MOLECULAR CHARACTERIZATION OF IL-8 GENE AND ITS ASSOCIATION WITH MILK PRODUCTION AND UDDER HEALTH TRAITS IN DEONI CATTLE

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE AWARD OF

DEGREE OF MASTER OF VETERINARY SCIENCE

IN

ANIMAL GENETICS AND BREEDING

BY

DR. SONALI THAKUR
(B.V.Sc. & A.H.)

DAIRY PRODUCTION SECTION

NATIONAL DAIRY RESEARCH INSTITUTE (I.C.A.R.)

SOUTHERN CAMPUS, 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 IL-8 GENE AND ITS ASSOCIATION WITH MILK PRODUCTION AND UDEER HEALTH TRAITS IN DEONI CATTLE"** submitted by ***Dr. SONALI THAKUR*** towards the partial fulfillment for the award of the degree of **MASTER OF VETERINARY SCIENCE** in **Dairying (ANIMAL GENETICS BREEDING)** of the **National Dairy Research Institute (Deemed University), Karnal (Haryana), India**, have submitted one research paper out of the dissertation work for publication in the journal '**Tropical Animal Health And Production'.**

(Dr. D. N. DAS)

Major Advisor

****

DEDICATED TO MY

BELOVED FAMILY,

And

GUIDE

****

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*I bow my head with great reverence to the '**Almighty God**', who is a cause behind every effect and without whose blessings this work would never have been accomplished I give all glory, honour and praise to the Lord, whose abundant love and grace enabled me to travel throughout life and come to this stage.*

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ABBREVIATIONS

A	Adenine
A260	Absorbance at 260 nm
A280	Absorbance at 280 nm
AP	Andhra Pradesh
APS	Ammonium Persulphate
Bp	Base Pair
C	Cytosine
CAPS	Cleaved Amplified Polymorphic Sequence
CL	Corpus Luteum
cp	Crossing Point
CRC	Colorectal cancer
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EC	Electrical Conductivity
EDTA	Ethylene Diamine Tetra Acetic Acid
EtBr	Ethidium Bromide
FL	Front Left
FR	Front Right
G	Guanine
GLM	General Linear Model
HL	Hind Left
HR	Hind Right
Kb	Kilobase
KDa	Kilodalton
Kg	Kilogram
LL	Lactation Length
LMY	Lactation Milk Yield
LPS	Lipopolysaccharide
Lys	Lysine

MAFSU	Maharashtra animal and fishery science university
MgCl2	Magnesium Chloride
Mm	Milimolar
mRNA	Messenger Ribonucleic Acid
MT	Million Tons
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NAP	Neutrophil Activating Protein
NCBI	National Centre For Biotechnology Information
ng	Nanogram
nm	Nanometer
OD	Optical Density
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PMN	Polymorphonuclear Cells
pmol	Picomol
QTL	Quantitative Trait Loci
RBC	Red Blood Cells
RE	Restriction Endonucleases
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolution Per Minute
SCC	Somatic Cell Count
SD	Standard Diveation
SDS	Sodium Dodecyl Sulphate
SE	Standard Error
SNPs	Single Nucleotide Polymorphisms
SPSS	Statistical Package For Social Science
T	Thymine
Taq	Thermos Aquaticus
TBE	Tris Borate EDTA

TBS	Tris Buffer Saline
Thr	Thrionine
UTR	Un-Translated Region
µl	Microlitre
µm	Micromolar

ABSTRACT

Interlukin-8/CXCL8, one of the most important potent chemo attractants is primerly released from mono-nuclear phagocytes, epithelial cells, endothelial cells, neutrophils, eosinophils, fibroblast, T-cells and hepatocytes. The present study was undertaken on characterization of IL-8 gene and its association with production and udder health traits in Deoni cows. Genomic DNA was isolated from all lactating cows maintained at Cattle yard of SRS, NDRI, Bangalore and livestock farm Prabhani, MAFSU, M.H., by high salt method. Quality and quantity of DNA were checked by 0.8% agarose gel electrophoresis and spectrophotometer respectively. A set of primers were designed by using primer-3 tool software covering all coding regions of IL-8 gene. PCR-RFLP was done for all samples of Deoni cattle using HpyCH4V, SspI and HaeIII restriction enzymes. Pattern of resolution of digested fragments by all restriction enzymes was found monomorphic in coding regions 1, 2, 4.1 and 4.2, while polymorphism was observed in coding region 3. In this exon 3, two genotypes (AA and AB) were detected on the basis of band patterns with the frequency 0.21 and 0.79 respectively. The allelic frequencies of A and B were 0.61 and 0.39 respectively. In the association study, genotype, parity and stage of lactation revealed no significant effect on udder health traits (somatic cell counts and electrical conductivity in milk), while genotype revealed significant effect on milk production traits (lactation length and lactation milk yield) at 5% level. The sequences were compared to the sequence of *Bos taurus* sequence at NCBI. BLAST analysis revealed sequence identity of coding region (IL-8 gene) of Deoni cattle with *Bos indicus* at NCBI was 98% to 100% , 99% with *Bos taurus,* 98% with *Bubalus bubalis* and 92% with *Ovies aries*. The relative quantification of IL-8 gene by real time PCR method was done in two groups i.e. subclinical mastitis (n=8) and healthy (n=8) classified based on SCC. mRNA was isolated by RNeasy Mini Kit (Qiagen) and purity was checked by Nanodrop2000/2000C which ranged from 3.1 to 9.2 with mean 4.22±0.37ng/µl and the optical density of mRNA at 260/280 observed was 1.84±0.08. cDNA synthesized from mRNA for IL-8 gene and GAPDH gene by reverse transcriptase PCR and checked the purity of cDNA by Nanodrop2000/2000C ranging from 2151.3 to 2583.3 with mean 2298.5±28.42 ng/µl. Fold change of IL-8 gene significantly (0.70±0.028) higher in milk of SCM group and lower (0.13±0.03) expression obtained in healthy group in milk of Deoni cattle.

सारांश

Interlukin-8/CXCL8, सबसे महत्वपूर्ण शक्तिशाली कीमो attractants की एक primerly मोनो परमाणु फ़ैगोसाइट, उपकला कोशिकाओं, endothelial कोशिकाओं, neutrophils, इयोस्नोफिल्स, fibroblast, टी कोशिकाओं और hepatocytes से जारी है. वर्तमान अध्ययन IL-8 जीन और Deoni गायों में उत्पादन और थन स्वास्थ्य लक्षण के साथ अपने सहयोग के लक्षण वर्णन पर किया गया था. जीनोमिक डीएनए उच्च नमक विधि द्वारा, एसआरएस, एनडीआरआई, बंगलौर और पशुधन खेत Prabhani, MAFSU, महाराष्ट्र के मवेशी यार्ड पर बनाए रखा सभी स्तनपान कराने वाली गायों से अलग किया गया था. डीएनए की गुणवत्ता और मात्रा क्रमश: 0.8% agarose जेल वैद्युतकणसंचलन और स्पेक्ट्रोफोटोमीटर द्वारा जांच की गई. प्राइमरों का एक सेट IL-8 जीन के सभी कोडिंग क्षेत्रों को कवर प्राइमर -3 उपकरण सॉफ्टवेयर का उपयोग करके बनाया गया. पीसीआर RFLP HpyCH4V, SSPI और HaeIII प्रतिबंध एंजाइमों का उपयोग Deoni पशुओं के सभी नमूनों के लिए किया गया था. सभी प्रतिबंध एंजाइमों से पच टुकड़े के संकल्प के पैटर्न बहुरूपता क्षेत्र 3 कोडिंग में मनाया गया, जबकि क्षेत्रों 1, 2, 4.1 और 4.2 कोडिंग में monomorphic पाया गया था. इस एक्सॉन 3 में, दो जीनोटाइप (ए.ए. और एबी) के आधार पर पाया गया क्रमशः आवृत्ति 0.21 और 0.79 के साथ बैंड पैटर्न की. ए और बी के allelic आवृत्तियों क्रमश: 0.61 और 0.39 थे. जीनोटाइप ($p<0.05$) में दूध उत्पादन लक्षण (स्तनपान लंबाई और स्तनपान दूध उपज) पर महत्वपूर्ण प्रभाव का पता चला है, जबकि संघ अध्ययन में, जीनोटाइप, समता और स्तनपान के चरण, थन स्वास्थ्य लक्षण (दैहिक सेल मायने रखता है और दूध में विद्युत चालकता) पर कोई महत्वपूर्ण प्रभाव का पता चला 5% स्तर. दृश्यों NCBI में बोस वृषभ अनुक्रम के अनुक्रम की तुलना में थे. ब्लास्ट विश्लेषण 100%, बोस वृषभ के साथ 99%, *Bubalus bubalis* साथ 98% और *Ovies aries* राशि के साथ 92% करने के लिए 98% थी NCBI में बोस इंडिकस साथ Deoni पशुओं के क्षेत्र (आईएल -8 जीन) कोडिंग के अनुक्रम पहचान का पता चला. पीसीआर विधि दो समूहों में किया गया था वास्तविक समय से आईएल -8 जीन के रिश्तेदार मात्रा का ठहराव (n= 8) subclinical स्तन की सूजन अर्थात और स्वस्थ (n = 8) वर्गीकृत एससीसी पर आधारित है. mRNA RNeasy मिनी किट (Qiagen) और पवित्रता से अलग किया गया था मतलब 4.22 ± 0.37ng/µl और मनाया 260/280 में mRNA की ऑप्टिकल घनत्व के साथ 3.1-9.2 लेकर जो Nanodrop2000/2000C द्वारा जाँच की थी 1.84 ± 0.08 था. सीडीएनए रिवर्स ट्रांसक्रिपटेस पीसीआर द्वारा आईएल -8 जीन और GAPDH जीन के लिए mRNA से संश्लेषित और मतलब 2298.5 ± 28.42 एनजी / µl के साथ 2151.3-2583.3 लेकर Nanodrop2000/2000C द्वारा सीडीएनए की शुद्धता की जाँच की. Deoni पशुओं के दूध में स्वस्थ समूह में प्राप्त (0.13 ± 0.03) अभिव्यक्ति एससीएम समूह के दूध में उच्च और निम्न (0.70 ± 0.028) काफी आईएल -8 जीन के परिवर्तन मोड़ो.

Chapter-1

Introduction

INTRODUCTION

India's milk production is continuously increasing after the white revolution; it enhanced from 17 million tonnes during 1951 to 133.79 million tonnes in 2013. India stands number one in milk production in world's map along with the highest cattle population which includes crossbred cattle also. The small holders have about 70 per cent of total milch animals in our country. As per 2007 census, there were about, 54.088 million breedable and 48.042 million milch cattle whereas, crossbreds comprise of 15.665 million breedable and 14.407 million milch animals. Out of all 37 registered cattle breeds in India, Deoni is an important dual-purpose breed of cattle. The breeding tract of Deoni cattle comprise of some parts of Medak district of Andhra Pradesh, northern part of Karnataka viz. Bidar, Bijapur, Gulberga district, Latur district and adjoining area of Prabhani, Nanded, Osmanabad district of Marathwada region in Maharashtra. The total populations of Deoni in the entire breeding tracts were estimated at 165,846, distributed in Maharashtra (44.1 per cent), Karnataka (44.5 per cent) and Andhra Pradesh (14.4 per cent). The average lactation milk yield, age at first calving, inter-calving period and fat per cent are 779.27±18.31 kg, 38.73±0.73 months, 447.2±6.64 days and 4.3 respectively in Deoni cows (Singh *et al.,* 2002 and Das *et al.,* 2011). Udder health becomes an important component for dairy industry from last few decades, because problems related to udder health always concerned with the decrease profitability, increase unexpected culling of dairy animals, increase cost of production *etc*. Udder health is governed by a number of environmental, managemental factors including pre-milking teat preparation, hands of operator, post milking teat dipping, reverse pressure of milking machine gradients which related to the entry of micro-organisms into the teat canal *etc*. (Dufour *et al.,* 2010). Apart from these factors, genetic aspects also play major role for udder health. Therefore, the selection of animal on the basis of molecular markers is very important now-a-days, especially, when there is a positive correlation with the production and udder health traits, which ultimately helps us to reduce losses due to udder health problems. Udder health problems occur when the first line of defense fails against infectious agents. Stimulation of inflammation reaction releases number of cytokines, chemokines, interferons against bacterial, viral, fungal infection.

Chemokines are group of small 8-12 KDa molecules able to induce chemotaxis in a variety of cells including neutrophils, monocytes, lymphocytes, eosinophils, fibroblasts & keratinocytes (Larry *et al.,* 2003). These chemokines are divided into four families based on the position of N-terminal cysteine residue: 1.CC, 2.XC 3.CXC, 4.CX3C. The CXC family including Interleukin-8/CXCL8, small protein (8.4kDa) which is the most important potent chemo attractant and primarily releases from mono-nuclear phagocytes, epithelial cells, endothelial cells, neutrophils, eosinophils, fibroblast, T-cells and hepatocytes (Graham *et al.,*2013). In the ovary, IL-8 is detected in the cells, granulosa, vascular endothelial in case of human and rabbits i.e. it is involved in the angiogenesis, cell proliferation & apoptosis (Belayet *et al.,* 2000). It induces neutrophil degranulation, the respiratory burst and adherence to endothelial cells by CD11b/CD18 (Larry *et al.,* 2003). IL-8 binds to the neutrophils receptors CXCR1 & CXCR2, promoting neutrophils transmigration across endothelial barrier resulting in neutrophils accumulation at the sites of inflammation and subsequently release of proteineases pro-inflammatory molecules. However, scanty work has been done on the IL-8 gene and its association with production and udder health traits in bovine. There is no work done before on this topic in Deoni cattle, so present study proposed to molecular characterization of IL-8 gene with two objectives:

1. Molecular characterization of the coding regions of IL-8 gene in Deoni cattle
2. Investigation on the association of genetic variability in IL-8 gene with milk production and udder health traits in Deoni cattle

Chapter - 2

Review of literature

REVIEW OF LITERATURE

Deoni is an important medium sized dual purpose indigenous breed of cattle. The name derived from the Deoni Taluk of Latur district of Maharashtra, where this breed originated. The breed is also known as Surti, Dongarpati and Dongri. The breeding tracts of Deoni cattle comprise of some parts of Medak district of Andhra Pradesh, northern part of Karnataka viz. Bidar, Bijapur, Gulberga district, Latur district and adjoining areas of Prabhani, Nanded, and Osmanabad district of Marathwada region in Maharashtra. They are found in three different colours including Wannera (clear white body with black patchy area on side of face), Balankya (clear white body with black patchy area on lower side of the body) and Shevera (white body with irregular black spots). These animals are maintained in semi intensive system of management and for feed they depend on grazing lands including fallow lands, dry lands or bunds of farmers. Almost 50 per cent of farmers rear them in open area, 3 per cent keeps them in pucca (cemented) area and remaining keeps them in katchha (muddy) house thatched with dry grass (Singh *et al.,* 2002). The average lactation milk yield, age at first calving, inter-calving period and fat per cent are 779.27±18.31 kg, 38.73±0.73 months, 447.2±6.64 days and 4.3 respectively in Deoni cows (Singh *et al.,* 2002; Das *et al.,* 2011). The overall mortality reported was 2.17 per cent in Deoni cattle (Das *et al.,* 2011). Milk production in Deoni herd maintained under semi-intensive management of SRS, NDRI, Bangalore is 870 Kg per lactation. Improvement on phenotypic performance is based on production traits and udder health traits are practiced in general.

With advancement of genomics genes controlling QTL are important for bringing improvement in production as well as udder health traits. IL-8 is such gene under all QTLs which is concerned with production traits and udder health traits.

2.1 Production traits

Milk production traits involve monthly test day milk yield, lactation length, lactation yield, 305 days or less milk yield, 305 or less wet average. Milk production of cattle depends on many factors like local environment, way of treating the animal during milking, animal health condition, proper management by milker during and after milking etc. In India, 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 huge population of animals. Mastitis is the third most common reason after low milk yield and reproductive disorders for culling of dairy animals and accounts to 6-17% of total culled animals. Losses due to culling are of great concern as animals with good genetic makeup are lost. Clinical cases of mastitis are characterized by the presence of the symptoms viz. abnormal milk, udder swelling, and systematic signs including an elevated temperature, lethargy and anorexia. Whereas subclinical cases of mastitis shows no visible sign in appearance of milk but milk production decreases, composition of milk alters and bacteria may be present in the secretion (Erskine, 2001). Cornell University showed that clinical mastitis tends to strike high producing animals in second plus lactation (Wilson *et al.,* 2004). In India, on an average economic loss on clinical mastitis is estimated nearly Rs. 1,700 crores (Joshi and Gokhale, 2006).

2.1.1 Lactation Length

Lactation length is an important trait which influences the magnitude of milk yield obtained in each lactation. First lactation length of Deoni cattle was reported as 293.3±2.9 days in established farm in Prabhani and Udgir (Deshpande *et al.,* 1977a). First and overall least squares means of lactation length in Deoni cattle under semi intensive management were observed as 186.61 ±3.02 and 189.43 ±5.03 days respectively (Das *et al.,* 2011).

2.1.2 Lactation Milk Yield

Lactation milk yield is an important production as well economic trait in cows which gives the information of dairy performances. Pooled lactation milk yield was in the range from 238.86±22.44 kg (Chakravarthy *et al*., 2002) to 957.90±29.23 kg (Kakde *et al.,* 1980). FLMY in Deoni cattle was reported as 942.7±16.6 kg (Deshpande *et al.,* 1977b) in established farm in Parbhani and Udgir. Pooled lactation milk yield of Deoni cattle in Maharashtra observed was 868.24±49.56 kg in ICAR network project (1999). First and overall least squares mean lactation milk yield of Deoni cattle under semi intensive

management was observed 715.87±32.11 and 779.27± 18.31 kg, respectively (Das *et al.,* 2011).

2.2 Udder health traits

Udder health has been an important component in dairy industry to enhance the production and productivity of dairy animals. Problems related with udder health are always concerned with reduced profitability and productivity of animals. Udder health status is generally affected by number of factors including presence and pathogenicity of microorganisms, environment and management, cow factors like susceptibility/resistance capability for microorganisms, immunological response, treatment and prevention strategies. Udder health traits are more important when the positive correlation exist between milk production of dairy animal on one hand and somatic cell count and clinical mastitis incidence on another hand (Poso and Mantyasaari, 1996). A healthy udder should have lower level of somatic cell counts (Table 2.1).

Approximately 20% of cows (dairy cows) are experiencing one or more cases of clinical mastitis per lactation (Barkema *et al.,* 1998 and Elbers *et al.,* 1998) and the incidences are highest in early lactation which may increase with pairity.

Udder health problems cannot be treated as a single trait but it's a combination of multiple disease process like pairity, environment and pathogen specific. To simplify it, it is useful to consider the risk factors that include the breed, infectious micro-organisms, environmental factors, milking practices, housing, nutrition *etc.* (Sudhan and Sharma, 2010).

2.2.1 Somatic Cell Count

Milk somatic cells are white blood cells including non-granulocytes (neutrophils, eosinophils, basophils) and granulocytes (monocytes, lymphocytes) and polymorphonuclear cells (PMN). Somatic cell counts increase when the mastitis causing agents are present in the udder (Nash *et al.,* 2003) and the inflammatory response is initiated in presence of bacteria in mammary glands which enter through teat canal

(Atakan, 2008). SCC level is important in milk not only for detection of mastitis incidence in herd but also for human health and overall quality management (Mostert *et al*., 2004). Lower level of SCC have been shown to be related to higher milk production and good quality of milk products which increase the economic value of dairy producer (Yalchin *et al*., 2000). Several studies found a negative association between milk yield and SCC (Juozaitiene *et al.,* 2006). SCC has higher heritability range of 0.15 to 0.25 in normal milk (Emanuelson *et al.,* 1988; Lindmark- Mansson *et al.,* 2006), 0.11 in subclinical mastitis and 0.04 in case of clinical mastitis (Mrode and Swanson, 1996). During the early stages of mastitis, neutrophils count increases to more than 95 per cent in somatic cell count (Persson *et al.,* 1992 and Kehrli *et al.,* 1994). Increase in somatic cells specifically neutrophils serves as a mechanism against infection of mammary gland (Sandholm *et.al.*, 1995).

2.2.2 Electrical Conductivity

Electrical conductivity (EC) is mainly based on the concentration of sodium and chloride ions, which increases in milk during mastitis. The electrolyte change in milk is detectable earlier than SCC. Electrical conductivity is good test because it measures actual injury to udder in comparison to SCC and NAGase test. For early detection of mastitis, EC is better especially in case of *S. aureus*, and *S. uberis.* The range of electrical conductivity in milk is shown in table 2.2 under normal and SCM condition of udder in Deoni cattle.

Table 2.2 Electrical Conductivity in cattle - Draminski mastitis detector manual (Das *et al.,* 2012)

Status	Range
Healthy	Above 300 units
SCM	300-250 units
Clinical mastitis	Below 250 units

2.3 Candidate genes of mastitis

A candidate gene is a gene suspected to be involved in particular disease, condition or abnormality, located in a chromosome region supposed to be involved in the expression

Table No. 2.1 Somatic Cell Counts in milk by California method

Status		Average Somatic Cell Count (Cells per milliliter)	Description of reaction
Negative	N	0 - 200,000	No thickening, Homogeneous
Trace	T	200,000 - 400,000	Slight thickening. Reaction disappears in 10 seconds.
Subclinical Mastitis	1	400,000 – 1,200,000	Distinct thickening, no gel formation.
Clinical Mastitis	2	1,200,000 – 5,000,000	Thickens immediately, begins to gel, levels in the bottom of cup.
Extreme Clinical Mastitis	3	Over 5,000,000	Gel is formed, surface elevates, with a central peak above the mass.

Table 2.3 Reports of association of candidate gene polymorphism and mastitis in dairy cattle

S.No.	Gene	Reference
1.	BoLA-DRB3	Sharif *et al.,*1998; Rupp *et al.,* 2007
2.	CXCR2	Youngermam *et al.,* 2004
3.	FEZ	Sugimoto *et al.,* 2006
4.	Lactoferin	Wojdak-Makysmiec *et al.,* 2006
5.	TLR4	Sharma *et al.,* 2006; Wang *et al.,* 2007; Wang *et al.,* 2008
6.	CPY11B1	Kaupe *et al.,* 2007
7.	CCL2, IL8	Leyva-Baca *et al.,* 2007
8.	CARD-15	Pant *et al.,* 2007
9.	CXCR1	Leyva-Baca *et al.,* 2008
10.	S100A8	Ogorevc *et al.,* 2009
11.	CD14	Beecher *et al.,* 2010
12.	BRCA1	Yuen *et al.,* 2012

of the traits. These genes may be structural genes or regulatory genes or genes affecting biochemical pathway of the expression. Several candidate genes with different functions in metabolism have been proposed as affecting milk production, composition of milk and susceptibility/resistance for disease in dairy cattle (Szyda and Komisarek, 2007). There are two different approaches for identification of candidate gene

- **The positional candidate gene approach**, by identification of genes within the vicinity of QTL.
- **The functional candidate gene approach**, by identification of trait associated expressed genes.

Mastitis resistance is a complex phenomenon/function including various biological pathways, involving many molecules, and cells. Identification of these genes and finding polymorphic alleles associated with higher or lower animal susceptibility for disease could lead towards better and faster decision for selection of superior animals (Rainard and Riollet, 2006). Some important reports of association between candidate gene polymorphism and mastitis in dairy cattle are listed in Table 2.3

2.4 Historical background of Interleukin-8 gene

IL-8, the chemokine is mostly associated with inflammation. It has been renamed CXCL8 by chemokine nomenclature subcommittee of International Union of Immunological Societies. However, IL-8 is an approved gene symbol as per Human Genome Organization (HUGO). It is also known as Neutrophil Chemotactic Factor which has two primary functions viz. (i) to induce chemotaxis especially neutrophil at the site of infection (ii) to induce phagocytosis. IL-8, a novel leukocyte chemotactic activating cytokine (chemokine), is produced by various types of cells due to inflammatory stimulation, and exerts many functions on leukocytes particularly neutrophils (Mukaida *et al.*, 1992). IL-8 is a chemotactic factor that attracts neutrophils, basophils, and T-cells, but not monocytes. It is also involved in neutrophil activation. It is released from several cell types in response to an inflammatory stimulus.

