Molecular Characterization of Argininosuccinate Synthetase Gene in Holstein Friesian and Jersey Males

Thesis submitted to the
National Dairy Research Institute, Karnal
(Deemed University)
in partial fulfillment of the requirements
for the award of the degree of

MASTER OF SCIENCE
IN
ANIMAL BIOTECHNOLOGY

by

P. HARITHA

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ADUGODI, BANGALORE - 560030, INDIA

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Approved by

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CERTIFICATE

This is to certify that the thesis entitled, "**Molecular Characterization of Argininosuccinate synthetase gene in Holstein Friesian and Jersey males.**" submitted by **P. HARITHA** towards the partial fulfilment of the award of the degree of **MASTER OF SCIENCE in ANIMAL BIOTECHNOLOGY of the NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY), Karnal** (Haryana), India, is a bonafide research work carried out by her under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Dated: 29th June, 2013

(K. P. Ramesha)
MAJOR ADVISOR & CHAIRMAN

DEDICATION

This thesis work is dedicated to my husband, Ananth,who has been a constant source of support and encouragement during the challenges of Post graduate school and life. I am truly thankful for having you in my life. This work is also dedicated to my parents, who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

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Dated: 29th June 2013

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Molecular characterization of Argininosuccinate synthetase gene in Holstein Friesian and Jersey males

Abstract

In the present investigation PCR-RFLP and PCR-SSCP analyses were carried out in Holstein Friesian and Jersey males to screen for the presence of mutant allele of citrullinemia in the population and to characterize Argininosuccinate synthetase (ASS) gene. Blood samples for DNA extraction from 131 Indian Holstein and 78 Jersey bulls used for AI were collected. Genomic DNA was extracted using High salt method from blood.

Samples were tested by Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) to identify carriers of mutant allele of citrullinemia. PCR reaction was performed by using primers reported earlier for the amplification of polymorphic region of the exon 5 of ASS gene on chromosome 11. No carriers of citrullinemia were detected in this study.

All 15 exons of ASS gene were amplified by PCR using a total of 14 sets of primers. The genetic variants were determined by Single Strand Conformation Polymorphism (SSCP) analysis of amplified PCR products. The exons 1, 5, 6, 8, 9, 11, 12, 13 and 14 of ASS gene showed monomorphism. Two unique SSCP patterns with a genotypic frequency of 0.5954 and 0.4045 respectively were observed in exon 2 of ASS gene in HF breed. Two SSCP patterns with a genotypic frequency of 0.7179 and 0.2820 were observed in Exon 3 of ASS gene in Jersey breed. The PCR-SSCP analysis of Exon 4 of ASS gene in Jersey revealed two unique SSCP patterns with a genotypic frequency of 0.7820 and 0.2179, respectively. Two unique SSCP patterns with a genotypic frequency of 0.7099and 0.2900, respectively were observed in exon 7 of ASS gene in HF. Two SSCP patterns with a genotypic frequency of 0.7480 and 0.2519 were observed in HF and Two SSCP patterns with a genotypic frequency of 0.6282 and 0.3717 were observed in Jersey for Exon 10 of ASS gene. The PCR-SSCP analysis Exon 15 of ASS gene in HF revealed two unique SSCP patterns with a genotypic frequency of 0.9084 and 0.0916, respectively.

Based on the differences in the SSCP patterns, allelic variants were selected and corresponding PCR products were sequenced to confirm polymorphisms. Our sequences were compared to reference sequence of ASS gene in ENSEMBL for cattle by CLUSTAL-W multiple sequence analysis. The analysis of Exon 2 in HF revealed A→G transition at position 7947 of ASS gene. The observed polymorphism (A → G) at position 7947 resulted

in substitution of an amino acid from Isoleucine to Valine. The detected polymorphisms in exon 3 and 4 in Jersey cattle, exons 7 and 15 in HF cattle and exon 10 in both HF and Jersey cattle resulted in silent mutations.

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A	Adenine
A_{260}	Absorbance at 260 nm
APS	Ammonium Persulphate
ASS	Argininosuccinate Synthetase
ASSP	Argininosuccinate Synthetase Pseudogene
A_{280}	Absorbance at 280 nm
AMP	Adenosine Mono Phosphate
ATG	Start codon
ATP	Adenosine Tri Phosphate
Ava	*Anabena variabilis*
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Cytosine
CH_3	methylene
^oC	degree centigrade
CGA	codon for Arginine
CTLN	Citrullinemia
cDNA	complementary DNA
C terminal	carboxy terminal
cen	centromere
cM	centiMorgan
CO_2	Carbon dioxide
DDH_2O	Double Distilled Water
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate
ddNTPs	Dideoxyribonucleotide Triphosphate
dATP	Deoxy Adenosine Triphosphate
dCTP	Deoxy Cytidine Triphosphate
dGTP	Deoxy Guanidine Triphosphate
dTTP	Deoxy Thiamine Triphosphate
E.C	Enzyme Commission
EDTA	Ethidium Diamine Tetra Acetic Acid
EtBr	Ethidium Bromide

E.coli	*Escherichia coli*
G	Guanine
HF	Holstein Friesian
HCO^{3-}	bicarbonate ion
HCL	Hydrochloric acid
K^{+}	Potassium ion
Kb	Kilobase
KD	Kilo Daltons
Kg	Kilogram
K_m	Michaelis constant
L	Litre
L-	Levo rotatory
Mg	Magnesium
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium Chloride
Min	Minute
ml	mililitre
mM	Milimolar
mRNA	messenger RNA
NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
NO	Nitric Oxide
NH^{4+}	ammonium ion
NH^{4}	ammonia
Ng	Nanogram
ORF	Open Reading Frame
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pBR	plasmid Boliver Rodriguez
pH	Negative logarithm of hydrogen ion concentration
RBC	Red Blood Cells
RFLP	Restriction Fragment Length Polymorphism
RNA	RiboNucleicAcid
rpm	Revolutions per minute

PAGE	Poly Acrylamide Gel Electrophoresis
Pmol	Pico mole
Pi	inorganic phosphate
PPi	Pyrophosphate
p	short arm of chromosome
q	long arm of chromosome
SSCP	Single-Strand Conformation Polymorphism
ssDNA	Single stranded DNA
SDS	Sodium Dodecyl Sulphate
SNPs	Single Nucleotide Polymorphisms
SV40	Simian Virus 40
T	Thymine
TGA	Stop codon/ nonsense codon
Taq	*Thermus aquaticus*
Tth	*Thermus thermophilus*
TBE	Tris Borate EDTA
TEMED	Tetra Methylene Diamine
ter	telomere
Tm	melting temperature
IU	International Unit
UV	Ultraviolet
U.S	United States
USA	United States of America
V	Volt
WBC	White Blood Corpuscles
w/v	weight by volume
µg	microgram
µl	Microlitre
µM	Micromolar
%	percent

CHAPTER - 1

Introduction

The availability of the bovine genome sequence and the use of DNA markers have widened our understanding of a number of hereditary diseases in cattle, leading to the development of techniques for their early diagnosis. Artificial insemination has, since its inception, revolutionised the cattle breeding industry. There is, however, an ever-present danger in its widespread application; undiagnosed genetic defects and less severe chromosome abnormalities may be spread rapidly through the use of a carrier bull's semen. Wide use of only a few elite bulls increased probability of genetic disorders due to inbreeding.

India has a vast reservoir of livestock of considerable genetic diversities. The productivity of the livestock is greatly dependent on their health status and it is indeed a challenging task to provide health care to such a gigantic population of our livestock. In order to increase the milk production from cattle a large scale cross breeding programmes with exotic germplasm was been started which led to higher milk production along with the emergence of various diseases in our livestock. There are various types of genetic disorders which cause huge economic losses in dairy sector. Among them one of the important diseases is citrullinemia, an autosomal recessive disorder.

Citrullinemia is (recessive autosomal) an inborn error of urea metabolism due to deficiency of the urea cycle enzyme, Argininosuccinate Synthetase (ASS). Homozygous recessive condition of citrullinemia is manifested by increasing ammonia and decreasing arginine concentrations in blood during the first 24 hours after birth. Affected calves display severe neurological dysfunction and death within one week as a result of a deficiency of the enzyme Argininosuccinate Synthetase. Problems of the central nervous system due to the build-up of ammonia may include unsteady gait, aimless wandering, apparent blindness and head pressing. Prior to birth the dam is able to remove the excess ammonia.

This disorder was first identified in the year 1962 in humans and later in dogs and Friesian calves in the year 1986. Incidence of heterozygotes for citrullinemia has been estimated at less than 5% among U.S. Holsteins. Linmack Kriss King, one of the most widely used sires for stockbreeding programmes in Holstein cattle, has been identified as heterozygotic carrier for citrullinemia.

The gene coding for ASS has been mapped to chromosome BTA11 in the bovine genome. Since Citrullinemia is caused by the presence of a point mutation in ASS gene, it can be

directly diagnosed by PCR RFLP technique. At molecular level, the disease is caused by a cytosine to thymine transition at codon 86 from exon 5 of the *ASS* gene that can be amplified by PCR and verified by digesting the resulting amplicon with *Ava* II.

Earlier studies (Robinson *et. al.,* 1993, Grupe *et. al.,* 1996, Patel *et al*. 2006, Oner *et. al*., 2010) have clearly shown that though the incidence of heterozygotes for citrullinemia has been estimated to be very less, but it leads to enormous economic loss to dairy sector. Therefore it is essential to eliminate this condition from the breed to avoid calf losses, which could be achieved only by early screening of animals particularly breeding males. Hence, the present investigation is proposed for molecular characterization of ASS gene and screening of citrullinemia in Holstein Friesian and Jersey males with the following objectives

1. To analyze genetic variation in Argininosuccinate Synthetase (ASS) gene exon 5 in Holstein Friesian and Jersey males.
2. Screening for Bovine Citrullinemia in Holstein Friesian and Jersey males.

CHAPTER - 2

Review of Literature

Genetic diseases

Genetic diseases are caused by the inheritance of an absent or otherwise non-functional (mutated or translocated) gene. The appearance of a genetic disorder due to the mutation of a single gene is not common, and most monogenic disorders are caused by a recessive allele. In this case, a cross between two carrier parents produces a genetically healthy (normal homozygote) animal in only 25% of the cases, a carrier in 50% of the cases, and a recessive homozygote in the remaining 25% of the cases. Genetic diseases, caused by the vertical transmission of defective genes to the offspring, can lead to significant losses in livestock sector. Known inherited disorders in cattle are mostly caused by autosomal recessively inherited genes. The characteristic feature of autosomal recessive genes is that they are only expressed as a diseased phenotype if both alleles are present. Therefore, unrecognized dissemination of such defective genes is possible and autosomal recessively inherited disorders are of greater concern in cattle breeding than disorders with dominant inheritance or recessive X-linked inheritance as these are easily recognized. Some of the most prevalent recessive autosomal disorders in cattle are- Bovine Citrullinemia (BC), Bovine Leukocyte Adhesion Deficiency (BLAD), Complex Vertebral Malformation (CVM), Deficiency of Uridine Monophosphate Synthase (DUMPS) and Factor XI deficiency. With the use of Artificial Insemination in cattle, the spread of undiagnosed genetic diseases tremendously increased. There is a need to screen breeding bulls for such genetic diseases, before selecting them for breeding programme.

2.1 Argininosuccinate Synthetase

Argininosuccinate synthase or synthetase (ASS) (E.C. 6.3.4.5) is an enzyme that catalyzes the synthesis of argininosuccinate from citrulline and aspartate.

ATP + citrulline + aspartate ⟷ **argininosuccinate + AMP + PP**

2.1.1 Description

ASS belongs to the Argininosuccinate Synthetase family, type I sub family. In humans, the gene that encodes for this enzyme, ASS, is located on chromosome 9q34. ASS is expressed mostly in periportal hepatocytes, but also in most other body tissues. The enzyme is a homotetrameric protein composed of 45-kD monomers and is involved in the synthesis of

arginine and catalyzes that condensation of citrulline and aspartate to argininosuccinate using ATP (summary by Engel et al., 2009).

2.1.2 Cloning

Bock et al., (1983) isolated clones corresponding to the ASS1 gene from a human cDNA library and deduced 412-residue protein which has a molecular mass of 46 kD. Haberle et al., (2002) provided a revised sequence for the ASS1 gene. Engel et al., (2009) described that the enzyme is having 3 domains: a nucleotide-binding domain, the synthetase domain, and a C-terminal oligomerization domain. Dennis et al., (1989) cloned and sequenced bovine cDNA for argininosuccinate synthetase and found 96% identity with the deduced human sequence at the amino acid level.

2.1.3 ASS1 gene structure

Haberle et al., (2002) determined that the ASS1 gene contains 16 exons. The start codon is in exon 3 and the stop codon in exon 16. Carritt et al., (1977) concluded that a gene for argininosuccinate synthetase (ASS) is carried by chromosome 9. In a study of 10 citrullinemic cell lines, no complementation was observed (Cathelineau et al., 1981). Northrup et al., (1989 identified three restriction fragment length polymorphisms (RFLPs) within the argininosuccinate synthetase (ASS) gene which maps to human chromosome 9q34-qter. Although RFLPs at pseudogene loci are detected by the cDNA, these are the first polymorphisms reported at the ASS locus. They found that the ASS gene is located about 0.04 cM from the ABO blood group locus and is probably centromeric to ABO.

2.1.4 Pseudogenes

Engel et al., (2009) noted that the ASS1 gene has 10 to 14 homologous copies scattered across the human genome. However, only the sequence on chromosome 9q34 seems to encode a functional protein. Using a cDNA probe for argininosuccinate synthetase, Beaudet et al., (1982) identified 10 or more distinct DNA sequences bearing homology. The only functional sequence is presumably that on chromosome 9, which is mutant in classic citrullinemia. Pseudogenes are situated on several autosomes (including ASSP2 on chromosome 6), on the X chromosome (ASSP4 and ASSP5), and perhaps on the Y chromosome (ASSP6). Su et al., (1984) mapped pseudogenes for ASS to 2cen-p25, 3qter-q12, 4qter-q21, 5 (2 loci), 6, 7, 9p13-q11, 9q11-q22, 11q, 12, Xpter-p22, Xq22-q26, and Ycen-q11. They emphasized the usefulness of cloned probes in cytogenetic analysis. Such

dispersion may have been mediated by a transposable element. McCarrey and Riggs (1986) proposed that determinator-inhibitor pairs are a mechanism for threshold setting in development, and that pseudogenes may serve as the source of intracellular inhibitors. They suggested that the system could function at the RNA level by the pairs taking the form of sense-antisense RNAs or at the protein level via a competitive inhibition mechanism. By PCR amplification of specific sequences in somatic cell hybrids, Todd and Naylor (1992) demonstrated that an ASS pseudogene, which they referred to as ASSP1, maps to 6p23-p12.

2.2 Role of ASS enzyme in amino acid and nitrogen metabolism

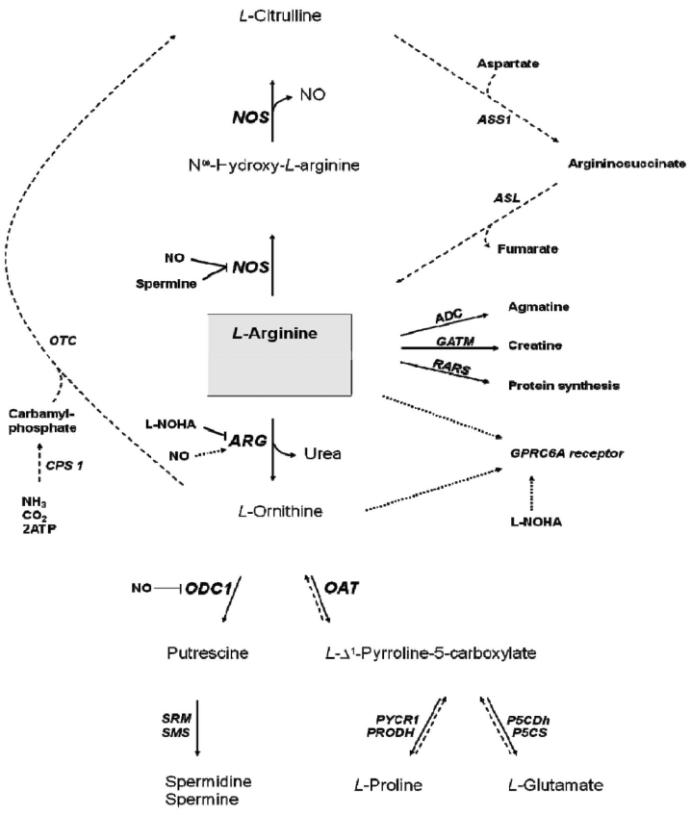
ASS enzyme plays an important role in the biosynthesis of the amino acid L-Arginine and also involved in nitrogen metabolism i.e. synthesis of urea from ammonia through urea cycle.

2.2.1 Biosynthesis of L-Arginine

L-Arginine has been characterized as semi-essential amino acid, in that it is non-essential in the healthy adult organism of most mammals, but has to be supplemented in the growing organism, after trauma or during various disease states. Normal L-arginine plasma levels are in the range of 100 – 200 microM. In adult mammalian organisms not all of the enzymes required for *de novo* synthesis of L-arginine are expressed in every tissue. Synthesis of L-arginine as described by Racke and Warnken, 2010 in Figure 2.1. Three steps in the L-arginine biosynthesis, differentially compartmented in the mammalian organism may be discriminated: Biosynthesis of I) L-ornithine, II) L-citrulline and III) L-arginine.

I) The biosynthesis of L-ornithine form food or blood derived L-glutamine and L-proline occurs almost exclusively in the small intestine as the L-Δ1- pyrroline-5-carboxylate (P5C) synthetase (P5CS) (one of the enzyme required for the conversion of L-glutamine into L-ornithine), is exclusively located in the intestinal mucosa. Likewise, proline dehydrogenase (PROHD), which catalyses the formation of P5C from proline, is also mainly expressed in the intestinal mucosa, although some activity is also detected in the liver and kidney. L-ornithine is also produced by the arginases using L-arginine as substrate, but this reaction may be considered as L-arginine consuming rather than as initial step in the L-arginine biosynthesis

II) The biosynthesis of L-citrulline from L-ornithine depends on the presence of ornithine carbamoyl transferase (OTC) and carbamoylphosphate synthetase 1 (CPS1). In the liver, this



ARG- arginase
ASL-argininosuccinate lyase
ASS1- argininosuccinate synthetase 1
OTC- ornithine carbamoyl transferase
CPS1- carbamoyl-phosphate synthetase 1
ADC- arginine decarboxylase
GATM- glycine aminotransferase
RARS- arginyl-tRNA-synthetase
NOS- nitric oxide synthase
ODC1- ornithine decarboxylase
OAT- ornithine aminotransferase
PYCR1-pyrroline-5-carboxylate reductase1
P5CDh=ALDH4A1, aldehyde dehydrogenase family 4, member A1
P5CS- pyrroline-5-carboxylase Synthetase (= ALDH18A1)
PRODH, proline dehydrogenase (oxidase) 1 SRM, spermidine synthase
SMS, spermine synthase

Figure: 2.1. Synthesis of L – Arginine (Adopted from: Racké and Warnken 2010)

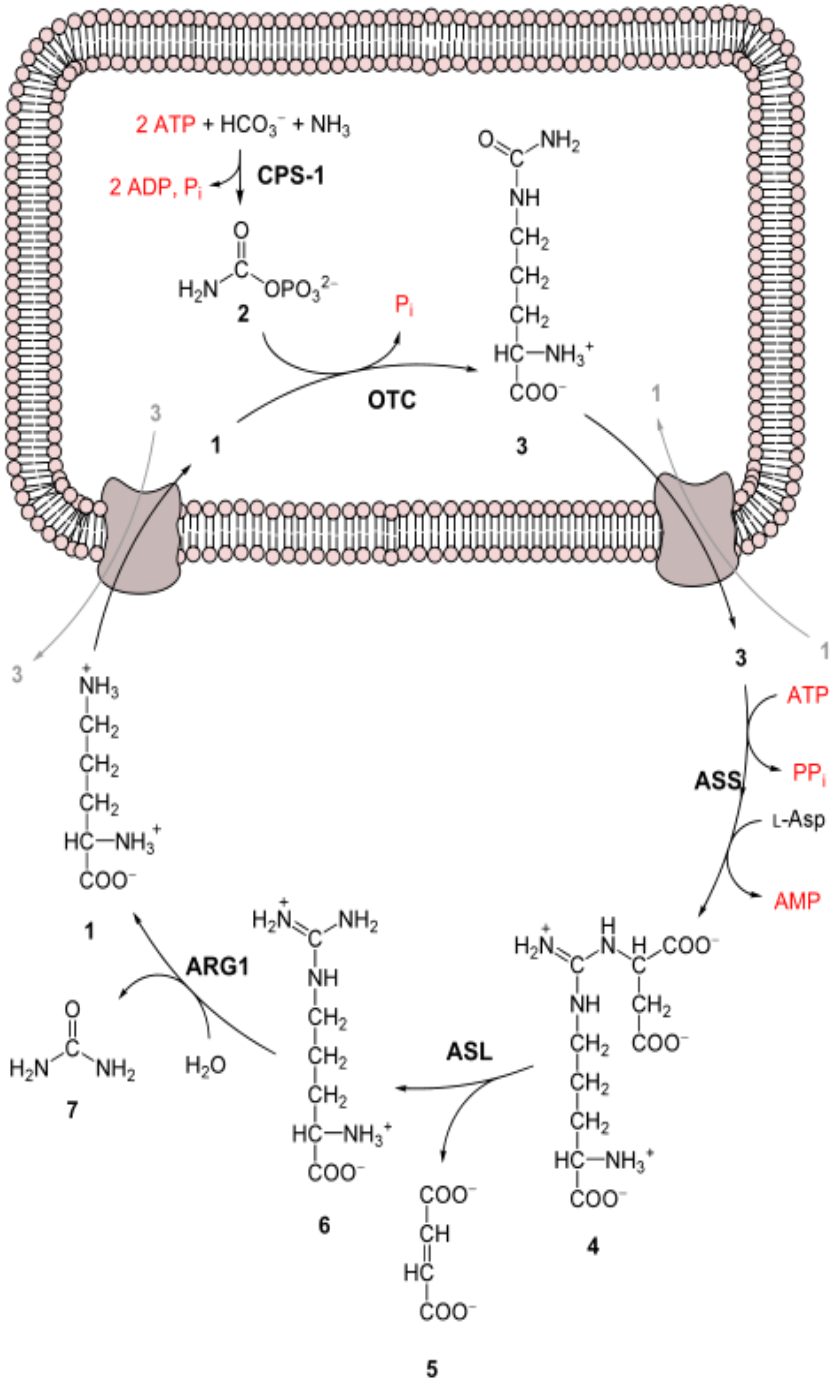
reaction is part of the urea cycle, whereas L-citrulline produced in the intestine is released into the circulation. Most of this circulating L-citrulline is taken up by cells of the proximal tubules of the kidney converted into L-arginine and finally released into the circulation for the benefit of other cells.

