work. The organism’s capacity to respond to specific infections may be impacted by this gene’s polymorphism. This may result in low milk output, poor milk quality, and issues being conceived. Humans who consume milk and dairy products produced from animals with lysozyme gene variants may also be impacted by the mutation. These

cattle, which are genetically modified organisms (GMOs), are a major issue because they may have an impact on the ecosystem and the entire food chain. The cattles with single nucleotide polymorphism (SNP) are susceptible for developing mastitis disease in the future. Mastitis is an inflammation of the mammary gland in cows that can be caused by trauma or infection. It's a common and costly disease in dairy cows. Mastitis in cattle is a multifactorial disease influenced by both genetic and environmental factors. While single nucleotide polymorphisms (SNPs) in the lysozyme gene can weaken the immune response and contribute to increased susceptibility, they are not the sole cause for mastitis. Cattles with SNPs are more prone to respiratory and other bacterial infections. Certain cattle breeds may show genetic variations that either enhance or impair their resistance to infections, impacting overall herd health, milk yield, and meat quality. Therefore, understanding these polymorphisms is essential for developing selective breeding strategies aimed at improving disease resistance and ensuring better livestock management.

3.AIM AND OBJECTIVE

AIM OF THE STUDY:

To study the comparative polymorphic analysis of Lysozyme gene (LYZ) variants in two breeds of cattle. (*Holstein friesian&Bostaurus taurus*).

OBJECTIVE OF THE STUDY:

- To design and synthesise primers specific for lysozyme gene in two breeds of cattle.
- To amplify the PCR product of lysozyme gene.
- To analyse the mutations in lysozyme gene using Sanger sequencing method.

RELEVENCE OF THE STUDY:

By studying the single nucleotide polymorphism of lysozyme gene in two breeds of cattle (*Holstein friesian&Bostaurus taurus*) it helps to identify how the mutations can affect the immunity in these cattles. Mutations in lysozyme gene can cause mastitis like bacterial diseases, respiratory diseases and enteric diseases. Lysozyme is the gene which have bactericidal activity. Thereforethese mutations can induce a susceptibility of developing mastitis, a bacterial infection in the future. This will reduce their resistance towards diseases and also affect their milk production including milk yield and milk quality.

Thereforeunderstanding these polymorphisms is essential for developing selective breeding strategies aimed at improving disease resistance and ensuring better livestock management.

4.REVIEW OF LITERATURE

The lysozyme gene is a vital component of the innate immune system of cattle, and it plays an important role in disease resistance, especially in the fight against mastitis. Lysozyme is a bactericidal enzyme with the capability to hydrolyze the peptidoglycan layer of bacterial cell walls, thus reinforcing the natural defense mechanism of the animal. Genetic variations in the lysozyme gene, including single nucleotide polymorphisms (SNPs), have been associated with variations in immune response, which could play a role in susceptibility to mastitis—a prevalent and economically important disease of dairy cattle. Further, levels of lysozyme in milk have been found to differ according to physiological stages, including colostrum production and mastitis infection, confirming its function in milk quality and production characteristics. This review seeks to investigate the genetic diversity of the lysozyme gene and its effect on bovine immunity, resistance to mastitis, and milk production, giving clues to its usefulness as a genetic marker for breeding in cattle.

Several studies were conducted to examine the relationship between lysozyme and mastitis, lysozyme and milk production, lysozyme and its role in immunity and about lysozyme gene in cattle itself. Some of the studies are mentioned below:

4.1 LYSOZYME AND MASTITIS

Studies were conducted to analyse relationship between lysozyme gene and mastitis resistance. These studies mainly focused on the potential of co-expressing two important genes, lactoferrin and lysozyme, which could be used to confer mastitis resistance and improve udder health of cows. Molecular characterization of natural gene variants was performed to identify alleles with high performance and the study included transgenic experiments to explore the application potential of the identified genes to prevent mastitis effectively under physiological conditions. Various studies indicate that use of lactoferrin and lysozyme in genetic strategies can enhance bovine udder health and disease resistance. (Seyfert et.al.,1996).

Sahooet.al,(2012)executed toexplore lysozyme as a genetic marker for disease resistance, particularly in relation to mastitis. In livestock, two primary types of lysozyme exist: g-type (goose-type) and c-type (chicken-type), distinguished by their origin, structure, and enzymatic properties. The enzyme plays a significant role in antibacterial defense, with its gene structure and biological functions. This study also focused on modulating lysozyme's antimicrobial activity by controlling its gene expression in both native and recombinant forms.

Similarly, research was carried out in transgenic cows to produce antimicrobial peptides that have increased resistance to mastitis disease. Scientists used zinc-finger nucleases (ZFNs) to create targeted double-strand breaks, allowing for the efficient integration of the human lysozyme (hLYZ) gene into the bovine β-casein locus. The milk produced from these cows

contained antibacterial activity against *Staphylococcus aureus.* This study points to a genetic engineering approach to creating disease-resistant livestock, with potential benefits to both animal health and dairy industry productivity. (Liu et.al.,2014).

Likewise,Osman et.al. (2010) conducted investigations to study the bovine udder reaction through the measurement of nitric oxide (NO) and lysozyme concentrations in infected mastitis milk samples of cattle due to *Clostridium perfringens*. The results showed a significant relationship between increased NO and lysozyme concentrations with the severity and development of the disease especially in subclinical and clinical forms. Acute mastitis induced by *C.perfringens* tends to be followed by gangrenous mastitis where there is immediate swelling blue-black discoloration, and a sharp boundary between necrotic and healthy tissue. The infected area turns cold, with peeling skin, exuding serous fluid, and milk being bloody and watery, pointing towards the seriousness of this infection

In a similar way, Goudswaard et.al,(1978) conducted experiment to study the preliminary results of lysozyme estimations in bovine milk, results show that lysozyme levels are higher in colostrum and mastitis milk than in normal milk. It was because lysozyme show antibacterial resistance against mastitis disease.

4.2 LYSOZYME AND MILK PRODUCTION

Several studies were conducted to elucidate the gene sequence of lysozyme enzyme in Indian Sahiwal & Holstein Friesian crossbred cattle and to explore the polymorphism of the gene as well as their milk production and somatic cell traits. This study found out single stranded confirmation polymorphism which had significant association with total milk yield, daily milk yield, and somatic cell score. (Salehin et.al.,2009).