Initially, IL-8 was isolated and characterized in human (Schroeder *et al*., 1987; Yoshimura *et al*., 1987). Bovine IL-8 was identified by Morsey *et al*. (1996) and it shares

96 per cent, 87 per cent and 76 per cent amino acid homology with Ovine, Porcine and Human IL-8 respectively.

2.5 Chromosomal Location of IL-8 Gene

In cattle, IL-8 gene is located on chromosome number 6 (http://www.ncbi.nlm.nih.gov) (BTA6) near alpha S1 and Kappa casein genes (CSN) (Heaton *et al.,* 1999) which corresponds to the human chromosome number 4 (q11-13) and pig chromosome number 8. (Table 2.4)

2.6 Molecular Organization of Bovine IL-8 Gene

IL-8 gene is protein coding gene. The total length of IL-8 gene is 3.77kb and contains four exons of different numbers of nucleotides in their length (Fig. 2.1). The transcript length is 1,481bps and the translation length is 101 residues. Multipoint linkage analysis indicated that most likely position for both IL-8 and epithelial cell inflammatory protein-2 (EICP-2) was between markers ILSTS087 and BM2460 (Kappes *et al.,* 1997). The gene accession no.: NC 007304.3 and Exon 1 from 1-734 (1-600 is 5'UTR and 601-734 coding region), Exon 2 from 735-2443 (735-2307 non coding region and 2708-2443 coding region), Exon 3 from 2444-2800 (2444-2716 non-coding region and 2717-2800 coding region), Exon 4.1 2801-3706 (3241-3706 coding region and 2807-3240 non-coding region) and Exon 4.2 3707-4966 (3707-4366 coding region, 4367-4966 is 3' UTR).

2.7 Structure of IL-8 protein

Interlukin-8 is a basic protein with the molecular weight of 8-8.5 Kda (Baggiolini et al., 1989). It has specific motif of Glutamic acid-Leucine-Arginine (ELR) immedietly before first cysteine of CXC motif (ELR positive). IL-8 has conserved amino acids which are important for creating their 3-dimensional or tertiary structure, such as four cysteine which interact with each other in pairs to create greek key shape, intramolecular di sulphide bonds typically join the first to third, second to fourth cysteine residues, number as they appear in the protein sequence of chemokine (Laing and Secombes, 2004). In beginning, two cysteine amino acids are situated close together near the N-terminal end

Table No.2.4 Chromosomal location of IL-8 gene in cattle, human and pig

S. No.	Species	Chromosomal location
1.	Cattle	Chromosome 6
2.	Human	Chromosome 4
3.	Pig	Chromosome 8

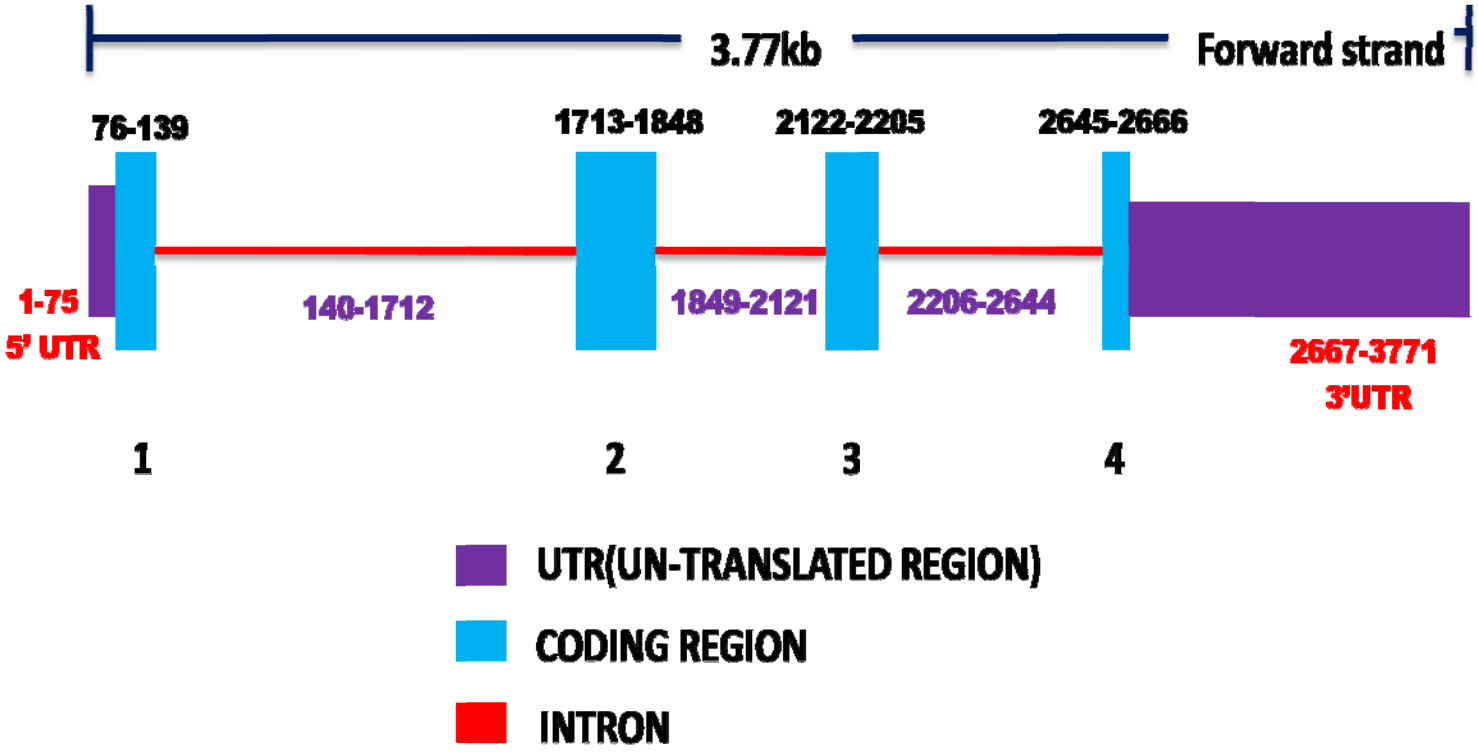


Fig. 2.1 Molecular Organization of IL-8 Gene

of the mature protein, the third cysteine residing in the centre of molecule and the fourth remains close to C-terminal end. The two n-terminal cysteine is separated by one amino acid, called 'X'. a loop of around ten amino acids follow the first two cysteines, called N-loop, which follow by a single turn helix, called a310-helix, three β-strands and a C-terminal α-helix. These helix and strands are connected by turns called 30s, 40s and 50s loop (Fig. 2.2).

2.8 Cellular expression of IL-8 gene

IL-8 is an inflammatory cytokine which is produced by various cell types including lymphocytes (Gregory *et al*., 1988), neutrophils (Strieter *et al*., 1990), monocytes/macrophages (Schroeder *et al*., 1987), and epithelial cells (Elner *et al*., 1990), human mammary gland epithelial cells (Palkowitz *et al.,* 1994), tumor cell lines (Van-Damme, 1994), including bovine mammary epithelial cells (Boudjellab *et al.,* 1998, Barber *et al.,* 1998).

The cells that produce IL-8 play a pivotal role in inflammation, immunity and wound healing. IL-8 mRNA reaches a maximum production two to three hours after stimulation of inflammation then gradually decreases, but high levels are still detectable at 24 hours of infection (Mielke *et al.,* 1990). IL-8 was produced in a time and dose related manner with maximal IL-8 mRNA production at 12hours and maximal protein production at 48 hours after LPS stimulation (10pg/ml) of bovine pulmonary epithelial cells (Boudjellab *et al*., 1998). Subcutaneous injection of IL-8 has been found to be a potent inducer of neutrophil accumulation in bovines (Caswell *et al.,* 1999) and sheep (Mulder and Colditz, 1993). In case of human, it produced from many different cell types, including macrophages, neutrophils and epithelial cells in response to other cytokines, endotoxin

and secreted bacterial products. IL-8 (Chang *et al*., 1998; and Yoshino *et al*., 2003) is

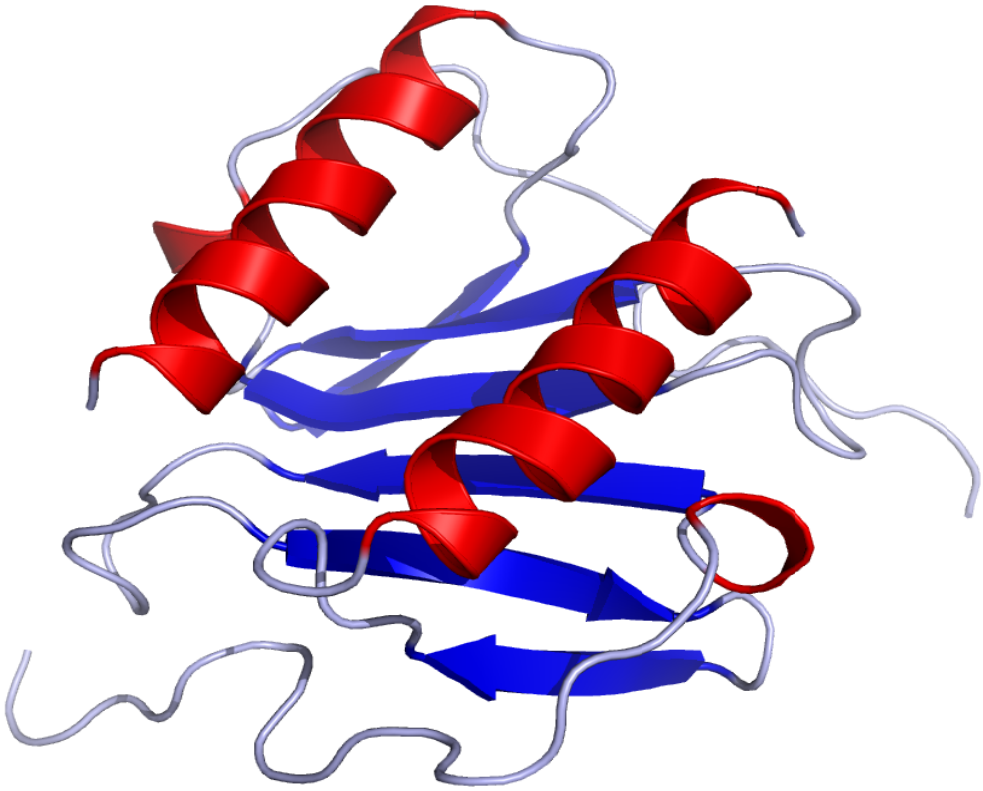


Fig.2.2 Three-dimensional structure of interleukin 8 in solution (Clore, et *al*., 1990)

found in high concentrations in the follicular fluid in the pre-ovulatory phase. In the ovary, IL-8 is detected in theca, granulosa, granulosa-lutein and vascular endothelial cells in humans (Arici *et al*., 1996 and Polec *et al*., 2009).

2.9 Biological role of IL-8 gene

IL-8 plays an important role in recruiting and activating neutrophils (Harada *et al.,* 1994 and Caswell *et al*., 1999), induces neutrophil degranulation (Schroder *et al*., 1987). IL-8 is also known as Neutrophil Activating Protein-1(NAP-1), since it stimulates the secretion of neutrophils at the site of inflammation. During the acute phase of coliform mastitis, the concentration of IL-8 is greatly increased in mastitis affected milk (Bannerman *et al*., 2004). Interleukin-8 is therefore considered to be involved in the infiltration of neutrophils into mammary secretions during mastitis (Fig. 2.3)

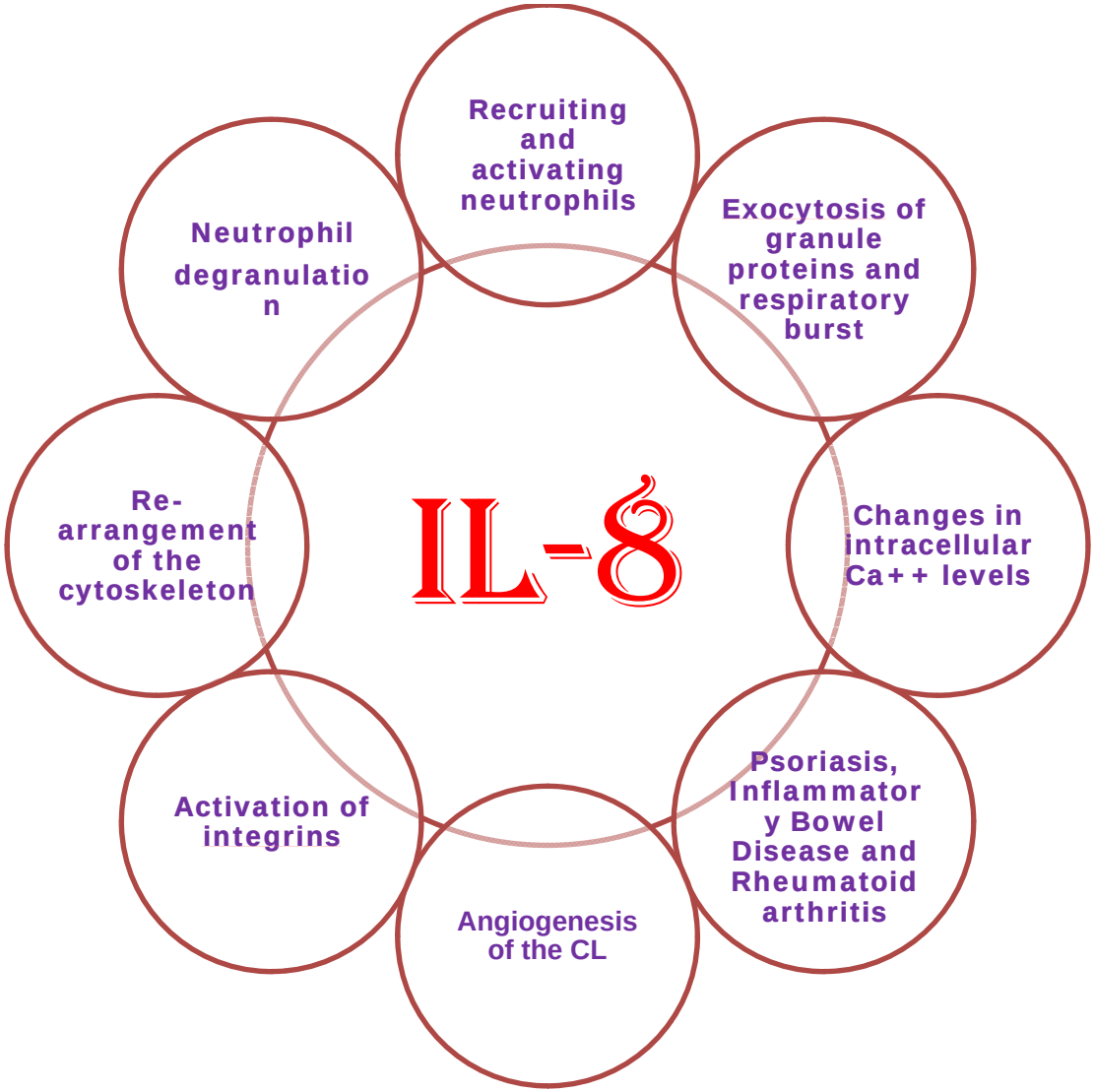


Fig.2.3 Biological role of Interlukin-8 gene

IL-8 also induces the re-arrangement of the cytoskeleton, changes in intracellular Ca++ levels, activation of integrins, exocytosis of granule proteins and respiratory burst (Baggioloni *et al*., 1989; Detmers *et al*., 1991; and Paccaud *et al*., 1990).

IL-8 has a central role in many human inflammatory conditions (Harada *et al*., 1994) including psoriasis, inflammatory bowel disease and rheumatoid arthritis, probably plays a role in angiogenesis of the CL (Jiemtaweebeon *et al.,* 2011).

2.10 IL-8 gene study in humans

Frade *et al*. (2011) investigated polymorphism in TGFB1 and IL-8 genes which are cytokines known for their role in onset and severity of diseases. In this study, polymorphism were detected at TGFB1 -509C/T and +869 T/C and IL-8 -251A/T which were analyzed by PCR-RFLP technique in 198 patients with visceral leshmaniasis, 98 individuals with asymptomatic Infection positive for delayed type hypersensitivity test and in 101 individuals with no evidence of infection.

Lu *et al*. (2005) investigated the genetic polymorphisms of IL-1B, IL-1RN, IL-8, IL-10 and TNFα with the risk of gastric cancer and their association with environmental factors. They conducted case-control study to assess the association between polymorphisms in IL-8 and TNFα which are involved in H. *pylori* infection and gastric cancer. Genotypes were determined by PCR-based denaturing high performance liquid chromatography analysis and direct sequencing in 250 incident cases with gastric cancer and 300 controls recruited in northern China. They found that the risk of gastric cancer significantly elevated two fold in subjects with IL-8-251 AA genotypes in comparison to other AG and GG genotypes.

Gray *et al.* (1997) described the use of high-resolution field emission scanning electron microscopy (FESEM) in conjunction with immune-gold labeling to visualize the cell surface distributions of receptors for the chemo-attractants C5a or IL-8 on human neutrophils. Cells double labeled for C5a and IL-8 receptors showed similar clustered distributions of gold particles bound to these receptor populations, indicating that clustering is characteristic of receptors for two major neutrophil chemo-attractants (Girard and Girard 1994 and Horuk, 1994).

2.11 IL-8 gene study in animals

Sharma *et al*. (2013) carried out the study on the IL-8 gene polymorphism and its association with mastitis in Murrah buffalo by using HaeIII and Hinf1 restriction enzymes. All monomorphic bands were found. Target sequence IL-8 gene of Murrah buffalo was sequenced which has been found to span over 754 bp long. The targeted sequence of IL-8 gene showed 98 per cent homology to that of *Bubalis bubalis* and found no association of IL-8 gene with mastitis in their study.

Landi *et al*. (2003) studied on animal models, the association study between SNP in IL-6(-174 G>C), IL-8 (-251 T>A), TNF-α (-308G>A) and PPARG (Pro121a) genes and risk of colorectal cancer (CRC) in a group of 377 cases and 326 control from Barcelona, Spain. The PPARGBA1a12 and IL-8-251A genotypes are associated with reduced risk of disease, whereas, IL-6-174C genotype is associated high risk. This is the first report which states that IL-6, IL-8 and PPARG genes are important in relation to inflammation related risk of sporadic CRC.

Meade *et al*. (2012) characterized the promoter region of the bovine IL-8 gene towards understanding its regulation and the effect of promoter polymorphism on gene expression levels and identified twenty nine polymorphic sites across a 2.1 kb upstream promoter region of IL-8 gene including two distinct promoter haplotypes (IL8-h1 and IL8-h2) which were present significantly at different frequencies in two divergently selected HF and Norwegian Red breeds of cattle.

Jiemtaweebeon *et al*. (2011) hypothesized that neutrophils infiltrate in developing CL from just after ovulation and may play a role in angiogenesis of the CL. They detected IL-8 gene in CL tissue by periodic acid - Schiff's (Pas) staining and it was measured in supernatant of CL tissue culture, considerable amounts of PMNs and high level of IL-8 were observed during early luteal phase. Polymorphonuclear cells (PMNs) and IL8 were low level in mid and late luteal phase but IL-8 was increased during luteal regression. IL-8 stimulated proliferation of CL derived endothelial cells and both the supernatant of activated PMNs and IL-8 stimulated formation of capillary like structure of LECs. PMNs migrate into early CL partially due to its major chemo attractant IL-8 produced at high

level of CL. PMNs are the potential regulator of angiogenesis together with IL-8 in developing CL in cow.

McClenahan *et al*. (2006) studied IL-8 expression by mammary gland endothelial and epithelial cells by experimentally inducing mastitis infection with E-coli by in-situ hybridization with an IL-8 riboprobe. They showed that level of IL-8 expression in epithelial cells decreases at 12hrs of post infection and increases at 8, 12 hrs post infection, and while in endothelial cells was low but slightly increases at 24 hrs post infection. Both endothelial cells and epithelial cells of mammary glands contribute to production of IL-8 in coliform mastitis.

Lahouassa *et al*., (2008) studied RNA ligase mediated (RLM)-RACE method to clone a novel bovine interleukin-8 receptor of bovine species and revealed that both bovine IL-8 are functional induced migration of HEK-293 cells expressing IL-8R.

Peli *et al.* (2004) studied quantitation of cytokines TNF-α, IL-8 and L-10 in bovine milk using real time TaqMan® PCR in Italian Friesian dairy cows at mid lactation. A comparision was made between healthy cows and animals affected by mastitis, divided into three groups of five animals each: group A showing average SCC 4.097×10^3 and the presence of *corynebacterium* sp., *staphylococcus* sp., and *streptococcus* sp., in two, one and two animals respectively; group B showing average SCC 74×10^3 and the presence of *e.coli* and *streptococcus* sp. In two and three animals respectively; group C showing average SCC 59×10^3 and no bacteria detected in milk. In the samples with higher SCC (group A), level of IL-8 were higher than the low SCC groups B and C.

Polymorphism of bovine IL-8 gene

Seven SNP markers were identified by sequencing two IL-8 DNA segments amplified from panel of 17 US popular cattle breeds. Exon-2 and exon-4 have revealed high degree of polymorphism in HF and US beef cattle and haplotypes were detected in breeds influenced by germplasm from Bos *indicus*. American bison, related showed same homozygous genotypes for all seven SNP sites (at nucleotide position 282: A cattle, G in bison) (Heaton *et al.,* 2001).

Hazare (2009) studied on IL-8 gene polymorphism in 179 lactating Sahiwal cattle involving whole gene including coding and non-coding regions with the product size of 500, 408, 584, 426, 418, 499, 581 & 578bp. First seven parts were monomorphic in PCR-RFLP using AluI and DraI restriction enzyme. In the segment of 578 bp polymorphisms exhibited CC and CD genotypes with 69 and 31 per cent frequency respectively. The allelic frequencies of C and D alleles were 84.6 and 15.4 per cent. it was observed that CD genotypes (40%) was less susceptible to mastitis than CC (62.9%) genotype.

Singh (2008) studied DNA polymorphism at interleukin-8 gene in Murrah buffaloes with the PCR-RFLP technique. PCR products of five sets of primers I-V were 685, 1354, 529, 444 and 435 respectively analysis using HaeIII and DraI restriction enzymes. The study revealed monomorphism from I-Iv using both enzymes, while primer V revealed polymorphism exhibiting AA, AB and BB genotypes. Multiple sequence alignment of IL-8 coding sequences of *Bos taurus* and *Bubalis bubalis* has revealed variation at five nucleotide where two nucleotide variation result into replacement of two amino acids histidine and lysine in buffalo.

Jagadeeshan (2008) investigated the characterization of coding and non-coding regions of IL-8 gene in Sahiwal cattle by custom sequencing by PCR-RFLP analysis. Four sets of primer used to amplify four coding (I-IV) regions and analysis with HaeIII and DraI restriction enzyme. PCR-RFLP analysis revealed the monomorphisms in I-III contig regions but contig IV revealed two different band patterns AA (42bp, 118bp and 275bp) and AB (42 bp, 118bp, 275 bp and 435 bp). Sequence analysis showed single mutation at 1654 nt (T to A) position in AA genotype.

2.12 Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

RFLP is a powerful tool for the identification of genetic variability, providing valuable markers for genetic improvement in dairy animals. Restriction Fragment Length Polymorphism (RFLP) is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA

samples in question with specific restriction endonucleases. RFLP, as a molecular marker, is specific to a single clone/restriction enzyme combination. Most of the RFLP markers are co-dominant viz. both alleles in heterozygous sample will be detected and highly locus-specific. PCR-RFLP Isolation of sufficient DNA for RFLP analysis is time consuming and labour intensive. However, PCR can be used to amplify very small amounts of DNA, usually in 2-3 hours, to the levels required for RFLP analysis. Therefore, more samples can be analyzed in a shorter time. An alternative name for the technique is Cleaved Amplified Polymorphic Sequence (CAPS) assay. The use of PCR to amplify the DNA sequence of interest followed by restriction enzyme digestion to reveal a RFLP is termed as PCR-based RFLP or PCR-RFLP, which is faster and more sensitive than traditional RFLP (Ivinson and Taylor, 1992). RFLP does not involve any kind of radioisotope. This method is more applicable because it allows the use of very small quantity of PCR amplified DNA as low as 50 ng (Ivinson and Taylor, 1992).