III) The biosynthesis of L-arginine from L-citrulline is performed by the cytosolic enzymes argininosuccinate synthetase 1 (ASS1) and argininosuccinate lyase (ASL). The reaction catalyzed by ASS requires L-aspartate as co-substrate and is the rate-limiting step. In contrast to the mitochondrial enzymes of the urea cycle OTC and CPS1, ASS1 and ASL appear to be expressed in many cells, although the degree of expression and the efficiency of this pathway appear to differ considerably between different cells.

2.2.2 Biosynthesis of urea

Urea cycle (Figure 2.2), is the biochemical process by which potentially toxic ammonia (a by-product of catabolism of proteins) is converted to urea, which is excreted in urine (Goodwin et al., 2004). If amino groups are not reused for the synthesis of new amino

acids or other nitrogenous products, they are channeled into a single excretory end product. Urea production occurs almost exclusively in the liver and is the fate of most of the ammonia channeled there. The urea passes into the bloodstream and thus to the kidneys and is excreted into the urine.



1 L- ornithine
2 Carbamoyl phosphate
3 L-citrulline
4 Arginino succinate
5 Fumarate
6 L-arginine
7 Urea
L-Asp L-aspartate
CPS-1 carbamoyl phosphate synthetase I
OTC- ornithine carbamoyl transferase
ASS- argininosuccinate synthetase
ASL argininosuccinate lyase
ARG1 arginase I

Figure: 2.2. Urea cycle (http://en.wikipedia.org)

Urea is produced from ammonia in five enzymatic steps

The urea cycle begins inside liver mitochondria, but three of the subsequent steps take place in the cytosol; the cycle thus spans two cellular compartments. The NH4 generated in liver mitochondria is immediately used, together with CO_2 (as HCO^{3-}) produced by mitochondrial respiration, to form carbamoyl phosphate in the matrix. This ATP-dependent reaction is catalyzed by carbamoyl phosphate synthetase I**,** a regulatory enzyme. Carbamoyl group donor now enters the urea cycle. The cycle has four enzymatic steps. First, carbamoyl phosphate donates its carbamoyl group to ornithine to form citrulline, with the release of Pi. Ornithine plays a role resembling that of oxaloacetate in the citric acid cycle, accepting material at each turn of the cycle.

The reaction is catalyzed by ornithine transcabamoylase**,** and the citrulline passes from the mitochondrion to the cytosol. The second amino group now enters from aspartate (generated in mitochondria by transamination and transported into the cytosol) by a condensation reaction between the amino group of aspartate and the ureido (carbonyl) group of citrulline, forming argininosuccinate. This cytosolic reaction, catalyzed by argininosuccinate synthetase**,** requires ATP and proceeds through a citrullyl-AMP intermediate. The argininosuccinate is then cleaved by argininosuccinase to form free arginine and fumarate, the latter entering mitochondria to join the pool of citric acid cycle intermediates. This is the only reversible step in the urea cycle. In the last reaction of the urea cycle, the cytosolic enzyme arginase cleaves arginine to yield urea and ornithine. Ornithine is transported into the mitochondrion to initiate another round of the urea cycle.

2.3 Urea cycle disorders

People with genetic defects in any enzyme involved in urea formation cannot tolerate protein rich diets. Amino acids ingested in excess of the minimum daily requirements for protein synthesis are deaminated in the liver, producing free ammonia that cannot be converted to urea and exported into the bloodstream, which is highly toxic. The absence of a urea cycle enzyme can result in hyperammonemia or in the build-up of one or more urea cycle intermediates, depending on the enzyme that is missing. Most urea cycle steps are irreversible, the absent enzyme activity can often be identified by determining which cycle intermediate is present in especially elevated concentration in the blood and/or urine. The synthesis of urea in the liver is the major route of removal of ammonia. Some of these genetic defects become evident a day or two after birth, when the afflicted infant becomes lethargic and vomits periodically. Coma and irreversible brain damage may soon follow. High levels

of NH^{4+} is toxic because the elevated levels of glutamine formed from NH^{4+} and glutamate produce osmotic effects that lead directly to brain swelling.

Anomalies of the urea cycle cause urea cycle disorders including

- Ornithine transcabamoylase deficiency
- Cabamoyl phosphate synthetase deficiency
- Argininosuccinic aciduria
- Argininemia
- Hyperornithinemia, hyperammonemia, homocitrullinuria syndrome (HHH syndrome, ornithine translocase deficiency)
- Lysinuric protein intolerance
- Citrullinemia
- N-Acetyl glutamate synthase deficiency

2.4 Role of ASS enzyme in Citrullinemia

Deficiency of Argininosuccinate synthetase enzyme causes citrullinemia. It is inherited in an autosomal recessive pattern (Figure 2.3), which means both copies of the gene in each cell have mutations. The parents of an individual with an autosomal recessive condition each carry one copy of the mutated gene without displaying symptoms themselves. Citrullinemia was first reported in humans in the year 1962 (Mc Murray et al., 1962). Its name derives from the marked elevation of citrulline in blood of affected individuals. This disorder has also been called "citrullinuria" because of the increased excretion of citrulline in urine and "argininosuccinic acid (argininosuccinate) synthetase deficiency" to denote its enzyme deficit. Heterogeneity is seen clinically, biochemically, and at the molecular level.

2.4.1 ASS enzyme deficiency in humans

In humans complete deficiency of ASS enzyme causes classic citrullinemia (McMurray et al., 1962; Su et al., 1984; Beaudet et al., 1986) or type I citrullinemia (CTLN I). Along with this CTLN I, CTLN II is also prevalent in humans. But the cause of this type II citrullinemia is not ASS enzyme deficiency. Mutations in the *SLC25A13* gene are responsible for type II citrullinemia.

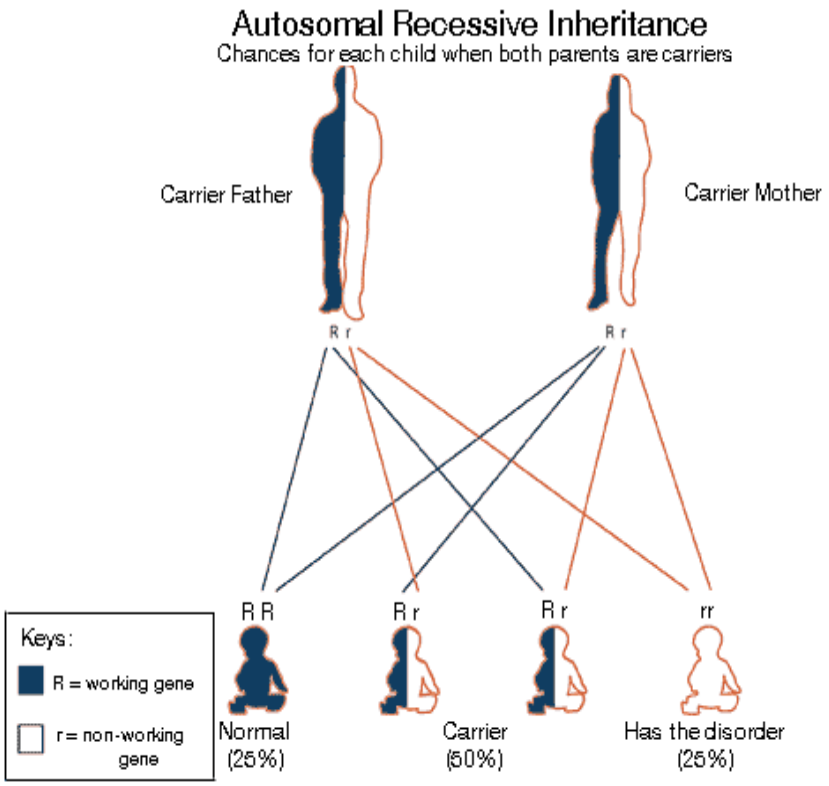


Figure 2.3: Pattern of inheritance of autosomal recessive disorders

(http://blatnicabiology.wikispaces.com)

Type I Citrullinemia

This disorder is typically associated with elevations in citrulline above 1,500 µM/L which is only ≥ 30 µM/L in normal healthy individual and is associated with significant long-term neurological disability. However, it has been recognized that some patients with milder deficiency may present with later onset of learning disabilities, in acute crisis in pregnancy or as an incidental finding with good long term follow up.

Type I citrullinemia (also known as classic citrullinemia) usually becomes evident in the first few days of life. Type I citrullinemia is the most common form of the disorder, affecting about one in 57,000 births worldwide. Mutations in the ASS gene cause type I citrullinemia. Most of these mutations change single amino acids in the argininosuccinate synthetase 1 enzyme. These genetic changes likely alter the structure of the enzyme, impairing its ability to bind to molecules such as citrulline and aspartate. A few mutations lead to the production of an abnormally short version of the enzyme that cannot effectively play its role in the urea cycle.

Type II Citrullinemia

The symptoms of type II citrullinemia usually appear during adulthood and mainly affect the central nervous system. Characteristic features include confusion, abnormal behaviors (such as aggression, irritability, and hyperactivity), seizures, and coma. These symptoms can be life-threatening, and are known to be triggered by certain medications, infections, and alcohol intake in people with this type.

Type II citrullinemia is primarily found in the Japanese population, where it occurs in an estimated one in 100,000 to 230,000 individuals. Type II has also been reported in people from East Asian and Middle Eastern populations. Mutations in the *SLC25A13* gene are responsible for type II citrullinemia. This gene makes a protein called citrin, which normally shuttles certain molecules in and out of mitochondria. These molecules are essential for the urea cycle and are also involved in making proteins and nucleotides. Mutations in *SLC25A13* typically prevent the production of any functional citrin, which inhibits the urea cycle and disrupts the production of proteins and nucleotides. The resulting buildup of ammonia and other toxic substances leads to the symptoms of type II citrullinemia. Researchers have found many infants with neonatal intrahepatic cholestasis have the same mutations in the *SLC25A13* gene as adults with type II citrullinemia (Takaya et al., 2005, Jung Min Ko et al., 2007, Jae Sung Ko et al., 2008, Hai Yan Fu et al., 2010, Thong et al., 2008 and Treeponqkaruna et al., 2012). Thirteen novel mutations (one insertion, two deletion, three splice site, two nonsense, and five missense) including a retrotransposal insertion in SLC25A13 gene were identified in patients with citrin deficiency (Tabata et al., 2008).

2.4.2 Molecular Genetic basis for mutations in ASS1 gene

Engel et al., (2009) provided a review of mutations in the ASS1 gene. They listed 87 mutations, including 27 novel mutations, in patients with citrullinemia. However, the G390R mutation in exon 15 was found to be the single most common mutation in patients with the classic phenotype. Engel et al., (2009) also provided a map of the geographic distribution of ASS1 mutations worldwide. Kobayashi et al., (1989) found that since most patients with citrullinemia express stable mRNA in fibroblasts, the disorder is ideally suited for gene amplification with PCR and sequence analysis of mutant cDNA. Kobayashi et al., (1990) further demonstrated the marked heterogeneity of mutations causing citrullinemia: among 13 unrelated patients with the neonatal form of the disease, they found 10 different mutations. In

the course of studying the molecular nature of mutations in Japanese patients with classic citrullinemia, Kobayashi et al., (1994) found that 10 of 23 affected alleles had the same mutation, deletion in exon 7 which is different from the situation in the United States, where far greater heterogeneity of mutations have been reported.

Kobayashi et al., (1995) reported that 20 mutations had been identified in ASS mRNA in classic citrullinemia. In an extension of their previous studies, Kobayashi et al., (1995) reported that 19 of 33 Japanese ASS alleles had the IVS6AS-2 mutation. Most reported patients with citrullinemia have presented with the classic form of the disease. There are also patients with a mild form of citrullinemia in whom the exact molecular basis and clinical relevance are uncertain. Mutations in the ASS gene had not been described in mildly affected or asymptomatic patients with citrullinemia until the work of Haberle et al., (2002), who described the entire genomic DNA sequence and mutations in the ASS gene of the patients with both classic and the mild form of the disease. In a study of 38 patients with classic citrullinemia, Gao et al., (2003) identified 16 novel mutations in the ASS gene.

2.5 Genomic organization of ASS gene in cattle

ASS gene is located on chromosome 9 in human and on chromosome 11 forward strand in cattle (Ensembl RefSeq: p14568), it is 51997 bp approximately 52 kb in length from 100,791,339 – 100,843,336 nt (Figure 2.4), having 15 exons and 14 introns.

2.5.1 Protein structure and molecular biology of ASS

ASS gene of 52 kb (Gene ID-ENSBTAG00000020747) transcribes into single transcript with 1497 bp (Transcript ID-ENSBTAT00000027649) that encodes an enzyme called Argininosuccinate synthetase and it is one of the six enzymes involved in urea cycle. The translated protein has 412 amino acids (Protein ID-ENSBTAP00000027649). This enzyme is mostly present in cytoplasm of liver and kidney cells.

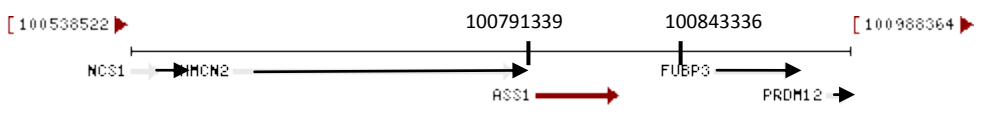


Figure 2.4: Genomic organization of ASS gene in cattle (http://www.ncbi.nlm.nih.gov)

The crystallographic structure of human Argininosuccinate synthetase is shown in the Figure 2.5. It exists as a homotetramer consisting of four identical polypeptide chains linked by disulfide bonds. The molecular weight of catalytically active enzyme of bovine is 185,000. The kinetic mechanism of bovine liver argininosuccinate synthetase has been determined at pH 7.5. The mechanism is also in accord with the formation of citrulline-adenylate as a reactive intermediate. An arginine seems to be important for the enzyme's catalytic mechanism. The sequences of ASS from various prokaryotes, archaebacteria and eukaryotes show significant similarity.

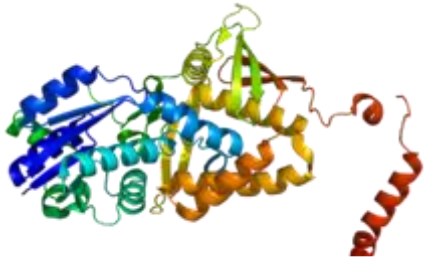


Figure 2.5: Crystallographic structure of human argininosuccinate synthetase (http://en.wikipedia.org)

Nucleotide and amino acid homology between different species range from 99-77% as shown in Table 2.1

Table 2.1 ASS gene homology between different species

Organism	Chromosome	Locus	Protein (aa)	Human Protein Similarity	NCBI Accession
Human *(Homo sapiens)*	9	ASS1	412	100%	**Gene ID**: 445
Chimpanzee *(Pan troglodytes)*	9	ASS1and LOC473355	452	99.8%	**Gene ID**: 743223
Cow *(Bos taurus)*	11	ASS1	412	98.1%	**Gene ID**: 280726
Mouse *(Mus musculus)*	2,10	Gm5424 and ASS1	412	98.5%	**Gene ID**: 11898
Rat *(Rattus norvegicus)*	3	ASS1	412	98.8%	**Gene ID**: 25698

2.6 ASS deficiency in cattle

Genetic disorders are hereditarily caused physical or functional anomalies of the norm with a negative impact on validity. In animals, an important effect is a decrease in or loss of performance. In most cases, mutated recessive genes are the reason for genetic disease in breeding animals (Zabek and Rys, 1998). In cattle, many of the autosomal recessive genetic diseases are breed-specific. Some of them are Holstein-specific, which include bovine citrullinaemia (Harper et al., 1986).

An inherent deficiency of ASS gene deficiency has been observed in Holstein- Friesian cattle in Australia. Animals that are deficient in ASS may have many symptoms. Citrullinemia is manifested by increasing ammonia from < 60 to > 150 micro moles and decreasing arginine concentrations in blood during the first 24 hours after birth and rapidly developing nonspecific neurological signs like seizures, coma and cerebrocortical edema described pathologically. It is also characterized by extreme elevation of citrulline in the plasma. Affected calves display severe neurological dysfunction and death within 1 week as a result of a deficiency of the enzyme Argininosuccinate Synthetase. Problems of the central nervous system due to the build-up of ammonia may include unsteady gait, aimless wandering, apparent blindness and head pressing. Prior to birth the dam is able to remove the excess ammonia. Ammonia interrupts nerve conduction by directly substituting for K^+ in ion exchange mechanisms (Binstock and Lecar, 1969). Ammonia has been shown to alter carbohydrate and fat metabolism and ATP levels, not only in the brain, but in other tissues as well (Wiechetek et al., 1979). Elevated levels of ammonia modify the properties of the blood brain barrier (Sears et al., 1985), interfere with amino acid transport (Mans et al., 1983), disrupts cerebral blood flow (Andersson et al., 1981), impede excitatory amino acid neurotransmitter metabolism, particularly that of glutamate and aspartate (Hindfelt et al., 1977) and cause morphological changes in astrocytes and neurons (Gregorios et al., 1985). Homogenous crystalline ASS prepared from bovine liver has been characterized with respect to amino acid composition and other chemical and physical properties (Ratner, 1982). Fleischer et al., 1983 demonstrated that an increased citrulline concentration in cell free amniotic fluid is important adjunctive evidence for the prenatal diagnosis of citrullinemia. Ratner et al., 1985 identified that cystein and arginine amino acid residues are required for activity that is involved in catalytic mechanism of ASS. Approximately 1000 recessive defects have been recorded in humans (Beaudet et al., 1989; Mc Krusik, 1990) compared with fewer than 200 in cattle (Houston, 1993).

In Friesian cattle in Australia, Harper et al., (1986, 1989) reported that citrullinemia-affected calves had a clinical disease similar to the acute neonatal form of citrullinemia in humans. Dennis et al., (1989) cloned and sequenced bovine cDNA for argininosuccinate synthetase and found 96% identity with the deduced human sequence at the amino acid level and found that a C-to-T transition converting arginine-86 (CGA) to a nonsense codon (TGA). Blood ammonia levels greater than 0.05 m mol^{-1} can be toxic to the central nervous system of most of the mammals (Meijer et al., 1990). ASS deficiency was first described in man (Mc Murray et al., 1962), then in dogs and was reported in Friesian calves (Healy et al., 1990, Viana et al., 1998). The bovine model of citrullinemia was first reported in dairy cattle in Australia (Healy et al., 1991). The allele frequency of the mutant allele was found to be high in Australia (Healy et al., 1991). Descendents of Linmack Kriss King (LMKK) which was a carrier for citrullinemia form 50% of the Australia National Friesian herd and 30 % of AI bulls in AI centers (Healy et al., 1991).

Robinson et al., 1993, detected only one heterozygote among 376 Holstein bulls tested in USA and Germany. Affected homozygous calves are unable to excrete ammonia and display neurological symptoms that become progressively worse, leading to death within one week of birth (Grupe et al., 1996, Lin et al., 2001). The loss of an AvaII site could be used for rapid, economical, nonradioactive detection of heterozygotes for bovine citrullinemia. Only one Holstein bull calf was tested heterozygote (carrier) for ASAS locus among 330 cattle and 135 buffalo bulls screened for (citrullinemia) mutant allele (Padeeri et al., 1999). The widespread use of elite sires by means of artificial insemination in livestock breeding leads to the frequent emergence of recessive genetic defects, which cause significant economic and animal welfare, concerns (Charlier et al., 2008). In India and Turkey, no carriers of citrullinemia were reported (Patel et al., 2006, Oner et al., 2010). Carriers for citrullinemia were not detected among 350 Holstein cows of Turkey (Meydan et al., 2010). Only one citrullinemia carrier was detected in 615 Chinese Holstein cattle with a frequency of 0.16% (Jianbin Li et al., 2011). Based on the analysis of 330 Holstein cows and native cattle, reared in Khuzestan province in Iran, Eydivandi et al., 2012 reported that, all cows possessed normal genotypes.

Padeeri et al., (1999) carried out PCR based DNA analysis for screening of breeding bulls for citrullinemia in various cattle and buffalo breeds of India and identified one carrier Holstein Friesian bull. Their findings have been given in Table 2.2

2.7 Molecular basis of ASS deficiency in cattle

The mutation responsible for citrullinemia has been characterized as a single-base substitution (C-T) (Table 2.3), in exon 5 of argininosuccinate synthetase (ASS), which converts the CGA codon that codes for arginine-86 to TGA, a translational termination codon. This conversion results in a truncated peptide product (85 amino acids instead of 412) deprived of functional activity (Dennis et al., 1989). This mutation leads to the removal of restriction site for restriction enzyme *Ava II*. In homozygous normal, this enzyme is able to cut at restriction sites and gives two bands of DNA on electrophoresis. In carrier (heterozygous mutant), as one allele is normal and the other is mutant, the enzyme is able to cut only the normal one but not the mutated. This on electrophoresis gives three bands of DNA i.e. two bands for normal allele and one band for mutated allele. In affected (homozygous mutant), as both the alleles are mutated, no restriction site for the enzyme, unable to cut the DNA. This on electrophoresis gives only one single band of DNA.

Table-2.2 Bovine ASS gene deficiency in India

Breed	No. of Animals	Affected/Carriers
CATTLE		
Holstein Friesian	150	1
Jersey	50	NONE
Sahiwal	40	
Gir	40	
Cross breeds	50	
BUFFALO		
Murrah	75	
Surti	40	
Jaffarbadi	20	

Table: 2.3. Alignment of bovine Citrullinemia sequences from normal and mutant

Normal: CAAGGAGTTTGTGGAGGAGTTCATCTGGCCGCCATCCAGTCCAGCGCACT
Mutant: CAAGGAGTTTGTGGAGGAGTTCATCTGGCCGCCATCCAGTCCAGCGCACT
Normal: GTACGAGGA**C**GATACCTCCTGGGCACCTCTCTCGCCAGGCCCTGCATCGCCC
Mutant: GTACGAGGA**T**GATACCTCCTGGGCACCTCTCTCGCCAGGCCCTGCATCGCCC

2.8. DNA isolation

Genomic DNA is the starting material for most of the molecular biology techniques. The convenient sources of nucleated cells for the extraction of genomic DNA from farm animals are blood and semen. The primary objective of the isolation process is to recover the maximum yield of high molecular weight DNA devoid of protein and enzyme inhibitors (Sambrook and Russel, 1989). Modified Phenol-chloroform extraction method (Thomas and Anil Kumar, 2008) and High salt method (Miller et al., 1988) are commonly used for extraction of DNA from blood of farm animals. Many researchers followed High salt method with minor modifications for the isolation of genomic DNA with an additional chloroform extraction method from blood samples of cattle (Ramesha et al., (2002) and Shergojry et al., (2011).