Similarly, studies were executed to investigate the potential of transgenic technology to improve the quality of milk and to produce biopharmaceutical within the mammary gland. This study found that transgenic cow milk have similar nutritional benefits as human milk and further technologies can improve the quality of milk. (Yang et.al.,2011).

Likewise, Krol et.al,(2010) performed a study by examining the antibacterial protein content of bovine milk compared immunoglobulin G (IgG), lactoferrin, and lysozyme concentrations in four breeds of cows raised under intensive production systems in Poland. The research identified breed-based differences in protein concentration that Simental and Jersey cows proved to have maximum levels of lactoferrin, lysozyme, and IgG, reflecting their better contribution toward antibacterial protection in milk.

Similarly, studies were managed to confirm the potential of genetic engineering in enhancing milk quality and udder health by minimizing bacterial proliferation. Lysozyme was present in the transgenic milk and it hadbacteriostatic activity against major dairy pathogens like *Escherichia coli,Staphylococcus aureus, and Pseudomonas fragi* but did not affect the growth of Lactococcus lactis, a cheese-producing organism. This study proved how genetic engineering can enhance the potential of milk safety, quality and human health. (Maga et.al.,2006).

4.3 LYSOZYME AND IMMUNITY

Grun et.al. 1985) conducted studies to analyse the enzyme lysozyme as one of the factors of the non specific defence mechanism of the mammary gland. It represents a regular constituent of milk, which despite its very low content in milk determines the health condition of the udder and its defending ability against infectious agents. Therefore, lysozyme is significant for the bactericidal effects of milk, its changes can result in mastitis.

In similar fashion, studies were executed to determine breed specific variations in serum lysozyme and complement activity in cows raised in various Bulgarian regions. It was found lysozyme as one of the principal factors to attain natural immunity. It has bacteriocide effect on gram positive bacteria and some gramnegative bacteria due to its lytic, cationic, and hydrophobic properties. (Sotirov et.al.,2007).

Kaiser et.al,(2017)performedstudies to understand the relationship between lactoferrin and lysozyme based on their strong antimicrobial properties and being vital components of the nonspecific defense system in human milk. Due to the limited availability of human milk, this study aimed to introduce both genes into bovine milk. The resulting transgenic milk displayed lysozyme activity comparable to human milk and nearly ten times higher than bovine lysozyme. This study marked significant progress in enhancing protein production through animal pharming. It also contributed to designing humanized milk with improved biological value for infant nutrition and development

Similarly, investigations were conducted to introduce genetic engineering to improve agricultural animals. The application involved the production of altered or novel proteins in the milk of transgenic dairy animals. Human milk contains high levels of antimicrobial proteins like lactoferrin and lysozyme. Transgenic animals that can synthesise these proteins were produced for a sustainable livestock management that can contribute to immunity of these animals. (Cooper et.al.,2015).

Perssonet.al,(1992) conducted studies to investigate the relationship between Immunoglobulins (Ig) and antibacterial proteins like lysozyme and lactoferrin and their importance in the humoral defense mechanism against infections. The study concluded that Ig most probably comes from both serum and local plasma cells, whereas lysozyme seems to be synthesized by leukocytes in inflammation, and lactoferrin is secreted by the mammary epithelium.

Likewise, investigations were pursued for analysing investigating the role of lysozyme in killing and lysing coliform bacteria in bovine milk. Tests to determine serum lysis of coliform bacteria revealed that there was no association between bacterial sensitivity to serum killing and lysozyme-mediated lysis. Although EDTA increased serum lysis in the presence of lysozyme, lysozyme alone had no direct lytic action. These results indicate that lysozyme might have a minimal role as a protective agent against coliform bacteria in vivo. (Carroll 1979).

Similarly Ferraboschiet.al,(2021) conducted experiments to examine the lysozyme amino acid sequences and enzymatic characters obtained from cow and sheep kidney homogenates. The study analysed the property of lysozyme to be used as an antibiotic. Also it focused on the applications of lysozyme in medicine, (the treatment of infectious diseases, wound healing, and anti-biofilm), veterinary, feed, food preservation, and crop protection.

Other experiments were directed to detect the lysozyme in tears of llama, sheep and cattle. Results of spectrophotometric assay suggested that llama and sheep tears had high concentrations of lysozyme, whereas cattle tears had low concentrations. The study concluded that the lysozyme concentration in tears may vary among species and this may contribute differing susceptibilities to ocular diseases such as infectious keratoconjunctivitis. (Juliet et.al.,2000)

4.4 LYSOZYME GENE

Studies were conducted to observe the function of lysozyme gene extracted from purified fresh raw bovine milk. It was found that the isolated enzyme exhibited a specific activity of 0.35 compared to 1.0 for egg-white lysozyme and a purification of 36,000 times on a milk protein basis. Salts were necessary for the enzyme to function, and sodium citrate was the most effective activator. (Chandran et.al.,1995).

Several experiments were executed to study the polymorphism of selected genes TNF-α, LTF, Mlyz for possible interactions between the genetic variants of the selected genes in determining various SCC values in milk. The study involved 171 Jersey cows. The results presented the existence of complex functional interactions between lactoferrin, lysozyme and tumor necrosis factor and suggest that the alleles of the genes that encode them might interact with each other. (Wodjak et.al.,2012).

Similarly, Studies were doneto find how lysozyme is regulated at the surface of the respiratory tract. The results indicated that multiple lysozyme mRNAs are expressed in the cow trachea and that the lysozyme encoded by cDNA 5a is the major form expressed in the tracheal gland serous cell. The sequential differences show resistance to proteolysis and affinity for large polyanions found in the respiratory tract lumen. The study concluded as explaining the major role of lysozyme to anticipate bacterial invasion at body surfaces and explaining the serous gland cell as the primary source of the enzyme. (Takeuchi et.al.,1993).

Also, experiments was done to evaluate clones from a cow stomach cDNA library using a lysozyme cDNA probe in order to look into the genesis of stomach expression of lysozyme in ruminants. In that library, 10% of the clones were lysozyme specific. Seven different forms of lysozyme mRNA sequences were discovered when thirty of the clones of lysozyme were sequenced. It was found gene duplications that happened throughout the evolution of ruminants are responsible for the many lysozyme genes produced in the stomach of cows. (Irwin et.al.,1989).

