Chapter II

REVIEW OF LITRATURE
2.1 Backdrop

Mastitis is a complex disease; there is no simple solution to its control. Some aspects are well understood and documented in the scientific literature. Others are controversial, and opinions are often presented as facts. To simplify understanding of the mastitis complex, it is useful to consider that three major factors are involved in this disease: the microorganisms as the causative agent, the cow as host, and the environment, which can influence both the cow and the microorganisms.

More than 100 different microorganisms can cause mastitis, and these vary greatly in the route by which they reach the cow and in the nature of the disease they cause. Cows contract udder infection at different ages and at different stages of the lactation cycle. Cows also vary in their ability to overcome an infection once it has been established. Therefore, the cow plays an active role in the development of mastitis.

The cows’ environment influences both the numbers and types of bacteria they are exposed to and their ability to resist these microorganisms. However, through appropriate management practices, the environment can be controlled to reduce this exposure and enhance resistance to udder disease (Schroeder, 1997).

Subclinical mastitis is subtle and more difficult to detect. The cow appears healthy, the udder does not show any signs of inflammation and the milk seems normal. However, microorganisms and white blood cells (somatic cells) that fight infections are found in elevated numbers in the milk. The cows that have subclinical mastitis are reservoirs of organisms that lead to infection to other cows. Most clinical cases start as subclinical; thus, controlling subclinical mastitis is the best way to reduce the clinical cases.

The incidence level of clinical and subclinical mastitis in various parts of the country ranged from 11.51 to 23.55%, 3.94 to 17.25% and 1.99 to 12.28% (Shinde et al., 2001) in crossbred cows, local cows, and buffaloes, respectively. Joshi and Gokhale (2006) studied the prevalence of mastitis in Indian cattle and buffaloes and reported that
subclinical mastitis was found more important (varying from 10–50% in cows and 5–20% in buffaloes) than clinical mastitis (1–10%). The incidence was highest in purebred Holsteins and Jerseys and lowest in local cattle and buffaloes. The incidence level of clinical and subclinical mastitis in various parts of the country ranged from 11.51% (Sethi and Balaine, 1978) to 23.55%, (Taneja et al., 1989) 3.94% to 17.25%, (Sethi and Balaine, 1978) and 1.99% (Kumar, 1988) to 12.28% in crossbred cows, local cows, and buffaloes, respectively.

2.2 Economic losses

Annual losses in the dairy industry due mastitis was approximately 2 billion dollars in USA and 526 million dollars in India, subclinical mastitis and clinical mastitis are responsible for approximately 70% and 30% respectively of dollars losses (Varshney et al., 2004). These estimates do not include additional costs arising from mastitis-associated problems related to antibiotic residues in human foods, milk quality control, dairy manufacturing, nutritional quality of milk, degrading of milk supplies due to high bacteria or somatic cell counts, and interference with genetic improvement of dairy animals.

As per the field survey conducted by the veterinary dispensary, Somenahalli, Kolar district, Karnataka shows that, data on 305 clinical mastitis cases (88 fresh cases and 217 repeat cases) recorded over a period of one year from April 2006 to March 2007. Average number of treatment days per clinical mastitis case was obtained by dividing total cases by fresh cases, which was 3.64 days per clinical mastitis case. Production loss included decrease in milk production during infection and discarding of the milk during and few days post-infection based on the antibiotics used, but discarding of milk post infection is not being practiced (even though it is advocated), hence was not considered here. Various antibiotics, analgesics, anti inflammatory drugs and intramammary infusions were used for treating clinical mastitis cases. Average per day cost of treating clinical mastitis worked out to be Rs. 73.00. Veterinarian and labour charges were excluded as cases were treated by government doctors, and farmers themselves were both the owners and laborers for the animals. On an average 1.3 quarters were affected in mastitis infected animals and 7% of the mastitis affected
quarters ended up in complete fibrosis. Culling of mastitis animals is rarely practiced, but animal with fibrosis udder is a definite loss to the farmers. As per market opinion, a complete fibrosis of one quarter causes on an average decrease in animals’ market value by Rs. 4000, 2000, and 2500 for crossbred, indigenous cows and buffaloes, respectively. Based on population statistics as per 17th livestock census (2003) and incidence of clinical mastitis obtained by field survey, the economic loss per annum due to clinical mastitis in Kolar district was estimated to be Rs. 1.48 crores (Naveen Kumar et al., 2010).

