Chapter 1

Introduction
1.1. Mastitis

Mastitis (Greek, Mastos = breast + it is = inflammation) is a multietiological complex disease, defined as inflammation of parenchyma of mammary glands and is characterized by physical, chemical and usually bacteriological changes in milk and pathological changes in glandular tissues (Viguier et al., 2009; Gunther et al., 2011). This Intra-mammary (IM) infection in cattle can result in different clinical outcomes that range from being acute and life-threatening to those that are chronic and sub-clinical (Wellnitz et al., 2012).

In addition to heavy losses in milk quality and quantity, mastitis also causes irreversible damage to the udder tissue and less occasional fatalities. Mastitis destroys the milk secreting cells. Scar or connective tissue replaces the milk secreting tissue, resulting in a permanent loss of productive ability.

There are two types of mastitis (Awale et al., 2012) (Figure 1.1)

1. Contagious Mastitis: It is caused by bacteria living on the skin of the teat and inside the udder. Contagious mastitis can be transmitted from one cow to another during milking.

Contagious mastitis can be divided into three types:

i). Clinical mastitis: It is characterized by the presence of gross inflammation signs (swelling, heat, redness, pain). Clinical mastitis can again be divided into three types:

a. Peracute mastitis: It is characterized by gross inflammation, reduction in milk yield and changes in milk composition, Systemic signs like fever, depression, shivering and loss of appetite and loss of weight.

b. Acute mastitis: Similar to peracute mastitis, but with lesser systemic signs like fever and mild depression.

c. Subacute mastitis: In this type of mastitis, the mammary gland inflammation signs are minimal
and no visible systemic signs.

ii). Subclinical mastitis: This form of mastitis is characterized by change in milk composition with no signs of gross inflammation or milk abnormalities. Changes in milk composition occur.

iii). Chronic mastitis: An inflammatory process that exists for months, and may continue from one lactation to another. It exists as subclinical but may exhibit periodical flare-ups subacute or acute form, which last for a short period of time.

2. Environmental mastitis: It is caused by organisms such as Escherichia coli which do not normally live on the skin or in the udder but which enter the teat canal when the cow comes in contact with a contaminated environment. The pathogens normally found in feces bedding materials, and feed. Cases of environmental mastitis rarely exceed 10% of the total mastitis cases in the herd (Awale et al., 2012).

Figure 1.1. Types of mastitis

1.2. Impact of mastitis

Mastitis is a global threat as it adversely affects animal health, quality of milk and economics of milk production and every country including developed ones suffer huge financial losses (Wellnitz et al., 2012). Costs associated with mastitis include milk production losses, pharmaceuticals, discarded milk, veterinary services, labour, milk quality deficits, investment in
mastitis management materials and infrastructure, diagnostic testing, and cattle replacement.

India is the highest milk producer in the world but the per capita availability of milk still remains half of the world average. The estimated annual economic losses due to bovine mastitis has increased 135 folds in about almost 5 decades from 1962 (INR 529 million/annum) to 2009 (INR 71655.1 million/annum) (Bansal and Gupta 2009). Decreased milk production accounts for approximately 70% of the total cost of mastitis (Sharma et al., 2012; Awale et al., 2012).

Prevalence pattern reports show, most mastitis occurs as a low grade infection, a subclinical state, increasing milk leukocyte count, reducing milk production and increasing bacterial count in milk. It has been reported that subclinical mastitis is 50-70% times more common than clinical mastitis and causes greatest overall losses in most dairy herds (Awale et al., 2012). In India subclinical mastitis is more important (varying from 10-50% in cows) than clinical mastitis (1-10%) (Awale et al., 2012)). More of a concern as the presence of such infections is a significant risk to uninfected animals in the herd.

The vehement research on bovine mastitis is comporting since several decades but unfortunately the problem is still challengeable for the bovine mastitis researchers and particularly for field veterinarians to treat and control it (Sharma et al., 2012).