2.12.1 Restriction Endonucleases (RE)

Restriction endonucleases (RE) are enzymes which bacteria generally use to destroy foreign DNA, usually of viruses. These enzymes originally were so named because they restricted phage infection among strains of bacteria. These enzymes recognize certain nucleotide sequences found on the foreign DNA, usually 4-10 bases, and cleave the DNA at all sites containing that specific sequence (Zabeau and Roberts, 1979). This site specifically is important since it enables the bacteria to protect similar sites present in their own DNA from the attack of same endonucleases by methylation (Table 2.5).

2.12.2 RFLP

DNA base change such as deletion causing elimination of nucleotides; insertion or rearrangements of base pairs creating translocation re-cleavage sites results into RFLP (Botstein *et al.,* 1980). A point mutation, deletion or insertion can create or abolish the recognition sites for a particular RE at the locus and thereby change the size of restriction fragments. Thus, change in DNA sequences associated with allelic change at a locus will be visualized by the mobility of restriction fragment on gel electrophoresis. Individual carrying different allelic variants of gene will show different band distribution

patterns. These differences in band numbers and locations that result from changes in fragment size are termed as RFLP.

RFLPs are inherited in Mendalian fashion, and since gene expression is not required for RFLP analysis, variation in the flanking regions or introns of genes may also be detected. Consequently the RFLP approach of analysis of genomic variation is potentially much more powerful than other available strategies (Theilmann *et al*., 1989).

2.12.3 Uses of RFLP markers

RFLP is one of the most efficient tools to find out genetic biodiversity in man and animals. In human, RFLPs have been used in prenatal diagnosis of Haemoglobinopathy (Little *et al*., 1980) and extended the utility of polymorphic markers in detection of genes responsible, for other genetic disorders like Duchenne Muscular Dystrophy (Murray *et al*., 1982), Huntington's chorea (Gusella *et al*., 1983) and Retinitis Pigmentosa (Bhattacharya *et al*., 1984). In livestock, RFLP mainly used in genes that are correlated with economic traits, to improve strain crosses, and for within population (Soller and Beckmann, 1983). The use of RFLP markers generated interest determining the genetic variability at DNA level and analyzed whether these variation directly and indirectly by linkage to behavioral and phenotypic characters (Beckmann and Soller, 1987).

2.12.4 Limitations of PCR-RFLP

The analysis requires knowledge of the sequence surrounding the RE sites. In some cases, differential amplification, i.e., allelic drop-out of one or either allele may take place when a sequence prevents one oligonucleotide from binding to one chromosome, resulting in PCR product being generated from only one chromosome (Fujimera et al., 1990). Besides, incomplete RE digestion of PCR-amplified products could leads to mis-intepratation of samples appearing as heterozygous. Use of known controls both homozygous and heterozygous avoids this problem (Ivinson and Taylor, 1992). Another, PCR-RFLP made Heteroduplexes which are PCR by-products that results from pairing between allelic complementary DNA strands that out competes the hybridization of oligonucleotides with their template strands (Mayers et al., 1989). This can be result in appearance of extra bands on the gel that belongs neither of the allelic

Table 2.5 Types of Restriction Endonucleases

S. No.	Characteristics	Type I	Type II	Type III
1.	Restriction and modification activities	Single, multifunctional enzymes	Separate endonucleases and methylase	Separate enzymes with a subunit in common
2.	Protein structure of res	Three different subunits	Simple	Two different subunits
3.	Requirements for restriction	ATP,Mg^{2+}, S-adenosyl methionine	Mg^{2+}	ATP,Mg^{2+} , S-adenosyl methionine
4.	Sequence of host specificity site	EcoB:TGAN*8TGCT EcoB:AACN6GTG	Rotational symmetry	Ecop1:AGACC Ecop15:CAGCAG
5.	Cleavage sites	Random, atleast 1000bp from the host specificity site	At or near host specificity site	24-26 bp to 3' end of host specificity site
6.	Enzymatic Turnover	No	Yes	No
7.	DNA Translocation	Yes	No	No
8.	Site of methylation	Host specificity site	Host specificity site	Host specificity site

pattern (Van Eijk et al., 1992). Since heteroduplexes form primerly in the later cycle of the PCR when concentration of DNA high, it can be avoided by standardization of DNA template concentration and an optimal number of cycles in PCR.

2.13 Real time PCR (Quantitative PCR)

Real time polymerase chain reaction is a laboratory technique of molecular biology which is based on the principle of polymerase chain reaction. It is used for amplification and simultaneously quantification of the targeted sequence of plasmid DNA, genomic DNA, cDNA or RNA. This technique also called quantitative PCR (qPCR). Real-time reverse-transcription PCR is often denoted as: qRT-PCR (Udvardi *et al.,* 2008). The acronym "RT-PCR" commonly denotes reverse transcription polymerase chain reaction and not real-time PCR, but not all authors adhere to this convention (Logan *et al.,* 2009).

There are three types of quantitation assays:

• DNA/cDNA quantitation

• RNA quantitation using one-step reverse transcription polymerase chain reaction (RT-PCR)

• RNA quantitation using two-step RT-PCR

Reverse transcription RT-PCR based assay are the most common method for characterizing or confirming gene expression patterns and comprising mRNA levels in different sample populations (Orlando *et al.,* 1998). The quantification can be done by two types (1) absolute quantification assay is used to quantitate unknown samples by interpolating their quantity from a standard curve and (2) relative quantification assay is used to analyze changes in gene expression in a given sample relative to another reference sample (such as an untreated control sample). Target gene mRNA levels are measured in relative to their respective competitor templates and are normalized to reference genes to control for cDNA loaded into the reaction (Bustin, 2002). The two common method used for the detection of products in quantitative PCR are (1) non-specific flurochromes and (2) hybridization probes

2.13.1 Non-specific flurochromes

Non-specific flurochromes, which binds with any double stranded DNA in PCR, causing florescence of dye. There is direct relation between the quantification of DNA and fluorescence intensity in each cycle. dsDNA dyes such as SYBER Green will bind to all dsDNA PCR products (specific or non-specific) and potentially interfere with, or prevent, accurate quantification of the intended target sequence. As the PCR progresses, more amplicons are created. Since the SYBR Green dye binds to all double-stranded DNA, the result is an increase in fluorescence intensity proportionate to the amount of PCR product produced. There are two requirements for a DNA binding dye for real-time detection of PCR

- Increased fluorescence when bound to double-stranded DNA
- No inhibition of PCR

Advantages of SYBR Green Dye

The advantages of the SYBR Green dye are as follows:

- It can be used to monitor the amplification of any double-stranded DNA sequence.
- No probe is required, which reduces assay setup and running costs.

Disadvantage of SYBR Green Dye

The primary disadvantage of the SYBR Green dye is that it may generate false positive signals; i.e., because the SYBR Green dye binds to any double-stranded DNA, it can also bind to nonspecific double-stranded DNA sequences. The primary disadvantage to these dyes is that they detect accumulation of both specific and nonspecific PCR products.

2.13.2 Hybridization probe or florescent reporter probes

Real-time systems for PCR were improved by the introduction of fluorogenic-labeled probes that use the 5´ nuclease activity of *Taq* DNA polymerase. The availability of

these fluorogenic probes enabled the development of a real-time method for detecting only specific amplification products

It can be used as multiplex assays for detection of several genes in the same reaction which based on specific probes with different colored labels provide to all targeted genes amplified with similar efficiency. It enables to quantify the targeted sequence even in the presence of non-specific DNA amplification and also prevent the interference of measurements caused by undesirable potential by-products like primer-dimers. Two types of *TaqMan* probes viz.

• TaqMan® probes (with TAMRA™ dye as the quencher dye)

• TaqMan® MGB probes

Advantages of TaqMan

The advantages of the TaqMan chemistry are as follows

• Specific hybridization between probe and target is required to generate fluorescent signal

• Probes can be labeled with different, distinguishable reporter dyes, which allows amplification of two distinct sequences in one reaction tube

• Post-PCR processing is eliminated, which reduces assay labor and material costs.

Disadvantage of TaqMan

The primary disadvantage of the TaqMan chemistry is that the synthesis of different probes is required for different sequences.

2.13.3 Real-Time PCR Applications

Real-Time PCR can be applied to traditional PCR applications as well as new applications that would have been less effective with traditional PCR. With the ability to collect data in the exponential growth phase, the power of PCR has been expanded into applications such as viral (Fend *et al.,* 2000) and bacterial (Goerke *et al.,* 2001)

quantitation, quantitation of gene expression (Emmert *et al.,* 1996 and Bustin *et al.,* 2000), monitoring transcription in vitro (Liu *et al.,* 2002), direct detection of effects of receptor signaling (Yuen *et al.,* 2002), quality control and assay validation (Baugh *et al.,* 2001), pathogen quantification (Ohyama *et al.,* 2000), and predictive genetic testing and the identification of relevant SNP (Simone *et al.,* 1998 and Luo *et al.,* 1999).

2.13.4 Advantages of using Real-Time PCR

Traditional PCR is measured at End-Point (plateau), while Real-Time PCR collects data in the exponential growth phase, less labour and more throughput, multiplex approach possible (Mifflin *et al.,* 2000). An increase in Reporter fluorescent signal is directly proportional to the number of amplicons generated. The cleaved probe provides a permanent record amplification of an Amplicons, Increase dynamic range of quantification, high technical sensitivity (<5copies) and high precision (Emmert *et al.,* 1996 and Fend *et al.,* 2000) and no-post PCR processing is required

Chapter - 3

Materials and Methods

MATERIALS AND METHODS

3.1 Experimental Animals

The present study was conducted in a herd of lactating Deoni cows (Figure 3.1) maintained at Cattle Yard, NDRI, Southern Campus, Bangalore and Livestock farm of Prabhani, MAFSU, Maharashtra. A total of 95 animals comprising 54 lactating Deoni cattle were utilized for present study.

3.2 Blood Collection

About 10 ml of blood was collected from each animal aseptically from Jugular vein in a Vaccutainer tube containing 0.5 per cent EDTA. After collection, the samples were stored at 4°C and DNA was isolated within 24 hrs of collection.

3.3 Milk Collection

About 15 ml of milk was collected from lactating animals to estimate the somatic cell count and electrical conductivity. Electrical conductivity and Somatic Cell Count (SCC) in milk was estimated on the same day of milk collection.

3.3.1 Measurement of Somatic Cell Count (SCC)

3.3.1.1 SCC estimation through digital reader (Portacheck)

The required number of porta-strips were removed from the pouches and placed on a flat surface with the sample well facing up.

i. The cow /sample ID was written on the strip
ii. The samples were mixed and drawn into the pipette by squeezing and holding the bulb of the pipette.
iii. The tip of the pipette was placed into the milk sample, so that the tip is completely submerged.
iv. The bulb was slowly released to draw milk into the pipette.

v. A single drop of milk sample was dispensed by holding the tip approximately ¼ inch above the sample well and gently squeezing the bulb, and care was taken not to allow the pipette tip to touch the sample well during drop formation.
vi. After the milk gets soaked completely into the sample well pad, 3 drops of activator solution was added into the sample well from the dropper bottle, and allowed for 45-60 minutes for colour development.
vii. A blank strip was slided (sample well down and forward) into the digital reader.
viii. The blank strips were removed and waited for flashing strip symbol to appear in the display. A developed test strip was slided fully into the Portacheck digital reader with the sample well down and forward.
ix. The result was recorded, and the displayed number was multiplied by 1,000,000 to get SCC value.

3.3.1.2 SCC by Nucleocounter SCC100 (IL-8 gene expression study)

i. Taken 500µl of milk sample (no. of samples=16) into the PCR tubes and add equal amount of reagent-C (lysis buffer), mix well with the pipette.
ii. Taken SCC- cassette from the box and dip into the well mixed tube, press it tightly so that milk sample runs into the cassette.
iii. Loaded cassette into the Nucleocounter scc100 and run.
iv. Display shows the SCC/ml.
v. On the basis of SCC/ml, animals were divided into two group: healthy and subclinical for further expression study of IL-8 gene

3.3.2 Measurement of Electrical Conductivity

Electrical conductivity of milk is one of the udder health trait, which measured by the electrical conductivity meter. The small quantity of milk was taken from each quarter of animal in the well of electrical conductivity meter and take reading. The electrical conductive meter based on the principle of salt concentration present in the milk. The udder health influenced by the salts containing ions viz. Na^+, K^+ etc.

3.4 Isolation of somatic cells from milk

i. Take 15 ml milk in a 50 ml Tarson tube.
ii. Make a fresh 100mM EDTA stock solution. Add 75µl EDTA which prevents casein precipitation.
iii. Mixed by inverting the tube several times. Spin at 2000xg for 15 mins at 4°C.
iv. Discarded supernatant.
v. Wipe the fat layer with alcohol soaked cotton.
vi. Resuspended cells (using pipette) in 20ml PBS-EDTA.
vii. Centrifuge at 2000xg for 15mins at 4°C
viii. Discard suernatent and wipe off fat layer carefully. If supernatant is not clear then repeat steps 6 and 7.
ix. Add 10ml PBS and resuspended pallet completely.
x. Centrifuge at 2000xg for 15mins at 4°C.
xi. Discarded supernatant. Resuspended pallet in 2ml PBS.
xii. Transfer the complete solution in 2ml eppendorf tubes.
xiii. Centrifuge at 2000xg for 15mins at 4°C.
xiv. Discarded supernatant. Resuspended (using pipette) somatic cell pellet in 500µl PBS
xv. Store at 4°C.

3.5 DNA extraction

i. DNA was isolated from blood using a modified High Salt method (Miller *et al.*, 1988).
ii. Volume of 10 ml of blood was collected in a vaccutainer tube.
iii. Blood was transferred to 50 ml centrifuge tube and 25 ml of RBC lysis buffer (chilled) was added into it and inverted several times. Incubated in ice with shaking for 10-15 minutes for complete lysis of cells.
iv. Nuclear material was pelleted by centrifugation at 4,000 rpm at room temperature for 10 minutes and supernatant was discarded. Nuclear pellet was washed with ten ml of RBC lysis buffer and centrifuged again.

v. The step number 3 was repeated three to four times until a clear nuclear pellet was obtained.

vi. Nuclear pellet was resuspended with a Pasteur pipette and washed with 10 ml Tris Buffer Saline (TBS) at 4000 rpm for 10 mins. Repeat it twice.

vii. Volume of 9 ml of TE buffer (pH 8.0) was added to the pellet and vortexing was performed.

viii. Takes all samples in the laminar flow, for further process. Then 50 µl of Proteinase – K enzyme and 500 µl of 0.5M EDTA was added to the above solution and mixed properly.

ix. 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.

x. Volumes of 4.3 ml of Saturated NaCl was added to the above mixture and shaken vigorously and add equal volume of Chloroform - Isoamyl alcohol (24:1), mixed and centrifuged twice at 4000 rpm for 15 mins.

xi. Take an Aqueous upper phase and transferred into fresh tube and step involving chloroform - isoamyl alcohol addition was repeated two times.

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

xiii. Precipitated DNA strands were transferred to a micro centrifuge tube containing 1 ml of ice-cold 70 per cent ethanol and centrifuged at 2,000 rpm for 5 minutes.

xiv. Supernatant was discarded and the pellet was air-dried. Dried DNA pellet was resuspended in 400µl TE buffer.

xv. Then samples were stored at -20°C after checking its purity.

3.6 Determination of quality and quantity of genomic DNA

DNA quality was checked by gel electrophoresis by loading 2 µl of DNA on 0.8% agarose gel electrophoresis unit using 1x TBE buffer as running buffer at 150 volts for about two and half hours. Gel was stained with Ethidium Bromide solution (0.5µg/ml), and gel photographed under gel documentation system and files were stored in

Table 3.1 Details of Primer sequences used (5’ to 3’ Sequences)

5’ UTR+EXON1	Sequence	Annealing Temperature
FP	GGAGTTCTCTGCCCAACAGA	**57.0**
RP	GGCTTGTTATCCAGGCATGA	**57.0**
EXON 2	**Sequence**	**Annealing Temperature**
FP	GCCAAGCTGTGCTTATGGAT	**56**
RP	GCTGGATTCTTCCACGTCTC	**56**
EXON 3	**Sequence**	**Annealing Temperature**
FP	GACTTGAATGGCAAGGTGGT	**56.5**
RP	TTCAGCAGCAGCAGAAAATG	**56.5**
EXON 4.1	**Sequence**	**Annealing Temperature**
FP	CAGAGCTGAGAAGCAAGATCC	**60**
RP	GTGCTTCCACATGTCCTCAC	**60**
Exon 4.2	**Sequence**	**Annealing temperature**
FP	GTGAGGACATGTGGAAGCAC	**57**
RP	CAAACTCCTGATGACTCTGACAA	**57**

Table 3.2 PCR protocol (25µl reaction mix)

S.No.	Components	Quantity
1	Sigma water	16.5 µl
2	10x Buffer (1X) with Mgcl2	2.5 µl
3	2.5 Mm dNTPs (100 µM each)	0.5 µl
4	Forward Primer (20 pmol/µl)	0.5 µl
5	Reverse Primer (20 pmol/µl)	0.5 µl
6	DNA Template (50 ng/ µl)	2.0 µl
7	Taq DNA Polymerase	0.5 µl
	Total Volume	**25.0 µl**

computer. The quality and purity of DNA were checked and quantitation was done by UV- spectrophotometer.

3.6.1 Spectrophotometric measurement

The purity and concentration of DNA samples were estimated by UV spectrophotometer. 2 μl of DNA was dissolved in 48 μl of Millipore water (1 in 100 dilutions) and optical density (OD) values were measured at 260 nm and 280 nm with distilled water as blank. DNA samples having the OD_{260} /OD_{280} ratio between 1.7 and 2.0 were considered better for PCR reaction. The concentration of DNA was estimated using the following formula:

$$\text{Concentration of DNA (ng)} = OD_{260} \times 50 \times 100 \text{ (dilution factor)}$$

The ratio between OD 260 and 280 was calculated. The samples possessing ratio of less than 1.7 and more than 2.0 was subjected to Proteineases K digestion and DNA extracted with high salt as described previously.

3.6.2 Preparation of Template DNA for PCR

The stock DNA was then diluted with autoclaved distilled water to arrive at a final concentration of 50 ng/μl. This was designated as the working solution.

3.7 Primer design

Primers were selected at random base distribution and GC content was similar to that of the amplified fragment. In particular, primers with 3' overlaps were avoided to reduce incidence of primer dimer formation. The PCR primers were designed using web-based software primer-3 (http:/www.genome.wi.mit.edu/cgibin/primer/primer3-www.cgi) for each of the exons namely Exon 1, Exon 2, Exon 3, Exon 4.1 and Exon 4.2 of known *Bos taurus* sequences species available in GenBank (www.ncbi.nlm.nih.gov.in/genbank). The primers procured from Amnion (Bangalore), (Table 3.1). The PCR reaction master mixture 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).

3.8 PCR amplification of Interlukin-8 alleles from genomic DNA

A normal PCR was performed to amplify the IL-8 alleles of Exon1, Exon2, Exon3, Exon 4.1 and Exon4.2. The reactions were carried out in a thermal Master Cycler (Eppendorf, Germany).

3.8.1 Method

PCR tubes containing the mixture were tapped gently and quickly spun at 10,000 rpm for few seconds. The PCR tubes with all the components were transferred to Master cycler (Eppendorf, Germany) and programmed for 35 cycles. The cycling conditions were as follows:

Steps and conditions of thermo cycling for PCR

S.No.	Step	Temperature	Time
1	Initial denaturation	95°C	3 min
2	Denaturation	95°C	30 sec
3	Annealing	**	45 sec
4	Extension	72°C	45 sec
Repeat the cycle for 35 times for Exon 1 & Exon 3 and 30 times for Exon 2, 4.1 & 4.2			
5	Final Extension	72°C	10 min

3.8.2 Agarose gel electrophoresis to check PCR product amplification

i. The correct amount of agarose (1.80 gm) was weighed in a conical flask and 120 ml of 1X TBE buffer was added to prepare 1.5 per cent solution.
ii. It was placed in a microwave oven until the agarose was dissolved completely.

iii. The solution was cooled and Ethidium Bromide (0.5 μg/ml) was added and mixed thoroughly.

iv. The comb was positioned 0.5 – 1 mm above the plate so that a complete well was formed when the agarose was added.

v. As soon as the gel was completely set after 30-45 minutes at room temperature, the comb removed carefully and the electrophoresis tank filled with TBE buffer.

vi. To confirm the targeted PCR amplification, 5 µl of PCR product from each tube was mixed with a two drops of gel loading dye (6X) and electrophoresed on 1.5 per cent agarose gel containing ethidium bromide (one per cent solution at the rate of 5 µl/100 ml) at constant 150 V for 30 minutes in 1X TBE buffer.

vii. The lid of the gel tank was closed and attached with the electrical leads so that the DNA would migrate from cathode (black lead) toward the anode (red lead).

viii. After half an hour, the power supply was turned off and the leads and lid were removed from the gel tank.

ix. The gel was visualized as a single compact band of expected size under the UV trans-illuminator and photographed using gel documentation system. The quality of the DNA was assessed based on the bands developed. Good quality DNA was used for further analysis through PCR-RFLP technique.

3.9 DNA separation and polymerase chain reaction- restricted fragment length polymorphism (PCR-RFLP)

The PCR-RFLP was performed to see the polymorphic patterns in the interleukin-8 allele. Briefly, PCR-RFLP consists of three stages, a hemi-nested PCR amplification, restriction enzyme digestion of the amplified fragment and then Gel Electrophoresis in a horizontal electrophoresis unit.

The PCR amplification resulted in a 251, 251, 239, 470 and 691 bp fragment for Exon 1, Exon 2, Exon 3 Exon 4.1 and Exon 4.2 of the IL-8 gene. The fragments were then digested separately with restriction enzymes with their respective enzymes (Table 3.3).

Digestion reactions were incubated overnight and digestion products were resolved at 2-2.5 per cent agarose gel using electrophoretic system (Consort, Belgium) by running at 200 V for 3-3 1/2 hrs and visualised through UV transilluminator.

3.9.1 Restriction digestion of PCR products

To determine the polymorphism in amplified PCR products specific restriction enzymes were utilized and the digested fragments were resolved in agarose gel.

3.9.2 Restriction digestion protocol

Restriction Digestion of PCR product with HPYVIII, HaeIII and SspI enzyme

S. No.	Reagents	Quantity
1.	PCR Product	10.0 μl
2.	Double Distilled water	7.5 μl
3.	Specific RE buffer	2.0 μl
4.	Restriction enzyme	0.5μl
	Total volume	20.0 μl

The above restriction digestion components in 0.2 ml PCR tubes were incubated at restriction enzyme specific temperature for a period of about 8 to 12 hrs. Horizontal electrophoresis on 2-3% agarose gel was used to resolve restriction fragment and visualized by Ethidium Bromide staining. The EtBr was added to the agarose gel @ of 1X buffer at 150volts for 45, 60, 90 minutes till complete separation and visualization of all fragments of RE digested gene fragment and PCR marker. The restricted digested gene fragments were visualized on UV transilluminator and photographed with gel documentation system. To the restriction digested products 5μl of 6x gel loading dye was added, the digested DNA fragments were mixed well and loaded into the wells of gel.

3.10 DNA sequencing

Amplified PCR products for each sets of primer were subjected to custom DNA sequencing from both ends 5' to 3' ends. Representative samples from each of the

Table 3.3 Restriction Enzymes of different Exons and their cutting sites

Exons	Restriction enzyme	Sites
Exon-1	HpyCH4V	5'…TG↓CA…3' 3'…AC↑GT…5'
Exon-2	HaeIII	5'…GG↓CC…3' 3'…CC↑GG…5'
Exon-3	SspI	5'...AAT↓ATT...3' 3'...TTA↑TAA...5'
Exon-4.1	HaeIII	5'…GG↓CC…3' 3'…CC↑GG…5'
Exon-4.2	HaeIII	5'…GG↓CC…3' 3'…CC↑GG…5'

variants obtained by RFLP analysis were also custom sequenced from Applied Biosystem, Amnion, Bangalore.

3.10.1 Multiple sequences analysis and SNP detection

Sequence data was analysed using c:\DNA Baser Assembler\ software. The all 4 coding regions of IL-8 gene was subjected to BLAST to know the sequence homology with corresponding regions of other spp. Sequence data from variants of different regions were subjected multiple sequence alignment for identifying the SNPs.