Mastitis is of great economic importance to milk producers, because the disease has negative impact on several important aspects of cow and herd performance. Incurred costs are of both direct and indirect nature (Kossaibati and Esslemont, 1997). Direct costs include veterinary costs, increased labour requirement, discarded milk (during the course of treatment), and reduced milk yield and quality. Indirect costs are those that are not always obvious to the milk producer, and are therefore referred to as hidden costs. They include increased risk of subsequent disorders, reduced fertility, increased risk of culling and occasionally, mortality. The total cost of mastitis can, consequently, be much higher than the direct cost (Kossaibati and Esslemont, 1997). The cost associated with each component is likely to vary between herds; partly because of differences in performance parameters (yield level, fertility, etc.) and partly because of different preferences of farmers influencing, for instance, their inclination to contact a veterinarian when mastitis is detected.

2.2.1 Discarded milk

Milk produced when a cow shows signs of mastitis, or while a cow is treated with antibiotics, is discarded. The withdrawal period includes the days when a cow actually receives drugs and a waiting time, usually consisting of some additional days, when there is a risk of antibiotic residues in the milk. The length of the withdrawal period depends on the production system (i.e. conventional or organic), and the drug used. The cost of discarded milk is comparable to that of milk loss, but with one important difference: discarded milk is produced by the cow and is therefore associated
with feed costs. The cost per unit of discarded milk is thus higher than the corresponding cost of milk not produced (Hogeveen and Osteras, 2005; Halasa et al., 2007).

2.2.2 Extra Labour

Clinical mastitis (CM) is associated with extra labour requirement, for instance in form of attendance of the visit by the veterinarian and administration of medicine. Also, CM may affect the order in which cows are milked, and thus gives rise to less efficient milking routines. The time requirement associated with a case of CM is likely to amount to two hours. The amount of time needed to treat Sub-clinical mastitis (SCM) can be expected to be less than that associated with CM, because SCM is not always detected, and, when detected, is not always treated. Extra labour requirement should be valued based on the opportunity cost of labour, i.e. the value of the next best alternative foregone as the result of having to assign time to mastitis. Opportunity cost of labour in agriculture is often difficult to assess, and is likely to differ between farms (Hogeveen and Osteras, 2005; Halasa et al., 2007).

2.2.3 Culling

Clinical mastitis increases the risk of culling (Grohn et al., 1998; Rajala-Schultz and Gröhn, 1999a; Schneider et al., 2007), as well as mortality (Bar et al., 2008a). The extent to which CM affects the risk of culling depends on lactation stage at clinical onset (Dohoo and Martin, 1984b; Beaudeau et al., 1995; Schneider et al., 2007). SCC above 3,00,000 cells/ml has been reported to increase the risk of culling in primiparous cows (Beaudeau et al., 1995), and in late lactation, SCM is the most important disease influencing culling decisions regardless of parity of the cow (Dohoo and Martin, 1984b).

2.2.4 Other Effects

Any kind of pathology involves some degree of poor animal welfare (Broom, 2006). Mastitis is a very painful condition and is one of the major welfare problems of dairy cows (Webster, 1999; Broom and Fraser, 2007). In European countries, there is a high level of consumer concern for animal welfare (Moynagh, 2000; Harper and Henson, 2001), which results in major public demand for improvements in animal
welfare. Indeed, consumers are prepared to pay considerably more for welfare-friendly production practices (Moynagh, 2000). If milk is produced from cows with high incidence of mastitis, consumers’ acceptance of dairy production, and thereby their willingness to buy dairy products, may be adversely affected.

2.2.5 Economic Assessment of Mastitis

The total economic cost of disease consists of two distinct components; production loss and control expenditures (McInerney et al., 1992). Losses include benefits that are taken away and benefits that are not realized. The former can be exemplified by milk that must be discarded following treatment with antibiotics and the latter by milk that is never produced as a result of disease. Expenditures are extra inputs needed to limit losses, either by reducing the impact of an unplanned event, such as treatment of a mastitic cow, or by preventing such events from occurring, as in the case of investments into preventive measures. Mastitis control can be practiced at different levels; udder quarter, cow, herd, or national. The cost of mastitis can be estimated at all of these levels, and the level of choice depends on the nature of the decision that is to be supported.

At udder-quarter level, decisions are concerned with whether or not to dry off an infected udder quarter. Cow-level decisions are directed at managing occurrences of mastitis, and the options are no treatment, treatment or culling. Treatment decisions impact also on herd level, as treatment reduces spread of infection to healthy cows. In the same way, culling might serve to reduce the overall incidence of mastitis in the herd. Mastitis control at herd level aims at reducing the incidence of mastitis, and consists of various proactive and reactive measures. Information on the national consequences of mastitis is needed to answer whether subsidized veterinary services and targeted research are necessary in order to reduce the incidence of mastitis.