1.3. Multiple etiological agents in mastitis

Mastitis is a multi-etiological complex disease. The primary cause of mastitis is a wide spectrum of bacterial strains; however, incidences of viral, algal and fungal mastitis were also reported (Awale et al., 2012).

Mastitis can be caused by over 250 different contagious and environmental microorganisms such as Gram-positive cocci, Gram-negative cocci (Coliforms specifically E. coli, Enterobacter, Klebsiella spp.) and other miscellaneous organisms, which include Nocardia, Prototheca and Yeast. Generally, the mastitis due to fungi and yeast is uncommon or rare. But a low prevalence of fungal mastitis of 2 to 7% has been reported. The predominance of a bacterial
species may vary according to the geographical region under scrutiny. Changes of pathogen trends have been both temporal as well geographical (Sharma et al., 2012).

**Staphylococcus aureus- chief etiological agent in mastitis**

Amongst bacteria, Staphylococci is the predominant group with *Staphylococcus aureus* being the most common pathogen causing mastitis. Recently the emergence of Coagulase negative Staphylococci (CNS) as mastitis pathogens has been recognized as a growing problem in mastitis. Even though the prevalence of the mastitis causing pathogens varies between the countries, *S. aureus* has been reported as the chief etiological agent of mastitis worldwide including India by various researchers. This emphasizes that *S. aureus* mastitis needs prior attention for prevention and control.

*S. aureus* is a versatile pathogen with a wide range of virulence strategies and causing a wide range of diseases (Rasigade and Vandenesch 2013). *S. aureus* can provoke clinical mastitis but more frequently cause subclinical infections that tend to become chronic, can persist for the life of the animal and difficult to eradicate by conventional antimicrobial therapies (Bannerman et al., 2009; Yang et al., 2008; Welehan et al., 2011). The ability of bacteria to generate genetic variations is crucial for their survival. Determining *S. aureus* population structure is necessary and the knowledge generated from population genetics has the potential to inform strategies to assist in the prevention or treatment of this powerful and successful pathogen (Enright 2008).

Molecular tools provide opportunities for understanding infectious diseases and increasing our understanding of the factors that determine the spatial and temporal distribution of pathogens and disease (Mullner et al., 2011). The population structure of *S. aureus* has been defined using a number of well established typing methods. In general, those that continue to be used widely today may be divided into band based (PFGE) and sequence based typing (MLST, spa typing etc) (Grundmann et al., 2002; Hasman et al., 2010). Whole understanding of pathogen biology of *S. aureus* is perquisite to understand the wide range of behavioural changes exposed by it. Same
pathogen has the capacity to behave differently in diverse environments. Strain specific characteristics can be expected to affect the probability of cure of \textit{S. aureus} IMI, and studies to that effect are starting to emerge (Barkema et al., 2006). Studies are necessary to determine the distribution of the clones associated with bovine intramammary infections in India and to confirm whether a few specialized clones are responsible for the majority of mastitis cases.

Until now, very little is understood about the pathogen biology in \textit{S. aureus} mastitis in Indian context. Lacks of systematic epidemiological investigation with methods varying between labs have led to incomparable results. As a result, a clear epidemiological picture is difficult to obtain from the existing pool of published informations. This emphasizes the need for molecular characterization of \textit{S. aureus} isolates in the province.

1.4. Host Pathogen Interaction

1.4.1. Pathogenesis

Pathogenesis of bovine mastitis is a result of a very complicated interplay between host and microbe. The bacteria involved in IM bacterial infections activate the mammary immune system in different ways which can influence the severity of the outcome. The successful establishment and persistence of an intramammary infection are mediated by both intrinsic virulence factors of the bacterial pathogen and the rapidity and nature of the immune response of the cow to the pathogen (Bannerman et al., 2009). A comprehensive understanding of the pathogenicity of mastitis is a key for the development of appropriate intervention strategies (Gunther et al., 2011).