3.11 Association Studies

3.11.1 Source of Data

The production data of Deoni was collected from history-cum-pedigree sheets maintained at Cattle Yard, NDRI, Southern Campus, Bangalore, livestock farm of Prabhani, MAFSU, Maharashtra, and Deoni DNA stock. The fresh data were collected from the available lactating Deoni Cattle for somatic cell count and EC.

3.11.2 Collection of Data

The following information on production traits for Deoni breed was collected and recorded

Animal number

Stage of lactation

Parity

Udder health traits

SCC

Electrical Conductivity

Production traits

Lactation Length

Lactation Yield

3.11.3 Standardization of Data

The records of animals with normal lactation were included in the present study. A lactation of at least 100 days long and in which the cow calved and dried under normal physiological conditions were considered normal.

3.11.4 Classification and Standardization of Data

3.11.4.1 Stage of Lactation

The 305 days milk yield was divided into three stages *viz.*

Stage	Days	Code
1st	7-90 days	I
2nd	91-180 days	II
3rd	181-305 days	III

3.11.4.2 Parity

Parity	Code
1st	I
2nd	II
3rd	III
4th	IV
5th	V
6th	VI
7th	VII

3.12 RNA extraction by RNeasy MINI KIT (QIAGEN)

i. Take the isolated somatic cell suspension.
ii. Centrifuge at 2000xg for 15mins at 4°C. Discard the supernatant.

iii. Add 175µl RLT lysis buffer (containing β-mercaptoethanol per ml buffer RLT) as per manufacturers' guidelines (cell counts/ml), mix well with pipette for proper homogenization. Add the other 175µl RLT buffer, mix by tapping the tube as well pipetting. Give a slight vortexing.

iv. Incubate the tubes at room temp. for five minutes. Spin at 10,000rpm for two minutes.

v. Carefully remove the supernatant by pipetting and transfer it to a new microcentrifuge tube. Use only this supernatant (lysate) in subsequent steps.

vi. Add 1 volume of 70% ethanol to the cleared lysate, and mix immediately by pipetting.

vii. Transfer upto 700µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2ml collection tube (supplied). Close the lid gently, and centrifuge for 25s at>10,000 rpm. Discard the flow through.

viii. Add 700µl buffer RW1 to the spin column. Close the lid gently and centrifuge for 25s at>10,000rpm to wash the spin column membrane. Discard the flow through.

ix. Add 500µl buffer RPE to the RNeasy spin column. Close the lid gently and centrifuge for 25s at>10,000rpm to wash the spin column membrane.

x. Add 500µl buffer RPE to the RNeasy spin column. Close the lid gently and centrifuge for 25s at>10,000rpm to wash the spin column membrane.

xi. Empty spin by centrifugation at full speed for 1 min. place the RNeasy spin column new 1.5 ml collection tube. Add 30-50µl RNase free water directly to the spin column membrane. Allow it to stand for 5 minutes at room temperature. Centrifuge for 1min at 10,000rpm for 1 min to elute the RNA.

xii. Quantify and check the purity of RNA using nanodrop2000/2000C software.

3.13 cDNA synthesis (Revert Aid H-Minus First Strand cDNA Synthesis Kit)

i. Prepare the following reaction mixture in a tube on ice:

S.No.	Components	Quantity
1	Template	10µl
2	Oligo(dT) primer (0.5µg/µl)+ random hexamer	1+1µl

3	DEPC Treated Water	-----
Total		12µl

ii. Mix gently and spin down for 3-5 secs in a microcentrifuge.

iii. In a cycler set the programe cDNA 1:

70°C: 15secs

70°C: 10mins

4°C : 2mins

iv. Finally snap chilling on ice and collect the drops by short spin.

v. Again place those tubes on ice and add the following in indicated order:

vi.

S.No.	Components Per Reaction Tube	Quantity
1	5x reaction buffer	4µl
2	RiboLock Ribonuclease inhibitors (20µ/µl)	1µl
3	10mM dNTP mix	2µl
Total		7µl

vii. Dispense 7µl in each reaction tube and incubate at room temperature for 10 mins.

viii. Add 1µl Revert Aid H minus M-MuLV to each reaction tube (making the total reaction to 20µl) mix it and give it a short spin to collect the droplets and set the cycler program(cDNA 2):

42°C: 1hour

70°C: 10mins

4°C : 5mins

ix. Keep it in 4°C.

x. Quantify and purity check of the first strand of cDNA should be done with nanodrop2000/2000C.

3.13.1 Real time PCR (Relative Quantification Method)

i. Thaw and spin all tubes.

ii. Dilute primer from 100pmoles/µl to 10pmoles/µl

iii. Complete set up is done in dark and without airflow.

iv. Mark plates carefully.

v. Switch on the RT-PCR machine prior to preparation of samples

vi. Mock run Taq-man assay with two genes(GAPDH, IL-8)

GAPDH- Reference gene

IL-8 – Test gene

vii. cDNA is taken out just before using. Give a short spin.

Table 3.4 Primer and probe sequences used for IL8 Real time assay

Primer Name (Max. 15 Characters)	Primer Sequence 5' to 3' - Do not put the "5' to 3' "for each row	Scale (µmol)	Purification	No. of Bases
102IL8- F	ACTGCGCCTTGGTTTCTTTA	0.01	HPSF	20
102IL8- R	GTGCTTCCACATGTCCTCAC	0.01	HPSF	20
147GAPDH - F	AATTCTGGCAAAGTGGACATC	0.01	HPSF	21
147GAPDH - R	GACCATGTAGTGAAGGTCAATGAA	0.01	HPSF	24

viii. Prepare master mix, label two different master mix

S.No.	Components	Quantity
1	Water	3.8µl
2	Probe mastermix	10µl
3	Primer (forward)	2µl
4	Primer (reverse)	2µl
5	Probe	0.2µl
6	Total	18µl

ix. Add the master mixture in 2 µl of template cDNA and make it upto 20µl.

x. Tape the vials well to mix.

xi. Spin the tubes.

xii. Take out PCR plate (the one racked last among the lot).

xiii. Note down the plate number and jot down the date and genes on the plates

xiv. Make a plates set up record. Keep NTC at first well or last well. While adding master mix, add the last part to the well meant for NTC.

xv. Spin cDNA and add to each well.

xvi. Mix well using pipette and add to each well and mix again with pipette.

	1	2	3	4	5	6	7	8	9	10	11	12
A(GAPDH)	A1	A2	A3	A4	NTC							
B(IL-8)	B1	B2	B3	B4	NTC							
C												
D												
E												
F												
G												
H												

xvii. Seal the plate with the transparent sticker using the one edge and scroll across the plate. Trim the edges of the sticker

xviii. Then seal the plate on the edges

xix. Place it on the plate holder (or machine) in correct orientation

xx. Software used: light cycler 480 software release 1.5.0 SP3

	Temp°C	**Time**	**No. of cycle**
Pre incubation	95	7min	1
PCR quantification	95	10s	35
	60	30s	
	72	1s	
Melting curve	95	5s	1
	60	1min	
	95	continuous	
Cooling	40	10s	

xxi. Take out the plate and note down the Cp values for IL-8 gene expression assay.

xxii. On the basis of Cp value in the software get the fold change in IL-8 gene expression in subclinical and healthy samples.

3.14 Statistical analysis

The statistical analysis was carried out using statistical software packages SAS 9.3. The General Linear Model (GLM) was used for analysis of variance to get the effect of 3 independent variables viz, Stage of lactation, parity and genotypes on four dependent variables viz, LL, LMY, SCC and EC. In order to overcome the problem of non-orthogonality of effects due to unequal and disproportionate sub-class frequencies, least squares analysis of fitting constants as suggested by Harvey (1987) was taken into account for the analysis of data.

3.14.1 Calculation of allele and genotype frequencies

POPGENE version 3.2 software was used to evaluate the gene and genotypic frequencies, level of heterozygosity, effective number of alleles and X^2 values (Yeh *et al*., 2006)

3.14.1.1 Genotype frequency

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

$$\text{Genotype frequency} = \frac{\text{The Proportion of animals with a particular genotype}}{\text{Total number of animals (N)}}$$

3.14.1.2 Gene frequency

The genetic constitution of a population, referring to the gene it carries, is described by the array of gene frequencies. The allelic frequencies of each allele were calculated in the studied population (Deoni population). Allele frequencies were calculated by using the following formula given below

$$\text{(2 X no. of homozygote) + (no. of heterozygote)}$$

$$\text{Frequency of an allele (p)} = \frac{}{2 \times \text{total no. of individual in the population}}$$

Presence of Hardy – Weinberg equilibrium in Deoni purebred for all coding regions of IL8 gene were checked by calculating the expected genotypic frequencies and compared them with observed genotypic frequencies. Observed genotypic frequency was calculated with the formula given below

$$\text{Observed genotypic frequency} = \frac{\text{Number of heterozygote animals}}{\text{Total number of animals (N)}}$$

All the requirements of HW equilibrium were assumed to be true while calculating the expected genotypic frequencies, which were calculated using POPGENE as follows:

$$\text{Expected frequency of homozygote} = p^2 \text{ and } q^2$$

Expected frequency of heterozygotes (Expected heterozygosities) = 2pq

Where, $p + q = 1$

The significance of the deviation from HW equilibrium was tested by using χ^2 test by comparison between the observed and expected frequencies of each distinct genotype in Deoni (Yeh *et al*., 2006). The effective number of alleles (n_e) was also estimated using POPGENE v 3.2 software based as per method given by Ohta and Kimura (1973).

3.15 Association study – General linear model (GLM) and Multivariate analysis of variance (ANOVA)

Least squares analysis of fitting constants as suggested by Harvey (1982) and multifactor dispersive analysis (ANOVA) data was used to find out the significant difference among genetic (IL8 gene Exon 1, 2, 3, 4.1 and 4.2) and non genetic factor (parity and stage of lactation) on economic traits namely pooled LL, polled LMY, SCC and EC. The analysis was done by using following model

The least square analysis for SCC, EC for FL, FR, HL, HR and pooled EC was done by using the following model:

$$Y_{ijk} = \mu + P_i + S_j + G_k + e_{ijk}$$

Where,

μ = Overall mean for herd or population

Y_{ijk}= SCC and EC of animals belonging i^{th} parity, j^{th} stage of lactation and k^{th} genotype

P_i = Effect of i^{th} parity (where i = 1 to 7)

S_j = Effect of j^{th} stage of lactation (j = 1, 2, 3)

G_k = Effect of k^{th} genotype (where k = AA and AB for Exon 3)

e_{ijk} = Random error associated with Y_{ijk} observations and assumed to be NID (0, $\sigma^2 e$)

The least square analysis for lactation length and lactation milk yield was done by using the following model:

$$Y_{ik} = \mu + P_i + G_k + e_{ik}$$

μ = Overall mean for herd or population

Y_{ijk}= LL and LMY of animals belonging i^{th} parity and k^{th} genotype

P_i = Effect of i^{th} parity (where i = 1 to 7)

G_k = Effect of k^{th} genotype (where k = AA and AB for Exon 3)

e_{ijk} = Random error associated with Y_{ijk} observations and assumed to be NID (0, $\sigma^2 e$)

The decision will be to reject the null hypothesis if the test statistic from the table is greater than the F critical value with k-1 numerator and N-k denominator degrees of freedom. If the decision is to reject the null, then at least one of the means is different. As, ANOVA does not depict where the difference lies. Hence, “Tukey” (Post-hoc test) test was applied.

3.16 Post-hoc test

The post hoc multiple comparison tests were performed for each dependant variable separately. The differences of means between parity, stage of lactation (non-genetic factors) and allelic mean differences (genetic factors) within the SCC was tested for significance by applying ‘Tukey’ Post-hoc test.

Chapter - 4

RESULTS & DISSCUSSION

RESULT AND DISSCUSSION

The study was carried out for molecular characterization of IL-8 gene using PCR-RFLP analysis in 95 Deoni cows including 54 milking animals maintained at cattle yard of National Dairy Research Institute, Southern Regional Station, Bangalore and Livestock farm of Prabhani, MAFSU, Maharashtra. The results obtained from the experiments are presented along with the discussions under following sections.

4.1 Isolation, Yield and Purity of genomic DNA

The isolation of genomic DNA was carried out in 95 Deoni cattle, maintained at SRS, NDRI, cattle yard, Bangalore, by high salt method of Miller (1988). The yield of genomic DNA samples of Deoni cattle ranged from 300.2 to 1927.6ng/µl with overall mean of 788.18 ± 38.52ng/µl (Table 4.1). The range of optical density of DNA samples at A260/280nm in the range of 1.66 to 2.26 with the mean value of 1.95 ± 0.015. Similarly Rao *et al.* (2012) reported, the yield of DNA isolated from blood of Deoni cattle by Millers' High salt method was in the range from 360 to 1330 ng/µl, where mean OD value was 1.78 ± 0.02 at 260/280 nm which was close to present study. The mean yield of DNA (ng/µl) and purity of the DNA samples obtained from blood samples of Deoni cattle indicated good deproteinization. Extraction of DNA from whole blood by high salt method yielded good quality of high molecular weight DNA as determined by agarose gel electrophoresis (Fig 4.1).

4.1.1 Description of gene under study

All four exons of IL-8 gene were investigated for detection of genetic variation. PCR products of exon-1, exon-2, exon-3, exon-4.1 and exon-4.2 were 251, 251, 239, 470 and 691 bp respectively and they were analyzed for detection of polymorphisms with their respective restriction enzyme.

4.1.2 PCR amplification of coding regions of IL-8 gene

The composition of PCR mix required for thermal cycling was optimized for PCR amplification of all coding regions of IL-8 gene. The amplified products were checked at 1.5 per cent agarose gel prepared with 1X TBE buffer. The PCR amplification resulted in the respective base pairs (Fig. 4.2, 4.4, 4.6, 4.8 and 4.10).

4.1.3 Genotyping of the coding regions of IL-8 gene through PCR-RFLP

Molecular characterization of all exons of IL-8 gene of Deoni cattle were carried out through PCR-RFLP technique. For RFLP studies, three restriction enzymes were used for digestion of respective PCR products of DNA samples. HpyCH4V (TG↓CA) restriction enzyme was used for detection of polymorphism in exon-1 of IL-8 gene. HaeIII (GG↓CC) restriction enzyme was used for detection of polymorphism in exon-2, exon-4.1 and exon-4.2; SspI (AAT↓ATT) was used for detection of polymorphism in exon-3 of IL-8 gene. The gel photographs (Fig. 4.3, 4.5, 4.7, 4.9 and 4.11) indicated representative band patterns of different genotypes observed for all exons of IL-8 gene in the studied population. In this study, exon-1, exon-2, exon-4.1 and exon-4.2 were found to be monomorphic (Fig. 4.3, 4.5, 4.9 and 4.11), while band pattern exhibited in exon-3 as polymorphic (Fig. 4.7). PCR-RFLP was also carried out using DraI RE (Fig.4.12 and 4.13) for exon-4.2 comprising fragment (578 bp) of exon-4 and 3'UTR. This fragment was also found monomorphic. However, Hazare (2009) reported this fragment as polymorphic in sahiwal breed of cattle. In the Sahiwal cattle, Jagdeeshan (2008) revealed polymorphism in contig region four by the technique PCR-RFLP with HaeIII and DraI RE Similarly, Sharma *et al.* (2013) revealed monomorphic pattern for fragment (853 bp) of IL-8 gene comprising exon-4 in Murrah buffalo. The genotypic and allelic frequencies for these loci are presented in Table 4.2.

4.1.4 Polymorphism at exon-3 of IL-8 gene

The gel photograph obtained through PCR-RFLP technique for IL-8 gene reflected polymorphism at exon-3 in Deoni cattle (Fig. 4.7). Polymorphic band patterns exhibited two distinct genotypes AA and AB at third exon with genotypic frequencies of 0.21 and 0.79 respectively in the studied population (Fig.4.7). The resulting allelic frequency obtained for A allele was 0.61 and for B allele was 0.39 (Table 4.2). Thus, the present study indicated that the A type is more frequent in the studied population of Deoni cattle.

4.1.5 Population genetic analysis of exon-3 IL-8 gene

The genotypic patterns obtained for bovine IL-8 gene in Deoni cattle population were determined by manual segregation. The degree of heterozygosities as well as Chi-

Table4.1 Mean yield and purity of DNA of Deoni cattle

Descriptive Statistics	DNA concentration (ng/µl)	Optical density (260/280nm)
Mean	788.18	1.95
Standard error	38.52	0.015
Standard deviation	375.46	0.15
Minimum	300.2	1.66
Maximum	1927.6	2.26
N (no. of observation)	95	95

Table4.2 Genotype and allele frequencies of IL-8 gene

Genotype (N)	Genotype Frequency	Allele	Allelic Frequency
AA (20)	0.21	A	0.61
AB (75)	0.79	B	0.39

N= number of cattle under genotype

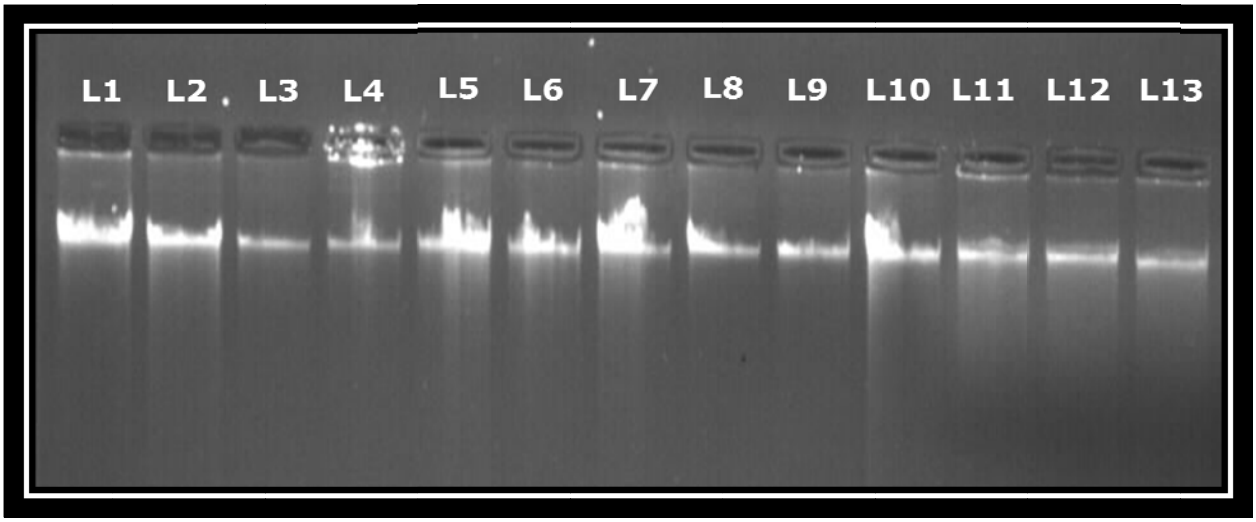
Table 4.3 Heterozygosity statistics for IL-8 gene

Heterozygosity		Effective Number Of Allele (Ne)
Observed	Expected	
0.79	0.48	1.9151

Table 4.4 HW equilibrium statistics for IL-8 gene

Gene	Chi-squares value	Probability
IL-8	39.8	<0.01

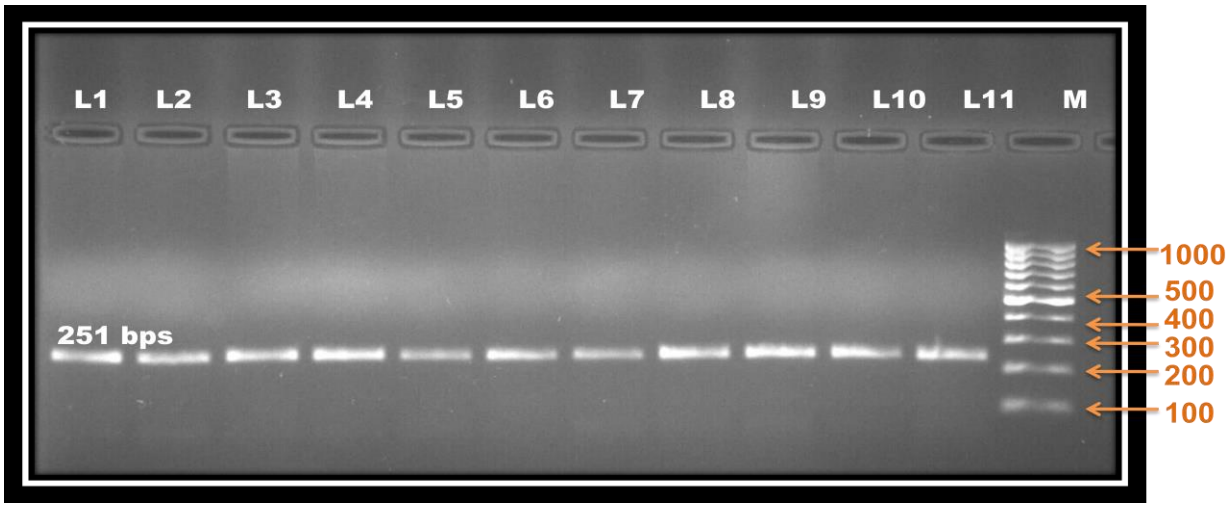
Fig 4.1 Quality of DNA of Deoni cattle at 0.8 per cent agarose gel



Lane 1, 2, 5, 6, 7, 8, 9 & 10 = Concentrated DNA

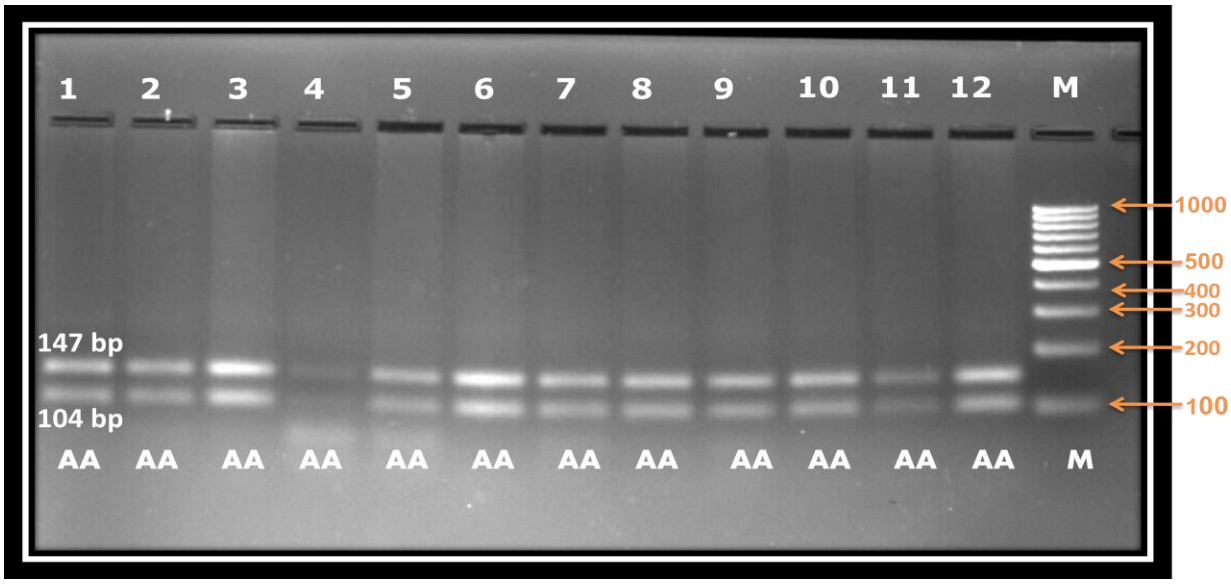
Lane 3, 4, 11, 12 & 13 = Optimum DNA Concentration