Likewise, Lie et.al,(1989) conducted experiments to study the lysozyme activity in serum and colostrum of primiparous cows using lysoplate method. Samples from 336 animals were collected. It was found a highly significant correlation between serum and lysozyme. There was no association between serum and colostrum lysozyme activity. Therefore the study concluded the genetic association between lysozyme activity in the two body fluids is exclusively reliant upon the chief gene defined. Therefore, collection of bulls for serum lysozyme activity will effect the colostrum or milk lysozyme activity in the cow population, providing that the major gene is seen in the population.

Other studies conducuted for the examination of restriction fragment length polymorphism (RFLP) of the lysozyme gene cluster in a Norwegian bovine family separating a single dominant Mendelian factor for high lysozyme activity in serum. The study discovered a genetic marker for the high lysozyme activity trait. (Olsaker et.al.,1993).

Steinhoff et.al,(1994)conducted experiments to analyse the functional Lyz action in bovine milk. The cDNA copies of a bovine lysozyme (bLys)-encoding gene (Lys) were isolated from libraries specific for granulocytes, as well as the lactating mammary gland. Through sequence comparisons, they found evidence that this segment has been removed during evolutionary divergence of the stomach Lys. Based on their observations,the thoughtful of Lys evolution were discussed. As well as the possibility, that the product of this gene may be responsible for the functional Lys action in bovine milk.

In similar fashion investigations were carried out to show the lysozyme activity in two half sub families of Polish Black and White Lowland cattle using alleles at a microsatellite locus within the macrophage that expressed lysozyme gene. The result concludes that the bimodal distribution of lysozyme activities in both progeny groups is concordant with the occurrence of the alternative paternal alleles. The microsatellite is linked to a locus for high lysozyme activity that accounts for 70±95% of the phenotypic variation of both offspring groups considering the lysozyme activities of animals being older than 1 month. (Pareek et.al.,1998).

Also, Studies were conducted to produce large scale production of an animal model of human lysozyme(rhLZ) by, considering the medicinal value and market demand for human lysozyme. . In this study, they generated transgenic cloned cows with the marker-free vector pBAC-hLF-hLZ, which was shown to efficiently express rhLZ in cow milk. These results provided a solid foundation for the large-scale production of rhLZ in the future. (Dan Lu et.al.,2016).

Along with that, experiments were conducted to look over some properties of the lysozyme in serum and colostrum from cows with high and low lytic power against *Micrococcus lysodeikticus***.**The work described the lytic characteristics of bovine serum lysozyme (BSL) and bovine colostrum lysozyme (BCL). The study concluded that the dramatic differences in lysozyme activity in high- and low-level animals are probably the result of genetic influences on enzyme amount and activity. This association between BSL and BCL activity further suggests that genetic mechanisms may play a role in regulating lysozyme expression and function. (Lie et.al.,1986).

5.METHODOLOGY

1.SAMPLE COLLECTION:

The blood samples of two cattle breeds (*Holstein Friesian &Bos taurus taurus*) were collected from the veterinary department of Veta Genomics

2.PRIMER SYNTHESIS:

Primer synthesis was the initial step in this work. From the NCBI website the FASTA sequence of the lysozyme gene was obtained. Later, the FASTA sequence was pasted in the PRIMER BLAST to obtain the primer product. Primers with single primer product wasselected. After that, using PRIMER STAT, the efficiency of the primer was studied. Primer without any warning was selected and out sourced for primer synthesis.

3.DNA ISOLATION:

The cattle breeds blood samples were used to isolate their DNA. The QIAGEN QiAmp Blood mini kit was utilized for isolation procedure. For the lysis procedure 20µlof protease enzyme was added to the vial. To this 200µl of blood sample was added. After adding the blood sample ,200µl of lysis buffer was also added. There were three distinct layers created. A vertical vortex was used to combine them into a single layer. Then the vial was incubated in a dry bath for ten minutes at 58°C. Precipitation followed the lysis. For precipitation 200µl of 100% ethanol was added. It was followed by vertical vortex. Later, the components in the vial are subsequently moved into the column tube. It underwent centrifugation at 8000rpm for a minute. After centrifugation the collection tube was discarded and replaced with a new one. Two washing buffers are then added to complete purification process. After adding 500µl of washing buffer (AW1), the mixture was centrifuged for one minute in 8000 rpm. The components gathered in the collection tube were disposed for following centrifugation. After that,500µl of washing buffer (AW2) was added and the mixture was centrifuged at 1400rpm for three minutes. A second cycle of centrifugation was carried out without the addition of any buffer (Empty Spin) after the components gathered in the colleting tube were removed. A micro centrifuge tube was later used to hold the column tube. To the vial 100µl of elution buffer (AWE) was added. It was then allowed to sit in a room temperature for five minutes. Then centrifuged at 8000rpm for a minute. Afterwards the concentration of DNA was measured using Qubit Fluorometer. To the Qubit tube 199µl of high sensitivity buffer and 1µl of DNA isolated was added. Then it is subjected to Pulse vortex and the tube was placed inside the Qubit fluorometer. The concentration of DNA was measured.

4.PCR:

The DNA products were amplified using the Polymerase chain reaction. The kit used was Carmine Taq PCR Kit. The components needed for the reaction was added to the vial. These elements consist of: Cattle breed's DNA, Forward and Reverse primer,Master mix, and

Nucleus free water. PCR was done in two days for each breed of the cattle. PCR was conducted for about two hours at 55°C.

5.GEL ELECTROPHORESIS:

The amplified DNA was subjected to agarose gel electrophoresis. 0.5g of agarose and1xTAE buffer were used to create agarose gel. It was heated for one minute and dye was added. It was moved to a gel rig, where the comb was positioned to create wells. The comb was taken out after an hour, and the gel was carefully put to the tank. This was followed by the addition of buffer solution to submerge the gel 2-5mm. Voltage was applied and the wells were positioned near the cathode. Other wells included the amplified DNA of two cattle breeds, whereas the first well featured a ladder. The DNA was size separated when the voltage was applied.

6.SEQUENCING:

Under the direction of Veta Genomics lab the DNA was sequenced from an external source.