Most intramammary infection results from bacteria overcoming the anatomical-physical barrier of the teat canal (Wellnitz et al., 2012). Normally, the teat canal is tightly closed by sphincter muscles, preventing the entry of pathogens. It is lined with keratin, a waxy material derived from stratified squamous epithelium that obstructs the migration of bacteria and contains antimicrobial agents, such as long-chain fatty acids, that assist in combating the infection.
However, the efficiency of keratin is restricted (Paulrud et al., 2005). Fluid accumulates within the mammary gland as parturition approaches, resulting in increased intramammary pressure and mammary gland vulnerability caused by the dilation of the teat canal and leakage of mammary secretions (Sordillo and Streicher 2002). Additionally, during milking, the keratin is flushed out and there is distention of the teat canal (Rainard and Riollet 2006). The sphincter requires about 2 hour for returning back to the contracted position (Capuco et al., 1992).

Once inside the teat, bacteria must also elude the cellular and humoral defence mechanisms of the udder (Sordillo and Streicher 2002). If they are not eliminated, they start multiplying in the mammary gland (Figure 1.2). They liberate toxins and induce leukocytes and epithelial cells to release chemoattractants, including cytokines such as tumour necrosis factor-α (TNF-α), interleukin (IL)-8, IL-1, eicosanoids (like prostaglandin F2α), oxygen radicals and acute phase proteins (APPs) (e.g. haptoglobin, serum amyloid A). This attracts circulating immune effector cells, mainly polymorphonuclear neutrophils (PMNs), to the site of infection (Zhao and Lacasse 2008).

PMNs act by engulfing and destroying the invading bacteria via oxygen-dependent and oxygen-independent systems. They contain intracellular granules that store bactericidal peptides, proteins, enzymes (such as myeloperoxidase) and neutral and acidic proteases (such as elastase, cathepsin G, cathepsin B and cathepsin D) (Paape et al., 2002). The released oxidants and proteases destroy the bacteria and some of the epithelial cells, resulting in decreased milk production and release of enzymes, such as N-acetyl-b-D-glucosaminidase (NAGase) and Lactate Dehydrogenase (LDH). Destruction of most of the PMNs take place by apoptosis, once their task is fulfilled. Subsequently, macrophages engulf and ingest the remaining PMNs (Paape et al., 2002; 2003). The dead and sloughed off mammary epithelial cells, in addition to the proteinases and dead leukocytes, are secreted into the milk, resulting in high milk SCCs (Somatic cell count) (Leitner et al., 2006).
Figure 1.2. Schematic representation of mastitis development in an infected udder. Environmental and contagious microorganisms invade the udder through the teat cistern. They then multiply in the udder where they are attacked by neutrophils while damaging the epithelial cells lining the alveoli. The epithelial cells also secrete anti-microbial compounds. Considerable tissue damage is observed once the immune effector cells begin to combat the invading pathogens.

If the infection persists, internal swelling within the mammary epithelium, not normally detectable by an external examination, can occur. The mammary gland alveoli become damaged and start losing anatomical integrity. The blood-milk barrier is breached, causing extracellular fluid components, such as chloride, sodium, hydrogen, potassium and hydroxide ions, and plasminogen to enter the gland and mix with the milk (Zhao and Lacasse 2008). When extensive damage to the blood-milk barrier has occurred, blood might be detected in the milk. This leads to visible changes on the udder, such as enhanced external swelling and reddening of the gland. Changes also occur in the milk, including increased conductivity, increased pH, raised water content and the presence of visible clots and flakes (Zhao and Lacasse 2008). This marks the initial stage of clinical symptoms, and the most severe infections might ultimately result in the
1.4.2. Host Immune response patterns

The ability of bacteria to establish such infection is determined in part by the nature and rapidity of the corresponding host innate immune response. Because the clearance of bacterial pathogens from the gland is often governed by responses that occur in the immediate hours and days after initial infection, the innate arm of the immune system represents the primary host determinant for dictating the outcome of intramammary infections.