Fig 4.2 PCR Product of Bovine IL-8 Gene at Putative Exon-1 on 1.5% Agarose Gel



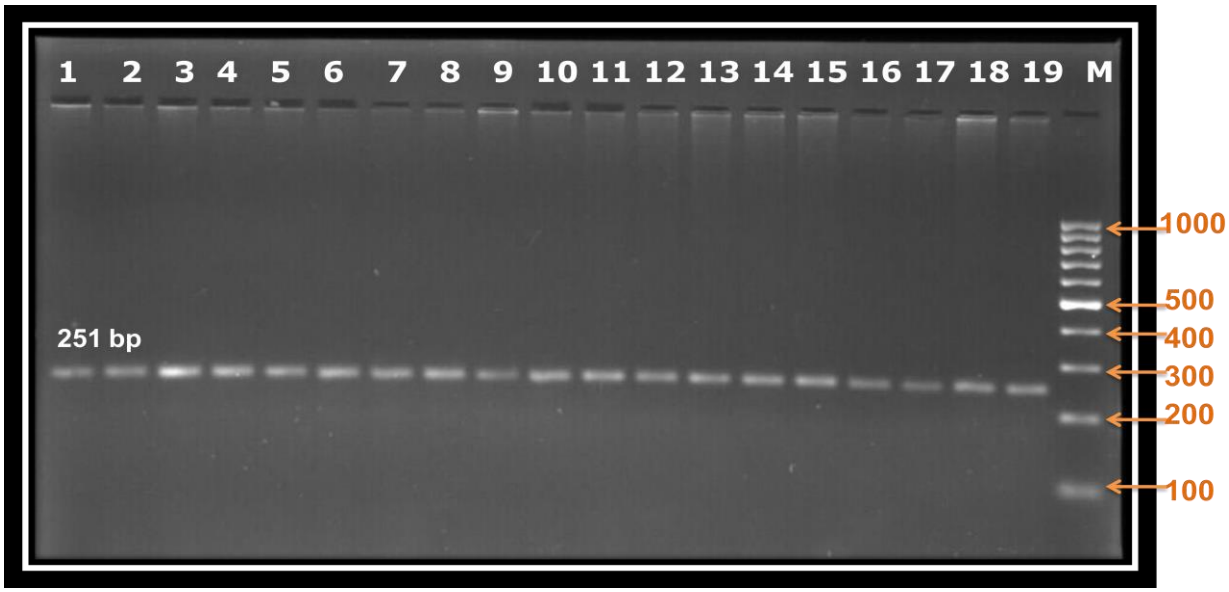
M=100 bp DNA marker**,** **L=** Lane number

Fig 4.3 HpyCH4V Digested PCR-RFLP Pattern at Putative Exon-1 in IL-8 Gene of Deoni Cattle



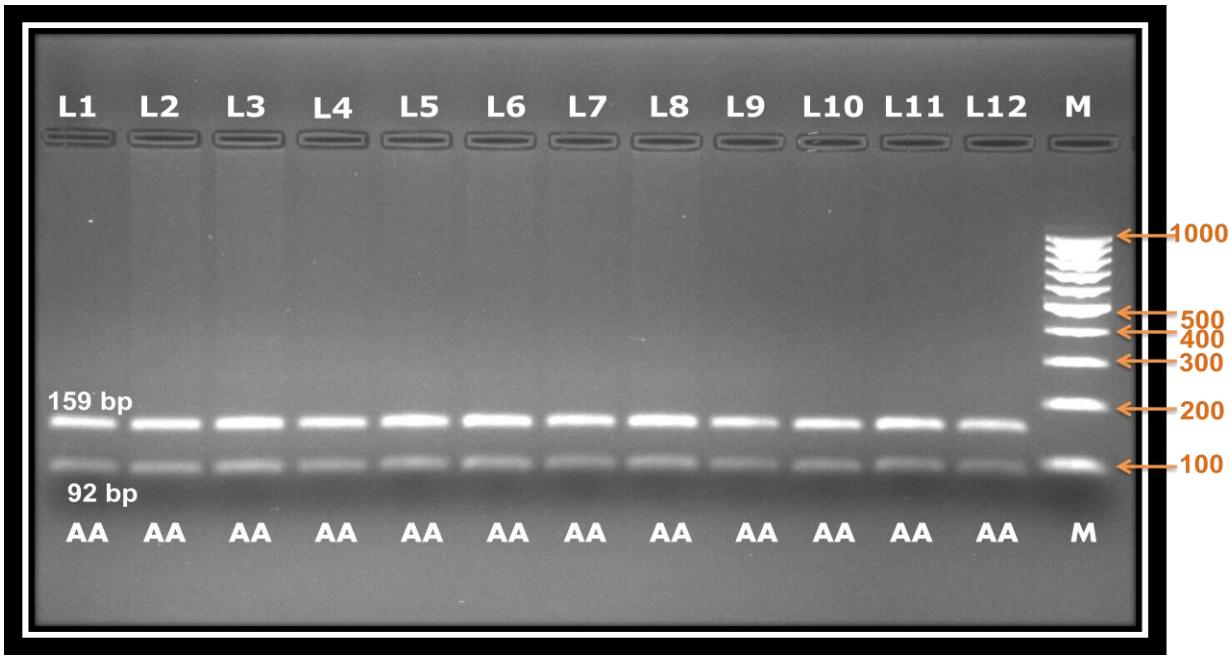
Lane L1-l12 = AA Genotypes, **M =** 100 bp DNA Marker

Fig 4.4 PCR Product of Bovine IL-8 Gene at Putative Exon-2 on 1.5% Agarose Gel



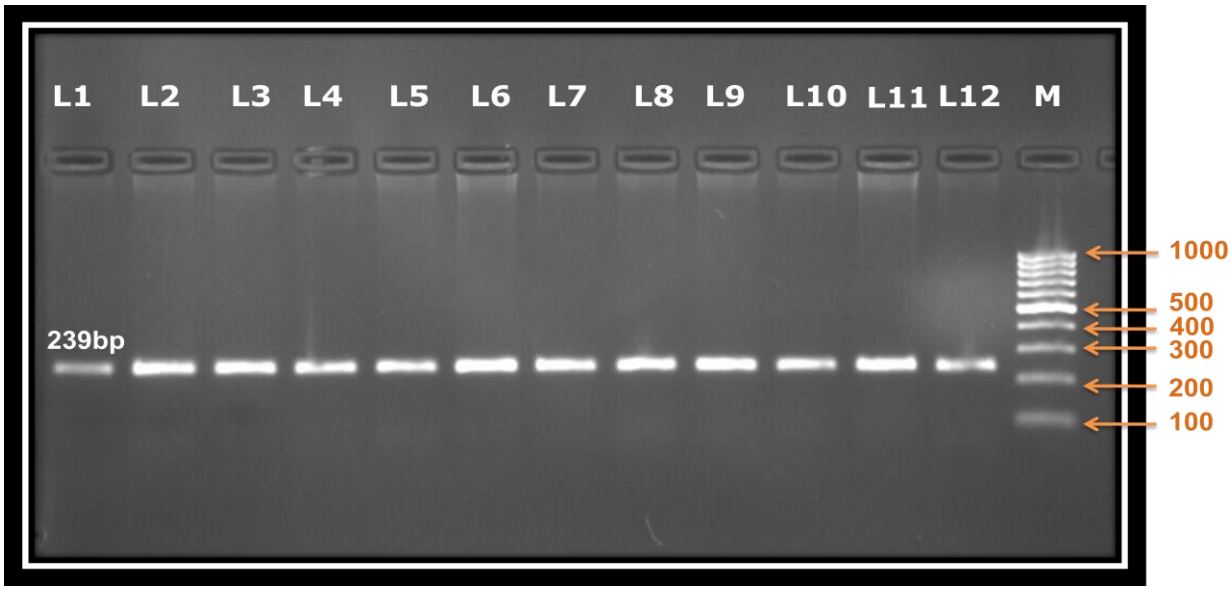
L = Lane Number, **M=**100 bp DNA Marker

Fig 4.5 HaeIII Digested PCR-RFLP Pattern at Putative Exon-2 in IL-8 Gene of Deoni Cattle



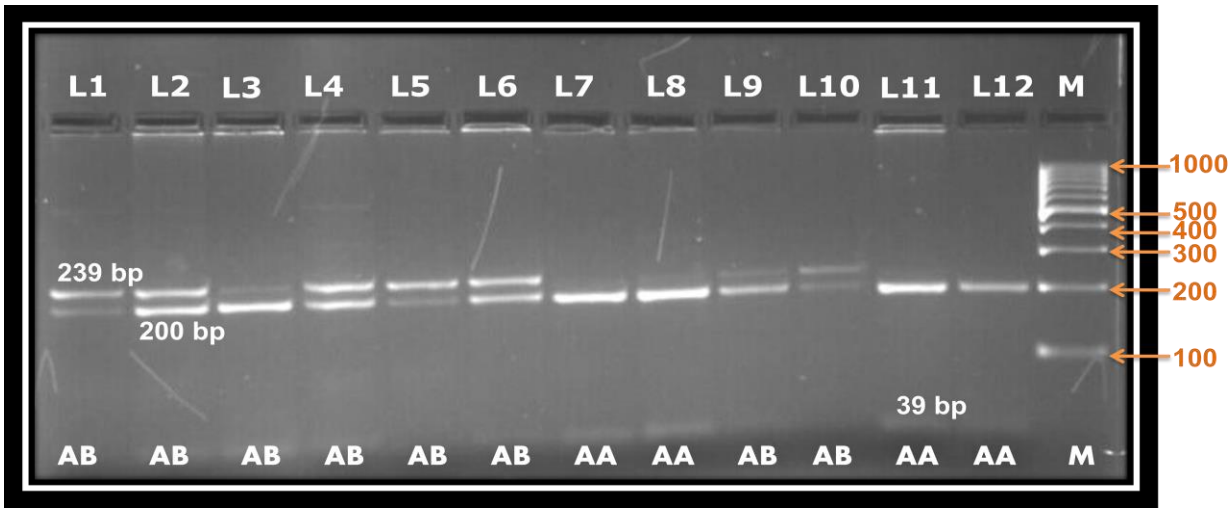
Lane L1-L12 = AA Genotypes, **M =** 100 bp DNA Marker

Fig 4.6 PCR Product of Bovine IL-8 Gene at Putative Exon-3 on 1.5% Agarose Gel



L = Lane number, **M=**100 bp DNA marker

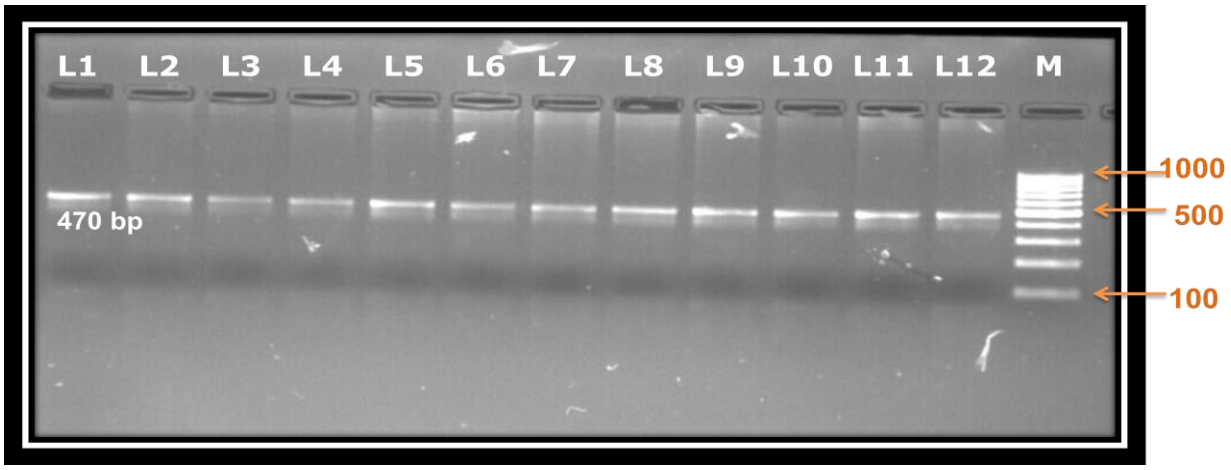
Fig 4.7 SspI Digested PCR-RFLP Pattern at Putative Exon-3 in IL-8 Gene of Deoni Cattle



Lane L1-L6, L9, L10 = AB Genotypes, **M =** 100 bp DNA Marker

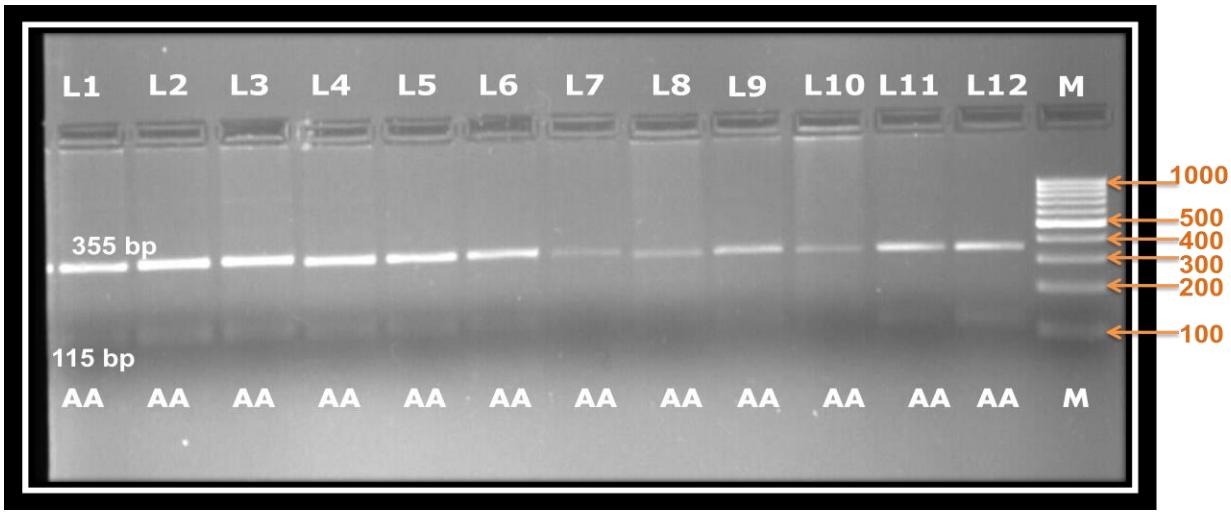
Lane L7, L8, L11, L12 = AA Genotypes

Fig 4.8 PCR Product of Bovine IL-8 Gene at Putative Exon-4, Part-1 on 1.5 % Agarose Gel



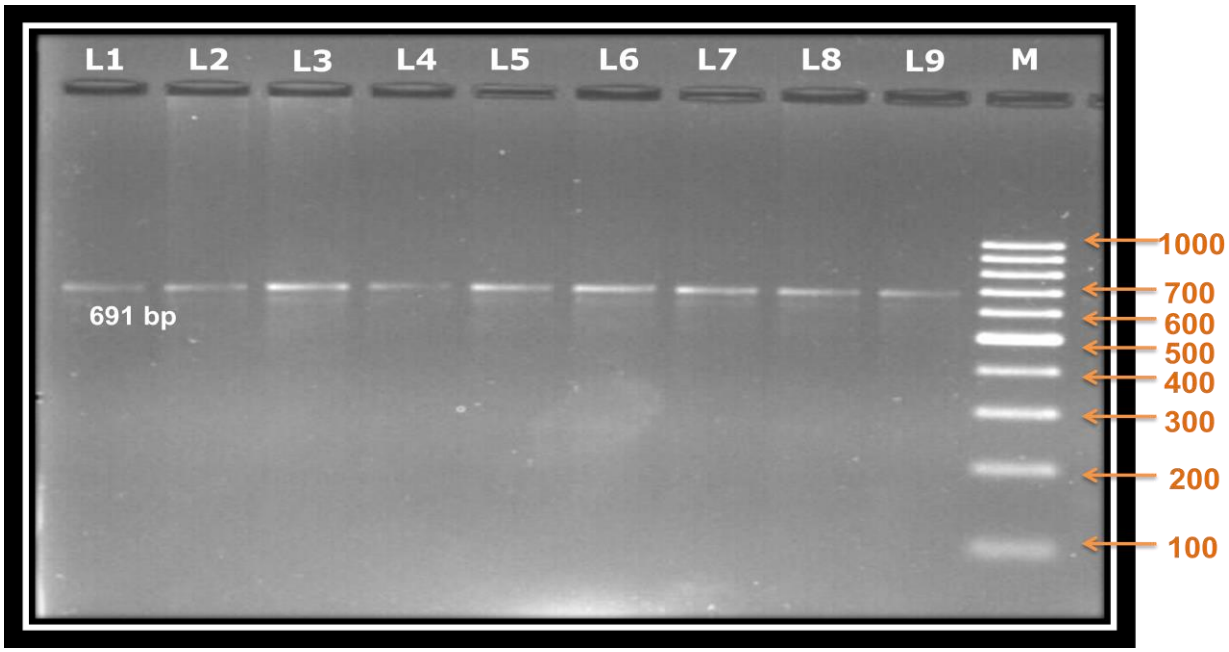
L= Lane number, **M=**100 bp DNA marker

Fig 4.9 HaeIII Digested PCR-RFLP Pattern at Putative Exon-4 Part-1 in IL-8 Gene of Deoni Cattle



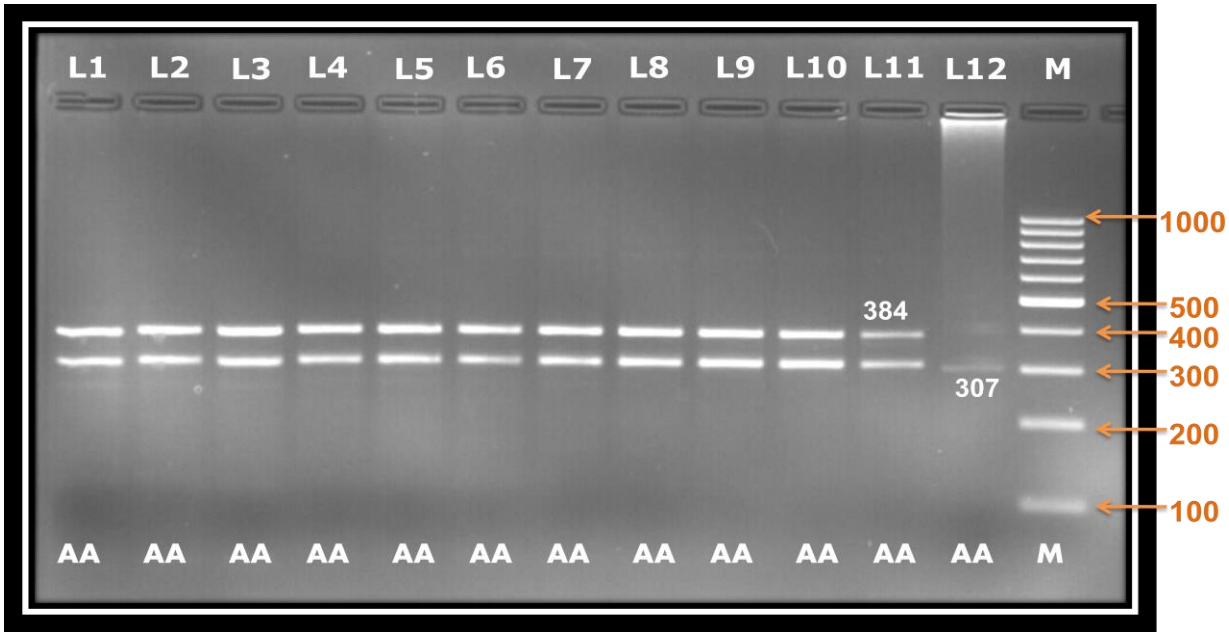
Lane L1-L12 = AA Genotype, **M =** 100 bp DNA Marker

Fig 4.10 PCR Product of Bovine IL-8 Gene at Putative Exon-4, Part-2 on 1.5 % Agarose Gel



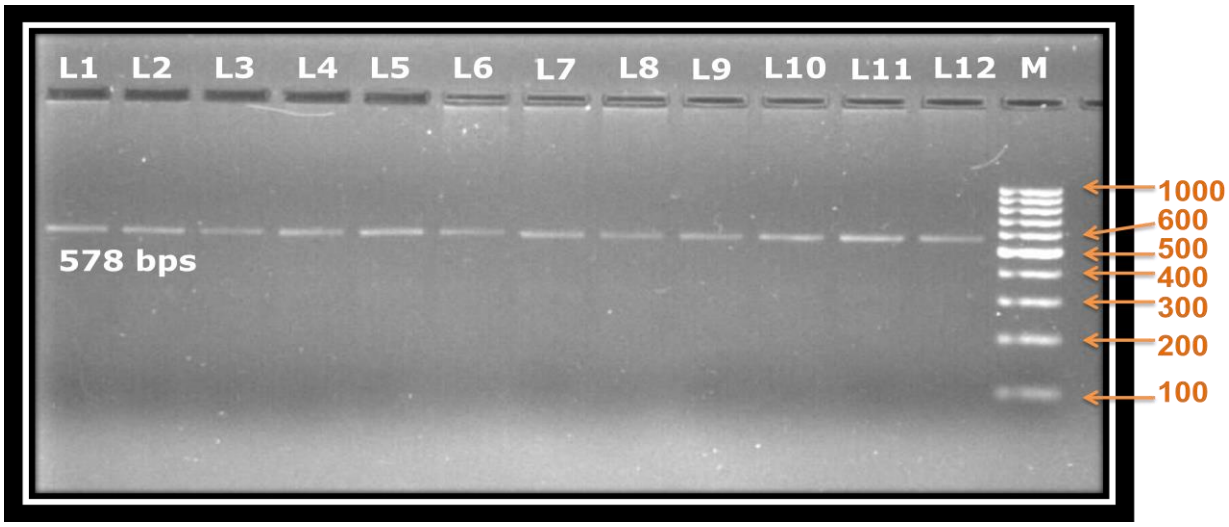
M=100 bp DNA marker, **L=** Lane number

Fig 4.11 HaeIII Digested PCR-RFLP Pattern at Putative Exon-4, Part-2 in IL-8 Gene of Deoni Cattle



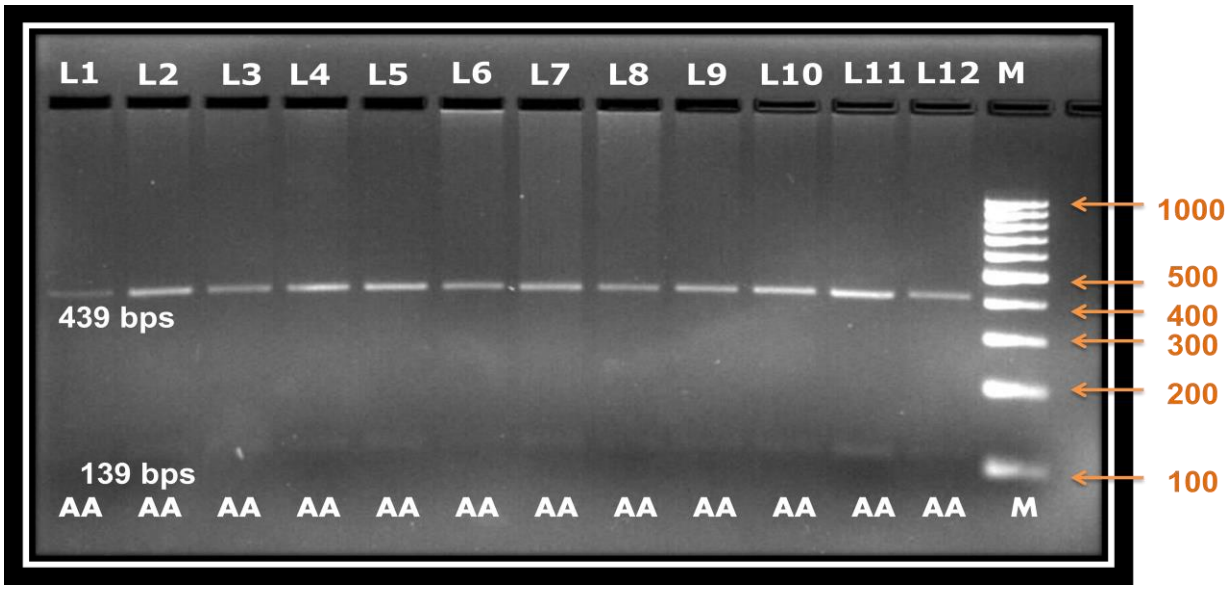
Lane L1-L12 = AA Genotype, **M =** 100 bp DNA Marker

Fig 4.12 PCR product of Bovine IL-8 Gene at putative fragment of Exon-4 on 1.5 % Agarose gel of Deoni Cattle



Lane L1-L12 = AA Genotype, **M =** 100 bp DNA Marker

Fig.4.13 DraI Digested PCR-RFLP Pattern at Putative fragment of Exon-4 in IL-8 Gene of Deoni Cattle



Lane L1-L12 = AA Genotype, M = 100 bp DNA Marker

squares for Hardy-Weinberg equilibrium for the cattle population were performed using population genetics analysis software POPGENE version 1.31 (Yeh *et al.,* 2006).