6.RESULT

In the present study on the topic “Comparative polymorphic analysis of Lysozyme gene (LYZ)variants in two breeds of cattle; (*Holstein friesian&Bostaurus taurus*)” the results obtained are given below.

PRIMER DESIGNING AND SYNTHESISING

The primers for lysozyme gene of the cattle breed was obtained from NCBI Primer Blast. In the present study,Primer designing and synthesis ensures that only lysozyme gene is amplified from cattle DNA.This helps in obtaining clear and accurate PCR by the amplification of lysozyme genewithout amplification of non-specific or unwanted regions. The primers used for the amplification obtained were:

Forward Primer: TCCATGCCACCCTGTAGAGA

Reverse Primer: ATGGGATGGAATGAAGAGTTCCC

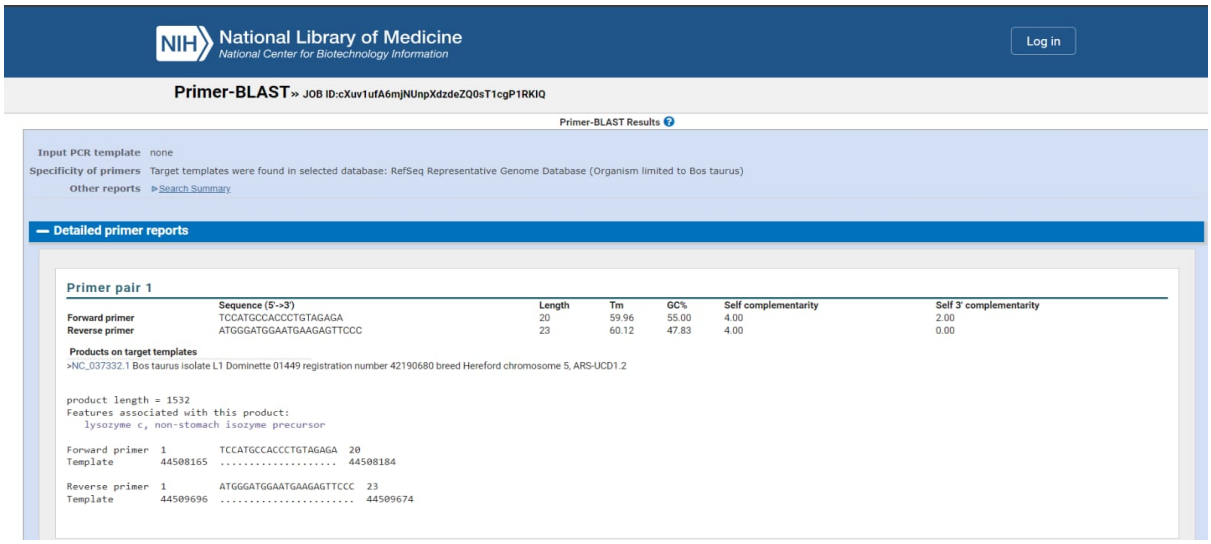


Figure 6.1: - Unique primer obtained from Primer BLAST NCBI for specific region of target gene with product length 1532.

A report, detailing each primer's characteristics, such as melting temperature, % GC content, and PCR compatibility is provided by PCR Primer Stats in exchange for a list of PCR primer sequences. To assess appropriate PCR primers, PCR Primer Stats is employed. Thus, the obtained primer satisfies the ideal parameters of PCR primer as mentioned in Figure 6.2.

PCR Primer Stats results

```
Global settings:
-The primers do not have a 5'-phosphate group.
-Combined concentration of K+ and Na+ in the reaction = 50 millimolar.
-Mg+2 concentration in the reaction = 1.5 millimolar.
-Primer concentration in the reaction = 200 nanomolar.

------------------------------------------------------------
------------------------------------------------------------
General properties:
-------------------
                    Primer name:  Forward
                Primer sequence: TCCATGCCACCCTGTAGAGA
                Sequence length: 20
                    Base counts: G=4; A=5; T=4; C=7; Other=0;
                 GC content (%): 55.00
     Molecular weight (Daltons): 6061.99
                      nmol/A260: 5.26
                micrograms/A260: 31.89
           Basic Tm (degrees C): 54
   Salt adjusted Tm (degrees C): 49
Nearest neighbor Tm (degrees C): 65.15

PCR suitability tests (Pass / Warning):
-----------------------------------
               Single base runs: Pass
         Dinucleotide base runs: Pass
                        Length: Pass
                    Percent GC: Pass
          Tm (Nearest neighbor): Warning: Tm is greater than 58;
                     GC clamp: Pass
                Self-annealing: Pass
              Hairpin formation: Pass
------------------------------------------------------------

------------------------------------------------------------


------------------------------------------------------------------
General properties:
-------------------
                        Primer name:  Reverse
                    Primer sequence: ATGGGATGGAATGAAGAGTTCCC
                    Sequence length: 23
                        Base counts: G=8; A=7; T=5; C=3; Other=0;
                     GC content (%): 47.83
     Molecular weight (Daltons): 7152.73
                          nmol/A260: 4.25
                    micrograms/A260: 30.40
               Basic Tm (degrees C): 55
   Salt adjusted Tm (degrees C): 50
Nearest neighbor Tm (degrees C): 65.24

PCR suitability tests (Pass / Warning):
-----------------------------------
                   Single base runs: Pass
             Dinucleotide base runs: Pass
                            Length: Pass
                        Percent GC: Pass
              Tm (Nearest neighbor): Warning: Tm is greater than 58;
                         GC clamp: Pass
                    Self-annealing: Pass
                  Hairpin formation: Pass
------------------------------------------------------------------
```

Figure 6.2: Primer STAT analysing the properties of forward and reverse primer.

SAMPLE PREPARATION: DNA ISOLATION

For the study, blood sample from two breeds of cattle(*Holstein friesian*&*Bostaurus taurus*)was obtained in an EDTA vial, and QIAGEN kit was used to isolate the DNA samples.

QUANTITATIVE AND QUALITATIVE ANALYSIS

Thermo Fisher Scientific's Qubit fluorometer analyses DNA concentration and measures it in ng/L. DNA sample concentration was 2.08 ng/µl and 10.2ng/µl for *Holstein friesian*&*Bostaurustaurus*respectively. The Qubit meter reading is in the Figure 6.3. Using Agarose Gel Electrophoresis the qualitative analysis of DNA was performed and was size separated.