2.8.1 Yield and quality of DNA

Shinde et al., (2008) extracted DNA from blood samples of cattle by high-salt method and obtained pure DNA in the range of 150 ±25 µg of DNA from 200µl of blood. Shergojry et al., (2011) isolated DNA from whole blood of Deoni cattle by High salt method with minor modification and the yield of DNA was in the range of 375 µg to 1330 µg / ml of blood with a mean yield of 596.50 ± 36.50 µg/ ml.

2.9. Polymerase Chain Reaction (PCR)

The PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest

in the target DNA. The PCR allows the amplification of a specific deoxyribonucleic acid (DNA) region that lies between two regions of known DNA sequence.

2.9.1 Components of PCR

PCR components are critical to a successful PCR amplification. These components include DNA template, oligonucleotide primers, magnesium concentration, deoxynucleotides, Taq DNA polymerase concentration, PCR buffers (Sambrook and Russel, 1989).

DNA polymerase

The most commonly used Taq DNA polymerase is isolated from Thermus aquaticus. A recommended concentration of Taq DNA polymerase varies between l.0 and 2.5 units per 100µl reaction mixture when other parameters are optimum. Too high concentration of DNA polymerase concentration results in the accumulation of nonspecific background products, while at too low concentration of enzyme, the yield may be insufficient (Shergojry et al., 2011).

Deoxy ribonucloeoside triphosphates

In the standard PCR protocol, each deoxynucleoside triphosphate (dNTP) concentration is 200 µM. It is important to keep the four dNTP concentrations above the estimated K_m of each dNTP (10 µM-15 µM) and balanced for best base incorporation. However, deviations may be beneficial in certain applications. For example, when random mutagenesis of a specific target is desired, unbalanced dNTP concentrations promote a higher degree of miss incorporation by Taq DNA Polymerase. Lowering the dNTP and magnesium ion by an equal molar concentration can improve fidelity.

Magnesium ion

In the presence of 0.8 mM total dNTP concentration, a magnesium chloride titration series in 0.5 mM increments over the 1 mM - 4 mM range will locate the optimum magnesium ion concentration, producing the highest yield of a specific PCR product. Generally excess Mg^{2+} will result in the accumulation of non-specific amplification products and insufficient Mg^{2+} will reduce the yield (Innis and Gelfand, 1990). A magnesium concentration of 1.5–2.0 mM is optimal for most PCR products generated with Taq DNA Polymerase. Optimization normally involves supplementing the magnesium concentration in 0.5 or 1.0 mM increments (Sam brook and Russel, 1989).

PCR primers

PCR primers are oligonucleotides, typically 20-30 bases long, hybridizing to opposite strands and flanking the region of interest in the target DNA. While choosing two PCR primers, it is important that they do not contain bases complementary to themselves or with each other. Primers are always present at an excess and equal concentration in conventional (symmetric). It is generally advisable to use purified oligomers of the highest chemical integrity. Complementarity at the 3' ends should especially be avoided to minimize the formation of an artifactual product, often called "primer-dimer" or "primer-oligomer." A 40%-60% G+C content is recommended for each primer to avoid internal secondary structure and long stretches of any one base. Also, primers should not sit on regions of secondary structure (within the target) having a higher melting point than the primer. Optimal annealing temperatures and primer concentrations must be determined empirically. Primer concentration between 0.1 and 0.5 μM are considered optimum (Innis and Gelfand, 1990). Taq DNA Polymerase has activity in the range 25°C-72°C.

PCR buffer

PCR buffer is an important component of PCR which affect the outcome of amplification. Standard buffers for PCR amplification with DNA polymerase is the 10X PCR Buffer. The 10X PCR buffer is composed of 500 mM potassium chloride, 100 mM Tris-HCl(pH 8.3 at room temperature), 15 mM magnesium chloride and 0.01% (w/v) gelatin. In particular, the concentration of $MgCl_2$ has a profound effect on the specificity and yield of amplification. Innis and Gelfand (1990) recommended a PCR buffer that contains 10-50mM Tris HCl with pH between 8.3 and 8.8 at 20°C.

DNA Template

Use of high quality, purified DNA templates greatly enhances the success of PCR reactions. The PCR sample may be single or double-stranded DNA or RNA. If the starting sample is RNA a reverse transcriptase is used to prepare first-strand cDNA prior to conventional PCR amplification. For RNA templates with high G+C content or complex secondary structure, the high temperature reverse transcriptase activity of thermostable Tth (Thermus thermophiles) DNA polymerase is effective. When using DNA as the starting template, nanogram amounts of cloned template, up to microgram amounts of genomic DNA are chosen to start optimization trials. Optimum concentration of template DNA per reaction

could vary substantially from typical conditions (100ng per reaction) depending on the primer-template combination use.

Taq DNA polymerase Concentration

Taq DNA Polymerase is normally used at a final concentration of 25 units/ml (1.25 units/50 µl reaction), but can range from 5–50 units/ml (0.25–2.5 units/50 µl reaction) in specialized applications (Shergojry et al., 2011).

Cycling Parameters

PCR is performed in three steps in a cycle of amplification, namely denaturation, annealing and extension. DNA denaturation is the critical step in the PCR process and is often the focus of attention if PCR experiments fail. The practical range of denaturation temperatures for most samples is 94°C-96°C for 15 seconds. Several workers have used template denaturation conditions of 94°C for 45 seconds (Ramesha et al., 2002), 94°C for 30 seconds (Yao et al., 2011) and 94°C for 1 minute (Shergojry et al., 2011). For difficult templates such as GC-rich sequences, a longer denaturation of 2–4 minutes at 94°C is recommended prior to PCR cycling to fully denature the template. Annealing temperature is based on the Tm (melting temperature) of the oligonucleotides chosen for PCR amplification. If unwanted bands are observed, the annealing temperature is raised in 2°C-5°C increments in subsequent optimization runs. While the primer annealing temperature range is often 37°C-55°C, it may be raised as high as the extension temperature in some cases. In fact, high-temperature annealing should result in enhanced specificity. Primer extension, in most applications, occurs effectively at a temperature of 72°C and seldom needs optimization. Typically, 25-35 cycles are required for extensive amplification of a specific target. The primers are allowed to anneal to their complementary sequences by briefly cooling (Innis and Gelfand, 1990). Annealing temperatures should be chosen to match the Tm values of the primer pair and are typically 45–68°C. If extra bands are observed, higher annealing temperatures should be considered. The absence of product can indicate the need for a lower annealing temperature. Annealing times of 15–60 seconds are usually adequate (Sam brook and Russel, 1989). Extensions are normally done at 72°C. As a general rule, extension times of one minute per kb should be used. A final extension of 5 minutes at 72°C is recommended (Sam brook and Russel, 1989).

2.10. Agarose gel electrophoresis

Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins. Most every molecular biology research laboratory routinely uses agarose gel electrophoresis for the preparation and analysis of DNA.

Electrophoresis is a method of separating substances based on the rate of movement while under the influence of an electric field. Agarose is a polysaccharide purified from seaweed. An agarose gel is created by suspending dry agarose in a buffer solution, boiling until the solution becomes clear, and then pouring it into a casting tray and allowing it to cool. The result is a flexible gelatin-like slab. During electrophoresis, the gel is submersed in a chamber containing a buffer solution and a positive and negative electrode. The DNA to be analyzed is forced through the pores of the gel by the electrical current. Under an electrical field, DNA will move to the positive electrode (red) and away from the negative electrode (black). Several factors influence how fast the DNA moves, including; the strength of the electrical field, the concentration of agarose in the gel and most importantly, the size of the DNA molecules. Smaller DNA molecules move through the agarose faster than larger molecules. DNA itself is not visible within an agarose gel. The DNA will be visualized by the use of a dye that binds to DNA.

Electrophoresis is used to determine the presence or absence of PCR products and quantify the size (length of the DNA molecule) of the product.

2.11. Identification of new Polymorphisms

Varieties of techniques are available to detect new mutation / polymorphisms. Techniques Such as PCR Restriction fragment length polymorphism (PCR-RFLP), Hetero-duplex analysis (HDA), Denaturing gradient gel electrophoresis (DGGE), Protein truncation test (PTT), Allele specific oligonucleotides (ASO), DNA microarray technology; Nucleotide sequencing can provide useful alternative methods for direct analysis of sequence variation. Single Strand Conformational Polymorphism (SSCP) is commonly used strategy for SNP screening. The SSCP technique involves - amplification of gene of interest by PCR, scan the PCR products for the presence of DNA variants by confirmation-based mutation scanning methods, and then sequence positive PCR products to identify SNPs. A single-nucleotide polymorphism (SNP, pronounced as snip) is a DNA sequence variation occurring when a single nucleotide — A, T, C or G in the genome (or other shared sequence) differs between members of a biological species or paired chromosomes in an individual. Each SNP

represents a difference in a single DNA building block, called a nucleotide. For example, a SNP may replace the nucleotide cytosine (C) with the nucleotide thymine (T) in a certain stretch of DNA. Single nucleotide polymorphisms may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions. They can act as biological markers which are useful in genome-wide association studies. The development of new generation DNA sequencers also allows direct heterozygote sequencing frequently used in SNP detection. Vast amount of human expressed sequence tags (ESTs) and genomic clones in the public domain; computer based sequence alignment and clustering also provide a rich source for SNP identification (Makino et al., 1992).

2.12 Restriction enzymes

Arber discovered restriction enzymes while studying a phenomenon known as host-controlled restriction of bacteriophages. Prior to Arber's work, researchers Salvador Luria and Mary Human had shown that various phages were host specific, with each phage surviving and flourishing only in one host bacterial strain and growing poorly in others (Luria & Human, 1952). Those phages that grew poorly were said to be "restricted" by their host. Arber proposed that bacterial cells were able to protect themselves against foreign DNA through some sort of enzymatically catalyzed genetic defense mechanism (Arber & Linn, 1969). Arber further proposed that there were specific sites in the genome at which restriction activities occurred. Arber and Linn referred to the enzyme responsible for this "endonucleolytic scission" as endonuclease R, a name later changed to EcoB. It didn't take long for other scientists to identify a second restriction enzyme in *E. coli*: EcoK (Meselson & Yuan, 1968).

Soon after the discovery of EcoB and EcoK, microbiologists Hamilton Smith and Kent Wilcox isolated and characterized the first restriction enzyme from a second bacterial species, *Haemophilus influenzae*. They also confirmed Arber's hypothesis by demonstrating that HindII, the name eventually given to the enzyme they discovered, degrades foreign phage DNA but not the bacterial host's DNA (Smith & Wilcox, 1970). Smith and his postdoctoral fellow, Thomas Kelly, later identified the actual nucleotide sequence of the specific site where HindII cleaves (known today as a restriction or recognition site), again confirming Arber's hypothesis that restriction enzymes are extremely selective with regard to where they make their cuts (Smith & Kelly, 1970). Since the early research of Arber, Smith, and others, scientists have isolated more than 800 different restriction enzymes from bacteria, which altogether recognize and cut more than 100 different restriction sites. Most restriction

sites are 4 to 6 bases long, and most are palindromic. *Ava II*, for example, is an *Anabaena variabilis* restriction enzyme that recognizes the sequence 5' GGTCC 3' (upper strand)/3' CCAGG 5' (lower strand) and cleaves between the two G's on both strands. (Here, the upper and lower strand sequences are the same but reversed). The first three letters of a restriction enzyme's name are abbreviations of the bacterial species from which the enzyme has been isolated and the fourth letter represents the particular bacterial strain. Roman numerals are also used as part of the name when more than one restriction enzyme has been isolated from the same bacterial strain.

Types of restriction enzymes

Scientists recognize three categories of restriction enzymes: Type I, Type II and Type III. Properties, function and mechanism of action of all three types are described in the Table 2.4. All restriction enzymes serve the purpose of defense against invading viruses. Bacteria protect their DNA by modifying their own recognition sequences, usually by adding methyl (CH_3) molecules to nucleotides in the recognition sequences and then relying on the restriction enzymes' capacity to recognize and cleave only unmethylated recognition sequences. Bacteriophages that have previously replicated in a particular host bacterial strain and survived are similarly modified with methyl-labeled nucleotides and thereby protected from cleavage within that same strain.

Table: 2.4 Types of restriction enzymes

	Type I	**Type II**	**Type III**
Protein structure	Bifunctional enzyme of 3 subunits	Separate endonuclease and methylase	Bifunctional enzyme of 2 subunits
Recognition site	Bipartite and asymmetric	4 – 6 bp sequence, usually palindromic	5 – 7 bp, usually asymmetric
Cleavage site	Non specific > 1000 bp recognition sequence	Same as or close to recognition site	24 – 26 bp downstream of recognition site
Restriction and methylation	Mutually exclusive	Separate reactions	Simultaneous
ATP needed for restriction	Yes	No	Yes
Examples	EcoB and EcoK	EcoRI, HindII, HindIII, AvaII, BamHI, TaqI	HzaI, Hph I, Mbo II

Ava II

The type II restriction endonuclease *Ava II* from *Anabaena variabilis* was isolated and described by Murray et al., (1976). Gregor J. Sutcliffe and George M.Church (1978) mapped eight *Ava II* sites of pBR322, as well as a unique site for *AvaI.*

Restriction enzyme reactions should be carried out at 37^{o} C and heat denaturation can be done by heating at 65^{o}C for 15 minutes (www.theLabRat.com).

Restriction site sequence for *Ava II*:

```
...GGTCC...
...CCAGG...
```

Restriction site sequence after cut by *Ava II*:

```
    ↓
...G GTCC...
...CCAG G...
      ↑
```

2.13 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism or RFLP analysis is used to identify a change in the genetic sequence that occurs at a site where a restriction enzyme cuts. RFLPs can be used to trace inheritance patterns, identify specific mutations, and for other molecular genetic techniques. These were among the first developed DNA markers. RFLPs are defined by the presence or absence of a specific site, called a restriction site, for a bacterial restriction enzyme. This enzyme breaks apart strands of DNA wherever they contain a certain nucleotide sequence. RFLP also has a number of other uses, particularly in criminology. Restriction enzymes are proteins isolated from bacteria that recognize specific short sequences of DNA and cut the DNA at those sites. The normal function of these enzymes in bacteria is to protect the organism by attacking foreign DNA, such as viruses.

Restriction fragment length polymorphisms (RFLPs) are special types of SNPs, ones which result in a restriction site being changed. When digested with a restriction endonuclease the loss of the site is revealed because two fragments remain joined together. A specific type of restriction endonuclease can cut the DNA molecule into a set of fragments, which is

reproducible for any DNA sample of that organism. The set of DNA fragments of various sizes produced by the restriction digestion of a DNA sample is known as the restriction pattern, which is reproducible for that individual and thus forms a molecular marker. The RFLP analysis is based on variations in the DNA sequences even between individuals of a species and will result in the variations in the distribution of restriction sites for one or more types of restriction endonucleases.

Different steps involved in RFLP analysis as diagnostic tool can be summarized in the following steps. Amplification of gene of interest by PCR, the DNA samples are digested by an appropriate restriction enzyme (single digestion) or by a mixture of two or more enzymes (double digestion, triple digestion, etc.), producing a number of DNA fragments of varying sizes with single-stranded overhangs (sticky ends) or blunt ends. DNA fragments can be separated according to their molecular weight on an agarose gel. In this process of two-dimensional gel electrophoresis, DNA (which has a negative charge) migrates through the gel towards the positive electrode. Large pieces of DNA move through the tangle of agarose carbohydrates slower than small fragments. DNA in the gel can be visualized under UV light after staining with fluorescent dye, ethidium bromide.

2.14 Single Strand Conformational Polymorphism (SSCP)

Single-strand Conformation Polymorphism (SSCP) analysis is one of the most widely used technique for mutation detection. SSCP analysis detects sequence variations (single-point mutations and other small scale changes) through electrophoretic mobility differences in polymerase chain reaction amplified DNA. These variations can potentially cause conformational changes in the DNA molecules. Under non-denaturing conditions and often reduced temperature, single stranded DNA molecules can assume unique conformations that vary depending on their nucleotide sequences. SSCP analysis is generally considered to be most suitable for the detection of mutations in short stretches of DNA. Hence, the size of PCR fragments investigated is usually in the range of 175-250 bp. Since the first reported use of SSCP in 1989 (Orita et al., 1989), this technique has been widely used to detect mutations. SSCP technique could detect even a single base substitution in a PCR fragment of several hundred nucleotides in length (Orita et al., 1989). SSCP requires relatively less labor and less expensive than most other approaches used to detect mutations. It is user friendly, low in cost, time efficient and potentially high-throughput platform.

SSCP relies on the principle that the electrophoretic mobility of a single-stranded DNA molecule in a non-denaturing gel is highly dependent on its size and structure. In solution, the single-stranded DNA molecules take on secondary and tertiary conformations due to base pairing between nucleotides within individual strands. These conformations depend on its sequence, the length of the strand and location and the number of regions of base pairing. Hence a mutation at a particular nucleotide position in the primary sequence can alter the conformation of the molecule. On separation in a non-denaturing gel matrix, DNA molecules which differ by a single nucleotide can be separated based on their mobility rates as a consequence of differing conformations.

The mutation detection rate of SSCP varies depending on parameters, such as base composition (some point mutations are non detectable because of A+T or G+C richness) of the sequence, size of the fragment, electrophoresis temperature, buffer systems, addition of neutral compounds to the gel and gel composition (pore size and cross linking) and unfamiliarity or inexperience of the operator. Generally successful differential separation of single strand conformers occur in the range of 200–300 bp. Addition of neutral compounds e.g., glycerol, 50–150 mL/L gives better electrophoretic separation of conformers in some cases (Glavac and Dean, 1993). In non-denaturing PAGE, the components used to synthesize DNA separating matrix are Acrylamide monomers, N,N-methylene bisacrylamide, Ammonium Per Sulphate (APS) and N,N,N',N'-Tetra methylene diamine (TEMED) which are dissolved in 1X TBE. Ammonium per-sulphate (APS) when dissolved in water generates free radicals, which activate acrylamide monomers inducing them to react with other acrylamide molecules forming long chains. These chains get cross linked with N, N-methylene bisacrylamide to form the DNA separation matrix. TEMED acts as catalyst for gel formation because of its ability to exist in free radical form. The casted non-denaturing PAGE gels are run in vertical gel electrophoresis unit containing 1X TBE for resolving DNA fragments in PCR-SSCP analysis.

2.15 Direct Sequencing

The DNA sequencing method that was used to sequence the human genome and many other genomes was an enzymatic sequencing method pioneered by Fred Sanger. It relies on random inhibition of chain elongation, creating newly synthesized DNA strands of various lengths that can be separated by size. The DNA needs to be in a single-stranded form that will act as a template for making a new complementary DNA strand in vitro by using a suitable DNA

polymerase. The substrate for DNA sequencing was often a recombinant DNA that would be denatured so that a strand-specific sequencing primer could be used to direct new strand synthesis, or DNA fragments would be cloned into phagemid vectors that were manipulated to produce single-stranded recombinant DNA. Alternatively, and increasingly commonly, DNA produced by PCR amplification is used and converted to a Single-stranded form to act as a sequencing template. The final product of either method is a population of many identical copies of the DNA to be sequenced. Sequencing is conducted in four parallel reactions, each containing the four dNTPs (dATP, dCTP, dGTP, and dTTP) plus a small proportion of one of the four analogous dideoxynucleotides (ddNTPs) that will serve as a base-specific chain terminator. A ddNTP is closely related to its dNTP counterpart but lacks a hydroxyl group at the 3' carbon position and also at the 2' carbon. It can be incorporated into the growing DNA chain by forming a phosphodiester bond between its 5' carbon atom and the 3' carbon of the previously incorporated nucleotide. However, because it lacks a 3' hydroxyl group, any dideoxynucleotide that is incorporated into a growing DNA chain cannot participate in phosphodiester bonding at its 3' carbon atom. Once a dideoxynucleotide has been incorporated, it causes the abrupt termination of chain elongation. By ensuring that one of the four dNTPs or the primer is labeled, the growing DNA strand becomes labeled. By setting the concentration of the dideoxynucleotide to be very much lower than that of the corresponding deoxynucleotide analog, there will be competition between a specific dideoxynucleotide and its deoxynucleotide counterpart for inclusion in the growing DNA chain. The deoxynucleotide is present in excess; when it is incorporated, chain elongation continues, but occasionally the dideoxynucleotide will be incorporated in the growing chain, ending polymerization and so causing chain termination. Each reaction is therefore a partial reaction because chain termination will occur randomly at one of the possible choices for a specific type of base in anyone DNA strand. Because the DNA sample is a population of identical molecules, each of the four base-specific reactions will generate a collection of labeled DNA fragments of different lengths. Each of the fragments in one reaction will have a common 5' end (defined by the sequencing primer). However, the 3' ends are variable because the insertion of the selected dideoxynucleotides occurs randomly at one of the many different positions that will accept that specific base. Fragments that differ in size by even a single nucleotide can be size-fractionated on a denaturing polyacrylamide gel, a gel that contains a high concentration of urea or other denaturing agent so that the migrating DNA remains single stranded.

2.16 Sequence data analysis

Sequence data analysis can be done by using certain bioinformatic tools like, DNA baser, Clustal W, BioEdit etc.

DNA Baser

DNA Baser was first released in 2004. Since then it was constantly updated. The list of features is based on 'user request' which means that the end user can ask for new features and vote in a poll which features to be implemented first. DNA Baser was the first program to offer automatic end trimming, automatic vector removal and automatic sequence assembly (batch assembly).

DNA Baser Assembler is unique bioinformatics software for manual and automatic DNA sequence assembly, DNA sequence analysis, automatic sample processing, contig editing, metadata integration, file format conversion and mutation detection.

DNA Baser concentrates on automation of sequence assembly process. With DNA Baser Sequence Assembler, one can:

- Assemble multiple DNA samples or align to a reference sequence
- Batch assemble or align in groups of sequences by name
- Automatically clip sample ends (low quality region trimming)
- Automatically detect and trim recognition (vector/primer) sequences
- Import and analyze sequences from ABI, SCF, FASTA and SEQ
- Preview chromatograms with a single click
- View and edit sequence traces
- Mark specific regions (like discrepancies, low-quality areas in chromatograms) with visible colors and quickly navigate to these regions
- Convert between different file formats (ABI, SCF, FASTA, multi-FASTA, GBK...)

ClustalW

ClustalW is a widely used multiple sequence alignment computer program. This program accepts a wide range of input formats, including NBRF/PIR, FASTA, EMBL/Swiss-Prot, Clustal, GCC/MSF, GCG9 RSF, and GDE. The output format can be one or many of the following: Clustal, NBRF/PIR, GCG/MSF, PHYLIP, GDE, or NEXUS.

There are three main steps involved in multiple sequence alignment-

1. Do a pairwise alignment
2. Create a guide tree (or use a user-defined tree)
3. Use the guide tree to carry out a multiple alignment

These are done automatically when the option "Do Complete Alignment" was selected.