4.1.5.1 Level of heterozygosity and effective number of allele (n_e) of Deoni cattle

Table 4.3 shows level of observed and expected heterozygosity and effective number of alleles in the Deoni cattle population for IL-8 gene. The distribution of genotypic frequencies in bovine IL-8 gene exon-3 in Deoni population revealed that the observed heterozygosity value was 0.79 which was higher than the expected heterozygosity value 0.48. This clearly indicated that there was prevelance of heterozygosity at higher degree in the studied population. The effective number of alleles (ne) in Deoni breed was found to be 1.915 for AB genotype using population genetics analysis software POPGENE version 1.31 (Yeh *et al.,* 2006).

4.1.5.2 Chi-squares test for Hardy-Weinberg equilibrium

Under the present study, chi-squares test was applied to test whether the genotypes under exon-3 of IL-8 gene in Deoni cows were in accordance to Hardy-Weinberg equilibrium or not. The results for exon-3 of IL-8 gene in Deoni cows presented in Table 4.4. The POPGENE analysis revealed that the estimated Chi-squares value 39.8 for genotype AB of IL-8 gene was highly significant ($p<0.01$). Hence, the population was not consistent with HW equilibrium.

4.1.6 Sequence data analysis for exon-3 of IL-8 gene of Deoni cattle

Representative PCR products in duplicate for homozygote patterns of PCR-RFLP of IL-8 gene were sent for custom sequencing (Applied Biosystem, Amnion, Bangalore). The partial sequence data of the gene was used for analysis by using c:\DNA Baser Assembler\software. For detection of SNP Clustal W multiple alignments were performed with respect to reference sequences of Bos *taurus* cattle.

4.1.6.1 Clustal W multiple alignment analysis

Chromatograph of sequence data of IL-8 gene at putative exon-3 of Deoni cattle are presented in Fig. 4.14 and 4.15. Sequence of size 501 AA and 521 AB genotypes of IL-8 were aligned with reference sequence of Bos *taurus*. Clustal W alignment

analysis showed one single nucleotide at 2738 position (C to A) change with change in amino acid Thrionine (Thr) to Lysine (Lys) in exon-3 region of IL-8 gene in Deoni cattle.

4.1.6.2 Sequence identities of exon-3 of IL-8 gene in Deoni cattle with different species

Sequence of IL-8 gene was subjected to basic local alignment search tool (BLAST) (http://www.ncbi.nih.gov/BLAST/) at NCBI data base to know the sequence identity with the corresponding region in different species. Sequence of 501 (AA) bases (Fig 4.16) of putative exon-3 at IL-8 gene in Deoni cattle exhibited 98 per cent identity similarity with the reference sequence (accession number AY849380.1) of *Bos taurus* cattle. The sequence of nucleotide had 93 per cent identity (accession number KC912524.1) in sheep (*Ovis aries*). The sequence of nucleotide had 98 and 90 per cent identity (accession number FJ595833.1) in *Bubalis bubalis* and *Sus scrofa* (accession number JF906514.1). Sequence of 521 (AB) bases (Fig 4.17) of putative exon-3 at IL-8 gene in Deoni cattle showed identity of 99 per cent with the reference sequence (accession number AY849380.1) of *Bos taurus* cattle. The sequence of nucleotide had 92 per cent identity (accession number KC912524.1) in sheep *(Ovis aries*). The sequence of nucleotide had 97 and 89 per cent identity (accession number FJ595833.1) in *Bubalis bubalis* and *Sus scrofa* (accession number JF906514.1).

4.2 Descriptive statistics of milk production traits and udder health traits

Present study focused on the milk production and udder health traits of Deoni cattle. The mean first lactation milk yield and pooled lactation milk yield was 808.07±45.26 kg and 839.90±26.64 kg respectively with mean first lactation length and pooled lactation length of 213.43±8.38 and 203.91±4.94 days respectively (Table 4.5). Kakde *et al.* (1980) and Das *et al.* (2011) had reported lower FLMY as 798.35±72 and 715.87±32.11 kg, respectively. Estimated first lactation length of the cattle is higher with the previous studies in Deoni (Das *et al.,* 2011) cattle as 189.43±5.03 days. Chakravarthy *et al.* (2002) reported lower estimates of pooled lactation length (149.43±33.52 days) than the present finding in Deoni cattle. Deshpande *et al.* (1977), Singhal *et al.* (1980) and Dhumal *et al.* (1993) reported higher pooled lactation length in Deoni cattle.

Fig. 4.14 Clustal W alignment of IL-8 gene of Deoni with IL-8 of *Bos taurus*

Alignment: Exon 3 of Deoni IL-8 gene with Exon 3 of IL-8 gene of *bos taurus*

```
                .|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
EXON-3          TGTTAAG CTTACCAATG GAAACGAGGT CTGCTTAAAC CCCAAGGAAA
D_14 (AB)       TGTTAAG CTTACCAATG GAAACGAGGT CTGCTTAAAC CCCAAGGAAA
D_16 (AA)       TGTTAAG CTTACCAATG GAAAAGAGGT CTGCTTAAAC CCCAAGGAAA

                ....|....| ....|....| ....|....| ....|..
                        110        120        130
EXON-3          AGTGGGTGCA GAAGGTTGTG CAGGTATTTG TGAAGAG
D_14 (AB)       AGTGGGTGCA GAAGGTTGTG CAGGTATTTG TGAAGAG
D_16 (AA)       AGTGGGTGCA GAAGGTTGTG CAGGTATTTG TGAAGAG
```

Fig. 4.15 Chromatogram of sequences of Exon 3 of IL-8 gene showing variation(C to A) in AA genotype

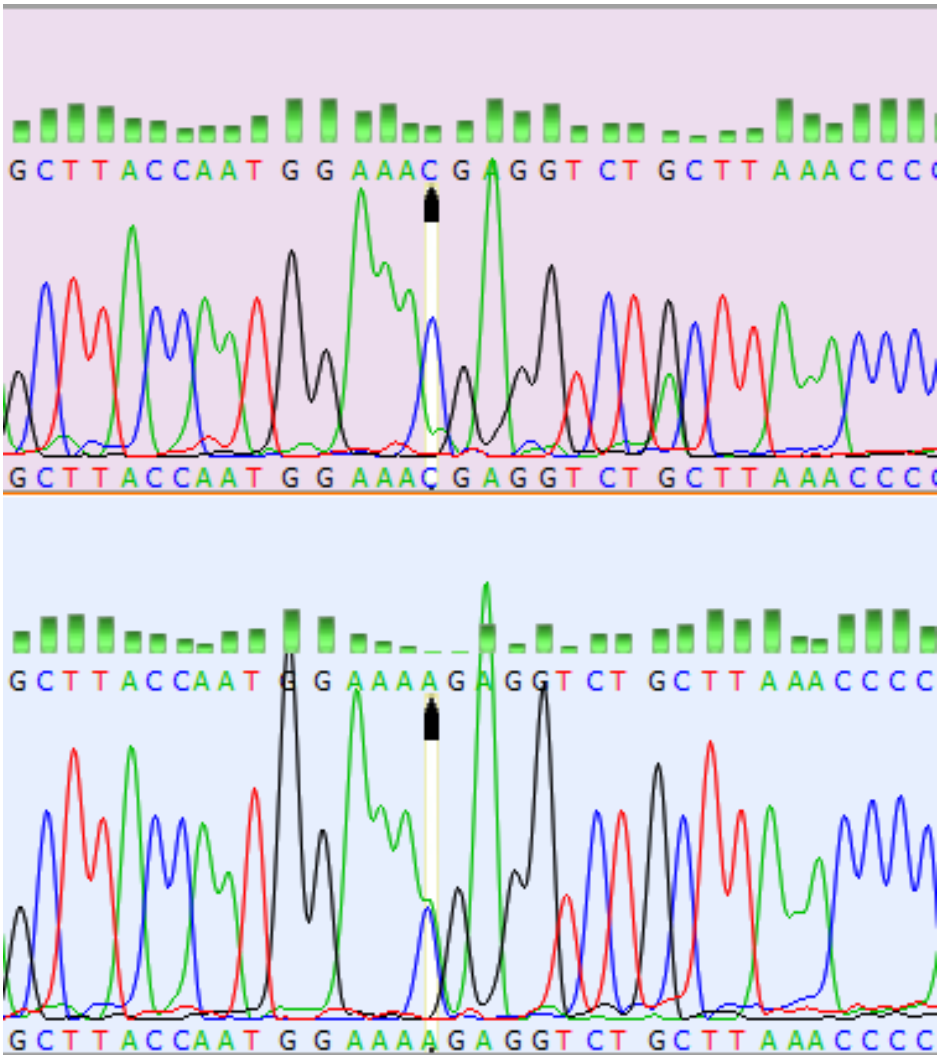


Fig 4.16 Nucleotide sequence of IL-8 gene at putative exon3 of Deoni cattle (AA)

CGAGGGTTATAAGGAGCATCCTGGCTTTATCATATCTCTCTCATTTCAGTGTTAAGCTTACC
AATGGAAAAGAGGTCTGCTTAAACCCCAAGGAAAAGTGGGTGCAGAAGGTTGTGCAGGTA
TTTGTGAAGAGGTAAGTTTTTCTTGAATTTATATTCTTCATTTATCCTGTGACATTTACTCCA
AAAGTGAGCCTTTACATTTTCTGCTGCTGCTGAAAAAAATGTAAAGGCTCCTTTTGGAGTAA
ATGTCCAGGATAAATGAAAAATATAAATTCAGAAAAACTTTCCTCTTCCCAATACTGCCAAC
TTTTGACCCCTTTTTCCTGGGGGTTTGGCAAACCCTTTTCCTTGGGAATTTAACCGGAAATA
AAAAAAAATTTTTTAAACCTTTTTTGGGGGCTAAAAACCCTGCCTTTAGGAAAAGGGTCTTC
TTTGTTTAGCCGTTGTCTGTTTTCCTGCCGGGCTTTTGGGGGGGGGGGGGGGAGCCCCCC
CCCAAGAG

Red marking indicated Exon 3 of bovine IL-8 gene

Fig 4.17 Nucleotide sequence of IL-8 gene at putative exon3 of Deoni cattle (AB)

CTAAAAGTAAACCTGCTAGGTGTCTCTGCTTTATCATATCTCTCTCATTTCAGTGTTAAGCTT
ACCAATGGAAACGAGGTCTGCTTAAACCCCAAGGAAAAGTGGGTGCAGAAGGTTGTGCAG
GTATTTGTGAAGAGGTAAGTTTTTCTTGAATTTATGTTCTTCATTTATCCTGTGACATTTACT
CCAAAAGTCAGCCTTTACATTTTCTGCTGCTGCTGAAAAAAATGTAAAGGCTGACTTTTGGA
GTAAATGTCCAGGATAAATGAAGAAATAAATTCCGGAAAAAATTACCTCTTCCAAATACTGC
CAACTTTTGCCCCCTTTTTCTTGGGGGTTAAAAAAACTCCTTTCCTTGGGAAGCTAAAATGA
AATAGAAAAAAATTGATTAAACGTATTTTTTGGGTTTTAAAAACCTTGGCATTTAGAAAAATT
GGTCCCCCCCTGGCCCTGGGCGGCGTGACGGTGCGGGGGGGGGGAGCCCCCCCCCCCT
AGTCGTGGGGGGACGCGCGGGGGCCGAT

Red marking indicated Exon 3 of bovine IL-8 gene

Table 4.5 Descriptive statistics of LL and LMY of Deoni cattle

Traits	Mean ± SE	SD	Minimum	Maximum	CV (%)
FLMY	807.24±45.17 (95)	440.35	72	1856.5	54.5
FLL	213.93±8.40 (95)	81.90	37	381	38.28
Pooled LMY	839.90±26.64 (214)	387.65	38.5	1856.5	46.16
Pooled LL	203.91±4.94 (214)	71.43	28	381	35.03

Parentheses indicated (N) number of observation under each trait

SE= Standard Error, SD= Standard Diveation, CV= Coefficient of Variance (%), FLL= First Lactaction Length (Days), FLMY= First Lactation Milk Yield (kg)

Table 4.6 Descriptive statistics of SCC, FL, FR, HL, HR and Pooled EC of Deoni cattle

Traits	Mean ± SE (N)	SD	Minimum	Maximum	CV (%)
SCC (million/ml)	0.463±0.08 (54)	0.67	0.05	3	**153.08**
FL (units)	423.15±8.03 (54)	59.04	290	570	**13.95**
FR (units)	423.88±7.15 (54)	52.50	320	610	**12.38**
HL (units)	425±7.73 (54)	56.79	320	590	**13.36**
HR (units)	416±7.78 (54)	57.16	300	520	**13.74**
Pooled EC (units)	422.23±6.82 (54)	50.11	315	555	**11.87**

Parentheses indicated (N) number of observation under each trait

SE= Standard error, SD= Standard diveation, CV= Coefficient of variance (%), SCC= Somatic cell count, FL= Front left quarter, FR= Front right quarter, HL= Hind left quarter, HR= Hind right quarter, Pooled EC=Pooled electrical conductivity

Table 4.7 Analysis of variance on lactation length (LL) and lactation milk yield (LMY) for genotype

	LL			LMY		
Source	**DF**	**MSS**	**Pr>F**	**DF**	**MSS**	**Pr>F**
Genotype	1	63306.67	0.0017	1	1196987	0.0104
Error	93	6064.38		93	174407.69	

Table 4.8 Analysis of variance on lactation length (LL) and lactation milk yield (LMY) for parity

	LL			LMY		
Source	**DF**	**MSS**	**Pr>F**	**DF**	**MSS**	**Pr>F**
Parity	5	10794.096	0.0643	5	223034.12	0.1952
Error	208	5103.12		208	150278.53	

The means of SCC and electrical conductivity of front left (FL) quarter, front right (FR) quarter, hind left (HL) quarter, hind right (HR) quarter and pooled samples were averaged for 54 Deoni cattle and are shown in Table 4.6. The SCC estimated by using Portacheck method was 0.408±0.078 million cells/ml in milk of Deoni cattle, which is higher than the report of Rangarajan *et al.* (2012) estimated the SCC mean was 1.325±0.060 lakh cells/ ml in milk of Deoni cattle.

The Electrical Conductivity of FL, FR, HL, HR and pooled samples were 423.15±8.035, 423.15±7.144, 425±7.73, 416.85±7.78 and 422.22±7.0 units respectively in milk of Deoni cattle.

4.3 Effect of genetic and non-genetic factors on milk production traits

The influence of genetic and non-genetic factors viz. genotype, parity and stage of lactation on lactation length (LL) and lactation milk yield (LMY) observed in Deoni cattle are presented in Table 4.8 and 4.9 respectively.

4.3.1 Effect of genotype on lactation length (LL) and lactation milk yield (LMY)

The ANOVA test reveled significant differences in LL and LMY ($p<0.05$) with respect to various genotypes. Least squares means of the AA and AB genotypes were 982.25±119.04 and 806.83±69.67 on LMY respectively and 262.53±22.19 and 208.63±12.99 are least square mean of AA and AB genotypes respectively for LL under present study are presented in Table 4.7. No previous report was available on the effect of IL-8 genotype on LL and LMY in Deoni cattle.

4.3.2 Effect of parity on lactation length (LL) and lactation milk yield (LMY)

The overall mean of LMY is 839.901 kg and LL is 203.911 days. Least squares means of the different parities on LMY and LL under present study are presented in Table 4.8. In Deoni cattle, lower LL was observed during first parity and upward trend from third parity was observed in subsequent parities. The ANOVA test reveled non significant in LMY and LL with respect to various parity.

4.4 Effect of genetic and non-genetic factors on udder health traits

4.4.1 Effect of genotype, parity and stage of lactation on somatic cell count (SCC)

The least squares mean of SCC for AA and AB genotypes in Deoni cows were 0.39±0.20 and 0.50±0.13 million cells/ml respectively. The ANOVA revealed non-significant effect between different genotypes of IL-8 gene over somatic cell count in Deoni population (Table 4.10). In Deoni cattle, higher somatic cell count was observed in third and fourth parity than the other parities (Table 4.12), similar to present study, Shekhar (2012) obtained that the SCC was higher in fourth and sixth parity than the earlier parities but there was no significant effect of parity on SCC.

The SCC was not found significant over stages of lactation in Deoni cattle (Table 4.12). On the contrary, Shekhar (2012) reported that 0.34±0.05, 0.51±0.08, 0.79±0.17 million cells/ ml in I, II and III stage of lactation respectively in Deoni cattle. In the present study, downwards trend of SCC was observed with the advancement of stage of lactation. However, Sender *et al.* (1987), Komisarek *et al.* (2006) and Rangarajan (2012) reported an upward trend of SCC was observed with advancement of stage of lactation. The non significant effect of genotype, parity and stage of lactation on SCC might be due to less number of observation.

4.4.2 Effect of genotype, parity and stage of lactation on electrical conductivity (EC)

Effect of genotype, parity and stage of lactation revealed non-significant on electrical conductivity of front left quarter (FL), front right quarter (FR), hind left quarter (HL), hind right quarter (HR) and pooled milk sample EC in milk of Deoni population (Table 4.11). No report is available on the effect of electrical conductivity to compare or contrast the present study in Deoni.

4.5 Relative expression study on Interleukin-8 gene in milk of Deoni cows

Relative expression study on interleukin-8 gene between healthy and subclinical mastitis (SCM) groups of Deoni cattle, classified on the basis of somatic cell count obtained from Nucleocounter SCC100 (Fig. 4.18) at NIVEDI, Hebbal, Bangalore. Out of total 16 animals, there were 8 animals in each group of Deoni cows. The reference gene GAPDH gene of bovine was considered for relative quantification of IL-8 gene. The mean of SCC of IL-8 gene in SCM group is 10.73±3.17 lakhs cells/ml with the range from 171000 to 2000000 and in healthy group the overall mean is 0.414±0.102 lakhs cells/ml (Fig. 4.18) with the range from 10000 to 810000. The

Table 4.9 Least Square means of genotype and parity on lactation length (LL) and lactation milk yield (LMY)

	LL	LMY
	LSM±SE	LSM±SE
	n=95	n=95
GENOTYPE	***	***
AA	262.53±22.19	982.25±119.04
AB	208.63±12.99	806.83±69.67
PARITY	**NS**	**NS**
1 (n=95)	213.94±7.33	807.24±39.77
2 (n=38)	196.79±11.59	830.12±62.89
3 (n=30)	177.40±13.04	800.28±70.78
4 (n=22)	193.32±15.23	852.10±82.65
5 (n=16)	195.50±17.86	900.72±96.91
>6 (n=13)	240.92±19.81	1103.11±107.52

Table 4.10 Analysis of variance of genotype, parity and stage of lactation on SCC

Source	DF	SS	MSS	F Value	Pr>F
Genotype	1	0.087	0.087	0.173	0.68
Parity	6	0.99	0.165	0.329	0.92
Lactation	2	0.08	0.042	0.083	0.92
Error	44	22.08	0.502		
Total	**53**	**23.24**			

Table 4.11 Analysis of variance for genotype, parity and stage of lactation on FL, FR, HL, HR & pooled EC

	FL			FR			HL			HR			Pooled EC		
SOURCE	**DF**	**MSS**	Pr>F	**DF**	**MSS**	Pr>F	**DF**	**MSS**	Pr>F	**DF**	**MSS**	Pr>F	**DF**	**MSS**	Pr>F
Genotype	1	4408.33	0.28	1	5208.33	0.19	1	5489.81	0.17	1	59.26	0.89	1	2625.52	0.31
Parity	6	2118.36	0.76	6	1362.99	0.83	6	3193.94	0.37	6	3049.16	0.44	6	1765.94	0.65
Lactation	2	1722.62	0.63	2	1762.19	0.55	2	8739.73	0.06	2	10210.48	0.05	2	4096.25	0.21
Error	44	3750.82		44	2933.85		44	2904.76		44	3095.72		44	2538.51	

Table 4.12 Least square means of genotype, parity and stage of lactation on SCC, FL, FR, HL, HR and pooled EC.

	SCC	FL	FR	HL	HR	pooled EC
	LSM±SE	LSM±SE	LSM±SE	LSM±SE	LSM±SE	LSM±SE
	n=54	n=54	n=54	n=54	n=54	n=54
Genotype	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**
AA (18)	0.39 ±0.20	442.55±17.75	444.31±15.70	447.25±15.62	425.54±16.12	439.91±14.60
AB (36)	0.50±0.13	415.40±11.98	409.61±10.60	412.09±10.55	412.43±10.89	412.38± 9.86
Parity	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**
1 (19)	0.55±0.18	427.32±15.75	438.23±13.93	426.95±13.86	424.46±14.31	429.24±12.96
2 (7)	0.28±0.26	397.56±23.26	400.47±20.57	388.79±20.47	385.07±21.13	392.97±19.14
3 (4)	0.72±0.39	463.63±33.41	451.90±29.55	469.55±29.40	460.52±30.36	461.40±27.49
4 (9)	0.52±0.24	416.28±20.59	424.95±18.21	448.54±18.12	427.53±18.71	429.32±16.94
5 (9)	0.24±0.25	438.77±21.44	417.52±18.96	414.20±18.87	391.97±19.48	415.60±17.64
6 (3)	0.26±0.42	425.51±36.28	408.19±32.09	405.62±31.93	412.58±32.96	412.98±29.85
7 (3)	0.52±0.47	433.82±40.47	447.45±35.79	454.08±35.61	430.75±36.76	441.52±33.29
Stage of lactation	**NS**	**NS**	**NS**	**NS**	**NS**	**NS**
1 (14)	0.51±0.20	415.39±17.65	413.53±15.61	399.84±15.54	398.12±16.04	406.72±14.52
2 (21)	0.43±0.18	432.45±15.53	436.29±13.73	450.56±13.66	446.92±14.10	441.55±12.77
3 (19)	0.39±0.21	439.08±18.35	431.07±16.23	438.63±16.14	411.91±16.67	430.17±15.09

purity of isolated mRNA from milk samples of Deoni cattle was checked by Nanodrop2000/2000C having mean 4.22±0.37 ng/µl with a range 3.1 to 9.2. The mean of optical density of mRNA obtained as 1.84±0.08 at A260/280 with the range from 1.57 to 3.03. The synthesized cDNA from the isolated mRNA of each sample through reverse transcriptase PCR and the purity was checked by Nanodrop2000/2000C. The mean of cDNA obtained 2298.49±28.42 ng/µl with the range of 2151.3 to 2583.3. The mean optical density of cDNA at A260/280 was 1.64±0.002 with the range from 1.62 to 1.65.

Real time PCR (relative quantification method) was used for the relative expression of IL-8 gene with the reference of GAPDH by the taqman® assay. The real time PCR plates were sealed on the edges and inserted into the light cycler 480 after loading all samples in separate wells. The cp value and fold changes were determined using Light Cycler 480 Software 1.5.0.SP3. The mean of cp value (Fig.4.19) for target (IL-8) and reference gene (GAPDH) was 29.32±0.90 and 28.57±0.70 respectively with the range from 22.77 to 35.48 and 24.47 to 32.55 respectively. The fold change in IL-8 gene was higher in cattle with SCM than healthy group (Fig. 4.20).

The result of relative expression study showed significantly higher expression of IL-8 gene in sub clinical mastitis affected group of cattle and lower in the healthy group. Bhatt *et al. (*2012) also reported that, mastitis affected milk of Kankrej, Gir and crossbred showed comparatively higher level of IL-8 gene in milk of crossbred than the indigenous breeds. Peli *et al.* (2004), obtained that, the expression level of IL-8 gene was higher in mastitis group than non-mastitis group in Italian Friesian dairy cows at mid lactation. Similarly, McClenahan *et al.* (2006) revealed similar kind of result in experimentally induced mastitis infection with E-coli by in-situ hybridization.

Hence, expression of IL-8 gene in SCM group was higher than the healthy group of Deoni cattle probably due to more invasion of micro-organisms causing inflammation at the site of infection.