Figure 6.3:- The Qubit fluorometer reading depicts the concentration of DNA was is 10.2 ng/µl for Jersey and 2.08ng/µl for HF.

POLYMERASE CHAIN REACTION

Carmine Taq PCR kit is used to carry out Polymerase Chain Reaction (PCR), which amplifies the target gene/gene of interest. DNA ladder is used in the Agarose Gel Electrophoresis, and the length of the PCR result is assessed,(Figure 6.4) the band had a length of approx.1538bp.

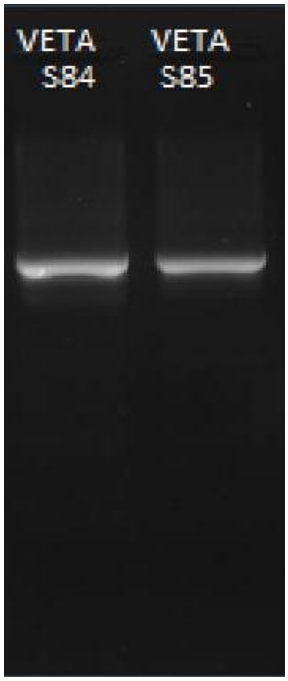


Figure 6.4:Agarose Gel Electrophoresis of amplified product by Amplification PCR. (VETAS84:HF, VETAS85:Jersey)

SANGER SEQUENCING

Bigdye x Terminator V3.1 sequencing kit was used to sequence the PCR product. As depicted in Figures, Sanger sequencing typically employs fluorescently labelled dideoxynucleotides that are detected by a laser following capillary electrophoresis to produce a sequence chromatogram with fluorescent peaks corresponding to incorporation of the four different fluorescent dyes coupled to ddATP, ddCTP, ddGTP, and ddTTP. It is useful for assessing the presence or absence of recurrent single nucleotide mutations or small insertions/deletions in a specific region of target gene.

SANGER SEQUENCE DATA ANALYSIS

To find any potential mutations, the Sanger Sequenced data was evaluated. Most often, a chromatogram in the.ab1 file type is the result of Sanger sequencing. Its identification as our target gene was confirmed by the FASTA format that was obtained from Nucleotide BLAST from NCBI. Finch TV analyses the quality of the sequences. It offers a graphical representation of chromatogram files, enables quick analysis and editing of DNA sequences, provides extra details about a sequence when accessing the nucleotide sequence, and allows to see the entire sequence as well as the total number of bases and lanes.

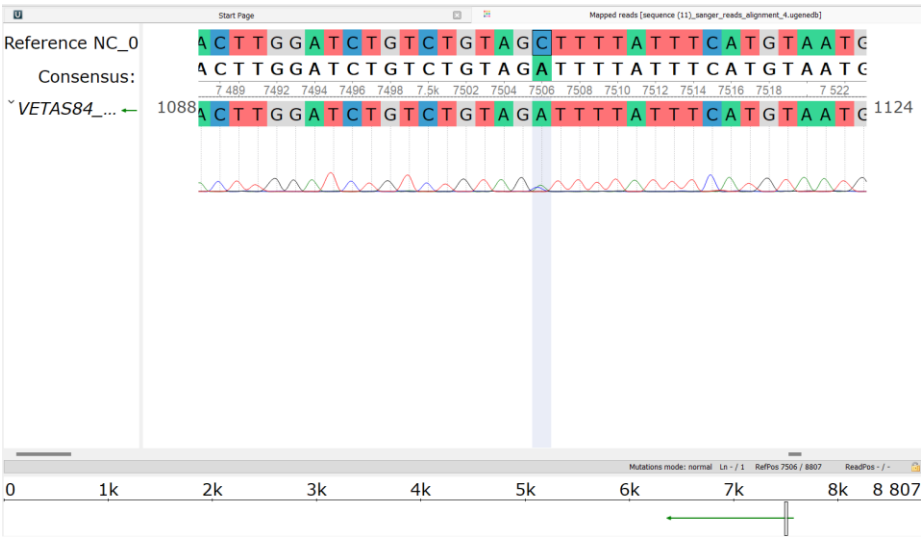
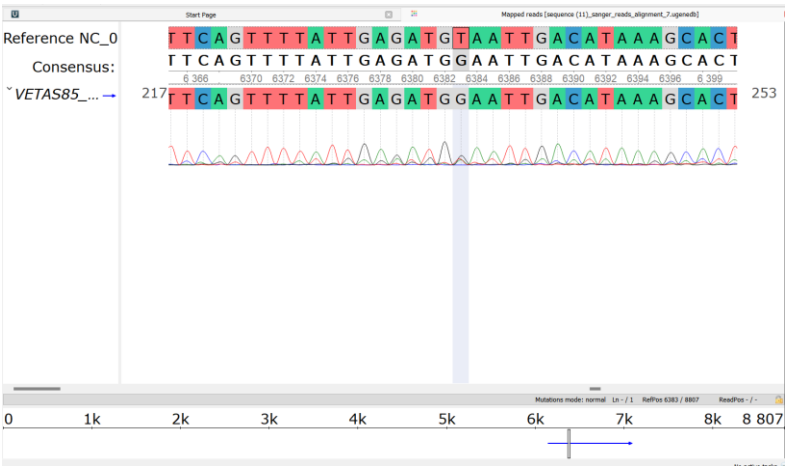
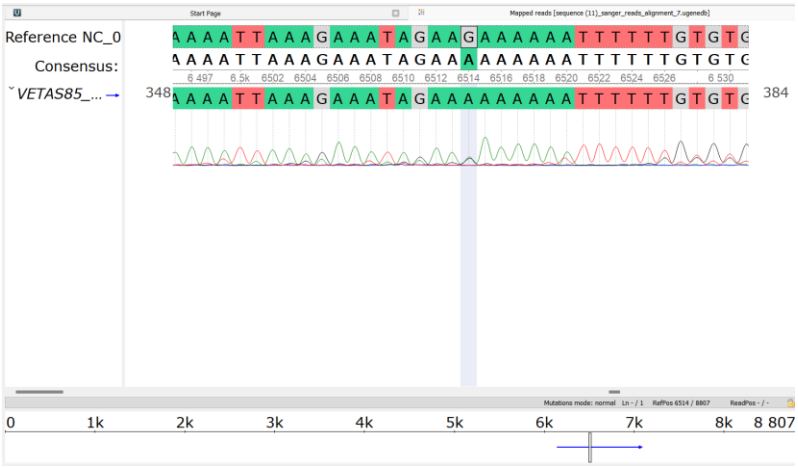


Figure 6.5: Analysis of Sanger Sequenced data by Multiple Sequence Alignment with reference sequence of cattle breed HF.