A rapid acting and effective innate immune response is predicted on early recognition of pathogens (Akira et al., 2006). Innate immunity is initiated when specific pattern recognition receptors (PRR) on the surfaces or within host cells bind the particular bacterial molecules termed Pathogen-associated molecular patterns (PAMP). PRR are expressed on leucocytes in milk and on the epithelial cells lining the mammary gland (Strandberg et al., 2005; Wellnitz et al., 2012). The toll like receptor (TLR) group are the best characterized of these receptors with 13 types identified in mammals of which 10 are known to occur in cattle. Activation of the PRRs initiates signal transduction pathways that culminate in the transcription of a wide range of immune genes including cytokines, which are synthesised by infiltrating cells (Lee et al., 2006; Schukken et al., 2009) as well as resident cells in response to bacterial infection (Lahouassa et al., 2007) and have been shown to orchestrate both the local and the systemic immune response (Elazar et al., 2010, Fitzgerald et al., 2007 and Mitterhuemer et al., 2010).

Several different mediators of inflammation are expressed at different times after pathogen or stimulus exposure. Cytokines are an important group of inflammatory mediators. Pro-inflammatory cytokines promote inflammation quickly after the perception of the pathogen, anti-inflammatory cytokines suppress and confine the activity of proinflammatory cytokines. Chemokines recruit cellular factors of immune defense to the site of infection by facilitating the passage of leukocytes from the bloodstream into the tissues. The chronologically coordinated
induction of their synthesis at the site of inflammation is decisive for an effective inflammatory response, including pathogen clearance, wound healing, and return to the normal state. Disturbances in the well-balanced order and extent of the inflammatory response often lead to chronic inflammation and/or infection.

The extent of the inflammatory reaction after pathogen contact must be controlled and calibrated in this large organ ensuring a sufficient but not an overshooting immune response. Otherwise, uncontrolled release of inflammatory mediators might eventually cause systemic illness with sometimes even fatal consequences caused by septic shock, for example. The inflammatory process must therefore be tightly regulated by many immunomodulatory mechanisms (Serhan and Savill, 2005).

Such conditions emphasize the role of the regulatory mechanisms involved in controlling immune response. Recently biopsies of the mammary epithelium have revealed much about the regulation of genes involved in the host response to an IMI (Buitenhuis et al., 2011; Mitterhuemer et al., 2010; Genini et al., 2011). The mounting of an inflammation involves many different regulatory steps. How such regulatory mechanisms control the host pathogen interaction, remains a significant area to comprehend.

**1.4.3. Epigenetic reprogramming of host genes in bacterial pathogenesis**

Pathogens evolve sophisticated molecular strategies to disturb and subvert host defences (Mogensen 2009). The link between bacteria and host chromatin remodelling is an emerging area of research. The bacterial impact on host epigenetics is yet another strategy used by bacterial pathogens to interfere with key cellular processes. Unravelling how pathogens provoke host chromatin changes will provide new insights into host epigenetic regulation mechanisms (Hamon and Cossart 2008).

Recent studies highlight that bacteria can affect the chromatin structure and transcriptional program of host cells by influencing diverse epigenetic factors (i.e., histone modifications, DNA
methylation, chromatin-associated complexes, noncoding RNAs, and RNA splicing factors) Bacteria provoke histone modifications (Figure 1.3) and chromatin remodelling in infected cells thereby altering the hosts transcriptional program and in most cases dampening the host innate immune response (Hamon and Cossart 2008).

Figure 1.3. Nucleosome Structure and Histone Tail Modifications
(A) The arrangement of the eight histone proteins in the nucleosome is shown schematically. One hundred and forty-seven base pairs of DNA are wrapped around the histone core. Histone H1 seals the nucleosome separating each nucleosome unit from each other.