Fig. 4.18 Graphical presentation of comparative somatic cell count in milk of subclinical mastitis and healthy group of Deoni cattle

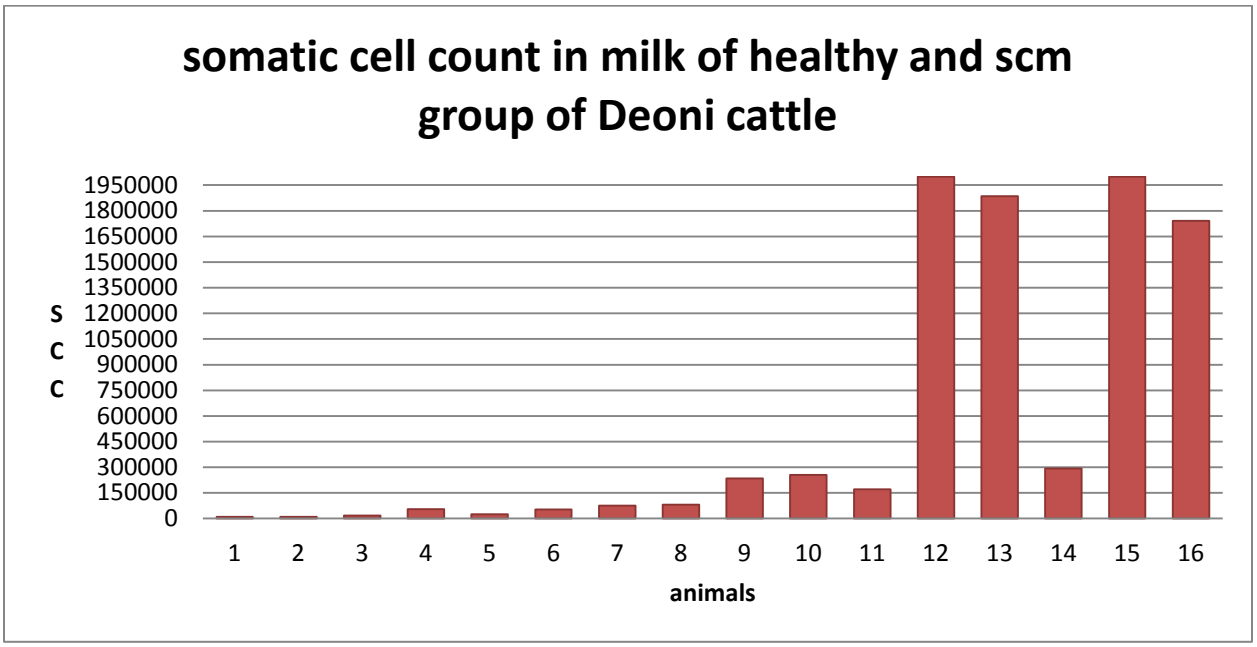


Fig. 4.19 Graphical presentation of cp value of IL-8 gene and GAPDH gene in milk of subclinical mastitis (SCM) and healthy group of Deoni cattle

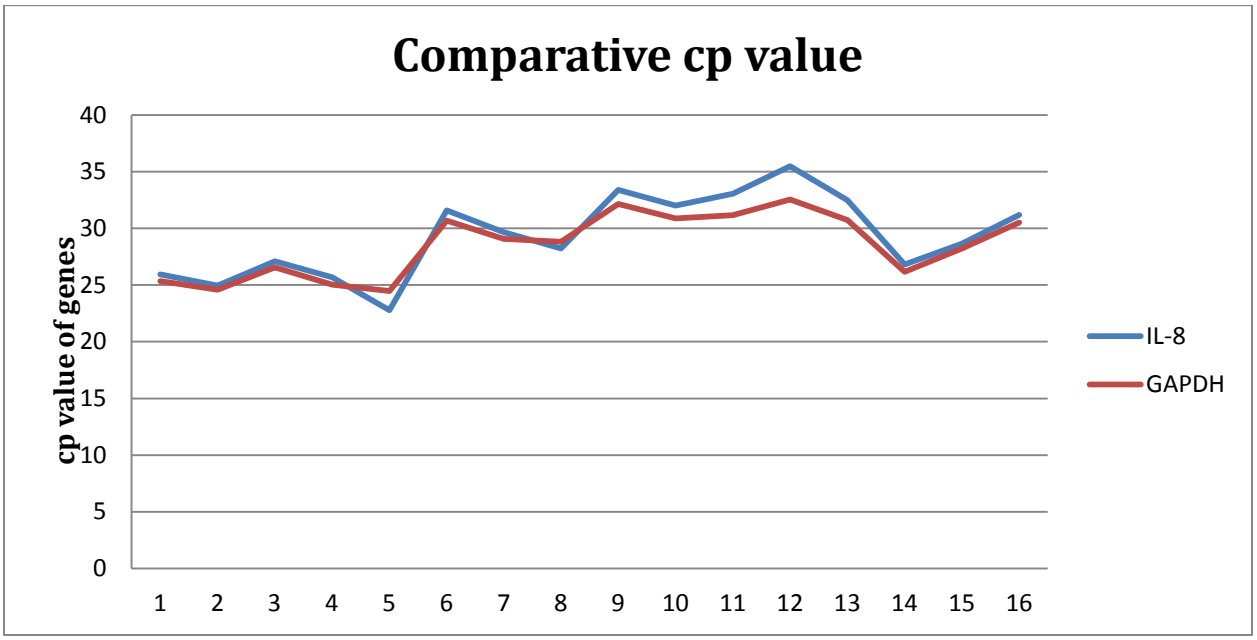
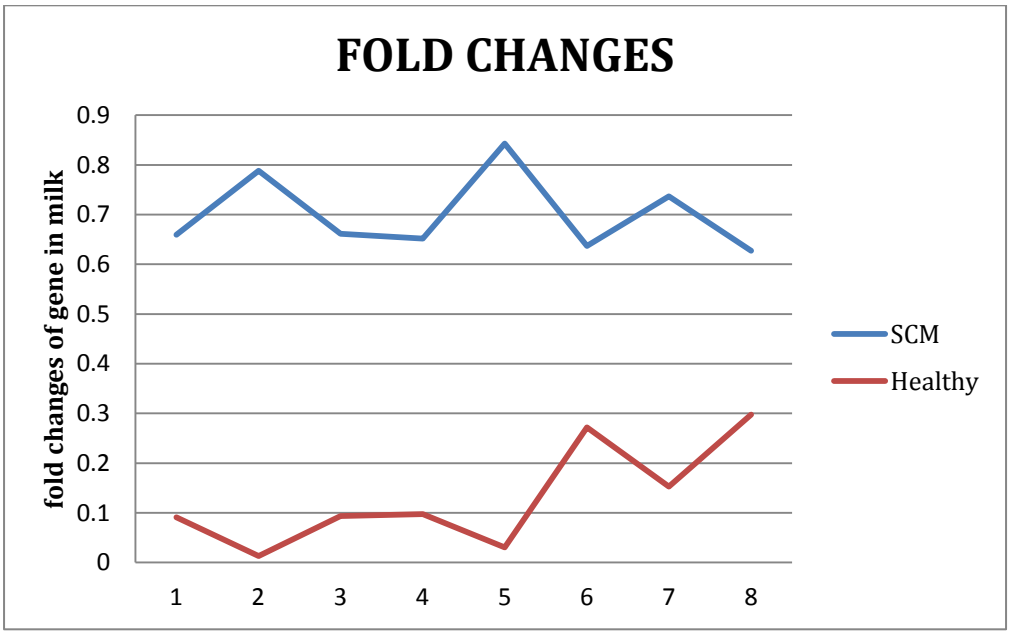


Fig. 4.20 Graphical presentation of relative quantification of IL-8 gene in milk of subclinical mastitis (SCM) and healthy group of Deoni cattle



Chapter - 5

Summary & Conclusion

SUMMARY AND CONCLUSION

The study was undertaken on Deoni, the medium sized dual purpose indigenous cattle breed of southern India, maintained at southern campus of NDRI, Bangalore and livestock farm of Prabhani, MAFSU, Maharashtra with the following objectives:

- Molecular characterization of the coding regions of IL-8 gene in Deoni cattle
- Investigation on the association of genetic variability in IL-8 gene with milk production and udder health traits in Deoni cattle

Molecular characterization of interleukin-8 gene was performed through PCR-RFLP technique in 95 DNA samples. The DNA samples were extracted through High Salt method and quality and quantity were determined by UV spectrophotometer. Primers designed from primer3 tool software were used for PCR amplification at putative exon 1-4.2 of IL-8 gene. The amplicons of putative exon1 of IL-8 gene was digested with HPYCH4V, exon2, 4.1 and 4.2 digested with HaeIII and exon3 digested with SspI restriction enzyme. Gel electrophoresis of the digested products was performed with 2-2.5 per cent agarose and visualization was done through gel documentation system. Two polymorphic band patterns (Fig. 4.5), namely AA and AB, were observed for the digested product of IL-8 gene putative exon 3. Frequencies of AA and AB genotypes were observed as 0.21 and 0.79, respectively with the allelic frequencies of 0.61 and 0.39 for A and B types. POPGENE (version 1.31) software was used to analyse the polymorphic data. Higher degree of heterozygosity was observed for the AB genotype for IL-8 gene than the expected one and chi squares test revealed that the population was not in H-W equilibrium for the same. Sequence data of the amplicons were analysed through DNA baser software where Clustal W analyses revealed one SNPs at contig sizes of 501 bases at 2738 nucleotide C to A with threonine to Lysine amino acid change of IL-8 gene putative exon 3.

Expression study was carried out in 16 animals of Deoni cattle divided into two groups viz. healthy and SCM, on the basis of SCC of milk. Somatic cell extracted from milk of each animal and isolated the mRNA from RNeasy Mini Kit (Qiagen). The purity of mRNA checked by Nanodrop2000/2000C ranging from 3.1 to 9.2 with mean 4.22±0.37 ng/µl and the optical density of mRNA at 260/280 observed 1.84±0.08. Then, cDNA synthesized from mRNA for IL-8 gene and GAPDH gene by reverse

transcriptase PCR and checked the purity of cDNA by Nanodrop2000/2000C ranging from 2151.3 to 2583.3 with the mean of 2298.5±28.42 ng/µl. The optical density of cDNA ranged from 1.62 to 1.65 with mean 1.64±0.002 at 260/280. On the real time PCR showed significantly higher fold change of IL-8 gene in SCM group while lower in healthy group.

Mean of FLMY, FLL, pooled LMY and pooled LL milk production trait of Deoni cattle was estimated as 807.24±45.17 kg, 213±8.40 days, 839.90±26.64 kg and 203.91±4.94 days respectively. Mean of udder health trait of Deoni cattle was estimated as 0.463±0.08 million cells/ml, 423.15±8.03, 423.88±7.15, 425±7.73, 416±7.78 and 422.23±6.82 for the trait of SCC, Electrical Conductivity of FL, FR, HL, HR quarters and pooled EC respectively. Effect of different genotype on LMY, LL and effect of genotypes along with non-genetic factors on SCC and EC were analysed through least squares method using GLM procedure of SAS software (version 9.2). Significant effect of genotypes was found with LMY and LL.

Association studies of the genotypes were performed with unadjusted, adjusted LL, LMY, SCC, FL, FR, HL, HR and pooled EC. Significant ($p<0.05$) association of the genotypes at putative EXON3 of IL-8 was observed with the data of LL and LMY. Based on the present study carried out the conclusions mentioned as follows:

1. The amplified fragments of putative Exon 3 in IL-8 gene of Deoni cattle exhibited DNA polymorphisms using PCR-RFLP method.
2. Allelic frequency of A allele (0.61) were higher than B alleles (0.39) at putative Exon 3 of IL-8 gene in studied population.
3. Clustal W alignment analysis showed one single nucleotide at 2738 position (C to A) change with change in amino acid Thrionine (Thr) to Lysine (Lys) in Exon 3 region of IL-8 gene in Deoni cattle.
4. Chi-squares test revealed the population was not in H-W equilibrium with respect to AB genotypes of IL-8 gene.
5. Mean lactation length and lactation milk yield was observed as 203.91±4.94 days and 839.90±26.64 kg respectively in Deoni cattle.
6. Mean SCC and electrical conductivity of FL, FR, HL, HR quarters and pooled EC was 0.463±0.08 million cells/ml, 423.15±8.03, 423.88±7.15, 425±7.73, 416±7.78 and 422.23±6.82 units respectively in Deoni cattle.

7. LL and LMY showed significant ($p<0.05$) association with genotypes at putative exon3.
8. Expression study of IL-8 gene carried out in milk of (n=16) Deoni cows by using real time PCR technique. Fold change of IL-8 gene was significantly (0.70±0.028) higher in milk of SCM and lower (0.13±0.03) expression was obtained in healthy milk of Deoni cattle.

Chapter - 6

Bibliography

Arici, A. Oral, E. Bukulmez, O. Buradagunta, S. Engin, O. and Olive, D. L. 1996. Interleukin-8 expression and modulation in human preovulatory follicles and ovarian cells. *Endocrinology,* **137 (**9): 3762-3769.

Atakan, K. O. C. 2008. A study of somatic cell counts in the milk of Holstein- Friesian cows managed in Mediterranean climatic conditions. *Turkish. J. Vet. Ani. Sci*., **32** (1): 13-18.

Baggioloni, M., Walz, A., and Kunkel, S. 1989. Neutrophil Activating Peptide/ Interlukin-8, a novel cytokine that activates neutrophils. *J. Clin. Invest.* **84**: 1045-1049.

Bannerman, D.D. Paape, M. J. Hare, W. R. and Hope, J. C. 2004. Characterization of the bovine innate immune response to intramammary infection with Klebsiella *pneumoniae*. *Journal of Dairy Science.* **87**: 2420–32.

Barber, M. R. and Yang, T. J. 1998. Chemotactic activities in non-mastitic and mastitic mammary secretion: presence of interleukin-8 in mastitic but not non-mastitic secretions. *Clin. Diagn. Lab. Immunol*. **5**: 82-86.

Barkema, H. W. Schukken, Y. H. Lam, T. J. G .M. Beiboer, M. L. Wilmink, H. Benedictus, G. and Brand. A. 1998. Incidence of clinical mastitis in dairy herds in three bulk milk somatic cell count cohorts. *J. Dairy Sci.* **81**:411–419.

Baugh, L. R. Hill, A. A. Brown, E. L. and Hunter C. P. 2001. Quantitative analysis of mRNA amplification by *in vitro* transcription*. Nucleic Acids Res.* **29.**

Beckmannn, J. S and Soller, M. 1987. Molecular markers in genetic improvement of farm animals. *Biotechnology.* **5**: 573-576.

Beecher, C. Daly, M. Childs, S. Berry, D. P. Magee, D. A. McCarthy, T. V. Giblin, L. 2010. Polymorphisms in bovine immune genes and their associations with somatic cell count and milk production in dairy cattle. *BMC Genet*. **11**: 99.

Belayet, H. M. Kanayama, N. Khatun, S. Asahina, T. Okada, Y. Kitamura, K. Kobayashi, T. and Terao, T. 2000. Pharmacologic doses of interleukin 8 suppositories induce follicular maturation in rabbits. *Cytokine*. **12** (4): 361-367.

Bhatt, V. B. Khade, P.S. Tarate, S. B. Tripathi, A. K. Nauriyal, D. S. Rank, D. N. Kunjadia, A. P. Joshi, C. G. 2012. Cytokine expression pattern in milk somatic cells of sub-clinical mastitis-affected cattle analyzed by real time PCR. *Korean J Vet Res*. **52**(4); 231-238.

Bhattacharya, S. S. Wright, A. F. Clayton, J. F. Price, W. H. Phillips, C. I. Mckeown, C. M.M. E. Jay, M. Bird, A. C. Pearson, P.L. Southern, E. M. and Evans, H. J. 1984. Close genetic linkage between X-linked retinitis Pigmentosa and a restriction fragment length polymorphism identified by recombinant DNA probe LI-28, *Nature*. **309**: 253-255.

Botstein, D. White, R. L. Skolnick, M. and Dabis, R. W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphism. *Am. J. Hum. Genet*. **32**:135-331.

Boudjellab, N. Chan-Tang, H. S. Li, X. and Zhao, X. 1998. Interleukin-8 responses by bovine mammary epithelial cells to Lipopolysaccharide stimulation. *Am. J. Vet. Res.* **59**: 1563-1567.

Bustin, S. A. 2002. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology*. **25**: 169-193.

Bustin, S. A., Gyselman, V. G., Siddiqi, S. and Dorudi, S. 2000. Cytokeratin 20 is not a tissue-specific marker for the detection of malignant epithelial cells in the blood of colorectal cancer patients. *International Journal Surgical Investigation.* **2**: 49–57.

Caswell, J. L. Middleton, D. M. M. Sorden, S. D. and Gordon, J. R. 1999. Production and functional characterization of recombinant bovine Interleukin-8 as a specific neutrophil activator and chemoattractant. *Vet. Immunopathol*. **67**: 327-340.

Chakravarthy, K. Bidarkar, D. K. Gupta, B. R. Rao, G. N. and Sudhakar, K. 2002. Production and reproduction traits of Ongole cattle. *Indian J. Anim. Sci*. **72** (10): 908-910.

Chakravarthy, M. K. Sasidhar, P.V. and Reddy, Y. R. 2002. Productive and reproductive performances of Deoni cattle. *Indian J. Dairy Sci.* **55** (1): 56-57.

Chang, R. J. Gougeon, A. and Erickson, G. F. 1998. Evidence for a neutrophil-Interleukin-8 system in human folliculogenesis. *Am. J. Obstet. Gynecol*. **178** (4): 650-657.

Clore, G. M. Appella, E. Yamada, M. Matsushima, K. and Gronenborn, A. M. 1990. Three-dimensional structure of Interleukin 8 in solution. *Biochemistry*. **29** Pp.1689-1696.

Das, D. N., Kataktalware, M. A., Ramesha, K. P. and Reddy, A. O. 2011. Productive and reproductive performances of Deoni cattle under intensive management system. *Indian Journal of Animal Science.* **81**(11): 1186-1188.

Das, D. N., Ramesha, K.P., Kataktalware, M. A. and Varalakshmi, S. 2012. Final report of the research project entitled "Bovine sub clinical mastitis in crossbred dairy cattle, early diagnosis and control for enhancement of milk production under field conditions: an integrated approach" funded by NABARD.

Deshpande, K. S. and Singh, B. P. 1977a. Genetic studies on Deoni cattle III. Lactation period and dry period*. Indian Vet. J.* **54** (9): 814-817.

Deshpande, K. S. and Singh, B. P. 1977b. Genetic studies on Deoni cattle. II, lactation milk yield, peak yield and days taken to reach peak yield. *Indian Veterinary Journal.* **54**: 727-31.

Detmers, P. A. Powell, D. D. Walz, A., Clark-Lewis, I. Baggionlini, M. and Cohn, Z. A. 1991. Differential effects of neutrophil-activating peptide/IL-8 and its homologues on leukocyte adhesion and phagocytosis*. J. Immunol*. **147**: 4211-4217.

Dhumal, M. V. Butte, S. V. and Haytnagarkar, D. D.1993. Non-genetic factors affecting lactation milk yield and lactation length of Deoni cattle. *Indian J. Anim. Prod. & Mgt.* **9** (2-3):135-137.

Dufour, S. and Scholl, D. 2010. Improving udder health on your farm best management practices. *Canadian Bovine Mastitis Research Network*. Conference. Université de montréal.

Elbers, A. R. W. Miltenburg, J. D. De Lange, D. Crauwels, A. P. P. Barkema, H. W. and Schukken, Y. H . 1998. Risk factors for clinical mastitis in a random sample of dairy herds from the southern part of the Netherlands. *J. Dairy Sci.* **81**: 420–426.

Elner, V. M. Streiter, R. M. Elner, S. G. Baggionlini, M. Lindley, I. and Kunkel, S. L. 1990. Neutrophil chemotactic factor (IL-8) gene expression by cytokine treated retinal pigment epithelial cells. *Am. J. Pathol*. **136**: 745-750.

Emanuelson, U. Danell, B. and Philipsson, J. 1988. Genetic parameters for clinical mastitis, somatic cell counts and milk production estimated by multiple-trait restricted maximum likelihood*. J. Dairy. Sci.* **71**: 467-476.

Emmert-Buck, M.R., Bonner, R.F., Smith, P.D., Chuaqui. R.F., Zhuang, Z., Goldstein, S.R., Weiss, R. A. and Liotta, L. A. 1996. Lasers capture micro-dissection. *Science*. **274:** 998–1001.

Erskine, R. J. 2001. Mastitis Control in Dairy Herds. Chap 10 in Herd Health: food animal production medicine. 3rd ed. Radostits editor. WB Saunders, Philadelphia.

Fend, F. Kremer, M. and Quintanilla-Martinez, L. 2000. Laser capture micro-dissection: methodical aspects and applications with emphasis on immuno-laser capture micro-dissection. *Pathobiology.* **68**: 209–214.

Frade, A. F. Oliveira, L. Costa, D. L. Costa, C. H. N. Aquino, D. Weyenbergh, J. V. Netto, M. B. Barral, A. Kalil, J. and Goldberg, A. C. 2011. TGFB1 and IL8 gene polymorphisms and susceptibility to visceral leishmaniasis. *Infection, Genetics and Evolution.* **11**: 912-916.

Fujimara, F. K. Northrup, H. Beaudet, A. L. and O' Brien, W. E. 1990. Genotyping errors with the polymerase chain reaction. *New England J. Med.* **322**:61.

Girard, C. and Girard, N. P. 1994. C5A anaphylatoxin and its seven transmembrane-segment receptor. *Annu. Rev. Immunol.* **12**: 775–808.

Goerke, C. Bayer, M. G. and Wolz, C. 2001. Quantification of bacterial transcripts during infection using competitive reverse transcription-PCR (RT-PCR) and Light Cycler RT-PCR. *Clinical and Diagnostic Laboratory Immunology.* **8**: 279–282.

Graham, Gerard, J. Locati, Massimo. 2013. "Regulation of the immune and inflammatory responses by the 'atypical' chemokine receptor D6". *The Journal of Pathology.* **229** (2): 168–75.

Gray,G. D. Sharon, R. Hasslen, Julia A. Ember, David F. Carney, Michael J. Herron, Stanley L. Erlandsen, and Robert D. Nelson. 1997. Receptors for the Chemoattractants C5a and IL-8 are clustered on the surface of human neutrophils. *J. Histochem. Cytochem.* **45**: 1461.

Gregory. H. Young, J. Schroder, J. M. Mirowitz, U. and Christophers, E. 1988 structure determination of a human lymphocyte derived neutrophil activating peptide (LYNAP). *Biochem. Biophys. Res. Commun.* **151**: 883-890.

Gusella, J.F. Wexler, N.S. Conneally, P.M. Nayor, S.L. Anderson, M. A. Young, A.B. Shoulson, I. Bonilla, E. and Martin, J. B. 1983. A polymorphic DNA marker genetically linked to Huntingon's disease. *Nature*. **306**: 234-238.

Harada, A. Sekido, N. Akahoshi, T. Wada, T. Mukaida, N. and Matsushima, K. 1994. Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J. Leuko. Boil.* **56**: 559-564.

Harvey, W. R. 1966. Least squares analysis of data with unequal subclasses number. ARS. USDS. Iowa State University. USA.

Hazare, R. 2009. Interleukin-8 gene polymorphism and its association with mastitis incidence in Sahiwal cattle. M.V.Sc. thesis submitted to National Dairy Research Institute, Karnal, India.

Heaton, M. P. Chitko- Mckown, C. G. Micheal, G. W. Keele, J. W. James, E. and William, W. Laegreid. 2001. Interleukin-8 haplotype structure from nucleotide sequence variation in commercial populations of U.S. beef cattle. *Mamm. Genome.* **12**: 219-226.

Heaton, M. P. William, W. Laegreid, Craig, W. Beattie, Timothy, P. L. Smith, S. and Kappes, M. 1999. Identification and genetic mapping of bovine chemokine genes expressed in epithelial cells. *Mamm. Genome*. **10**(1): 296-297.

Horuk, R. 1994. The interleukin-8-receptor family: from chemokines to malaria. *Immunol Today.* **15**:169–174.

http://en.wikipedia.org/wiki/cytokine

http://www.copewithcytokines.de

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

http:/www.genome.wi.mit.edu/cgibin/primer/primer3-www.cgi

ICAR Network Project, 1999, final progress report of a project on characterization, conservation and evaluation on Deoni cattle, Department Of Animal Husbandary and Dairying, Marthwada Agricultural University, Parbhani.