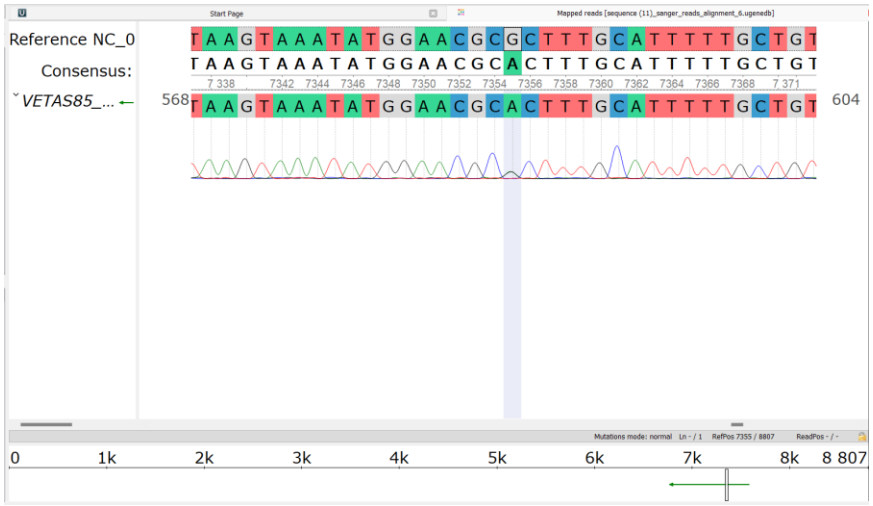


Figure 6.6: Analysis of Sanger Sequenced data by Multiple Sequence Alignment with reference sequence of cattle breed Jersey.

COMPARISON OF MUTATIONS IN LYSOZYME GENE OF TWO CATTLEBREEDS

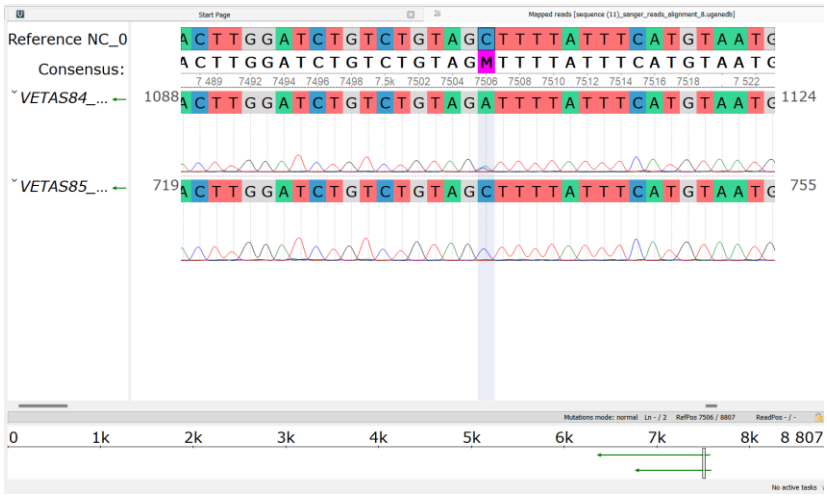


Figure 6.8: Mutation in 7506th position in HF.

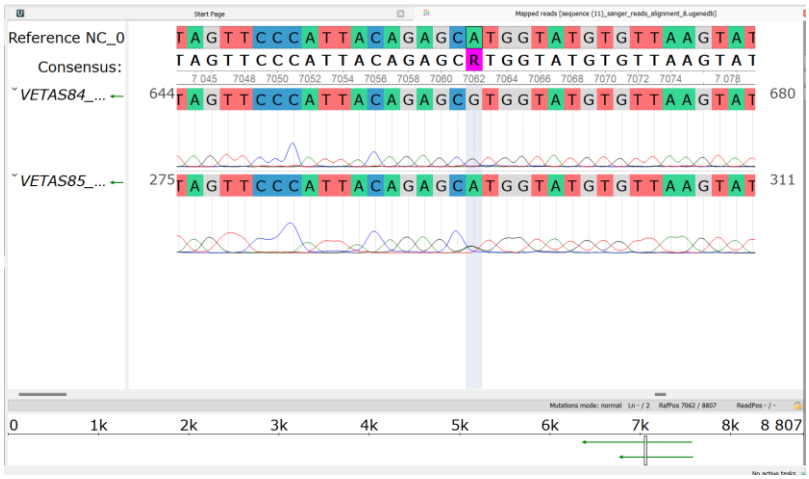


Figure 6.9: Mutation in 7062th position in HF.

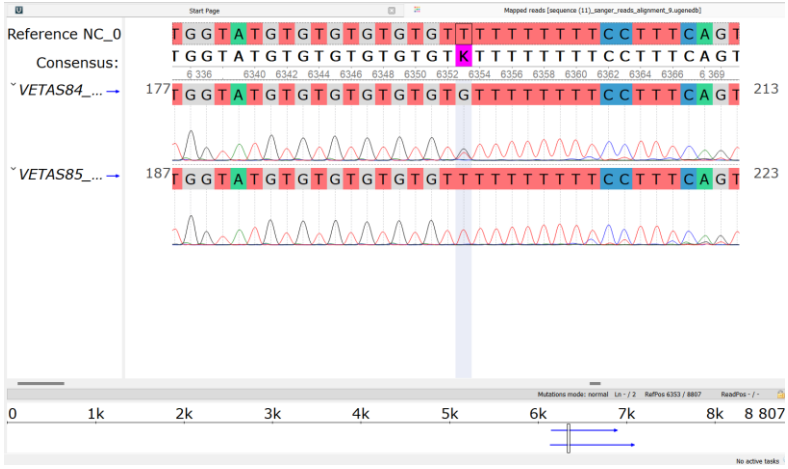


Figure 6.10: Mutation in 6353th position in HF.