Users can align the sequences using the default setting, but occasionally it may be useful to customize one's own parameters. The main parameters are the gap opening penalty, and the gap extension penalty

BioEdit

"BioEdit is a mouse-driven, easy-to-use sequence alignment editor and sequence analysis program intended to supply a single [tool] that can handle most simple sequence and alignment editing and manipulation functions that researchers are likely to do on a daily basis, as well as a few basic sequences analyses." (Hall, 2007a).

"BioEdit's features include" (Hall, 2007):

- Several modes of hand alignment.
- Automated ClustalW alignment.
- Automated Blast searches (local and [via internet]).
- Plasmid drawing and annotation.
- Accessory application configuration.
- Restriction mapping.
- RNA comparative analysis tools.
- Graphical matrix data viewing tools.
- Shaded alignment figures.
- Translation-based nucleic acid alignment.
- ABI trace viewing, editing and printing.
- And other features.

BLAST

In bioinformatics, **B**asic **L**ocal **A**lignment **S**earch **T**ool, or **BLAST**, is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold. Different types of BLASTs are available according to the query sequences. For example, following the discovery of a previously unknown gene in the mouse, a scientist will typically perform a BLAST search of the human genome to see if humans carry a similar gene; BLAST will identify sequences in the human genome that resemble the mouse gene based on similarity of sequence. The BLAST program was designed by Altschul et al., 1990.

CHAPTER - 3

Materials and Methods

3.1 Experimental animals

The present study was conducted on 131 Holstein Friesian and 78 Jersey bulls, maintained at different bull stations across Karnataka.

3.1.1 Holstein Friesian

Holstein Friesian, large sized dairy cattle breed originating in northern Holland and Friesland. Holstein Friesian cattle could be easily recognized by their distinctive black and white (Figure 3.1) or red and white color markings and outstanding milk production. A healthy Holstein Friesian calf weighs 30 Kg or more at birth. A mature Holstein cow weighs about 600-700 Kg and stands 58 inches tall at the shoulder. These cattle are believed to have been selected for dairy qualities for about 2,000 years. They are valued highly for their milk-producing ability. However, the milk has relatively low butterfat content. The average actual production of Holstein was 23,385 pounds of milk, 858 pounds of butterfat and 719 pounds of protein per year (Holstein Association USA, 2011). However, in India, graded Holstein cows produce about 20-30 Kg of milk per day with 3.5% fat and 8.5% SNF.

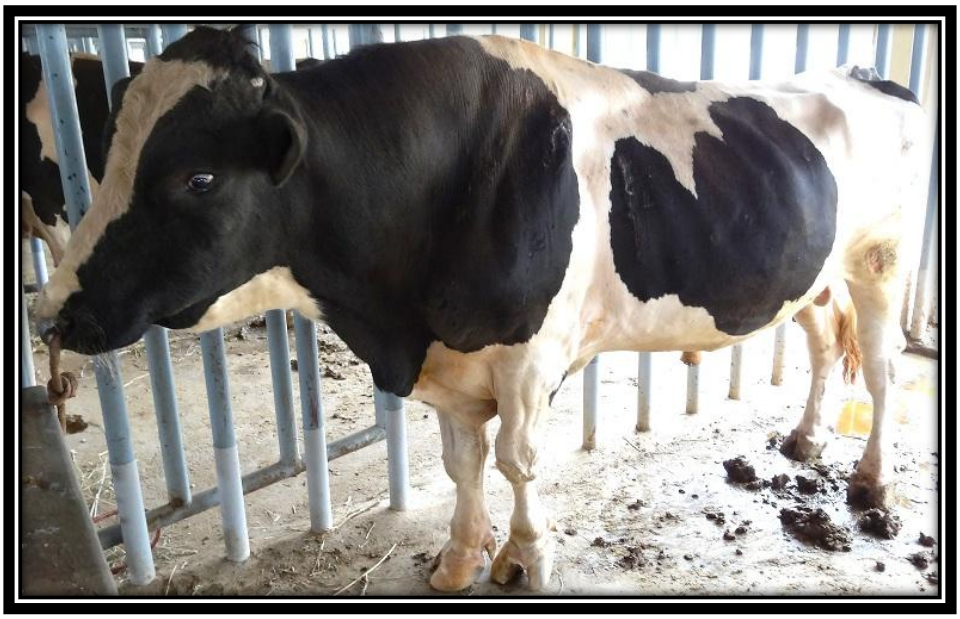


Figure 3.1 Holstein Friesian Bull

3.12 Jersey

The Jersey breed originated on the Island of Jersey, a small British island in the English Channel, off the coast of France. The Jersey is one of the oldest dairy breeds, having been

reported by authorities as being purebred for nearly six centuries. The breed was known in England as early as 1771 and was regarded very favorably because of its milk and butterfat production. Adaptable to a wide range of climatic and geographical conditions. Outstanding Jersey herds are found from Denmark to Australia and New Zealand, from Canada to South America, and from South Africa to Japan. They are more tolerant of heat than the larger breeds. With an average weight of 480 Kg, the Jersey produces more Kg of milk per Kg of body weight than any other breed. Most Jerseys produce far in excess of 13 times their body weight in milk each lactation. Cows show very marked refinement about their heads and shoulders, carry long, straight top lines, and usually carry out long and level at the rump. For their size, they are usually deep in the body and full and deep in the barrel.

Jersey bulls (Figure 3.2), are small as compared to the HF bulls, and are extremely masculine. They are quite muscular about their crests and shoulders and are considerably less refined throughout than are the females. The same general qualities of straight lines and diary conformation as are found in the cows are desired in bulls. They usually range in weight from 600-700 Kg. The color in Jerseys may vary from a very light gray or mouse color to a very dark fawn or a shade that is almost black. Both the bulls and females are commonly darker about the hips and about the head and shoulders than on the body (summary by The American Jersey cattle Association).

3.2 Blood Collection

About 10 ml of blood was collected aseptically from Jugular vein in a vacutainer tube containing 0.5 per cent anticoagulant Ethylene Diamine Tetra Acetic Acid (EDTA). After collection, the samples were stored at 4°C and DNA was isolated within 24 hours.

3.3 DNA Extraction by High Salt method

Genomic DNA was isolated from the collected blood samples using modified High salt method (Miller et al., 1988) as follows:

1. 10 ml of blood sample was added to 50 ml centrifuge tube. 25 ml of RBC lysis buffer was added into it and inverted several times and was incubated in ice flakes with continuous shaking for 10 minutes for complete lysis of cells.

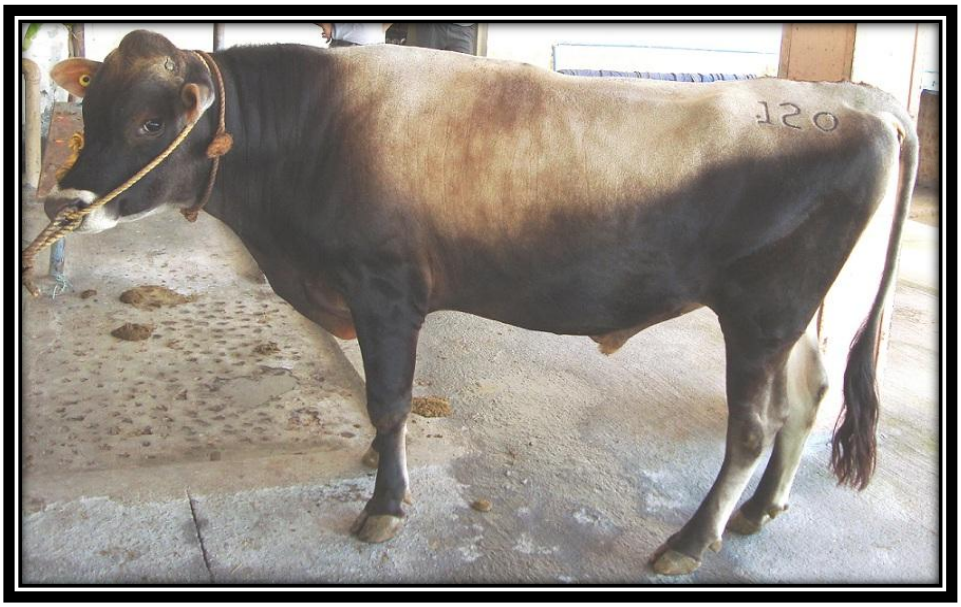


Figure 3.2 Jersey Bull

2. Nuclear material was pelleted by centrifugation at 4,000 rpm at room temperature for 10 minutes and the supernatant was discarded.

3. Nuclear pellet was washed with 10 ml of RBC lysis buffer and centrifuged again. This step was repeated 3 to 4 times until the clear nuclear pellet was obtained.

4. Nuclear pellet was resuspended with a Pasteur pipette and washed two times with 10 ml Tris Buffer Saline (TBS) at 3000 rpm for 10 minutes

5. A volume of 9 ml of Tris EDTA buffer (pH 8.0) was added to the pellet and vortexed.

5. Then 50 µl of Proteinase – K enzyme and 500 µl of 0.5M EDTA (Ethylene Diamine Tetra-acetic acid) was added to the above solution and mixed thoroughly. 20 per cent Sodium Dodecyl Sulphate (SDS) was added at the rate of 500 µl to above solution with gentle mixing and incubated at 50° C for overnight.

6. 4.3 ml of Saturated NaCl was added to the above samples and vigorously shaken, equal volume of Chloroform: Isoamyl alcohol (24:1 ratio) was added and centrifuged at 4000 rpm for 15 minutes.

7. Aqueous upper phase was transferred to fresh tube and the step involving addition of chloroform - isoamyl alcohol was repeated twice.

8. Finally, two volumes of 95% ethanol was added to the supernatant containing DNA at room temperature and inverted several times until the DNA was precipitated.

9. Precipitated DNA was transferred to a micro centrifuge tube containing ice-cold 70% ethanol and centrifuged at 2,000 rpm for 5 minutes. Supernatant was discarded and the pellet was air-dried. Dried DNA pellet was resuspended in 400µl TE buffer and the DNA samples were stored at -20°C.

3.4 Determination of Quality and Quantity of Genomic DNA

The quality of DNA was checked by agarose gel electrophoresis. The purity and concentrations were checked by UV- Spectrophotometer. About 30 µl of DNA was dissolved in 2970 µl of triple glass distilled water (1 in 100 dilution) and optical density (OD) values were measured at 260 nm and 280 nm with distilled water as blank. DNA samples having the OD260 /OD280ratio between 1.7 and 2.0 were considered pure and used for PCR reaction. The concentration of DNA was estimated using the formula:

Concentration of DNA (ng) = OD260 ×50 ×100 (dilution factor)

3.5 Preparation of template DNA for PCR

Working solution was prepared by diluting the stock genomic DNA with autoclaved distilled water to arrive at a final concentration of 50 ng / µl.

3.6 Primers for PCR amplification of ASS gene

A total of 14 sets of primers were designed for Argininosuccinate synthetase (ASS) gene using Primer3 software based on the 51997 bp sequence for ASS (Ensembl RefSeq: p14568). Oligonucleotide melting temperature, Oligonucleotide length, GC content, Primer-dimer possibilities, PCR product size, Positional constraints within the source sequence and miscellaneous constraints were considered while designing the primers.

Primers

The Wallace formula was used to calculate working approximation of Tm value.

$Tm= 4(G + C) + 2(A + T)$ °C

The Designed primers were procured from Amnion Biosciences Private Limited, Bangalore.

3.7 Polymerase Chain Reaction and Restriction Fragment Length Polymorphism PCR conditions were optimized for the reported Primer (Table 3.1) by testing a number of variables such as master mix concentration and temperature conditions. The combinations giving the best amplification were used for further studies.

Table 3.1 primer used for the amplification of exon 5 in the study of PCR-RFLP

Exon	Primer sequence (5' to 3' direction)
Exon no. 5	**F:** 5'GGCCAGGGACCGTGTTCATTGAGGACATC 3' **R:** 5' TTCCTGGGACCCCGTGAGACACATACTTG 3'

3.7.1 PCR Master Mix preparation

The PCR reaction master mix was prepared by adding the reagents in the following order into a sterile 0.2 ml PCR tube (Table 3.2). The contents were mixed thoroughly and spun for a few seconds in spinner (Spinwin MC-OO). The thermo cycling conditions optimized for the PCR amplification of exon 5 is summarized in Table 3.3.

Table 3.2 Composition of reaction mixture for PCR

S.No.	**Components**	**Quantity**
1	Sigma water	18 µl
2	10x Buffer (1X) with 1.5mM $Mgcl_2$	2.5 µl
3	2.5 Mm dNTPs (100 µM each)	2.0 µl
4	Forward Primer (20 pmol/µl)	0.25 µl
5	Reverse Primer (20 pmol/µl)	0.25 µl
6	DNA Template (50 ng/ µl)	1.0 µl
7	Taq DNA Polymerase	1.0 µl
	Total Volume	25.0 µl

Table 3.3 Thermo cycling conditions for PCR amplification of exon 5

S.No	Step	Temperature	Time
1	Initial denaturation	94°C	5 min
2	Denaturation	94°C	1 min
3	Annealing	58°C	1 min
4	Extension	72°C	1 min
Repeat the cycle for 35 times			
5	Final Extension	72°C	5 min
6	Hold	4°C	-

3.7.2 Agarose gel electrophoresis

DNA markers (100 bp DNA ladder) was loaded and run along with the samples in the same gel. The electrophoresis was performed at 100 V for 50 minutes. After the specified time, the gel was visualized under Gel Documentation system (Bio-Rad, USA).

1. 1.2 grams of agarose was weighed and transferred to a conical flask and 80 ml of 1X TBE buffer was added to prepare 1.5 per cent solution. It was placed in a microwave oven until the Agarose was dissolved completely

2. The solution was cooled to 60°C and ethidium bromide (0.5 µg/ml) was added and mixed thoroughly. The comb was positioned 0.5 – 1 mm above the plate so that a complete well was formed when the agarose was added.

3. As soon as the gel was completely set after 30 - 45 minutes at room temperature, both the comb and the tape were removed carefully and the gel was mounted in the electrophoresis tank with TBE buffer.

4. To confirm the targeted PCR amplification, 5 µl of PCR product from each tube was mixed with a small drop of gel loading dye (10X) and electrophoresed on 1.5 percent agarose gel containing Ethidium Bromide (one per cent solution at the rate of 5 µl/100 ml) at constant 100 V for 40 minutes in 1X TBE buffer. The lid of the gel tank was closed and attached with the electrical leads so that the DNA would migrate towards the anode.

5. After 40 minutes of running, the power supply was turned off and the lid was removed from the gel tank. The gel was visualized and photographed using gel documentation system (BIO RAD, USA).

6. The quality of the DNA was assessed based on the bands developed. Good quality DNA was used for further analysis through PCR-RFLP technique.

3.7.3 Restriction digestion

The RFLP digestion mix was prepared by adding the reagents in the following order into a sterile 0.2 ml PCR tube (Table 3.4).

3.7.4 Agarose Gel Electrophoresis

DNA markers (100 bp DNA ladder) along with the digested samples were loaded and run in the same 2.5% Agarose gel. The electrophoresis was performed at 100 V for 5 hours, followed by visualization of gel under Gel Documentation system (Bio-Rad, USA). The pattern of the restriction digestion was assessed based on the bands developed.

Table 3.4 composition of digestion mixture for RFLP

S.No.	**Components**	**Quantity**
1	Sigma water	3.0 µl
2	PCR product	10.0 µl
3	Buffer	1.5 µl
4	*Ava II* restriction endonuclease	0.5 µl
	Total Volume	25.0 µl
Incubate over night at 37^{o} C		

3.8 Polymerase Chain Reaction- Single Strand Conformational Polymorphism (PCR-SSCP)

PCR conditions were optimized for each Primer (Table 3.5) by testing a number of variables such as master mix concentration and temperature conditions. The combinations giving the best amplification were used for further studies. The thermo cycling conditions for PCR amplifications of different fragments are summarized in Table 3.9 to 3.11.

Table 3.5 Details of primers used in the study for PCR- SSCP analysis

Exons:	Primer sequence 5'-3'
Exon:1	**F**- GAGCTTATAACCCGGGATGC **R**-CCCAGGGAGCCAGGAATC
Exon:2	**F**-GGTCAGCTCCTCATGCTGAT **R**-TAAGACAGGCTTGGGTGAGG
Exon:3	**F**- AAAGACACCTGCCCAGGAC **R**- TGGAAGCAAGAGGACCAACT
Exon:4	**F**- TCAAGACCACCCTGTTAGCTG **R**-CGGGAACCAACGATTGTC
Exon:5	**F**-GGGTTTGTAGCACCCAGTTC **R**-GAGTGTCCCATGGAAGAAGG
Exon:6	**F**-AACCCTCGTGTCTCAGTTGG **R**- CCCCATCCTTGAGCCTTT
Exon:7 & 8	**F**-CGTGCCAACACTGTCTTACC **R**- CAGCCACCACCAAAAGACA
Exon:9	**F**- GATGGCAAGTGCCAGCAG **R**- CTTCCGGGAAACACAGGAG
Exon:10	**F**- ACATCCATTTAAGGCGTTGC **R**- CGGGACTCTTGTACAGAAGCA
Exon:11	**F**- GACGGAAGTTGCACACTGAG **R**- GTGAAAGGACTTGCCCAAAG
Exon:12	**F**- GCGTGAACATAATGGCAGAT **R**- AGGTGGCAGTGGGTAAGTTG
Exon:13	**F**- CTTGCTCCTCAGTGTGGTCA **R**- AAGTTCCACGAGGACAGAGG
Exon:14	**F**- GTTGTGGCTCCCTGCTCTC **R**- CTGTCAGCAAGTGTGCCTTC
Exon:15	**F**- TGAACTGAGTGCATGTTGTTATTG **R**- CACACACACACACATTCTTACCC

3.8.1 PCR Master Mix preparation

The PCR reaction master mix was prepared by adding the reagents in the following order into a sterile 0.2 ml PCR tube. The PCR mixture for PCR amplifications of different exons are summarized in Table 3.6 to 3.9 The contents were mixed thoroughly and spun for a few seconds in spinner (Spinwin MC-OO). The optimized thermo cyclic conditions for all fifteen exons are summarized in Table 3.10-3.13.

3.8.2 Agarose gel electrophoresis

DNA markers (100 bp DNA ladder) was loaded and run along with the samples in 1.5% Agarose gel. The electrophoresis was performed at 100 V for 50 minutes. After the specified time, the gel was visualized under Gel Documentation system (Bio-Rad, USA). The quality of the DNA was assessed based on the bands developed. Good quality DNA was used for further analysis through PCR-SSCP technique.

3.9 Poly Acrylamide Gel Electrophoresis (PAGE)

3.9.1 Preparation of acrylamide solution

The concentration of acrylamide and bis acrylamide concentrations (29:1) viz 30% was utilized for native PAGE gel preparation. Acrylamide: bis acrylamide were weighed in 29:1 ratio, then the contents were transferred to a clean 250 ml beaker and 60 ml of Double Distilled (DD) water was added and vortexed at 50ºC to dissolve the contents thoroughly. After dissolving, the volume was made up to 100 ml and the contents were filtered using Whattman Filter paper No. 1 and stored in tightly capped amber bottle to prevent formation of acrylic acid crystals.

3.9.2 Poly Acryl amide Gel preparation

1. The concentration of Polyacrylamide gel was determined based on the DNA sizes which are to be resolved. The gel casting with different acryl amide concentrations are shown in Table 3.14.

2. The various constituents required for 40 ml PAGE gel using 30 % acrylamide are given in Table 3.15.

Table 3.6 Composition of reaction mixture for PCR for exons- 3, 4, 6, 10, 11,12,13,14 and 15

S.No.	**Components**	**Quantity**
1	Sigma water	18 µl
2	10x Buffer (1X) with 1.5mM $Mgcl_2$	2.5 µl
3	2.5 Mm dNTPs (100 µM each)	2.0 µl
4	Forward Primer (20 pmol/µl)	0.25 µl
5	Reverse Primer (20 pmol/µl)	0.25 µl
6	DNA Template (50 ng/ µl)	1.0 µl
7	Taq DNA Polymerase	1.0 µl
	Total Volume	25.0 µl

Table 3.7 Composition of reaction mixture for PCR for exons- 1 and 2

S.No.	**Components**	**Quantity**
1	Sigma water	17 µl
2	10x Buffer (1X) with 1.5mM $Mgcl_2$	2.5 µl
3	2.5 Mm dNTPs (100 µM each)	2.0 µl
4	Forward Primer (20 pmol/µl)	0.25 µl
5	Reverse Primer (20 pmol/µl)	0.25 µl
6	DNA Template (50 ng/ µl)	2.0 µl
7	Taq DNA Polymerase	1.0 µl
	Total Volume	25.0 µl

Table 3.8 Composition of reaction mixture for PCR for exons 5, 7 and 8

S.No.	Components	Quantity
1	Sigma water	18.1 µl
2	10x Buffer (1X) with 1.5mM $Mgcl_2$	2.5 µl
3	2.5 Mm dNTPs (100 µM each)	2.0 µl
4	Forward Primer (20 pmol/µl)	0.2 µl
5	Reverse Primer (20 pmol/µl)	0.2 µl
6	DNA Template (50 ng/ µl)	1.0 µl
7	Taq DNA Polymerase	1.0 µl
	Total Volume	25.0 µl

Table 3.9 Composition of reaction mixture for PCR for exons 9

S.No.	Components	Quantity
1	Sigma water	18.6 µl
2	10x Buffer (1X) with 1.5mM $Mgcl_2$	2.5 µl
3	2.5 Mm dNTPs (100 µM each)	1.5 µl
4	Forward Primer (20 pmol/µl)	0.2 µl
5	Reverse Primer (20 pmol/µl)	0.2 µl
6	DNA Template (50 ng/ µl)	1.0 µl
7	Taq DNA Polymerase	1.0 µl
	Total Volume	25.0 µl

Table 3.10 Thermo cycling conditions for PCR amplification of exons 11,12,13,14 and 15

S.No	Step	Temperature	Time
1	Initial denaturation	94°C	5 min
2	Denaturation	94°C	1 min
3	Annealing	60°C	1 min
4	Extension	72°C	1 min
Repeat the cycle for 35 times			
5	Final Extension	72°C	5 min
6	Hold	4°C	-

Table 3.11 Thermo cycling conditions for PCR amplification of exons 4, 6 and10

S.No	Step	Temperature	Time
1	Initial denaturation	94°C	5 min
2	Denaturation	94°C	1 min
3	Annealing	52°C	1 min
4	Extension	72°C	1 min
Repeat the cycle for 35 times			
5	Final Extension	72°C	5 min
6	Hold	4°C	-

Table 3.12 Thermo cycling conditions for PCR amplification of exons 3, 7&8 and 9

S.No	Step	Temperature	Time
1	Initial denaturation	94°C	5 min
2	Denaturation	94°C	1 min
3	Annealing	55°C	1 min
4	Extension	72°C	1 min
Repeat the cycle for 30 times			
5	Final Extension	72°C	5 min
6	Hold	4°C	-

Table 3.13 Thermo cycling conditions for PCR amplification of exon 1, 2 and 5

S.No	Step	Temperature	Time
1	Initial denaturation	94°C	5 min
2	Denaturation	94°C	1 min
3	Annealing	54°C	1 min
4	Extension	72°C	1 min
Repeat the cycle for 30 times			
5	Final Extension	72°C	5 min
6	Hold	4°C	-

Table 3.14 Per cent of PAGE gels and sizes of denatured DNA

Acrylamide: Bis acrylamide ratio	Gel %	Denatured DNA (bp)
29:1	10	250-500

Table 3.15 Components in the preparation of PAGE gel

Components	10%
Acrylamide and Bis Acrylamide (29:1)	13.40ml
1 X TBE	26.21ml
10% APS	180 µl
TEMED	75 µl
Total volume	40 ml

3. The PCR - SSCP analysis of amplified gene fragments were carried out using vertical gel electrophoresis unit (Scie – Plas, UK). The two glass plates were washed thoroughly using tap water with detergent and rinsed initially under running tap water till no remnants of detergent are left. The plates were wiped two times with tissue paper soaked in distilled water first, 70 percent alcohol and then air-dried. The similar thorough cleaning treatment is given to spacers and comb to ensure proper alignment of 20 cm glass plates.