(B) Covalent modifications of histone tails as listed per histone. The sequences of the N-terminal tails of histones are shown with amino acid position indicated in gray underneath. Modifications shown above the sequence are associated with an activation of transcription and those indicated beneath are associated with transcriptional repression.

Figure Courtesy: Hamon and Cossart 2008
Bacterial-induced epigenetic deregulations may affect host cell function either to promote host defense or to allow pathogen persistence. Thus, pathogenic bacteria can be considered as potential epimutagens able to reshape the epigenome. Their effects might generate specific, long-lasting imprints on host cells, leading to a memory of infection that influences immunity and might be at the origin of unexplained diseases (Cossart et al., 2011). However relatively little information is currently available in mastitis, and we anticipate future studies addressing this issue will uncover their effects in pathogenesis and more importantly provide possible options for prevention and intervention.

In this battle of host-pathogen, recent developments in miRNA biology offer lucrative opportunities to better understand the disease. A flurry of recent reports have revealed a new world of RNAs that subvert the formula of central dogma: DNA makes RNA makes proteins and proteins are the cellular work horses that carry out all the crucial tasks, in which many small RNAs do not code for proteins but instead exercise control over those RNAs that do.

In recent years, the number of members in the RNA family has grown rapidly (Figure 1.4). Of these MicroRNAs (miRNAs/miRs) have engraved specific attention and have come into focus as powerful regulators of gene expression and fundamentally impact on the pathogenesis of different pathological events.

miRNAs are non-coding RNAs (~22 nucleotides) that lead to silencing of genetic information through post-transcriptional degradation of messenger-RNA and/or translational inhibition of protein expression. MiRNAs are highly conserved in different species and are thought to regulate at least 50% of the genome. MiRNAs are formed in a highly regulated process in the nucleus and are then transported into the cytosol, in which they are further processed (Figure 1.5).
Although the immune response is predominantly controlled at the transcriptional level, microRNAs-mediated RNA interference is emerging as an important regulatory mechanism. Numerous studies have underlined their critical importance for disease initiation and progression by influencing distinct disease-specific signal transduction pathways. As these small RNAs can control the transcription and translation of protein coding RNAs, many believe that they represent a newly discovered level of control over the workings of the genome. In addition to their involvement in a wide range of physiological and pathological processes, microRNAs are increasingly implicated in the eukaryotic response to bacterial pathogens. Recent studies have characterized changes in host microRNA expression following infection with Gram-negative bacteria. However, there is limited knowledge about cell expression and the regulatory role of microRNAs following other bacterial infections (Izar et al., 2012). Targeting non-coding genes such miRNAs, which have the capacity to regulate large sets of coding genes, represents the future of gene therapy (Sen et al., 2009). Hence focus on miRNA directed gene therapy seems prudent.
A comprehensive understanding of pathogen – host interactions in such intramammary infection is fundamental to devise wise strategy to combat mastitis.

Figure 1.5. The linear canonical pathway of microRNA processing. The miRNA processing pathway has long been viewed as linear and universal to all mammalian miRNAs. This canonical maturation includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 (Pasha) in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand is degraded.
1.5. Management of mastitis including treatment and vaccination

Several attempts and extensive research have been conducted over decades to fully describe the extent and nature of this problem worldwide but bovine mastitis still remains a complex disease and its management is an increasing challenge.

Typically when clinical mastitis is detected, the cow is milked out and then given an intramammary infusion of antibiotic directly into the infected gland. Clinical mastitis symptoms are recognized by the milker from detection of clots or flakes in the milk, from a cow that has a quarter sensitive to the touch, a quarter that is swollen or hot to the touch. However subclinical cases of mastitis remains unnoticed due to lack of any symptoms and hence ignored. Typically there is no treatment as such followed for subclinical mastitis.