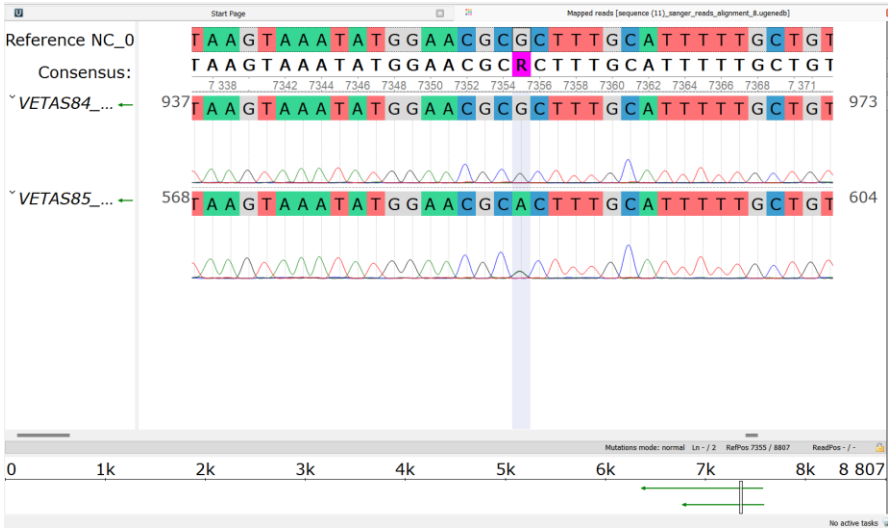


Figure 6.7: Mutation in 7355th position in Jersey.

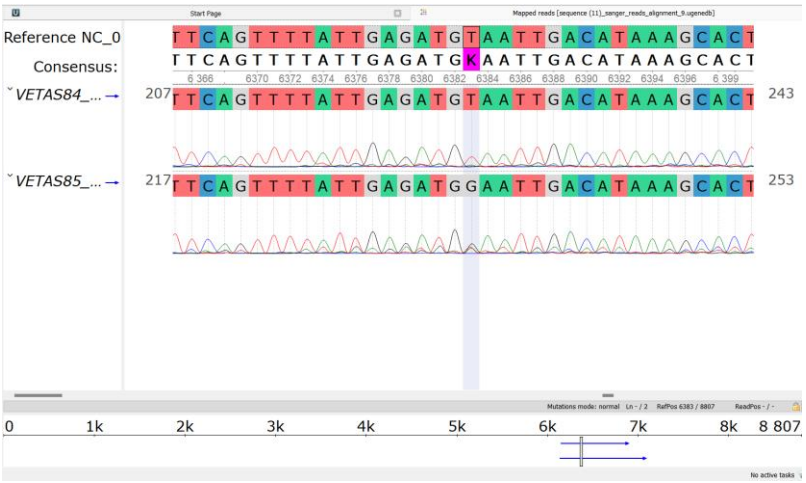


Figure 6.11: Mutation in 6383th position in Jersey.

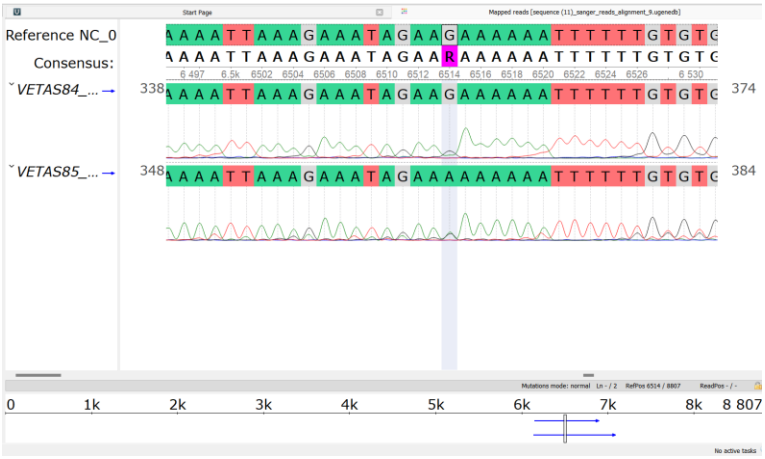


Figure 6.12: Mutation in 6514th position in Jersey.

Comparison of reference sequence and mutation sequence in both HF and Jersey. Mutations in single nucleotide sequence is marked in pink colour.

All sequence electropherograms were visually checked for quality and consistency before sequences were assembled and aligned using Unipro U GENE. The reference sequence and the mutation was analysed by Multiple Sequence Alignment. There were 6 single nucleotide mutations at positions 6353th,7062th, and7506th positions in HF and in 6383th, 6514thand 7355th positions in Jersey breed of cattle.

In HF breed of cattle (VETAS84) when the reference sequence and mutation sequence was compared there were three single nucleotide mutations was found. In the 6353th position it

was guanine instead of thymine. In the 7062th position it was guanine instead of adenine. In the 7506th position it was adenine instead of cytosine.

Also, in the Jersey breed of cattle (VETAS85) there were three single nucleotide mutations was discovered. In the 6383th position it was guanine instead of thymine. In the 6514th position it was adenine instead of guanine. In the 7354th position it was again adenine instead of cytosine.

The changes in the nucleotide sequences isthe reason for mutations. In this study of Comparative polymorphic analysis of Lysozyme gene (LYZ)variants in two breeds of cattle (Holstein Friesian and Jersey) the single nucleotide polymorphism wasexamined.

These mutations can induce drastic changes to protein production and regulation in genomic levels, and affect their immunity. These mutations can also make the cattle susceptible for developing mastitis disease, respiratory diseases and other bacterial diseases in the future.

7.DISCUSSION

In a study conducted to elucidate the gene sequence of lysozyme enzyme in Indian Sahiwal & Holstein Friesian crossbred cattle and to explore the polymorphism of the gene as well as their milk production and somatic cell traits. This study found out single stranded confirmation polymorphism which had significant association with total milk yield, daily milk yield, and somatic cell score. (Salehin et.al.,2009). Here HF breed is crossbred with Indian Sahiwal it may have a different lysozyme composition compared to an individual HF breed. Thus in this study it was found there were single nucleotide mutations which may be a cause of developing mastitis in the future combining with low quality milk yield.