4. The glass plates were assembled with spacers in between them and are fastened with the clips after affixing. The cassette was formed by two glass plates separated by spacers, typically 0.5 to 1 mm in thickness, and sealed with adhesive tape at the bottom of the glass plates. Native PAGE gels were prepared by mixing an Acryl amide / Bis - acryl amide monomers concentrate, buffer concentrate and water to achieve the desired gel concentration.

5. 10% Ammonium Per sulphate (APS) and N, N, N', N' – Tetra methyl Ethylene Diamine (TEMED) were then added to the acryl amide solution to initiate polymerization, the contents were mixed well and immediately the solution was poured between the two glass plates (cassettes) and the comb is placed for well formation. It was allowed to solidify for a period of 60 to 90 minutes.

6. After gel formation the bottom adhesive tape was removed and clips along with the comb was gently removed and the loading wells were washed thoroughly with 1x TBE. It was ensured that there were no air bubbles in the wells.

7. The glass plate assembly with the gel was then fixed to the vertical gel electrophoresis unit and the 1 X TBE buffer was poured in the upper and lower buffer tanks. The samples were mixed with appropriate gel loading / tracking dye (15 µl sample + 5 µl dye) and loaded into the wells without air bubbles. Then the cathode and anode of the unit was connected to power pack correctly and the gel was run at 220V for about 4 to 6 hrs.

8. After completion of the electrophoresis the glass plates were removed from the assembly and gels were subjected to silver staining to visualize SSCP band patterns.

9. In order to stain the gel it was immersed in a tray of appropriate size filled with 10 per cent acetic acid (500ml) for at least 10 minutes for fixing DNA bands so as to prevent diffusion of the DNA bands (care was taken to see gel remains dipped well in solution). The gel was agitated slowly for 10 minutes or until the tracking dye is no longer visible.

10. The acetic acid was decanted and 500 ml of distilled water was poured to the tray and rinsed thoroughly by placing the tray on oscillatory automatic shaker for 5 minutes.
11. Meanwhile 500 ml of 0.1 per cent silver nitrate solution was prepared in amber color bottle and 500 µl of 37 per cent formaldehyde was added and mixed. Distilled water was gently decanted from tray. The gel was stained for 30 minutes in silver nitrate solution containing formaldehyde solution with constant shaking in a dark room or covering the tray with black cloth.
12. Then the gel was rinsed briefly for 30 seconds in distilled water.
13. Quickly distilled water was decanted from the tray. The freshly prepared and chilled 3 per cent sodium carbonate solution (3 per cent Na_2Co_3 and 750 µl of 37 per cent formaldehyde + 0.1 per cent Sodium Thiosulfate) was added to the tray. The gel was kept immersed until sharp bands were developed.
14. The gel was given 10 percent Acetic acid (stop solution) treatment for 10 minutes. Then 500 ml distilled water was added to the tray.
15. The gel was finally transferred gently on blotting paper and covered with the transparency sheet; excess water was soaked with tissue paper, and dried in gel drier for 10 minutes.

Examination of Silver stained gels: The silver stained gels were examined using transilluminator and photograph was taken using Sony digital camera. The gels were also examined using Gel Doc system (Bio Rad, USA) and SSCP variants were recorded. Then gels were labeled and scanned for computer image analysis and documentation. The different band patterns/variants were characterized based on the number of bands and mobility shifts identified for the different fragments of ASS gene. Each pattern was represented by a code.

3.10 Custom sequencing

The unique SSCP patterns obtained for the representative PCR products were segregated and further analyzed by direct sequencing (Amnion Biotech Pvt. Ltd., Bangalore, India). The custom made forward and reverse allele specific PCR primer sets used for PCR-SSCP assay were utilized for direct sequencing. At least two individual animal samples representative of each unique PCR-SSCP patterns were given for direct sequencing to obtain representative sequences.

3.10.1 Sequence data analysis

The complementary sequences representative of unique PCR-SSCP pattern were analyzed using DNA Baser sequence analysis tool. The sequence chromatograms were first analyzed to resolve ambiguous bases and to trim sequence ends of each chromatogram. The finalized chromatograms were carefully analyzed. The retrieved sequences representing each of the unique PCR-SSCP patterns were further analyzed using Clustal-W multiple sequence alignment tool and DNA Baser for detecting Single Nucleotide Polymorphisms (SNP's) and their respective deduced amino acid variations. The sequences were further analyzed by NCBI nucleotide Basic Local Alignment Search Tool (BLAST) to determine their gene identity and the degree of homology with sequences representative to related bovine species.

3.11 Genotype frequencies

The genotype frequencies were calculated directly by counting the bands appearing in the gels.

$$\text{Genotype frequency} = \frac{\text{The proportion of animals with a particular genotype}}{\text{Total number of animals}}$$

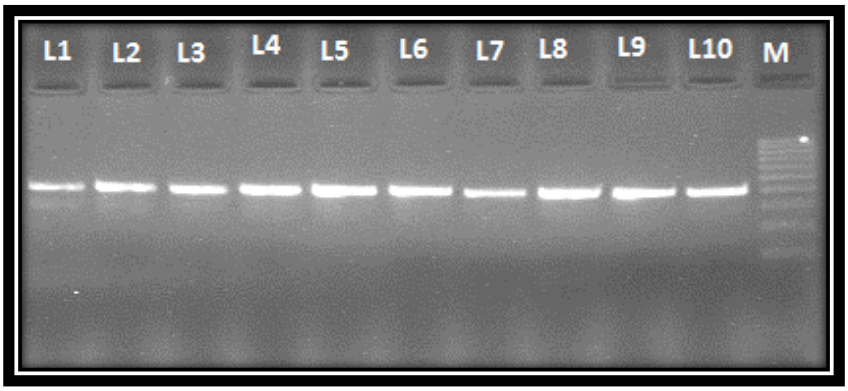
Results and Discussion

The present investigation was carried out to characterize Argininosuccinate Synthetase (ASS) gene and to find the deficiency of ASS gene if any in Holstein Friesian and Jersey bulls maintained at different bull stations across Karnataka state in India. The results obtained from the experiments are presented along with the discussions in this section.

4.1 DNA extraction, yield and purity

DNA was extracted from blood samples of 131 Holstein Friesian and 78 Jersey bulls by Miller's High Salt method with minor modifications. The yield of DNA ranged from 375 to 1330 µg/ ml of blood with a mean yield of 596.50 ± 36.50 µg / ml. The mean yield of DNA was comparable with the earlier reports of Shergojry et al., (2011) and Rao (2012). The purity of DNA (determined as O.D ratio at 260nm / 280nm) ranged between 1.7 and 1.9 in all the samples, with a mean of 1.80 ± 0.01 indicating high purity of the extracted DNA. The DNA samples were diluted to get the final concentration of 100 ng/ul in low Tris EDTA (TE) buffer for utilizing them as DNA template for further studies. Quality of DNA was checked by running PCR products on 0.8% Agarose gel (Figure 4.1).

Figure 4.1 Quality checking of DNA on 0.8% Agarose gel



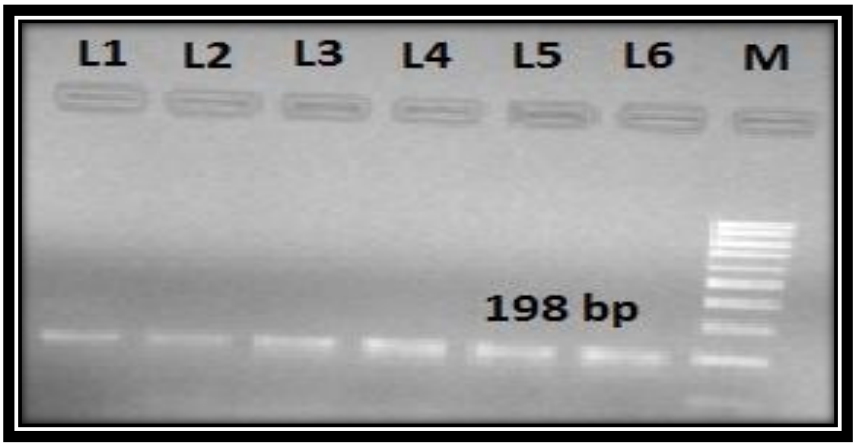
Lane 1-10 : Working DNA samples

4.2 Standardization of Polymerase Chain Reaction (PCR) for RFLP

PCR conditions were standardized for Primer by testing a number of factors such as primer concentration, number of cycles, concentration of $MgCl_2$, Taq DNA polymerase, template DNA and annealing temperature to obtain optimum amplification. Genotyping was carried out using primers according to Grupe et al., (1996). The PCR amplification was carried out at annealing temperature 58° C which is comparable with the earlier reports Oner et al., (2010)

and Meyden et al., (2010). The set of primer used for amplification of exon 5 gave a product of 198 bp. The PCR products were electrophoresed in 1.5% agarose gel at 100V for 45 minutes along with 100 bp DNA ladder as molecular weight marker and visualized under Gel Doc System (BIO RAD, USA). The amplified exon 5 PCR products (Figure 4.2) were subjected to Restriction Fragment Length Polymorphism (RFLP) analysis to screen for the presence of mutant allele.

Figure 4.2 Resolution of PCR amplified product of exon - 5 on 1.5% Agarose gel



Lane 1-6: PCR products (198 bp), Lane M: 100bp ladder molecular marker

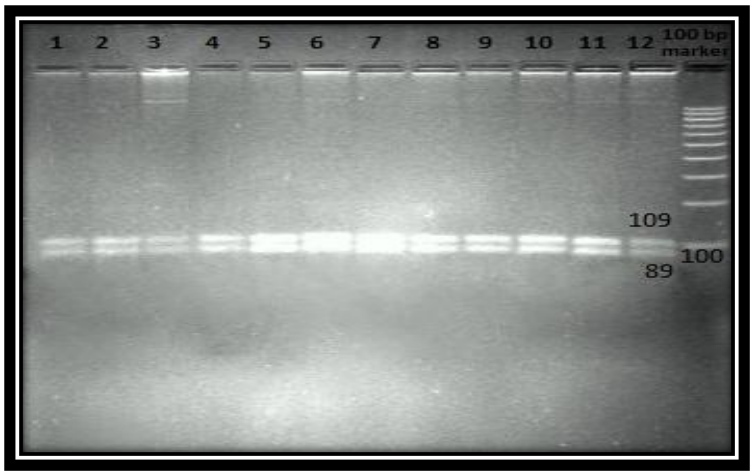
4.3 Standardization of Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) conditions were standardized by testing various factors such as enzyme concentration and duration of incubation (restriction digestion). For restriction digestion, *Ava II* enzyme was used with a concentration of 0.5 U and was incubated overnight, while Padeeri et al., (1999) used 5 U of *AvaII* and incubated for 3 hours. The digested products were electrophoresed in 2.5% agarose gel at 100V for 5 hours along with 100 bp DNA ladder as molecular weight marker and visualized under Gel Doc System (BIO RAD, USA) to analyze the bands (Figure 4.3).

Earlier workers has used 4% to 11% of gel for separation of digested products Robinson et al., (1993), Nassiry et al., (2005) and Jianbin Li et al., (2011). Jianbin Li et al., 2011 used 11% SDS PAGE to separate the digested products. In the present study we used EtBr for visualization of bands. Earlier workers (Robinson et al., (1993), Padeeri et al., (1999), Oner et

al., (2010)) have also used EtBr for visualization of gels, while Naassiry et al., (2005) used silver staining to detect bands.

Figure 4.3 PCR-RFLP digested products electrophoresed on 2.5% agarose gel



Lane 1-12: Digested products (109bp and 89 bp bp), Lane M: 100bp ladder molecular marker

The mutation that is responsible for citrullinemia results in the loss of restriction site for restriction enzyme *Ava II*. In homozygous normal, this enzyme is able to cut at restriction site and gives two bands 109 bp and 89 bp on electrophoresis. In carrier (heterozygous mutant), as one allele is normal and the other is mutant, the enzyme is able to cut only the normal one but not the mutant. This on electrophoresis gives three bands of DNA i.e. two bands for normal allele (109 bp and 89 bp) and one band for mutated allele (198 bp). In affected animals (homozygous mutant), as both the alleles are mutated, no restriction site is available for the enzyme. DNA from affected animals on digestion followed by electrophoresis gives only one single band (198 bp) of DNA. In the present study, on PCR-RFLP analysis, all animals showed two bands (109bp and 89 bp), revealing that none of them are carriers for citrullinemia. No carrier of citrullinaemia disease was observed among 131 Holstein Friesian and 78 Jersey bulls screened. The results obtained in the present study are in agreement with the earlier reports of Patel et al., (2006) in India, , Nassiry et al, (2005) and Eydivandi et al., (2012) in Iran, Oner et al., (2010), Meyden et al., (2010) in Turkey where they observed zero frequency for mutant allele of citrullinemia. Robinson et al., (1993) in United States Padeeri et al., (1999) Jianbin Li et al., (2011) in China reported low frequency of carrier for citrullinemia.

The availability of the bovine genome sequence and the ability of use of DNA markers have increased our efficiency in understanding a number of hereditary diseases in cattle, leading to the development of certain techniques for their early diagnosis. Due to increased practice of artificial insemination, there is an ever-present danger in its widespread application, such as undiagnosed genetic defects may be spread rapidly through the use of carrier bull's semen. Wide use of only a few elite bulls increased probability of genetic disorders. More attention is necessary towards screening of breeding bulls for genetic diseases to avoid using of carrier bulls in AI programme. Use of carrier bulls in breeding programs is harmful, because if a bull is carrying one copy of the mutant gene (a heterozygote) and is mated with an unaffected cow (homozygous wild type), they will produce 50% heterozygous carriers in the population. If both the parents (dam and sire) are carriers, then 25% of their progeny will be diseased, 50% will be carriers and only 25% will remain normal unaffected. It is therefore important to screen the animals, particularly breeding bulls at an early age to avoid great economic loss to cattle breeders.

The bovine model of citrullinemia was first reported in dairy cattle in Australia (Healy et al., 1991). The allele frequency of the mutant allele was found to be high in Australia (Healy et al., 1991). Descendents of Linmarck Kriss King (LMKK) which was a carrier for citrullinemia form 50% of the Australia National Friesian herd and 30 % of AI bulls in AI centers (Healy et al., 1991).

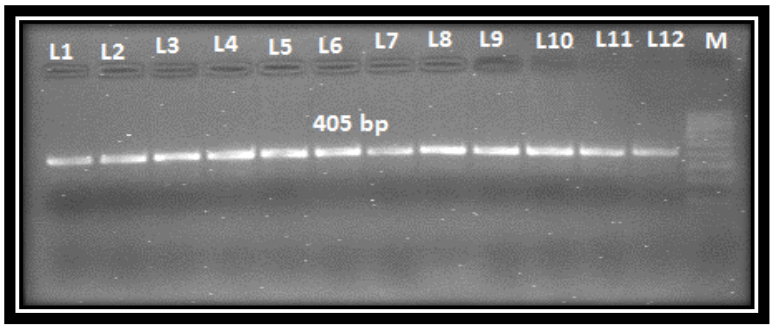
4.4 Standardization of Polymerase Chain Reaction (PCR) for SSCP

PCR conditions were standardized for each Primer by testing a number of factors such as primer concentration, number of cycles, concentration of $MgCl_2$, Taq DNA polymerase, template DNA and annealing temperature to obtain optimum amplification. Fourteen sets of primers were designed in such a way that each of the 15 exons of ASS gene was amplified and product size is below 500 bp (Table 4.1). The PCR conditions were optimized for fragment specific amplification of ASS gene. The PCR products were electrophoresed in 1.5% Agarose gel at 100V for 45 minutes along with 100 bp DNA ladder as Molecular weight marker and visualized under Gel Doc System (BIO RAD, USA). The amplified ASS gene PCR products (Figure 4.4 - 4.12) were subjected to single-strand conformation polymorphism (SSCP) analysis to determine the genetic variants.

Table 4.1 Details of annealing temperature and product size of different Exons

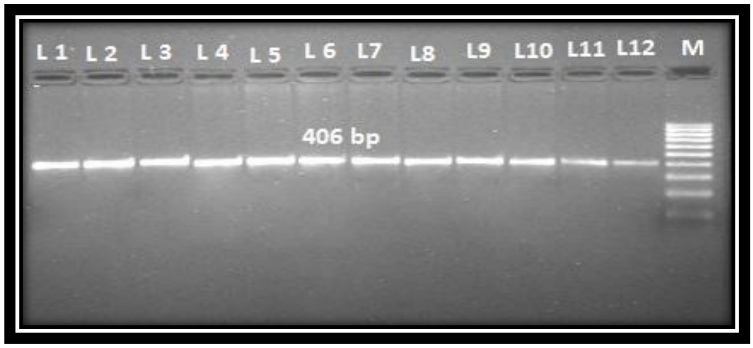
Exon	Location	Amplicon length (bp)	Annealing temperature
Exon 1	102 – 371	269	54°C
Exon 2	7671 – 8062	391	54°C
Exon 3	10001 - 10309	308	52°C
Exon 4	11382 - 11787	405	52°C
Exon 5	17708 - 18057	353	54°C
Exon 6	19578 - 20025	447	52°C
Exon 7 & 8	23078 - 23484	406	55°C
Exon 9	28343 - 28663	320	55°C
Exon 10	31093 - 31449	356	52°C
Exon 11	31613 - 31970	357	60°C
Exon 12	40694 - 41092	398	60°C
Exon 13	45929 - 46438	509	60°C
Exon 14	50464 - 50887	423	60°C
Exon 15	51868 - 52271	403	60°C

Figure 4.4 Resolution of PCR amplified product of exon - 4 on 1.5% Agarose gel



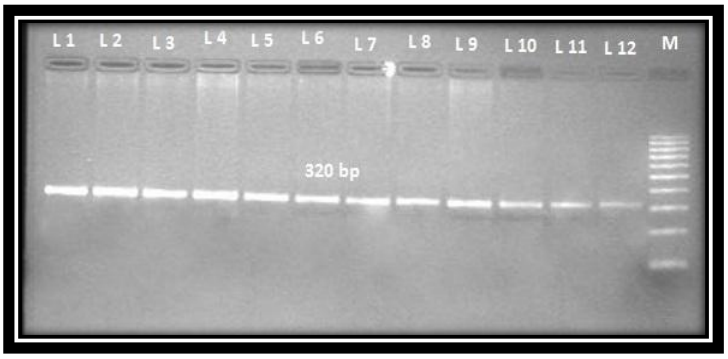
Lane 1-12: PCR product (405 bp), Lane M: 100bp ladder molecular marker

Figure 4.5 Resolution of PCR amplified product of exon- 7&8 on 1.5% Agarose gel



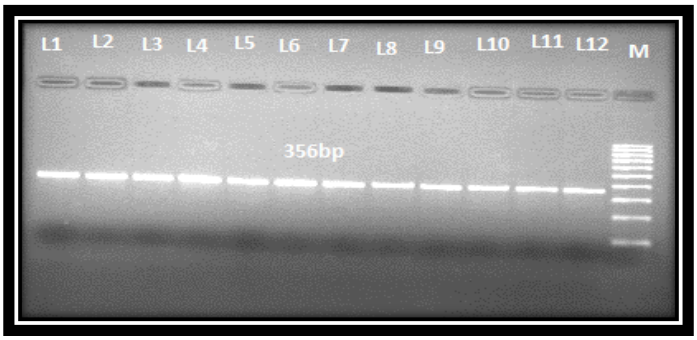
Lane 1-12: PCR product (406 bp), Lane M: 100bp ladder molecular marker

Figure 4.6 Resolution of PCR amplified product of exon- 9 on 1.5% Agarose gel



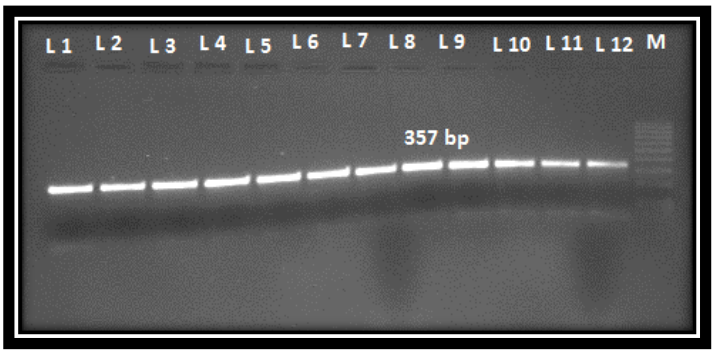
Lane 1-12: PCR product (320 bp), Lane M: 100bp ladder molecular marker

Figure 4.7 Resolution of PCR amplified product of exon- 10 on 1.5% Agarose gel



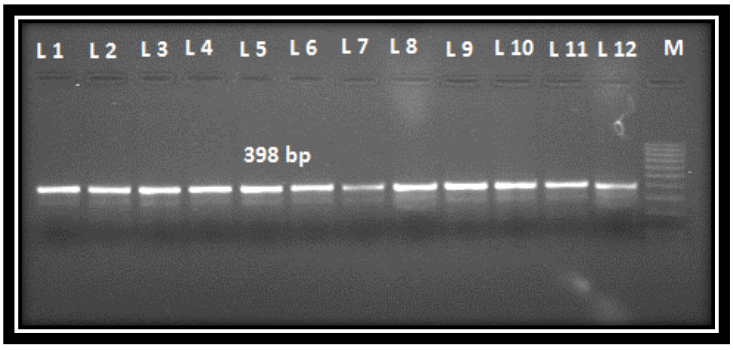
Lane 1-12: PCR product (356 bp), Lane M: 100bp ladder molecular marker