Ivinson, A. J. and Taylor, G. R. 1992. PCR in genetic diagnosis. In: PCR- a practical approach. Eds. Mj. Mcpherson, P.Quirke and G.R. Taylor. Pp.15-28. Oxford University Press.

Jagadeesan, K. 2008. Characterization of interleukin-8 gene in Sahiwal cattle. M.V.Sc. thesis submitted to National Dairy Research Institute, Karnal, India.

Jiemtaweeboon, S. Shirasuna, K. Nitta, A. Kobayashi, A. Schuberth, H. Shimizu, T. and Miyamoto, A. 2011. Polymorphonuclear neutrophils infiltrate into the developing corpus luteum and promote angiogenesis with interleukin-8 in the cow. *Reproductive Biology and Endocrinology. **9**: 79-89.*

Joshi, S. and Gokhale, S. 2006. Status of mastitis as an emerging disease in improved and peri-urban dairy farms in India. *Ann. N. Y. Acad. Sci.* **1081**: 74-83.

Juozaitiene, V. Juozaities, A. and Micikviencine, R. 2006. Relationship between somatic cell count and milk production or morphological traits of udder in black and white cows. *Turk. J. Vet. Anim. Sci.* **30**: 47-51.

Kakde, P. V. Rette, S. G. Deshpande, K. S. and Bonde, H. S. 1980. Peak yield and days to attain peak yield in Deoni cattle. *Indian Vet. J.* **57**(10): 816-820.

Kappes, S. M. Keele, J. W. Stone, R. T. McGraw, R. A. Sonstegard, T. S. Smith, T. P. Lopez-Corrales, N. L. and Beattie, C. W. 1997. A second-generation linkage map of the bovine genome. *Genome Res.* **7**(3):235-49.

Kaupe, B. Brandt, H. Prinzenberg, E. M. and Erhardt, G. 2007. Joint analysis of the influence of CYP11B1 and DGAT1 genetic variation on milk production, somatic cell score, conformation, reproduction, and productive lifespan in German Holstein cattle. *Journal of Animal Science.* **85:** 11–21.

Kehrli, M. E. and Shuster, D. E. 1994. Factors affecting milk somatic cells and their role in health of the bovine mammary gland. *Journal Dairy Sci.* **77**: 619-627.

Komisarek, J. Antkowiak, I. Rytlewski, J. Dorynek, Z. and Walkowicz, K. 2006. Effect of polymorphism in gene BTN1A1 on somatic cell count in milk of Jersey cows. *Pol. J. food Nutr. Sci*.**15**: 101-105.

Lahouassa, H. Rainard, P. Caraty, A. and Riollet, C. 2008. Identification and characterization of a new interleukin-8 receptor in bovine species. *Molecular Immunology.* **45:** 1153-1164.

Laing, K. and Secombes, C. 2004. Chemokines. *Dev. Comp. Immunol*. **28**(5): 443-60.

Landi, S. Moreno, V. Gioia-Patricola, L. Guino, E. Navarro, M. de Oca, J. Capella, G. and Canzian, F. 2003. For the Bellvitge Colorectal Cancer Study Group. Association of Common Polymorphisms in Inflammatory Genes Interleukin (IL)6, IL8, Tumor Necrosis Factor _, NFKB1, and Peroxisome Proliferator-activated Receptor _ with Colorectal Cancer. *Cancer Research.* **63**: 3560–3566.

Lindmark-Mansson, H. Branning, C. Alden, G. and Paulsson, M. 2006. Relationship between somatic cell count, individual leukocyte populations and milk components in bovine udder quarter milk. *Int. Dairy. J.* **6**: 717-727.

Larry, C. Borish, M. D. and John, W. Steinke. 2003. Cytokines and Chemokines *J. Allergy Clin. Immunol.* **111:** Number 2

Leyva-Baca, I. Schenkel, F. Martin, J. and Karrow N. A. 2008. Polymorphisms in the 5 upstream regions of the CXCR1 chemokine receptor gene, and their association with somatic cell score in Holstein cattle in Canada. *Journal of Dairy Science.* **91**: 407–17.

Leyva-Baca, I. Schenkel, F. Sharma, B. S. Jansen, G. B. and Karrow, N. A. 2007. Identification of single nucleotide polymorphisms in the bovine CCL2, IL8, CCR2 and IL8RA genes and their association with health and production in Canadian Holsteins. *Animal Genetics.* **38**: 198–202.

Little, P. F. R. Annison, G. Darling, S. Williamson, R. Camba, L. and Model, B. 1980. Model for antenatal diagnosis of β-thalassemis and other monogenic disorders by molecular analysis of linked DNA polymorphisms. *Nature*. **285**: 144-147.

Liu, J. Feldman, P. and Chung, T. D. 2002. Real-time monitoring in vitro transcription using molecular beacons. *Analytical Biochemistry.* **300:** 40–45.

Logan, J. Edwards, K. and Saunders. N. 2009. Logan, J., Edwards K, Saunders N, Ed. Real-Time PCR: Current Technology and Applications. Caister Academic Press. ISBN 978-1-904455-39-4.

Lu, W. Pan, K. Zhang, L. Lin, D. Miao, X. and You, E. 2005. Genetic polymorphisms of interleukin (IL)-1B, IL-1RN, IL-8, IL-10 and tumor necrosis factor and risk of gastric cancer in a Chinese population. *Carcinogenesis*. **26**(3): 631—636.

Luo, L. Salunga, R. C. Guo, H. Bittner, A. Joy, K.C. Galindo, J. E. Xiao, H. Rogers, K. E. Wan, J. S. Jackson, M. R. and Erlander, M. G. 1999. Gene expression profiles of laser-captured adjacent neuronal subtypes. *Nat. Med*. **5**: 117–122.

Mayers, R. M. Sheffield, V.C. and Cox, D. R. 1989. Mutation detection by PCR, Gc-clamps and denaturing gradient gel electrophoresis. In: OCR technology: principles and applications for DNA amplifications. Ed. H. A. Erlich. Pp.71-88. Stockton Press, Newyork.

McClenahan, D. Krueger, R. Yung Lee, H. Thomas, C. Kehrli Jr, M. E. Czuprynski, C. 2006. Interleukin-8 expression by mammary gland endothelial and epithelial cells following experimental mastitis infection with E. coli. *Comparative Immunology, Microbiology & Infectious Diseases.* **29**: 127–137.

Meade, K. G. O'Gorman, G. M. Narciandi, F. MacHugh, D. E. and O'Farrelly, C. 2012. Functional characterization of bovine interleukin 8 promoter haplotypes *in vitro*. *Molecular Immunology.* **50**: 108–116.

Mielke, V. Bauman, J. Sticherlin, M. Ibs, T. Zomershoe, A. Selimann, K. Hemeicke, H. H. Schroder, J. M. Sterry, W. and Cristophers, E. 1990. Detection of neutrophil activating peptide NAP/IL-8 and NAP/IL-8 mRNA in human recombinant IL1 and human recombinant tumor necrosis factor stimulated human dermal fibroblasts. *J. immunol*.**144**: 153-161.

Mifflin, T. E. Estey, C. A. and Felder, R. A. 2000. Robotic automation performs a nested RT-PCR analysis for HCV without introducing sample contamination. *Clinica Chimica. Acta.* **290**: 199–211.

Miller, S. A. Dykes, D.D. and Polesky, H. F. 1988. A simple salting out procedure for extraction of DNA from human nucleated cells. *Nucleic Acid Research*. **16**: 1215.

Morsey, M. A. Popowych, Y. Kowalski, J. Gerlach, G. Godson, D. Campos, M. and Babiuk, L. A. 1996. Molecular cloning and expression of bovine IL-8. *Microb. Pathog.* **20**: 203-212.

Mostert, B. E. Banga, C. Groeneveld, E. and Kanfer, F. H. J. 2004. Breeding value estimation for somatic cell score in South African dairy cattle. *South Afr. J. Anim. Sci.* **34**: 32-34.

Mrode, R. and Swanson, G. J. T. 1996. Genetic and statistical properties of somatic cell count and its suitability as an indirect means of reducing the incidence of mastitis in dairy cattle. *Anim. Breed. Abs.* **64**: 847-857.

Mukaida, N. Harada, A. Yasumoto, K. and Matsushima, K. 1992. Properties of Pro-inflammatory cell type-specific leukocyte chemotactic cytokines, interleukin8 (IL-8) and monocytes chemotactic and activating factor (MCAF). *Microbial. Immunol*. **36**: 773-789.

Mulder, K. and Colditz, I. G. 1993. Migratory responses of ovine neutrophils to inflammatory mediators in vitro and in vivo. *J. Leukoc. Boil.* **53**: 273-278.

Murrey, J. M. Davies, K. E. Harper, P.S. Meredith, L. Mueller, C. R. and Williamson, R. 1982. Linkage relationship of a cloned DNA sequence on the short arm of the X-chromosome to Duchene muscular dystrophy. *Nature*. **300**: 69-71.

Nash, D. L. Rogers, G. W. Cooper, J. B. Hargrove, G. L. and Keown, J. F. 2003. Heritability of intramammmary infections at first parturition and relationships with sire transmitting abilities for somatic cell score, udder type traits, productive life, and protein yield. *J. Dairy. Sci.* **86**: 2684-2695.

Ogorevc, J. Kunej, T. Razpet, A. and Dovc. P. 2009. Database of mammary gland genetic markers. *Animal Genetics* **40**: 832–851

Ohta, T. and Kimura, M. 1973. A new model for estimating the number of electrophoretically detectable alleles in a finite population. *Genet. Res.* **22**: 201-204.

Ohyama, H. Zhang, X. Kohno, Y. Alevizos, I. Posner, M. Wong, D. T. and Todd, R. 2000. Laser capture micro-dissection-generated target sample for high-density oligonucleotide array hybridization. *Biotechniques.* **29**: 530–536

Orlando, C. Pinzani, P. and Pazzagli, M. 1998. Developments in quantitative PCR. *Clinical Chemistry Laboratory Medicine.* **36:** 255–269.

Paccaud, I. Schifferli, I. and Baggiolini, M.1990. NAP-1/IL-8 induces up-regulation of CR1 receptors in human neutrophil leukocytes. *Biochem. Biophys. Res. Commun.* **166**:187-192.

Palkowitz, K. H. Royner, C. L. Garofalo, R. Rudolff, H. E. Schmalstieg, F. C. Jr. and Goldman, A. S. 1994. Production of interleukin-6 and interleukin-8 by human mammary gland epithelial cells. *J. Reprod. Immunol*. **26**: 57-64.

Pant, S. D. Schenkel, F. S. Leyva-Baca, I. Sharma, B. S. and Karrow, N. A. 2007. Identification of single nucleotide polymorphisms in bovine CARD15 and their associations with health and production traits in Canadian Holsteins. *BMC Genomics.* **8**: 421.

Peli, A. Luciani, A. De Santis, P. Polci, A. and Britti, D. 2004. Quantification of the cytokines TNF-α, IL-8 and IL-10 in bovine milk using Real-Time Taqman® PCR. *Veterinary Research Communications*. **28**: 359-362.

Persson, K., Sandgen, C. H. and Rodriguez, M. H. 1992. Studies of endotoxin-induced neutrophils migration in bovine teat tissues, using indium-111-labeled neutrophils and biopsies. *Am. J. Vet. Res*. **53**: 2235-2240.

Polec, A. Tanbo, T. and Fedorcsak, P. 2009. Cellular interaction regulates interleukin-8 secretion by granulosa-lutein cells and monocytes/macrophages. *Am. J. Reprod. Immunol.* **61**(1): 85-94.

Poso, J. and Mantysaari, E. A. 1996. Relationships between clinical mastitis, somatic cell score, and production for the first three lactations of Finnish Ayrshire. *J. Dairy Sci.* **79**: 1284-1291.

Rainard, P. and Riollet, C. 2005. Innate immunity of the bovine mammary gland. *Veterinary Research*. **37**: 369–400.

Rangarajan, K. 2012. Molecular characterization of bovine butyrophilin (BTN1A1) alleles in exon-3 and exon-8 and their association with fat content and SCC in milk of dairy cattle. Ph.D thesis submitted to National Dairy Research Institute, Karnal, India.

Rao, V. L. N. 2012. Molecular characterization of Glutathione S-Transferase PI (GSTP1) gene in Deoni cattle. M.Sc. thesis submitted to National Dairy Research Institute, Karnal, India.

Rupp, R. Hernandez, A. and Mallard, B. A. 2007 Association of bovine leukocyte antigen (BoLA) DRB3.2 with immune response, mastitis and production and type traits in Canadian Holsteins. *Journal of Dairy Science.* **90**: 1029–38.

Sandholm, M. Honkanen-Buzalski, T. Kaartinen, L. and Pyorala, S. 1995. The bovine udder and mastitis. *Gummerus Kirjapaino, Oy., Finland.* **312p**,

SAS Institute, Inc. 2003. Release 9.3 (TS3MO) of the SAS system for Microsoft Windows. SAS Institute. Inc., Cary. NC. USA.

Schroeder, J. M. Mrowietz, U. Mortia, E. and Chritophers, E. 1987. Purification and partial biochemical characterization of a human monocytes-derived, neutrophils activating peptide that lacks Interleukin-1 activity. *J. Immunol.* **139**: 474-483.

Sender, G. Bowna, M. G. and Bassalik- Chabielska L. 1987. Environmental factors determining somatic cell counts in cow's milk. *Zesz. Probl. Post. Nauk Roln*. **332**: 167-172.

Sharif, S. Mallard, B. A. Wilkie, B. N. Sargeant, J. M. Scott, H. M. Dekkers, J. C. and Leslie, K. E. 1998. Associations of the bovine major histocompatibility complex DRB3 (BoLA-DRB3) alleles with occurrence of disease and milk somatic cell score in Canadian dairy cattle. *Animal Genetics*. **29**: 185–93.

Sharma, B. S. Leyva, I. Schenkel, F. and Karrow, N. A. 2006. Associationof toll-like receptor 4 polymorphisms with somatic cell score and lactation persistency in Holstein bulls. *Journal of Dairy Science.* **89**: 3626–35.

Sharma, R. Sangwan, M. L. Singh, P. and Rana, A. 2013. Interleukin-8 Gene Polymorphism and Its Association with Mastitis in Murrah Buffalo. *Journal of Cell and Tissue Research.* **13**(1): 3591-3596.

Shekhar, S. C. 2012. Analysis of TLR4 gene polymorphism and its association with somatic cell count in milk of HF crossbred and Deoni cattle. M.V.Sc. thesis submitted to National Dairy Research Institute, SRS, Bangalore, India.

Simone, N. L. Bonner, R. F. Gillespie, J. W. Emmert-Buck, M. R. and Liotta, L. A. 1998. Laser-capture micro-dissection: opening the microscopic frontier to molecular analysis. *Trends Genet*. **14**: 272–276

Singh, G. Gaur, G. K. Nivsarkar, A. E. Patil, G. R. and Mitkari, K. R. 2002. Deoni cattle breed of India. A study on population dynamics and morphometric characteristics. *Animal Genetic Resources Information*.**32**: 35-43.

Singh, R. K. 2008. DNA polymorphism at interleukin-8 gene in Murrah buffaloes (*Bubalus bubalis*). M.V.Sc. thesis submitted to National Dairy Research Institute, Karnal, India.

Singhal, R. A. and Kaushik, S. N. 1980. Comparative performance of some India breeds of cattle. *Dairy Guide*. **2**(11): 25-28.

Soller, M. and Beckmannn, J. S. 1983. Genetic polymorphism in varietal identification and genetic improvement. *Theor. Appl. Genet*. **67**: 25-33.

Strieter, R. M. Kasahara, K. Allen, R. Showell, H. J. Standiford, T. J. and Kunkel, S. L. 1990. Human neutrophils exhibit disparate chemotactic factor gene expression. *Biochem. Biophys. Res. Commun.* **173**: 725-730.

Sudhan, N. A. and Sharma, N. 2010. Mastitis- an important production disease of dairy animals. In: farm management and diseases. SMVS dairy year book, pp: 72-88.

Sugimoto, M. Fujikawa, A. Womack, J. E. and Sugimoto, Y. 2006. Evidence that bovine forebrain embryonic zinc finger-like gene influences immune response associated with mastitis resistance. Proceedings of the National Academy of Sciences of the United States of America **103**: 6454–9.

Szyda, J. and Komisarek, J. 2007. Statatistical modelin g of candidate gene effects on milk production traits in dairy cattle. *J. Dairy. Sci.* **90**: 2971-2979.

Theilmann, J. L. Skow, L. C. Baker, J. F. and Womack, J. E. 1989. Restriction fragment length polymorphisms for growth hormone, prolactin, osteonectin, α-crystallin, γ-crystallin, fibronectin and 21 steriod hydrolyse in cattle. *Anim. Genet.* **20**: 257-266.

Udvardi, M. K. Czechowski, T. Scheible, W. R. 2008. "Eleven Golden Rules of Quantitative RT-PCR". *Plant Cell*. **20** (7): 1736–1737.

Van Damme, J.1994. Interleukin-8 and related chemotactic cytokines. In:Thomson a. ed. The cytokine handbook. New York, N.Y: Academic Press: pp.185-208.

Van Eijk, M. J. T. Stewart- Haynes, J. A. and Lewin, H. A. 1992. Extensive polymorphism of the Bola-DRB3 gene distinguished by PCR-RFLP. *Anim. Genet*. **23**: 483-496.

Wang, X. Maltecca, C. Tal-Stein, R. Lipkin, E. and Khatib, H. 2008. Association of bovine fibroblast growth factor 2 (FGF2) gene with milk fat and productive life: an example of the ability of the candidate pathway strategy to identify quantitative trait genes. *Journal of Dairy Science* **91**: 2475–80

Wang, X. Xu, S. Gao, X. Ren, H. and Chen, J. 2007. Genetic polymorphism of TLR4 gene and correlation with mastitis in cattle. *Journal of Genetics and Genomics*. **34**: 406–12.

Wilson, D. J. Gonzalez, R. N. N. Hertl, J. Schulte, H. F. Bennett, G. J. Schukken, Y. H. and Grohn, Y. T. 2004. Effect of clinical mastitis on the lactation curve: A Mixed Model Estimation Using Daily Milk Weights. *J. Dairy. Sci.* **87**: 2073-2084.

Wojdak-Maksymiec, K. Kmiec, M. and Ziemak, J. 2006. Associations between bovine lactoferrin gene polymorphism and somatic cell count in milk. *Veterinarni Medicina*. **51**: 14–20.

www.ncbi.nlm.nih.gov.in/genbank

Yalchin, C. Cevger, Y. Turkyilmaz, K. and Uysal, G. 2000. Estimation of milk yield losses from subclinical mastitis in dairy cows. *Turk. J. Vet. Anim. Sci*. **24**: 599-604.

Yeh, F. Yang, R. and Boyle, T. 2006. POPGENE version 1.31 Microsoft Windows-based freeware for population genetics analysis. University of Alberta and center for international forestry research, Canada. Available at (http://www.ualberta.ca/~fyeh/fyeh)

Yoshimura, T. Matsushima, K. Tanaka, S. Robinson, A. Appella, E. and Oppenheim, J. J. 1987. Purification of a human monocytes derived neutrophil chemotactic factor that has peptide sequence similarity to other defense cytokines. *Prac. Nat. Acad. Sci.* USA. **84**: 9233-7.

Yoshino, O. Osuga, Y. Koga, K. Hirota, Y. Yano, T. Tsutsumi, O. Fujimoto, A. Kugu, K. Momoeda, M. and Fujiwara, T. 2003. Upregulation of interleukin-8 by hypoxia in human ovaries. *Am. J. Reprod. Immunol*. **50**(4):286-290.

Youngerman, S. M. Saxton, A. M. Oliver, S. P. and Pighetti, G. M. 2004 Association of CXCR2 polymorphisms with subclinical and clinical mastitis in dairy cattle. *Journal of Dairy Science*. **87**: 2442–8.

Yuen, T. Zhang, W. Ebersole, B. J. and Sealfon, S. C. 2012. Monitoring G-protein-coupled receptor signaling with DNA microarrays and real-time polymerase chain reaction. *Methods in Enzymology.* **345:** 556–569.

Zabeau, M. and Roberts, R. J. 1979. The role of restriction endonucleases in molecular genetics. In: molecular genetics. Chromosome structure III. Ed. J. h. Taylor. Pp.1-63. Academic Press, New York.

ANNEXURE

Annexure

Solution/ Reagents For Isolation Of DNA

i. EDTA (10 per cent) solution

Ethylene Diamine Tetra Acetic Acid (Edta)	-	1 gm
Triple glass distilled water to make up to	-	10 ml

ii. RBC Lysis Buffer

Ammonium chloride	-	8.0235 g
Potassium chloride (10mM)	-	0.7455 g
EDTA	-	0.0372 g
Triple glass distilled water to make up to	-	1000 ml
Autoclaved and stored at 4°c		

iii. Tris Buffer Salines

Potassium chloride	-	0.0373 g
Sodium chloride (0.4m)	-	8.1800 g
Tris (hydroxymethyl) aminomethane HCL	-	0.0303g
Magnesium chloride (10mm)	-	1ml
Triple glass distilled water to make up to	-	1000 ml
Ph adjusted to	-	7.4
Autoclaved and stored at 4°c'		

iv. Tris-EDTA Buffer (TE Buffer)

Tris hydroxymethyl aminomethane HCL (10mm)		
ph7.6	-	1.2114 g
EDTA (0.1mm)	-	0.3722 g
Triple glass distilled water upto	-	1000 ml
Ph adjusted to	-	8.0
Autoclaved and stored at 4°c		

v. Sodium dodecyl sulphate (10 per cent SDS)

Sodium dodecyl sulphate	-	10 g
Triple glass distilled water to make up to	-	100 ml

vi. Saturated sodium chloride (6M)

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

vii. 0.5M EDTA

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

Dissolve in 80 ml DD water by keeping the beaker on.

Magnetic stirrer with heating raises the pH of solution to pH 8.

By adding NaOH, the contents are fully dissolved.

viii. Proteinase-k

Proteinase- K	-	20 mg
Triple glass distilled water to make up to	-	1 ml

Stored at 20°C

<u>Solutions/reagents for checking the genomic DNA</u>

i. **Tris acetate EDTA(TAE) buffer (50x)**

Tris (hydroxymethyl) amino methane HCL (10mM) pH 7.6	-	48.4g
Glacial acetic acid	-	11.42 ml
0.5 M EDTA (pH 8.0)	-	20 ml
Triple glass distilled water to make up to	-	1000 ml

ii. **Agarose (0.8 per cent)**

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

iii. **Ethidium bromide staining solution (10 per cent)**

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

iv. **Gel loading buffer**

Glycerol	-	5ml
Bromophenol blue	-	125 mg
Xylene cyanol	-	125mg
Triple glass distilled water to make up to	-	500ml

v. **TBE 5X buffer**

Tris 445mM	-	54g
Boric acid 445 Mm	-	27.5g
EDTA 10 mM	-	3.7224 g

Dissolve in 1000ml double distilled water

vi. **Tris buffer saline**

Potassium chloride	-	0.0373 g
Sodium chloride (0.4M)	-	8.1800 g
Tris (hydroxymethyl) aminomethane HCl	-	0.0303 g
Triple glass distilled water to make up to	-	1000 ml
pH adjusted to	-	7.4

Autoclaved and stored at 4°C

vii. **Tris-EDTA buffer (TE buffer)**

Tris (hydroxymethyl) amino methane HCL(10mM)		
pH 7.6	-	1.2114 g

EDTA (0.1 mM)	-	0.3722 g
Triple glass distilled water to make up to	-	1000 ml
pH adjusted to	-	8.0

Autoclaved and stored at 4°C

viii. **Sodium dodecyl sulphate (20% SDS)**

Sodium dodecyl sulphate	-	20 g
Triple glass distilled water to make up to	-	100 ml

Stir on magnetic stirrer filter and store at room temperature

ix. **0.5M EDTA**

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

Dissolve in 80 ml DD water by keeping the beaker on magnetic stirrer with heating raises the pH of solution to pH 8.

By adding NaOH, the contents are fully dissolved

x. **Ethidium bromide staining solution**

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

xi. **Gel loading dye (6X) for 10 ml solution**

Sucrose 40% W/V	-	4 g
Bromophenol blue 0.05% W/V	-	0.005 g
EDTA 0.1 M	-	2 ml of 0.5M EDTA
SDS 0.5% W/V	-	0.05 g

Triple glass distilled water to make up to 10 ml