In an experiment conducted to study the preliminary results of lysozyme estimations in bovine milk, show that lysozyme levels are definitely higher in colostrum and mastitis milk than in normal milk. (Goudswaard et.al.,1978). It may be because of, lysozyme having bactericidal activity was more in concentrartion to provide resistance against Mastitisis disease ,a bacterial infection . SNPs might be a reason for affecting the bacterial resistance in cattle.

In a study it was found the enzyme lysozyme is one of the factors of the non-specific defense mechanism of the mammary gland. It represents a regular constituent of milk, which despite its very low content in milk determines the health condition of the udder and its defending ability against infectious agents. Therefore, lysozyme is significant for the bactericidal effects of milk, its changes can result in mastitis.(Grun et.al.,1985). In the present study also, the SNPs may be a possibility of developing mastitis in cattles in the future.

In a study conducted to characterize two potential candidate genes (lactoferrin and lysozyme) for mastitis resistance and examine their relevance for an improvement of udder-health in cows. They developed defining candidate genes for mastitis resistance in cattle based on the role of lactoferrin and lysozyme. In the present study it hepls to analyse how single nucleotide mutations might be a reason to affect the udder health in cows.(Seyfert et.al.,1996)

Analysing single nucleotide transitions in the lysozyme gene of cattle and their contributions to immunity has profound and enlightening impacts in the study of genetic variation and its effects on genotype. Lysozymes are primary defensive enzymes in the body’s first line of defence against infection. They are responsible for the breakdown of bacteria as well as the action against other pathogenic microorganisms. Changes to the lysozyme gene result in changes to its structure and function which can have a bearing on the immune system response of the cattle.

Single nucleotide polymorphism in the lysozyme coding region could also lead to the substitutions of some amino acids with others, which may rearrange the stability and folding

of the enzyme or its activity. A change also depends on the type of change brought forward and it's position. Single nucleotide polymorphism can induce greater vulnerability to infections mastitis. In case the mutation compromises the functionality of lysozyme, cattle would become more vulnerable to bacterial infections like mastitis, pneumonia, and enteric disease. Also Changes in Innate and Adaptive Immunity like The gene for lysozyme plays a central role in innate immunity. Mutations in it would indirectly influence the effectiveness of other immune processes like macrophage activation and cytokine secretion. The presence of an inefficient lysozyme may disrupt the equilibrium of gut microbiota, which could result in gastrointestinal problems and systemic infections.

The comparative polymorphic analysis of the lysozyme gene variants in *Holstein friesian* and *Bostaurustaurus* cattle breeds reveals significant insights into genetic variation and its potential impact on immunity and disease resistance. The study identified mutations at key nucleotide positions, such as 7355, 6383, and 6514 in *Bostaurustaurus* and in 6353,7062, 7506 in *Holstein friesian*. Notably, adenine-to-guanine substitutions at these loci appeared to alter lysozyme protein structure, potentially impacting its antimicrobial properties. These mutations may affect the protein's binding affinity to bacterial peptidoglycan layers, thus influencing the overall immune response. It also make the cattles susceptible for mastitis like bacterial diseases in the future. In addition to genetic variation, environmental and management factors likely influence mastitis resistance.

Among the two breeds of cattle studies show *Holstein friesian* cattle generally have higher expression levels of lysozyme gene compared to Jersey cattle. to their higher lysozyme gene expression they show more active enzymatic defence mechanism. But more active mastitis resistance is found in *Bos taurus taurus*(Jersey) cattle despite of comparatively low lysozyme gene expression but because of their greater natural resistance,including more robust immune system, and high fat and protein content in milk.

8.CONCLUSION

In our study on the topic “Comparative polymorphic analysis of lysozyme gene variants in two breeds of cattle (*Holstein friesian*&*Bostaurus taurus*)” we successfully studied how single nucleotide polymorphism can effect the immunity of the cattles. As we all know, Innate immunity is essential in cattle since it is the first line of defence, and it is a quick, non-specific response to tissue damage and pathogens, usually averting the necessity for the slower, more specific adaptive immune response. The lysozyme gene contributes to the immunity in cattle. In cattle it also holds significance for both animal health and dairy production, as it encodes an enzyme with antimicrobial properties that could protect against bacterial infections like mastitis, a serious disease in dairy cows.

In this study we understood the different types of mutation that can happen to the lysozyme gene .This comprises generating primers using data gathered from NCBI, optimising PCR primers using gradient PCR, and showing specific amplification on Agarose Gel Electrophoresis at an annealing temperature of 60 °C. Based on the research, blood sample of the two cattle breed was prepared, and specifically the lysozyme gene was amplified, and it was Sanger sequenced to identify mutation, the mutation and examined by Unipro UGENE. Using specially designed primers, the mutations present in the lysozyme gene were found at the positions 6353^{th}, 7062^{th}, 7506^{th} in Holstein Friesian and at positions 7355^{th}, 6383^{th} and 6514^{th} in Jersey cow. (single nucleotide variation). Consequently, polymorphisms that alter the amino acid sequence may have an impact on the immunity.

The single nucleotide mutations can cause different bacterial, respiratory, and enteric diseases in cattle. Cattles with mutation in the lysozyme gene are susceptible for developing mastitis, a bacterial infection in the future. Because lysozyme which have the ability to break down the peptidoglycan cell wall of the bacteria will face a difficulty if there is a mutation happens in the nucleotide sequence. The SNPs can also affect the udder health, milk production including its quality and yield. Among the two breeds of cattle we have studied Holstein Friesian cattle generally have higher expression levels of lysozyme gene compared to Jersey cattle. Due to their higher lysozyme gene expression they show more active enzymatic defence mechanism. But more active mastitis resistance is found in Jersey cattle despite of comparatively low lysozyme gene expression but because of their greater natural resistance. Including more robust immune system, and high fat and protein content in milk.

We cannot state that the single nucleotide polymorphism is the only reason for developing mastitis in cattle. It is directly associated with bacterial infections. But SNPs could be a reason for developing mastitis. Further studies should be conducted to understand how lysozyme mutations can directly effect the immunity, health and milk production in cattles. Studies suggest that the enhancement of lysozyme expression in cattle, potentially through genetic engineering, could enhance milk quality and reduce the incidence of diseases like mastitis.

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