Figure 4.8 Resolution of PCR amplified product of exon- 11 on 1.5% Agarose gel



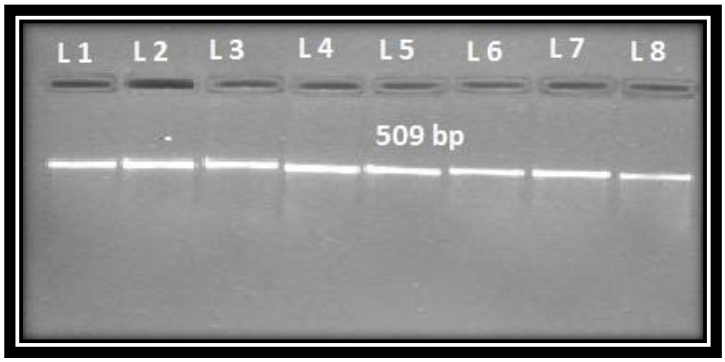
Lane 1-12: PCR product (357 bp), Lane M: 100bp ladder molecular marker

Figure 4.9 Resolution of PCR amplified product of exon- 12 on 1.5% Agarose gel



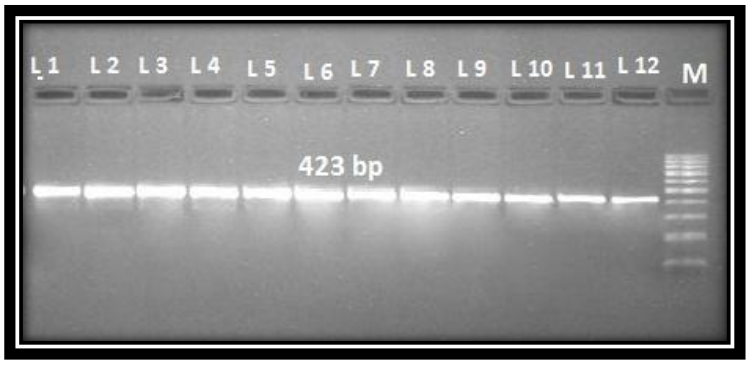
Lane 1-12: PCR product (398 bp), Lane M: 100bp ladder molecular marker

Figure 4.10 Resolution of PCR amplified product of exon- 13 on 1.5% Agarose gel



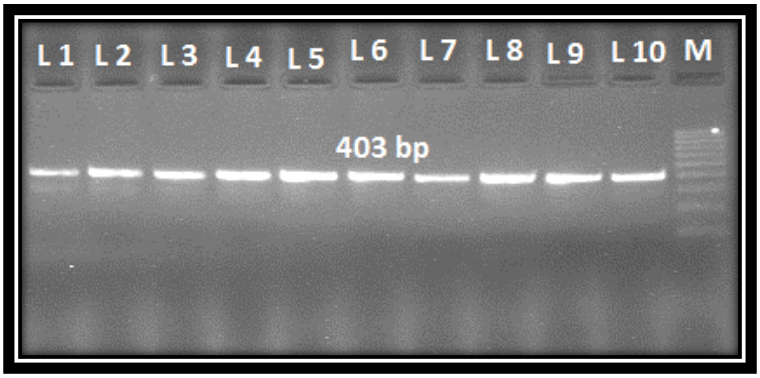
Lane 1-8: PCR product (509 bp), Lane M: 100bp ladder molecular marker

Figure 4.11 Resolution of PCR amplified product of exon- 14 on 1.5% Agarose gel



Lane 1-12: PCR product (423 bp), Lane M: 100bp ladder molecular marker

Figure 4.12 Resolution of PCR amplified product of exon- 15 on 1.5% Agarose gel



Lane 1-12: PCR product (403 bp), Lane M: 100bp ladder molecular marker

4.5 Single Strand Conformation Polymorphism (SSCP) analysis

The amplified PCR products of different fragments were analyzed by Single Strand Conformation Polymorphism (SSCP) analysis. Various factors such as amount of PCR product, denaturing solution, voltage, running time, acrylamide: bisacrylamide ratio, acrylamide concentration and temperature were optimized for SSCP analysis. Each PCR product was diluted in denaturing solution, denatured at 95°C for 8 min, chilled on ice and resolved on optimized concentration of non-denaturing polyacrylamide gels. The electrophoresis was carried out in a vertical electrophoretic system (SCIE-PLAS, U.K) in 1X TBE buffer. The optimized conditions for SSCP analysis using vertical electrophoresis is summarized in Table 4.2.

The percent of acrylamide used for PAGE in this study is comparable with the earlier reports of Nassiry et al., (2005) and Shergojry et al., (2011), where they have used acrylamide in the range of 6% to 10%. The gels were silver stained. Silver stained SSCP gels were dried and documented for detecting mobility shifts in different fragments of ASS gene in Holstein Friesian and Jersey breed of cattle. The different band patterns/variants were characterized by the number of bands and mobility shifts identified for the different fragments of ASS gene. Each pattern was represented by a code.

4.5.1 Distribution of PCR-SSCP patterns

The PCR-SSCP analysis of ASS gene amplicons revealed varying degree of genetic polymorphisms with respect to each of the ASS gene exons analyzed. PCR-SSCP analysis of amplicons of the exons-1, 5, 6, 8, 9, 11, 12, 13 and 14 showed monomorphism in Holstein Friesian and Jersey breed of cattle. The photographs of the representative exons were illustrated in the Figure 4.13 – 4.20. Thus the ASS gene exons 1, 5, 6, 8, 9, 11, 12, 13 and 14 showed absence of polymorphism indicating the probable absence/lack of mutation/s suggesting high degree of conservation of ASS gene in the above exons in Holstein Friesian and Jersey breed of cattle.

Polymorphisms in Holstein Friesian (HF) breed

PCR-SSCP analysis of amplicons of exons – 2, 7 and 15 showed polymorphism in HF breed. PCR-SSCP analysis of exon-2, 7 and 15 (Figure 4.21 – 4.23) in ASS gene of HF males revealed two unique SSCP patterns with different mobility shifts, viz. pattern I and

Table 4.2: Details of optimized conditions for SSCP analysis

ASS gene Fragments	Acrylamide : bisacrylamide Ratio	Acrylamide (%)	Amplified DNA product (µL)	Denaturing solution (µL)	Duration (h)
Exon -1	29:1	10%	10µl	10µl	5:00
Exon -2	29:1	10%	10µl	10µl	6:00
Exon -3	29:1	10%	10µl	10µl	5:00
Exon -4	29:1	10%	10µl	10µl	6:00
Exon -5	29:1	10%	10µl	10µl	6:00
Exon -6	29:1	10%	10µl	10µl	7:00
Exon-7&8	29:1	10%	10µl	10µl	6:00
Exon-9	29:1	10%	10µl	10µl	6:00
Exon-10	29:1	10%	10µl	10µl	6:00
Exon-11	29:1	10%	10µl	10µl	6:00
Exon-12	29:1	10%	10µl	10µl	6:00
Exon-13	29:1	10%	10µl	10µl	7:00
Exon-14	29:1	10%	10µl	10µl	7:00
Exon-15	29:1	10%	10µl	10µl	6:00

Figure 4.13 PCR-SSCP Patterns of exon 6 of ASS gene in Holstein Friesian breed

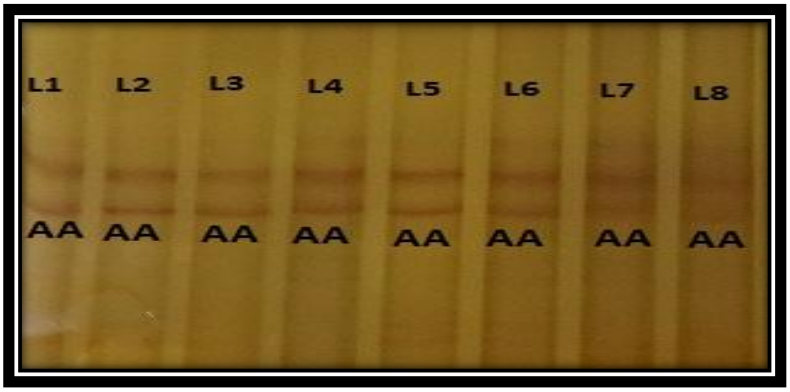


Figure 4.14 PCR-SSCP Patterns of exon 6 of ASS gene in Jersey breed

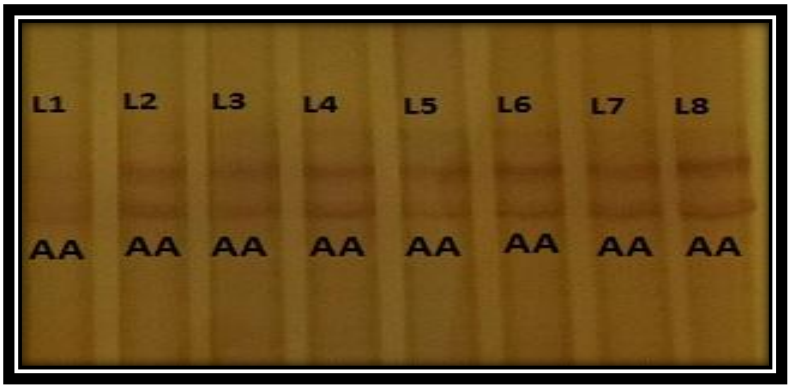


Figure 4.15 PCR-SSCP Patterns of exon 11 of ASS gene in Holstein Friesian breed

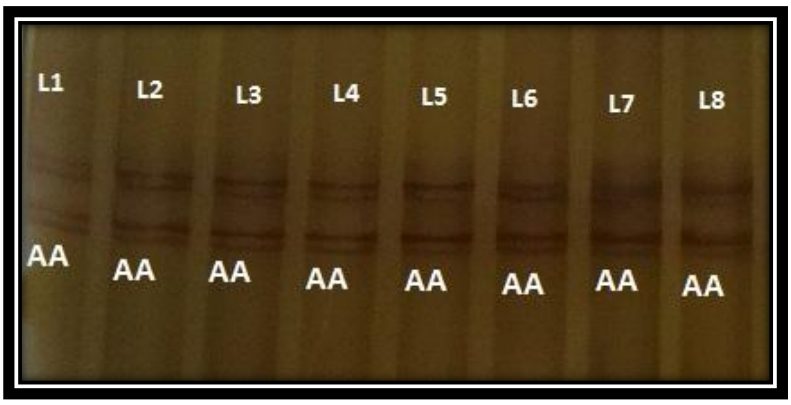


Figure 4.16 PCR-SSCP Patterns of exon 12 of ASS gene in Holstein Friesian breed

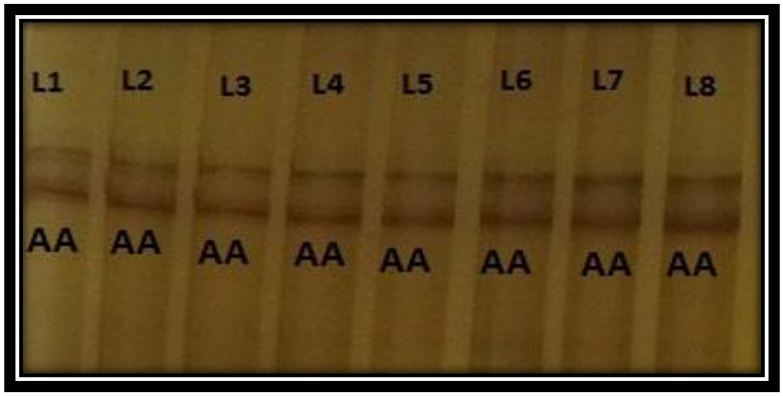


Figure 4.17 PCR-SSCP Patterns of exon 12 of ASS gene in Jersey breed

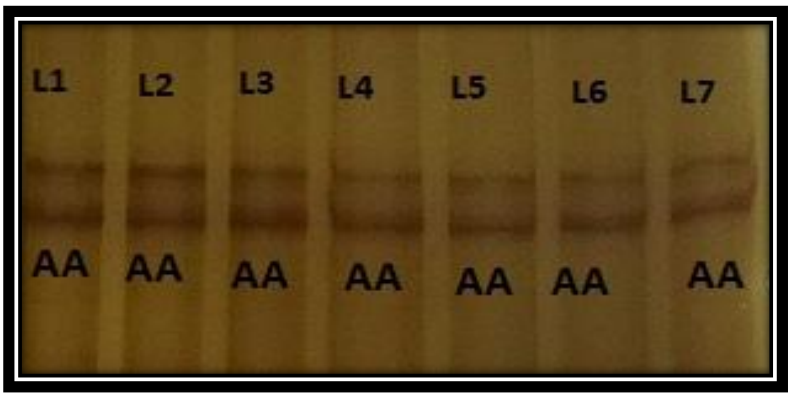


Figure 4.18 PCR-SSCP Patterns of exon 14 of ASS gene in Holstein Friesian breed

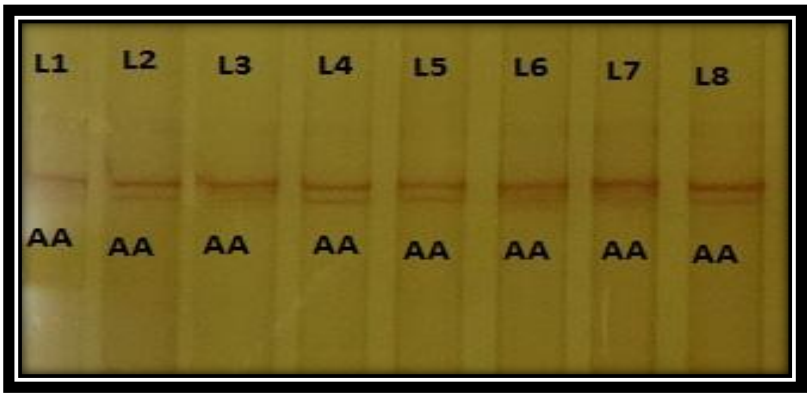


Figure 4.19 PCR-SSCP Patterns of exon 14 of ASS gene in Jersey breed

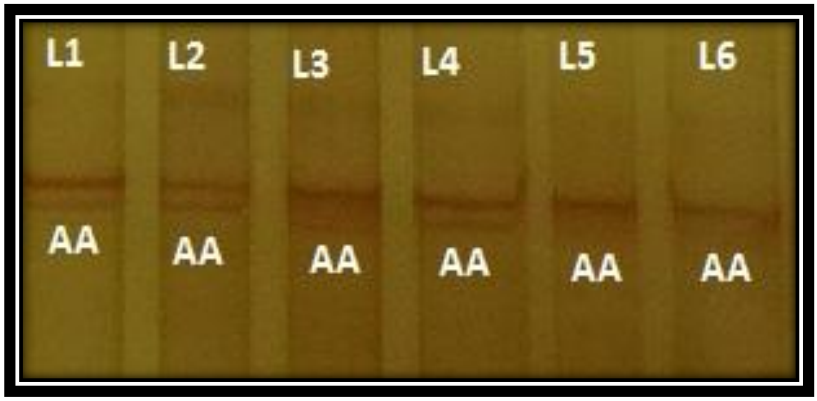
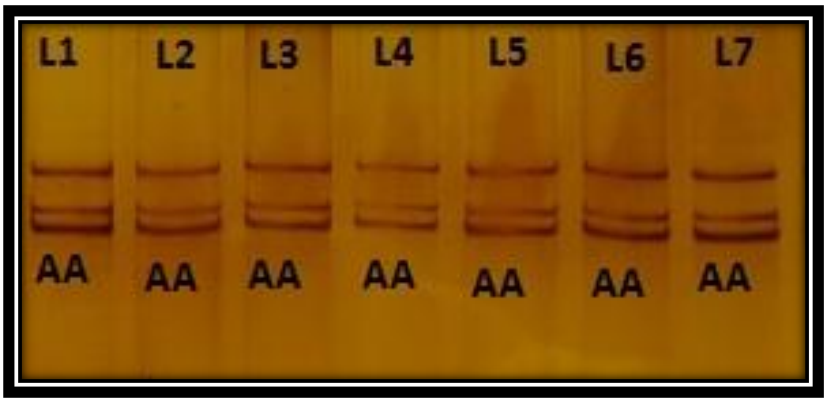


Figure 4.20 PCR-SSCP Patterns of exon 15 of ASS gene in Jersey breed



pattern II respectively for each exon. SSCP pattern I showed two distinct bands and pattern II showed three distinct bands in both exon 2 and 7. In exon 15 SSCP pattern I revealed three bands and pattern II revealed five bands. Out of the total 131 HF males genotyped, the genotypic frequency of pattern I, and pattern II for exons 2, 7 and 15 were 0.5954 and 0.4045, 0.7099 and 0.2900, 0.9084 and 0.0916 respectively (Table 4.3). No earlier report was available to compare or contrast the present findings.

Exons showing polymorphism in Jersey breed

PCR-SSCP analysis of amplicon of exons – 3 and 4 showed polymorphism in Jersey males. PCR-SSCP study of exon 3 (Figure 4.24) and 4 (Figure 4.25) in ASS gene of Jersey revealed two unique SSCP patterns with different mobility shifts, viz. pattern I and pattern II respectively. SSCP pattern I showed two distinct bands and pattern II showed three distinct

bands, respectively in both the exons 3 and 4. Out of the total 78 Jersey animals genotyped, the genotypic frequency of pattern I, and pattern II were 0.7179 and 0.2820 for exon 3 and 0.7820 and 0.2179 for exon 4 respectively (Table 4.4). No earlier report was available to compare the present study results.

Exon showing polymorphism in both Holstein Friesian and Jersey breeds

PCR-SSCP analysis of amplicon of exon 10 showed polymorphism in both HF and Jersey males. PCR-SSCP analysis of exon-10 in ASS gene of HF and Jersey bulls revealed two unique SSCP patterns (Figure 4.26 and 4.27), viz. pattern I and pattern II respectively for each breed with different mobility shifts. Out of the total 78 Jersey animals genotyped, the genotypic frequency of pattern I, and pattern II were 0.6282 and 0.3717 respectively (Table 4.5). Out of the total 131 HF animals genotyped, the genotypic frequency of pattern I, and pattern II was 0.7480 and 0.2519 respectively (Table 4.5). No earlier report was available to compare or contrast the present findings.

The genotype frequencies observed in the present investigation suggests that the Holstein Friesian breed of cattle have a diverse type of SSCP patterns for exon- 2, 7 and 15. Jersey males have a diverse type of SSCP patterns for exon- 3 and 4. Both HF and Jersey breeds of cattle have diverse type of SSCP patterns for exon 10, in the sampled population indicating the existence of variability.

Figure 4.21 PCR-SSCP Patterns of exon 2 of ASS gene in Holstein Friesian breed

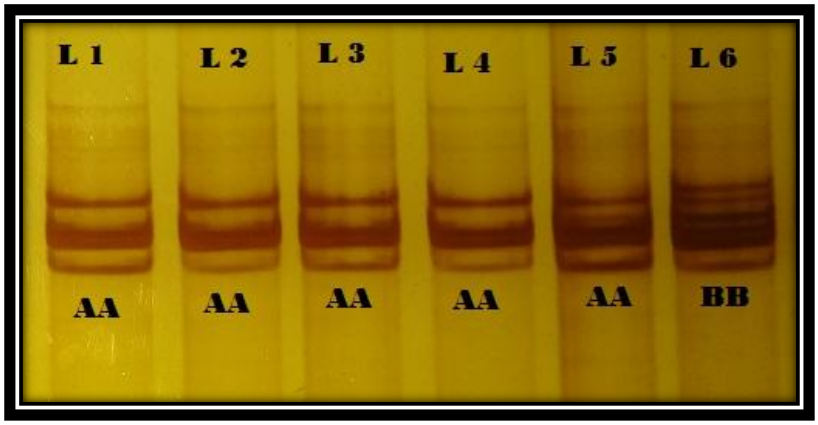


Figure 4.22 PCR-SSCP Patterns of exon 7 of ASS gene in Holstein Friesian breed

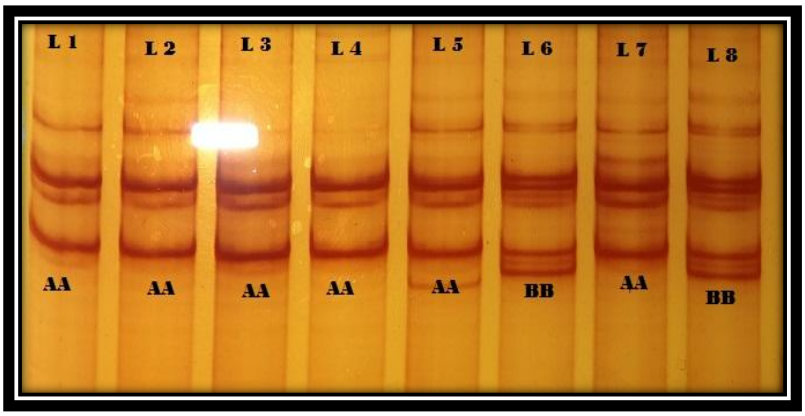


Figure 4.23 PCR-SSCP Patterns of exon 15 of ASS gene in Holstein Friesian breed

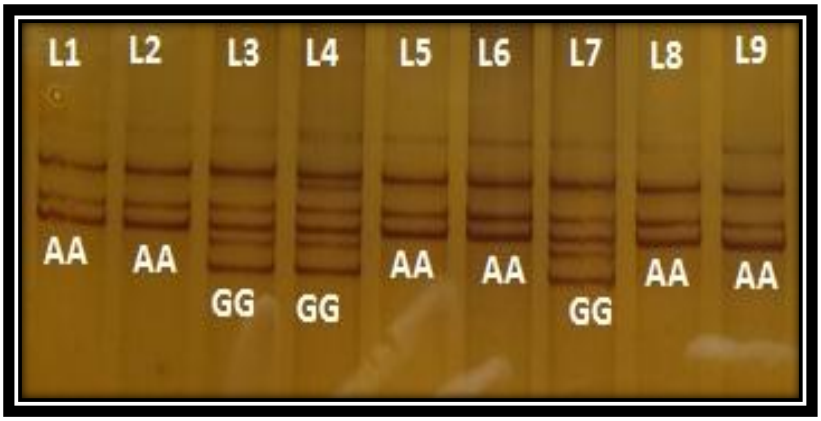


Figure 4.24: PCR-SSCP Patterns of exon 3 of ASS gene in Jersey breed

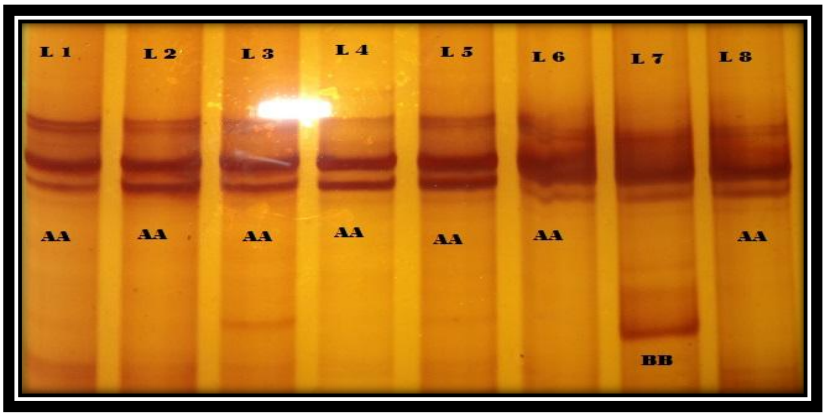


Figure 4.25: PCR-SSCP Patterns of exon 4 of ASS gene in Jersey breed

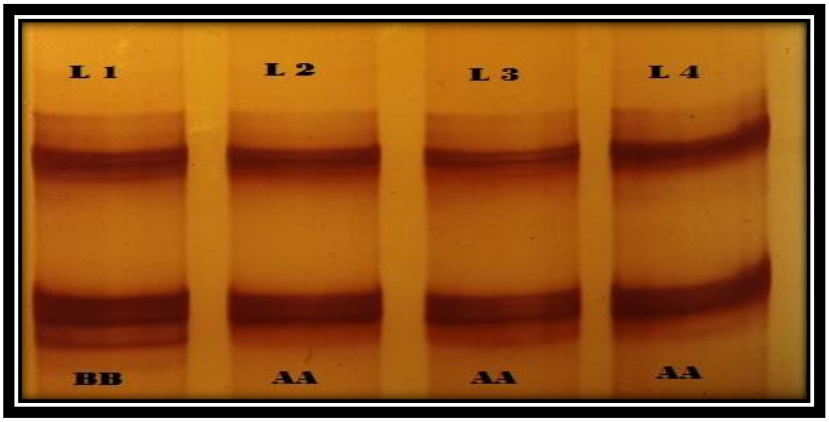


Figure 4.26: PCR-SSCP Patterns of exon 10 of ASS gene in Holstein Friesian breed

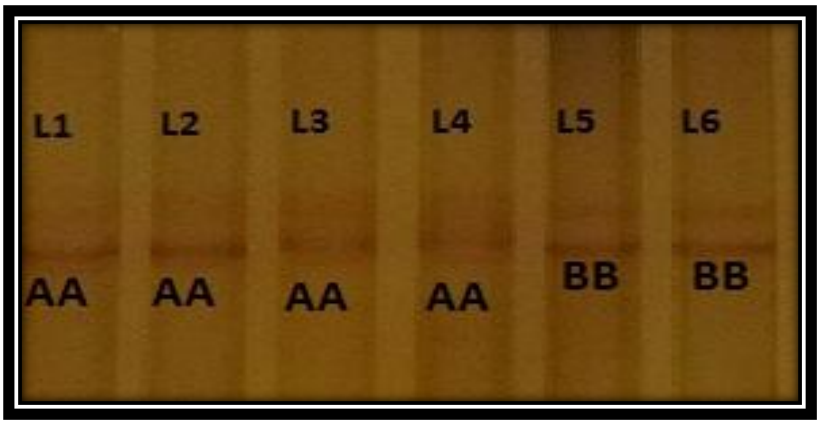


Figure 4.27: PCR-SSCP Patterns of exon 10 of ASS gene in Jersey breed

Table 4.3 Genotypic Frequencies and genetic diversity in exons- 2, 7 and 15 of ASS gene in Holstein Friesian cattle