Over the time indiscriminate usage of antibiotics has further resulted in resistance problem to many antibiotics. High attack rates and the ability of pathogens to develop resistance to all antibiotics in medical practice heighten the urgency of vaccine development (Daum and Spellberg 2012). However, despite ongoing efforts over the decades, no licensed S. aureus vaccine is currently available. Learning's from past clinical failures of vaccine candidates and a better understanding of the immunopathology of pathogen colonization and infection can aid the designing of new vaccine candidates (Anderson et al., 2012).

Over the past 125 years, mastitis has been one of the most studied conditions of dairy cows. This is confirmed by a search of peer reviewed literature covering this period, which revealed more that 12000 papers. Yet mastitis is still one of the most relevant and problematic diseases to treat and control in practice, which is testament to the complexity and mutability of the condition. Although progress has been made, recent research suggest that substantial areas require better understanding (Green and Bradley 2013). To attain any optimal therapeutic regimes and vaccine in mastitis, detailed elucidation of the underlying molecular events in this host pathogen interplay is fundamental.
In Indian context, research in mastitis is in incipient stage. At the current scenario, a systematic and coherent research approach on bovine mastitis will allow to shed some light to combat this existing challenge.

At the very outset, characterization of the pathogen *S. aureus* using molecular typing methods is crucial to identify the clones associated with the disease in the province. Further elucidation of the host immune response to this pathogen is essential. Until now, in mastitis research, the focus was majorly directed to specific pathogens, however strain directed infection response of host to *S. aureus* is insufficient. Although we know a great deal about *S. aureus* mastitis pathogenesis, all features of *S. aureus* which make this pathogen a successful parasite of the mammary gland have not been clearly identified. There is still a great deal to be learned about host-pathogen interaction of *S. aureus* in the context of bovine mastitis.

In this study, molecular characterization of *S. aureus* isolates obtained from subclinical mastitis milk was done to identify the predominant clones of *S. aureus* associated with the disease in the province. Subsequently, mice model was used to understand the time courses of the pathogen-specific reprogramming of the transcriptomes after challenge with two selected *S. aureus* strains found endemic in the province. To understand different layers of regulatory mechanisms, role of epigenetics associated with the host immune response was focussed. Concurrently the emerging role of microRNAs in mammalian host signalling and defence against bacterial pathogen was explored. We investigated different fractions of the complex transcriptome to facilitate our understanding of bacteria-host interaction and bacterial pathogenesis.
Objectives
1.6. OBJECTIVES

Staphylococcal mastitis is a complex disease. *Staphylococcus aureus* is a multifaceted pathogen which has the potential to express a myriad of virulence factors and is fully capable of evading immune surveillance and treatment compounds. These complexities are illustrated by the lack of efficacy of currently available vaccines and antimicrobial treatments. Being the leading pathogen causing mastitis, understanding of *S. aureus* pathobiology is of paramount importance. There is still lack of understanding of how strain-to-strain variations (bacterial genetics and virulence factors) and host-pathogen interactions lead to different clinical outcomes. To effectively combat this disease a multifaceted approach must be taken. Understanding of the diversity of *S. aureus* strains infecting dairy animals and subsequent understanding of molecular causes underpinning the pathogen specific immune response remains crucial to eventually develop innovative strategies to prevent and treat subclinical mastitis. This study aims to provide some insight into the interplay of pathogen and host as they interact in the mammary gland. This holistic approach to the understanding of the bovine immune response to *S. aureus* is anticipated to unravel some clue for therapeutic intervention.

Keeping in view the goal, the following objectives have been carried out in the study.

1. To characterize *S. aureus* isolates from subclinical mastitis milk by genotyping, toxin gene profiling and antibiotic sensitivity testing.
2. To induce intramammary infection (IMI) in mice model with selected strain of *S. aureus* and evaluate the immune gene expression in time course manner.
3. To investigate the alteration of mRNA levels by genome-wide gene expression profiling at selected time point.
4. To profile small RNA response to *S. aureus* IMI.
5. To study epigenetic alterations associated with host immune gene expression in *S. aureus* IMI.