Exon	Pattern I		Pattern II	
	No of Observations	Genotypic frequency	No of observations	Genotypic frequency
Exon 2	78	0.5954	53	0.4045
Exon 7	93	0.7099	38	0.2900
Exon 15	119	0.9084	12	0.0916

Table 4.4 Genotypic Frequencies and genetic diversity in exons- 3 and 4 of ASS gene in Jersey cattle

Exon	Pattern I		Pattern II	
	No of Observations	Genotypic frequency	No of observations	Genotypic frequency
Exon 3	56	0.7179	22	0.2820
Exon 4	61	0.7820	17	0.2179

Table 4.5 Genotypic Frequencies and genetic diversity in exons- 10 of ASS gene in both Holstein Friesian and Jersey cattle

Exon	Pattern I		Pattern II	
	No of Observations	Genotypic frequency	No of observations	Genotypic frequency
Exon 10 (HF)	98	0.7480	33	0.2519
Exon 10 (Jersey)	49	0.6282	29	0.3717

4.6 Sequencing and analysis

Representative samples in duplicate for each PCR-SSCP pattern of exon 2, 3, 4, 7, 10 and 15 were carefully chosen and the PCR products were custom sequenced (Amnion Biosciences Pvt. Ltd., Bangalore) using the automated ABI DNA sequencer to confirm the mobility shift in each pattern. Sequence data were analyzed using DNA baser and Bio edit software Clustal W multiple alignments for detecting single nucleotide polymorphisms (SNPs).

In order to detect possible polymorphisms, we compared our sequence to reference sequence of ASS in ENSEMBL with accession number p14568 for cattle. The Analysis of exon 2 of ASS gene in HF cattle revealed one A→G transition (Figure 4.28) at position 7947 (Table 4.7). The observed SNP resulted in change in amino acid from Isoleucine to Valine in the translated protein. The analysis of exon 3 of ASS gene in Jersey breed revealed one T→C transition (Figure 4.29) at position 10172 (Table 4.8), which was a silent mutation. The analysis of exon 4 of ASS gene in Jersey cattle revealed one G→A transition (Figure 4.26) at position 11506 and second G→A transition (Figure 4.30) at position 11518. Both the observed SNPs were silent mutations and resulted in no change in amino acid. The analysis of exon 7 of ASS gene in HF males revealed one C→T transition (Figure 4.31) at position 23153, which was also a silent mutation, results in no change in amino acid. The analysis of exon 10 of ASS gene in both HF and Jersey breed of cattle revealed one G→C transversion (Figure 4.32) at position 31215, which resulted in no change in amino acid in the translated product. The analysis of exon 15 of ASS gene in HF revealed four SNPs viz., T→G transversion at position 52105, C→T transition at position 52135, A→G transition at position 52136 and A→G transition at position 52153 (Figure 4.33). All the four SNPs were silent mutations.

In exon 2, due to A→G transition, a non polar amino acid (Isoleucine) was substituted by another non polar amino acid (Valine), results in no alteration in the properties of the protein. Not all mutations in DNA lead to a detectable change in phenotype. Mutation without apparent effect is called silent mutation. If a mutation in which the new codon specifying the same amino acid as the unmutated codon then it is a case of silent or synonymous mutation. This was same in the case of exons - 3, 4, 7, 10 and 15 even though base substitution has been taken place, due to degeneracy (one amino acid can be coded by more than one codon) of genetic code, change in amino acid has not taken place. In case of exon 15, four SNPs were detected. But the whole exon 15 is not getting translated i.e. only

some portion of it was undergoing translation. The detected SNPs were in the untranslated region and were silent mutations and did not show any effect on protein translation or its properties.

The present study revealed existence of variability in ASS gene in HF and Jersey breeding bulls. Though the present study as well as earlier studies has clearly shown that the incidence of heterozygotes for citrullinemia has been estimated to be very less, but it leads to enormous economic loss to breeding industry. Therefore it is essential to eliminate this condition from the breed to avoid calf losses, which could be achieved only by early screening of animals particularly breeding males.

Table 4.6 Summary of Single nucleotide polymorphisms observed in ASS gene in Holstein Friesian cattle

Gene	Position[a]	Variation[b]	Amino acid change
Exon 2	7947	GTCA/GTTG	Isoleucine to Valine
Exon 7	23153	CCCC/TAAG	No change
Exon 10	31251	AGCG/CTTG	No change
Exon 15	52105	GCCT/GCAG	No change
	52135, 52136	GCCC/TA/GGA	No change
	52153	CAGA/GCGG	No change

Table 4.7 Summary of Single nucleotide polymorphisms observed in ASS gene in Jersey cattle

Gene	Position[a]	Variation[b]	Amino acid change
Exon 3	10172	GCTT/CGGG	No change
Exon 4	11506	TGAG/AGGA	No change
	11518	CAAG/AGAG	No change
Exon 10	31251	AGCG/CTTG	No change

[a-] based on the sequence from the ENSEMBL reference sequence p14568

[b –] polymorphic residues underlined (the common nucleotide followed by the variant)

Figure 4.28: Clustal W multiple alignment sequence of exon 2

1. TCACGATGTCCGGCAAAGGCTCCGTGGTTCTGGCCTACAGTGGGGGCCTGGACACCTCCTGCATCCT
2. TCACGATGTCCGGCAAAGGCTCCGTGGTTCTGGCCTACAGTGGGGGCCTGGACACCTCCTGCATCCT

1. CGTGTGGCTGAAGGAGCAAGGCTATGACGTCATTGCCTACCTG
2. CGTGTGGCTGAAGGAGCAAGGCTATGACGTC**G**TTGCCTACCTG

1. Corresponds to Animal No. HF 20 ;
2. Corresponds to Animal No. Jy 22

Figure 4.29: Clustal W multiple alignment sequence of exon 3

1. GCCAACATCGGCCAGAAAGAAGACTTTGAGGAAGCCAGGAAGAAGGCGCTGAAGCTTGGGGCC
2. GCCAACATCGGCCAGAAAGAAGACTTTGAGGAAGCCAGGAAGAAGGCGCTGAAGCTTGGGGCC
3. GCCAACATCGGCCAGAAAGAAGACTTTGAGGAAGCCAGGAAGAAGGCGCTGAAGCT**C**GGGGCC
4. GCCAACATCGGCCAGAAAGAAGACTTTGAGGAAGCCAGGAAGAAGGCGCTGAAGCT**C**GGGGCC

1. AAAAAG
2. AAAAAG
3. AAAAAG
4. AAAAAG

1. Corresponds to Animal No. HF 22
2. Corresponds to Animal No. HF 95
3. Corresponds to Animal No. Jy 20
4. Corresponds to Animal No. Jy 168

Figure 4.30: Clustal W multiple alignment sequence of exon 4

1. GTGTTCATTGAGGACATCAGCAAGGAGTTTGTGGAGGAGTTCATCTGGCCGGCCATCCAGTCCAGCGCA
2. GTGTTCATTGAGGACATCAGCAAGGAGTTTGTGGAGGAGTTCATCTGGCCGGCCATCCAGTCCAGCGCA
3. GTGTTCATTGA**A**GACATCAGCAAGGAGTTTGTGGAGGAGTTCATCTGGCCGGCCATCCAGTCCAGCGCA
4. GTGTTCATTGAGGACATCAGCAA**A**GAGTTTGTGGAGGAGTTCATCTGGCCGGCCATCCAGTCCAGCGCA

1. GTGTACGAGGACCGATGCCTCCTGGGCACCTCTCTCGCCAGGCCCTGCATCGCCCGCAAGCAGGTGGAG
2. GTGTACGAGGACCGATGCCTCCTGGGCACCTCTCTCGCCAGGCCCTGCATCGCCCGCAAGCAGGTGGAG
3. GTGTACGAGGACCGATGCCTCCTGGGCACCTCTCTCGCCAGGCCCTGCATCGCCCGCAAGCAGGTGGAG

4. GTGTACGAGGACCGATGCCTCCTGGGCACCTCTCTCGCCAGGCCCTGCATCGCCCGCAAGCAGGTGGAG

1. ATCGCCCAGCGAGAAGGAGCC AAGTATGTGTCTCACGGCGCCACAGGAAAG
2. ATCGCCCAGCGAGAAGGAGCC AAGTATGTGTCTCACGGCGCCACAGGAAAG
3. ATCGCCCAGCGAGAAGGAGCC AAGTATGTGTCTCACGGCGCCACAGGAAAG
4. ATCGCCCAGCGAGAAGGAGCC AAGTATGTGTCTCACGGCGCCACAGGAAAG

1. Corresponds to Animal No. HF 20
2. Corresponds to Animal No. HF 33
3. Corsresponds to Animal No. Jy 38
4. Corresponds to Animal No. Jy 22

Figure 4.31: Clustal W multiple alignment sequence of exon 7

1. CAACATGGAATCCCCGTCCCAGTCACCCCCAAGAACCCGTGGAGCATGGACGAGAACCTG
2. CAACATGGAATCCCCGTCCCAGTCACCCC**T**AAGAACCCGTGGAGCATGGACGAGAACCTG

1. ATGCATATCAG
2. ATGCATATCAG

1. Corresponds to Animal No. HF 95 2. Corresponds to Animal No. Jy 168

Figure 4.32: Clustal W multiple alignment sequence of exon 10

1. GGGTCCCCGTGAAGGTGACCAACGTCGGGGATGGCACCACCCACAGCACAGC**C**TTGGAGCTTTTCCTGT
2. GGGTCCCCGTGAAGGTGACCAACGTCGGGGATGGCACCACCCACAGCACAGCGTTGGAGCTTTTCCTGT
3. GGGTCCCCGTGAAGGTGACCAACGTCGGGGATGGCACCACCCACAGCACAGC**C**TTGGAGCTTTTCCTGT

1. ACCTGAATGAAGTCGC
2. ACCTGAATGAAGTCGC
3. ACCTGAATGAAGTCGC

1. Corresponds to Animal No. HF 1009 3. Corresponds to Animal No. Jy 120
2. Corresponds to Animal No. Jy 40

Figure 4.33: Clustal W multiple alignment sequence of exon 15

1. GCTGAAGGAATATCATCGCCTCCAGAACAAGGTCACCGCCAAATAGACCAGCTGACAATGCAGAGCTGG
2. GCTGAAGGAATATCATCGCCTCCAGAACAAGGTCACCGCCAAATAGACCAGCTGACAATGCAGAGCTGG

3. GCTGAAGGAATATCATCGCCTCCAGAACAAGGTCACCGCCAAATAGACCAGCTGACAATGCAGAGCTGG
4. GCTGAAGGAATATCATCGCCTCCAGAACAAGGTCACCGCCAAATAGACCAGCTGACAATGCAGAGCTGG
5. GCTGAAGGAATATCATCGCCTCCAGAACAAGGTCACCGCCAAATAGACCAGCTGACAATGCAGAGCTGG
6. GCTGAAGGAATATCATCGCCTCCAGAACAAGGTCACCGCCAAATAGACCAGCTGACAATGCAGAGCTGG

1. GCCGCCTCACTTTACCCATCTCCCAAGTGTAGCTGCTAATTGTTGTGATAATTTGTGATTGTGACTTGTTCT
2. GCCGCCTCACTTTACCCATCTCCCAAGTGTAGCTGCTAATTGTTGTGATAATTTGTGATTGTGACTTGTTCT
3. GCCGCCTCACTTTACCCATCTCCCAAGTGTAGCTGCTAATTGTTGTGATAATTTGTGATTGTGACTTGTTCT
4. GCCGCCTCACTTTACCCATCTCCCAAGTGTAGCTGCTAATTGTTGTGATAATTTGTGATTGTGACTTGTTCT
5. GCCGCCTCACTTTACCCATCTCCCAAGTGTAGCTGCTAATTGTTGTGATAATTTGTGATTGTGACTTGTTCT
6. GCCGCCTCACTTTACCCATCTCCCAAGTGTAGCTGCTAATTGTTGTGATAATTTGTGATTGTGACTTGTTCT

1. CTGGGTCTGGCAGCGTAGTGGGGGTGCCAGGCCTCAGCTTTGTTCCAATAGTCCCCTGTAGCCCACAGA
2. CTGGGTCTGGCAGCGTAGTGGGGGTGCCAGGCCTCAGCTTTGTTCCAATAGTCCCCTGTAGCCCACAGA
3. CTGGGTCTGGCAGCGTAGTGGGGGTGCCAGGCCTCAGCTTTGTTCCAATAGTCCCCTGTAGCCCACAGA
4. CTGGGTCTGGCAGCGTAGTGGGGGTGCCAGGCCTCAGCTTTGTTCCAATAGTCCCCTGTAGCCCACAGA
5. CTGGGTCTGGCAGCGTAGTGGGGGTGCCAGGCC**G**CAGCTTTGTTCCAATAGTCCCCTGTAGCC**TG**CAGA
6. CTGGGTCTGGCAGCGTAGTGGGGGTGCCAGGCCTCAGCTTTGTTCCAATAGTCCCCTGTAGCCCACAGA

1. AGTGGTCCACAGACGGAGGGATGGGGCGGGGAGCGGGGGGCTGCAGTGAGG
2. AGTGGTCCACAGACGGAGGGATGGGGCGGGGAGCGGGGGGCTGCAGTGAGG
3. AGTGGTCCACAGACGGAGGGATGGGGCGGGGAGCGGGGGGCTGCAGTGAGG
4. AGTGGTCCACAGACGGAGGGATGGGGCGGGGAGCGGGGGGCTGCAGTGAGG
5. AGTGGTCCACAG**G**CGGAGGGATGGGGCGGGGAGCGGGGGGCTGCAGTGAGG
6. AGTGGTCCACAGACGGAGGGATGGGGCGGGGAGCGGGGGGCTGCAGTGAGG

1. Corresponds to Animal No. Jy 20
2. Corresponds to Animal No. Jy 541
3. Corresponds to Animal No. HF 574
4. Corresponds to Animal No. HF 18
5. Corresponds to Animal No. HF 32
6. Corresponds to Animal No. HF 34

CHAPTER - 5

Summary and Conclusions

The investigation was undertaken in Holstein Friesian (HF) and Jersey breeds of bulls to characterize ASS gene and to screen for citrullinemia disorder. The present investigation is the first Indian study aimed at molecular characterisation of ASS gene in cattle. In the present study, blood samples from 131HF bulls and 78 Jersey bulls maintained at different bull stations across Karnataka were collected and DNA was extracted using Miller's High Salt method with minor modifications.

A total of fourteen sets of primers were designed to cover the entire coding regions of ASS gene using Primer 3 software. The PCR amplification of fifteen exons was carried out after optimizing PCR conditions for each exon. The amplified PCR products were analyzed by Single-Strand Conformation Polymorphism (SSCP) analysis and the genetic variants in HF and Jersey breed bulls were determined after silver staining. The different band patterns in PCR fragments of ASS gene were characterized by the number of bands and mobility shifts.

The results of the present study indicated that the exons 1, 5, 6, 8, 9, 11, 12, 13 and 14 showed monomorphic patterns with no mobility shift indicating absence of any single nucleotide polymorphism. These exons showed monomorphism with similar pattern in all the 131 HF and 78 Jersey animals studied.

PCR-SSCP analysis of exon 2, 7 and 15 of ASS gene showed polymorphism with two different band patterns viz., pattern I and pattern II in Holstein Friesian breed. The pattern I and pattern II were observed with genotypic frequencies of 0.5954 and 0.4045, respectively for exon 2, 0.7099 and 0.2900 respectively for exon 7 and 0.9084 and 0.0916 respectively for exon 15.

PCR-SSCP study of Exon 3 and 4 of ASS gene showed polymorphism with two different SSCP patterns viz., pattern I and pattern II in Jersey breed. The pattern I and pattern II were observed with a genotypic frequency of 0.7179 and 0.2820, respectively for exon 3 and 0.7820 and 0.2179 respectively for exon 4.

PCR-SSCP assay of Exon 10 of ASS gene showed polymorphism with two different band patterns of pattern I and pattern II in both Holstein Friesian and Jersey breeds. The pattern I and pattern II were observed with a genotypic frequency of 0.7480 and 0.2519, respectively for exon 10 in HF and 0.6282 and 0.3717 respectively for exon 10 in Jersey cattle. It was observed that the whole population was not maintained under Hardy Weinberg equilibrium.

The genotype frequencies observed in the present investigation suggest that the Holstein Friesian breed of cattle have a diverse type of SSCP patterns for exon- 2, 7 and 15. Jersey

breed of cattle have a diverse type of SSCP patterns for exon- 3 and 4. Both HF and Jersey breeds of cattle have diverse type of SSCP patterns for exon 10, in the sampled population indicating the existence of variability

Bioinformatic tools like DNA baser, BioEdit and CLUSTAL-W multiple sequence analysis were carried out to find out polymorphisms in ASS gene. The obtained sequences were compared to reference sequence of ASS in ENSEMBL for cattle. The analysis of exon 2 displays A→G transition at position 7947, resulted in change in amino acid from Isoleucine to Valine. Exon 3 revealed T→C transition at position 10172, Exon 4 showed G→A transition at positions 11506 and 11518, Exon 7 revealed C→T transition at position 23153, Exon 10 showed G→C transversion at position 31251, Exon 15 revealed four SNPs - T→G transversion, C→T transition, A→G transition and A→G transition at positions 52105, 52135, 52136 and 52153 respectively of ASS gene. All the SNPs detected in the above exons resulted in silent mutations except in exon 2.

The Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) analysis was performed to identify carriers of mutant allele of Citrullinemia in bulls belonging to HF and Jersey breeds. On PCR-RFLP analysis, all animals showed two bands (109bp and 89 bp), revealing that none of them are carriers for citrullinemia. So, no animal was detected to be a carrier of citrullinaemia disease among 131 Holstein and 78 Jersey bulls, maintained by different semen stations of Karnataka state. The result of the present study is consistent with the earlier reports of the citrullinaemia gene deficiency, where low or no carriers for citrullinemia among Indian HF and Jersey cattle were detected.

Conclusions

1. PCR-SSCP analysis is a powerful technique to detect polymorphisms in ASS gene in cattle.
2. The exons 1, 5, 6, 9, 11, 12, 13 and 14 of ASS gene was found to be highly conserved.
3. The SSCP variants were observed in exons 2, 3, 4, 7, 10 and 15 of ASS gene in HF and Jersey breed of cattle.
4. In the present investigation no animal was found to be carrier for citrullinemia among 131 Holstein Friesian and 78 Jersey bulls, reared in various semen stations of Karnataka state in India.
5. High degree of variability in coding region of some exons of ASS gene proves that there is a need for further analysis in Indian cattle.

The DNA-based assay (PCR-RFLP), described in the present study is a powerful technique to identify the mutation responsible for Argininosuccinate synthetase deficiency. The present study showed that the frequency of carriers for genetic disorders among the studied populations is zero, however, screening of all the breeding bulls have to be carried out before including them for breeding programme in order to eliminate any chance of spreading of mutant allele for citrullinemia**.**

Bibliography

Altschul, S., Gish, W., Miller, W., Myers, E., Lipman, D. (1990). Basic local alignment search tool. Journal of Molecular Biology. **215** (3): 403–410

Arber, W. and Linn, S. (1969). DNA modification and restriction. Annual Review of Biochemistry. **38**: 467–500

Andersson, K.E., Brandt, L., Hindfelt, B. and Andljunggren, B. (1981). Cerebrovascular effects of ammonia in vitro. Acta physiology scandinavica. **113**: 349-353

Beaudet, A. L., O'Brien, W. E., Bock, H. G. O., Freytag, S. O. and Su, T. S. (1986). The human argininosuccinate synthetase locus and citrullinemia. Advanced Human Genetics. **15**: 161-196

Beaudet, A. L., Su, T. S., O'Brien, W. E., D'Eustachio, P., Barker, P. E. and Ruddle, F. H. (1982) Dispersion of argininosuccinate-synthetase-like human genes to multiple autosomes and the X chromosome. Cell. **30**: 287-293

Beaudet, A. L., Scriver, C.S., Sly, W.S., Valle, D., Cooper, D.N., Mckusick, V. A. and Schmidke, J. (1989). The Metabolic Basis of Inherited Disease 6th Ed. p 13.McGraw-Hill Publishing Co., New York.

Binstock, L. and Lecar, H. (1969). Ammonium ion currents in the squid giant axon. General Physiology. **53**: 342–361

Bock, H. G. O., Su, T.S., O'Brien, W. E. and Beaudet, A. L. (1983). Sequence for human argininosuccinate synthetase cDNA. Nucleic Acids. **11**: 6506-6512

Carritt, B., Goldfarb, P. S. G., Hooper, M. L. and Slack, C. (1977). Chromosome assignment of a human gene for argininosuccinate synthetase expression in Chinese hamster-human somatic cell hybrids. Experiment Cell. **106**: 71-78

Cathelineau, L., Dinh, D. P., Briand, P. and Kamoun, P. (1981). Studies on complementation in argininosuccinate synthetase and argininosuccinate lyase deficiencies in human fibroblasts. Human Genetics. **57**: 282-284

Charlier, Coppietersw, Rollinf, Desmechtd, Agerholmj. S., Cambisanon, Cartae, Dardanos, Divem, Fasquellec, Frennetj.C., Hansetr, Hubinx, Jorgensenc, Kariml, Kentm, Harvey K., Pearceb.R., Simonp, Taman, Nieh, Vandeputtes, Liens, Longerim, Fredholmm, Harvey

R.J. and Georgesm, (2008). Highly effective SNP-based association mapping and management of recessive defects in livestock. Nature Genetics. **40**: 449-454

Dennis, J.A., Healy, P.J., Beadudet, A.L. and O'brien, W.E. (1989). Molecular Definition of Bovine Argininosuccinate Synthetase Deficiency. Proceedings of the National Academy of Sciences of the United States of America. **86**, 7947-7951

Engel, K., Hohne, W. and Haberle, J. (2009). Mutations and polymorphisms in the human argininosuccinate synthetase (ASS1) gene. Human Mutations. **30**: 300-307

Eydivandi Cyrus, Amirinia Cyrus, Naser, Emam Jomeh Kashan, Mohammad Chamani, Jamal Fayazi and Hamid Reza Seyedabadi (2012).Study of Citrullinemia disorder in Khuzestan Holstein cattle population of Iran. African journal of Biotechnology. **11**:2587-2590

Fleischer D. Lynn, Catherine J. Harris, Deborah A. Mitchell and Henry L. Nadler (1983). Citrullinemia: Prenatal diagnosis of an affected fetus. Human Genetics. **35**:85-90

Gao, H.Z., Kobayashi, K., Tabata, A., Tsuge, H., Iijima, M., Yasuda, T., Kalkanoglu, H. S., Dursun, A., Tokatli, A., Coskun, T., Trefz, F. K., Skladal, D., and 24 others (2003). Identification of 16 novel mutations in the argininosuccinate synthetase gene and genotype-phenotype correlation in 38 classical citrullinemia patients. Human Mutations. **22**: 24-34

Glavac, D. and Dean, M. (1993). Optimization of the single - strand confirmation polymorphism (SSCP) technique for detection of point mutations. Human Mutations. **2**:404–414

Goodwin, B. L., Solomonson, L. P. and Eichler, D. C. (2004). Argininosuccinate synthase expression is required to maintain nitric oxide production and cell viability in aortic endothelial cells. Journal of Biological Chemistry. **279**: 53 -60

Gregorios, J. B., Mozes, L. W. and Andnorenberg, M. D. (1985). Morphologic effects of ammonia on primary astrocyte cultures. II. Electron microscopic studies. Neuropathology and experimental Neurology. **44**: 404–41

Gregor J. Sutcliffe and George M.Church (1978). The cleavage site of the restriction endonuclease Ava II. Nucleic Acids Research. **5(7):** 2313-2319

Grupe, S., Dietl, G. and Schwerin, M. (1996). Population survey of Citrullinemia on German Holsteins. Livestock Production Science. **45**:35-38

Haberle, J., Pauli, S., Linnebank, M., Kleijer, W. J., Bakker, H. D., Wanders, R. J. A., Harms, E. and Koch, H. G (2002). Structure of the human argininosuccinate synthetase gene and an improved system for molecular diagnostics in patients with classical and mild citrullinemia. Human Genetics. **110**: 327-333

Hai Yan Fu, Shao Ren Zhang, Hui Yu, Xiao Hong Wang, Qi Rong Zhu and Jian She Wang (2010). Most common SLC25A13 mutation in 400 Chinese infants with intrahepatic cholestasis. World Journal of Gasroenterology. **16(18):** 2278-2282

Hall, T. 2007. BioEdit: Biological sequence allignement editor for Win95/98/NT/2K/XP [Online].

Hall, T. 2007a. BioEdit v7.0.9 [Online].

Harper, P. A. W., Healy P.J., Dennis, J. A., O'Brien, J. J. and Rayward, H. D. (1986). Citrullinemia as a cause of severe neurological disease in neonatal Friesian calves. Australian Veterinary Journal. **63**:378-378.

Harper, P. A. W., Healy, P. J. and Dennis, J. A. (1989). Animal model of human disease: Citrullinemia (Argininosuccinate synthetase deficiency). American Journal of Pathology. **135**:1213-1215

Healy P.J., Dennis, J.A., Camilleri, L. M., Robinson, J.L., Stell, A.L. and Shanks, R. D. (1991). Bovine Citrullinemia Traced to the Sire of Linmack Kriss King. Australian Veterinary Journal. **68**: 155

Healy, P. J., Harper, P. A.W. and Dennis, J. A. (1990). Bovine citrullinaemia: a clinical, pathological, biochemical and genetic study. Australian Veterinary Journal. **67**: 255-258

Hindfelt, B., Plum, F. and Duffy, T. E. (1977). Effect of acute ammonia intoxication on cerebral metabolism in rats with portacaval shunts. Journal of clinical Investigations. 59, 386–396

Holstein Association (2011), 1 Holstein Place, Brattleboro, VT 05302-0808. Phone: (802) 254-4551

Houston, K (1993). Heritability and diagnosis of congenital abnormalities in food animals. Veterinary Clinics of North America. **9**:1

Innis, M.A. and Gelfand, D.H. (1990). Optimizaton of PCRs. In: PCR Protocols: A guide to methods and applications. Eds. Innis, M.A., Gelfand, D.H., Sninsky, J.J. and.White, T.J. Academic Press, San Diego.

Jae Sung Ko, Jung Han Song, Sung Sup Park and Jeong Kee Seo. (2008). Neonatal intrahepatic cholestasis caused by citrin deficiency in Korean infants. Journal of Korean Medical Science. **22(6):**952-6

Jianbin Li, Hongmei Wang, Yi Zhang, Minghai Hou, Jifeng Zhong and Yuan Zhang (2011). Identification of BLAD and Citrullinemia carriers in Chinese Holstein cattle. Animal Science Papers and Reports. **29(1)**: 37-42

Jung Min Ko, Gu Hwan Kim, Ju Hyun Kim, Jae Young Kim, Jin Ho Choi, Miharu Ushikai, Takeyori Saheki, Keiko Kobayashi and Han Wook Yoo (2007). Six cases of citrin deficiency in Korea. International Journal of Molecular Medicine. **20**: 809-815

Kobayashi, K., Jackson, M. J., Tick, D. B., O'Brien, W. E. and Beaudet, A. L (1990). Heterogeneity of mutations in argininosuccinate synthetase causing human citrullinemia. Journal of Biological Chemistry. **265**: 11361-11367

Kobayashi, K., Jackson, M. J., Tick, D. B., O'Brien, W. E. and Beaudet, A. L (1989). Characterization of nine mutant alleles causing citrullinemia. American Journal of Human Genetics. **45**: A201

Kobayashi, K., Kakinoki, H., Fukushige, T., Shaheen, N., Terazono, H. and Saheki, T (1995). Nature and frequency of mutations in the argininosuccinate synthetase gene that cause classical citrullinemia. Human Genetics. **96**: 454-463

Kobayashi, K., Shaheen, N., Terazono, H. and Saheki, T (1994). Mutations in argininosuccinate synthetase mRNA of Japanese patients, causing classical citrullinemia. American journal of Human Genetics. **55**: 1103-1112

Lin D.Y., Huang, Y.C., Chen, J.C., Yang, T.W., Shiao, T.F. and Chang, H.L. (2001). Investigation of citrullinaemia of dairy cattle in Taiwan. Journal of Taiwan Livestock Research. **34**: 279-284

Luria, S. E. and Human, M. L. (1952) A nonhereditary, host-induced variation of bacterial viruses. Journal of Bacteriology. **64**: 557–569

Makino, R., Yazyu, H., Kishimoto, Y., Sekiya, T. and Hayashi, K. (1992). Fluorescence based polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) analysis. PCR Methods and Applications. **2**:10-13

Mans, A. M., Biebuyck, J. F., and Hawkins, R. A (1983). Ammonia selectively stimulates neutral amino acid transport across blood–brain barrier. American Journal of Physiology. **245**: C74–C77

McCarrey, J. R. and Riggs, A. D (1986). Determinator-inhibitor pairs as a mechanism for threshold setting in development: a possible function for pseudogenes. Proceedings of National Academy of Sciences. **83**: 679-683

Mcmurray, W. C. F., Mohyuddin, R. J., Rossiter, J. C., Rathbun, G. H., Valentine, S. J. and Koegler, Jarfas, P. E. (1962). Citrullinemia: A new aminoaciduria associated with mental retardation. Lancet. **279**:138-138

McKusick, V. A. (1990). Mendelian Inheritance in Man: Catalogs of Autosomal Dominant, Autosomal Recessive and Sex-Linked Phenotypes. 9th Ed. Johns Hopkins University Press, Baltimore, MD

Meijer, A. J., Lamers, W. H. and Chamuleau, R. A. F. M. (1990). Nitrogen metabolism and ornithine cycle function. Physiology Review. **70**:701–748

Meselson, M. and Yuan, R. (1968). DNA restriction enzyme from E. coli. Nature. **217**: 1110–1114

Meydan, H., Ozdil, F., Gedic, Y. and Yildiz, M. A. (2010). Screening for bovine leukocyte adhesion deficiency, deficiency of uridine monophosphate synthase, complex vertebral malformation, bovine Citrullinaemia, and factor XI deficiency in Holstein cows reared in Turkey. Acta Veterinaria Scandinavica. **52**:56

Miller, S. A., Dykes, D. D. and Polesky, H. F. (1988). A sample salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research. **16**:1215

Murray, K., Hughes, S.G., Brown, J.G. and Bruce, S.A. (1976) Biochemical Journal. **159**:317-322

Nassiry Reza Mohammad, Amir Norouzy, Fereidoun Eftekhari Shahroudi, Ali Javadmanesh and Mohammad Ali Shad (2005). Investigation of two recessive disorders in breeder bulls of Abbas Abad animal breeding center. Iranian Journal of Biotechnology. **3(2):** 215-128

Northrup, H., Lathrop, M., Lu, S.Y., Daiger, S. P., Beaudet, A. L. and O'Brien, W. E (1989). Multilocus linkage analysis with the human argininosuccinate synthetase gene. Genomics. **5**: 442-444

Oner Y., Keskin A. and Elmaci A. (2010). Identification of BLAD, DUMPS, Citrullinemia, Factor XI deficiency in Holstein Cattle in Turkey. Asian Journal of Animal and Veterinary Advance. **5(1):** 60-65

Orita, M., Youchi, S., Sekiya, T. and Hayashi, K. (1989). Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics. **5**: 874–879

Padeeri, M., Vijaykumar, K., Grupe, S., Narayan, M.P., Schwerin, M. and Kumar, M.H. (1999). Incidence of hereditary Citrullinaemia and bovine leukocyte adhesion deficiency syndrome in Indian dairy cattle (Bos taurus, Bos indicus) and buffalo (Bubalus bubalis) population. Arch Tierz. **42**: 347-52

Patel, R. K., Singh, K. M., Soni, K. J., Chauhan, J. B. and Sambasiva Rao, K. R. (2006). Lack of carriers of Citrullinaemia and DUMPS in Indian Holstein cattle. Journal of Applied Genetics. **47(3)**: 239-242

Ramesha, K. P., Saravanan, T., Rao, M. K., Appannavar, M.M. and Obi Reddy, A. (2002). Genetic distances among South Indian Breeds of zebu cattle using Random Amplified DNA markers. Asian Australian Journal of Animal Science. **15(3):**309-314

Ratner, S. (1982). Argininosuccinate synthetase of bovine liver: chemical and physical properties. Proceedings of National Academy of science. **79**:5197-5199

Ratner, S., Swatantar Kumar and J. Lennane (1985). Argininosuccinate synthetase essential role of cystein and arginine residues in relation to structure and mechanism of ATP activation. Proceedings of National Academy of Science. **82**:6745-6749

Rao, T.V.L.N. (2012). Molecular characterization of glutathione s-transferase pi (GSTP1) in Deoni cattle. M.Sc, National Dairy Research Institute (SRS), Bangalore, India

Robinson, J.L., Burns, J.L., Magura, C.E. and Shanks, R.D.(1993). Low incidence of Citrillinemia carriers among dairy cattle of the United States. Journal of Dairy Science. **111(76)**: 853 – 858

Racke Kurt and Warnken Mareille (2010). L-Arginine Metabolic Pathways. The Open Nitric Oxide Journal. **2**: 9-19

Sambrook, J. and Russel, D.W. (1989). Molecular cloning -A laboratory manual. 3rdedn, pp 1.1-7.94 Cold Spring Harbor Laboratory Press, Newyork

Sears, E. S., Mccandless, D. W. and Chandler, M. D. (1985). Disruption of the blood–brain barrier in hyperammonemic coma and the pharmacologic effects of dexamethasone and difluoromethyl ornithine. Journal Neuroscienc Research. **14**: 255–261

Shergojry, S.A., Ganayi, B.A., Ramesha, K.P., Rengarajan, K., Srihari, G.V., Das, D.N. and Kataktalware, M.A. (2011). Association of Single Nucleotide Polymorphisms (SNPS) Of HSP90AA1 Gene with Reproductive Traits in Deoni Cattle. International Journal of Livestock Research. **1(1)**: 17-29

Shinde, D.B., Gujar, B.V., Patil, H.B., Satpute S.A. and Kashid, U.B. (2008). Protocol for isolation of genomic DNA from livestock blood for microsatellite analysis. Indian Journal of Animal Research. **42(4):**279-281

Smith, H. O. and Kelly, T. J. (1970) A restriction enzyme from Hemophilus influenzae. II. Base sequence of the recognition site. Journal of Molecular Biology. **51**: 393–409

Smith, H. O. and Wilcox, K. W. (1970) A restriction enzyme from Hemophilus influenzae. I. Purification and general properties. Journal of Molecular Biology. **51**: 379 – 391

Su, T. S., Nussbaum, R. L., Airhart, S., Ledbetter, D. H., Mohandas, T., O'Brien, W. E. and Beaudet, A. L. (1984). Human chromosomal assignments for 14 argininosuccinate

synthetase pseudogenes: cloned DNAs as reagents for cytogenetic analysis. American Journal of Human Genetics. **36**: 954-964

Tabata Ayako, Jian Sheng Sheng , Miharu Ushikai , Yuan-Zong Song Hong Zhi Gao, Yao Bang Lu, Fumihiko Okumura, Mikio Iijima, Kozo Mutoh, Shosei Kishida, Takeyori Saheki and Keiko Kobayashi. (2008) Identification of 13 novel mutations including a retrotransposal insertion in SLC25A13 gene and frequency of 30 mutations found in patients with citrin deficiency. Journal of Human Genetics. **53**:534-545

Takaya, J., Kabayashi, K., Ohashi, A., Ushikai, M., Tabata, A., Fujimoto, S., Saheki, T. and Kobayashi, Y. (2005). Variant clinical courses of 2 patients with neonatal intrahepatic cholestasis who have a novel mutation of SLC25A13. Metabolism. 54(12):1615-9

The American Jersey Cattle Association, 6486 East Street, Reynoldsburg, Ohio 43068-2362

Thomas, N. and Anilkumar, K. (2008). Genetic characteristics of five Microsatellite Markers associated with milk production traits in crossbred dairy cattle of Kerala. Veterinary World. **8**: 245-247

Thong, M. K., Boey C. C. M., Sheng J. S., Ushikai M and Kobayashi K. (2008). Neonatal intrahepatic cholestasis caused by citrin deficiency in two Malaysian siblings: outcome at one year of life. Singapore Medical Journal. **51(1):** 12- 14

Todd, S. and Naylor, S. L (1992). New chromosomal mapping assignments for argininosuccinate synthetase pseudogene 1, interferon-beta-3 gene, and the diazepam binding inhibitor gene. Somatic Cell Molecular Genetics. **18**: 381-385

Treeponqkaruna, S., Jitraruch, S., Kodcharin, P., Charoenpipop, D., Suwannarat, P., Pienvichit, P., Kobayashi, K. and Waltanasirichaigoon, D. (2012). Neonatal intrahepatic cholestasis caused by citrin deficiency: prevalence and SLC25A13 mutations among Thai infants BMC Gastroenterology.**12**:141- 148

Viana J.L., Fernandez, A., Iglesias, A. and Santamarina, G. (1998). DiagnÛstico y control de las principales enfermedades genÈticas (citrulinemia, DUMPS y BLAD) descritas en ganado Holstein-FrisÛn. Medical Veterinary Journal. **15**: 538-544

Wiechetek, M., Breves, G. and Holler, H. (1979). The effect of ammonia ion concentration on the activity of adenylate cyclase in various rat tissues in vitro. Journal of experimental Physiology.**64**: 169–174

Yao, J., Liao, K., Li, Q.L., Wang, C.F., Wang, H.M., Lai, S.J. and Zhong, J.F. (2011). mRNA expression of glutathione s-transferase pi (GSTP1) under heat stress and association of genotypes with heat tolerance ability in Holstein. Journal of Animal and Veterinary Advances. **10(6):** 757-763

Zabek, T. and Rys, A. (1998). Gene defect in farm animals.**36**: 5-13

http://blatnicabiology.wikispaces.com

http://en.wikipedia.org

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

http://www.theLabRat.com

Appendix

APPENDICES

Composition of reagents and buffers used in the study.

I. EDTA (10 per cent) solution

a.	Ethylene Diamine Tetra Acetic Acid (EDTA)	-	1g
b.	Triple glass distilled water to make up to	-	10 ml

II. RBC Lysis Buffer

a.	Ammonium Chloride	-	8.0235 g
b.	Potassium chloride (10 mM)	-	0.7455 g
c.	EDTA	-	0.0372 g
d.	Triple glass distilled water to make up to	-	1000 ml
e.	Autoclaved and stored at 4^0C		

III. Tris Buffer Saline

a.	Potassium chloride	-	0.0373 g
b.	Sodium chloride (0.4 M)	-	8.1800 g
c.	Trishydro xymethyl aminomethane HCl	-	0.0303 g
d.	Magnesium chloride (10 mM)	-	1 ml
e.	Triple glass distilled water to make up to	-	1000 ml
f.	pH adjusted to	-	7.4
g.	Autoclaved and stored at 4^0C		

IV. Tris-EDTA buffer (TE buffer)

a.	Tris hydroxymethyl aminomethane HCl		
b.	(10 mM) pH 7.6	-	1.2114 g
c.	EDTA (0.1 mM)	-	0.3722 g

d.	Triple glass distilled water to make up to	-	1000 ml
e.	pH adjusted to	-	8.0
f.	Autoclaved and stored at 4^0C		
g.	**Sodium dodecyl sulphate (10 per cent SDS)**		
h.	Sodium dodecyl sulphate	-	10 g
i.	Triple glass distilled water to make up to	-	100 ml

V. Saturated sodium chloride

a.	Sodium chloride	-	29.22 g
b.	Triple glass distilled water to make up to	-	100 ml

VI. 0.5M EDTA

a.	EDTA	-	18.612g
b.	Triple glass distilled water to make up to	-	100 ml

VII. Proteinase K

a.	Proteinase K	-	20 mg
b.	Triple glass distilled water to make up to	-	1 ml
c.	Stored at -20°C		

VIII. Agarose (0.8 per cent)

a.	Agarose	-	0.64 g
b.	TAE (50X) buffer	-	1.6 ml
c.	Triple glass distilled water to make up to	-	80 ml

VIX. Ethidium bromide staining solution (10 per cent)

d.	Ethidium bromide	-	100 mg
e.	Triple glass distilled water to make up to	-	1 ml

X. Gel loading buffer (6X)

f.	Glycerol	-	5 ml
g.	Bromophenol Blue	-	125 mg
h.	Xylene cyanol	-	125 mg
i.	Triple glass distilled water to make up to	-	500 ml

XI. TBE 5X buffer

j.	Tris 445 mM	-	54 g
k.	Boric acid 445 mM	-	27.5 g
l.	EDTA 10mM	-	3.7224 g
m.	Dissolve in 1000ml Double distilled water		

IX. ix Ethidium bromide staining solution)

a.	Ethidium bromide	-	10 mg
b.	Triple glass distilled water to make up to	-	1 ml

X. Gel loading buffer (6X) for 10 ml solution

a.	Sucrose 40% W/V	-	4 g
b.	Bromophenol Blue 0.05% W/V	-	0.005 g
c.	EDTA 0.1 M	-	2 ml of 0.5M EDTA
d.	SDS 0.5% W/V	-	0.05 g
e.	Triple glass distilled water to make up to	-	make up to 10 ml

XI. 10% Acrylamide/Bis-Acrylamide (29:1) 100 mL

a. Acrylamide - 29 g

b. Bis-acrylamide - 1 g

c.Ammonium Persulfate - 0.1 g

XII. 10% (w/v) Ammonium Persulfate (APS) 500 mL

a. Ammonium persulfate - 50 g

b. Make 50 mL aliquots. Store at -20°C.

XIII. Denaturing Solution - 1 L

a. NaCl - 87.66 g

b. NaOH - 20.0 g

XIV. SSCP electrophoresis gel

a. 10 % Poly acrylamide gel solution - 13.6 ml

b. TBE buffer - 26.4 ml

c. TEMED - 75 µl

d. Ammonium per sulphate (10%) - 180 µl
(Prepared fresh)

XV. Silver staining

a) Acetic acid solution(Fixative)

Glacial acetic acid - 50 ml

Distilled water - 450 ml

b) Silver nitrate solution

Silver nitrate - 0.5 gram

Formaldehyde - 750 µl

Distilled water - 500 ml

c) Sodium carbonate solution (Developer solution)

Sodium carbonate	- 15 gram
Formaldehyde	- 750 µl
Sodium thiosulphate (10%)	- 10 µl
(freshly prepared	
Distilled water	- 500 ml

d) Stop Solution

Glacial acetic acid 10 per cent	- 10ml
Distilled water	- 100ml