The level at which the impact of mastitis has been estimated obviously affects the results. Even though it has been suggested that the herd-level cost of mastitis can be obtained by aggregating the costs at cow level, this might impose bias on the results. Several of the direct costs of mastitis, such as treatment, discarded milk, increased labour and decreased milk production, can readily be assessed in individual cows.
Indirect consequences, however, frequently arise through herd dynamics, and often reflect management decisions taken by the farmer. Increased risk of culling and thus increased replacement costs, as well as penalties or loss of premiums connected with increased SCC in the bulk tank milk, are good examples of dynamic factors. The decision to cull a mastitic cow, in order to increase the average milk yield per cow and decrease SCC, incurs a replacement cost, and illustrates the way in which dynamics within the herd impact on herd level economy. Some herd-level effects can therefore not be assessed simply by summing up the cow-level effects, and this type of dynamics requires the economic impact of mastitis to be addressed at herd level (Seegers et al., 2003).

2.3 Role of somatic cells in bovine mastitis

Somatic cell count (SCC) testing in milk is the most sensitive methods for measurement of infection of bovine mammary glands. Somatic cell count presents a fast and reliable analytical tool. It is related to the immunological status of the udder and increases in response to an inflammatory stimulus like bacterial infection (O’Brien et al., 1999). Therefore, SCC is a widely used indicator for udder health and milk quality.

Somatic cells are a part of the natural defense mechanism and include lymphocytes, macrophages, polymorph-nuclear cells and some epithelial cells (Pillai et al., 2001). Somatic cells are therefore a reflection of the inflammatory response to an intramammary infection or another trigger of the immune system. Somatic cell count, or a parameter derived from this count, is often used to distinguish between infected and uninfected quarters. Somatic cells are mostly cells of the immune system (80% in uninfected quarters, 99% in mastitic quarters) (Sordillo et al., 1997). However, SCC cannot distinguish between the type of cells present in milk, and SCC varies with time and frequency of milking, stage of lactation, and season (Corbett, 1998; Kelly et al., 2000). Somatic cell count varies somewhat according to milking frequency, lactational stage, age and nutrition (Dohoo et al., 1984; Kelly et al., 2000). Somatic cell count measurement includes all types of cells in milk; the number and the distribution of lymphocytes, macrophages, PMNL, and epithelial cells depend on the immunological status of the mammary gland (Kehrli and Schuster, 1994; Kelly et al., 2000). In milk
from healthy udders, macrophages represent the major cell fraction (Burvenich et al., 1994; Paape et al., 2002; Sarikaya et al., 2004), and release chemo attractants such as tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) after contact with a pathogen (Hoeben et al., 2000; Wittman et al., 2002). This stimulus causes a rapid immigration of PMNL into the milk. Therefore, in mastitic milk, PMNL become the predominant cell fraction (Kehrli and Schuster, 1994). A number of mastitis detection methods have been developed previously with varying degrees of accuracy and automation based on electrical conductivity, milk yield, and milk temperature (de Mol et al., 1999; de Mol and Ouweltjes, 2001; de Mol and Woldt, 2001). However, these methods lack the combined time-series measurements of an indicator with other known mastitis predisposing factors of direct physiological relevance to the status of udder health throughout the lactation (Chagunda et al., 2006). Somatic cell count is related to the immunological status of the udder and increases in response to an inflammatory stimulus like bacterial infection (O’Brien et al., 1999; Leitner et al., 2000). The golden standard to measure inflammation is the cytological investigation; milk SCC (Hamann, 2002). The diagnosis of mastitis according to the International Dairy Federation (IDF) recommendations is based on the SCC and microbiological status of the quarter (Hillerton, 1999 and International Dairy federation, 1971). Clinical mastitis is easy to detect but also cows with subclinical mastitis should be identified. Bacteriological sampling is not feasible as a routine test to detect mastitis. Tests for indicators of inflammation are therefore necessary as screening tests to identify quarters with intramammary infections (IMI) and to select cows for subsequent bacteriological sampling (Ruegg and Reinemann, 2002). Although clinical signs of mastitis usually are not readily apparent until the SCC reaches 4 or 5 million cells/ml (Schalm et al., 1964) but observations of clots, swelling, or watery secretion have been reported for quarter milk as few as 1-2 million cells/ml.

Pillai et al. (2001) evaluated differential inflammatory cell count assay with individual quarter milk samples from 13 cows using flow cytometry. Cows were sampled at weekly intervals for 3 weeks and assayed for total cell count, mononuclear leukocyte count and polymorph-nuclear leukocyte count. Simultaneously, milk samples were evaluated by the conventional electronic SCC technique. Somatic cell count
positively correlated with total cell count \((r = 0.9)\), mononuclear leukocyte count \((r = 0.8)\), and polymorph-nuclear leukocyte count \((r = 0.89)\). Quarters with SCC > \(\log_{10} 5.4\) had a higher total cell count, mononuclear leukocyte count, and polymorph-nuclear leukocyte count and were more often culture positive compared with quarters with SCC < \(\log_{10} 5.4\). Quarters that were culture positive on all three test occasions had a higher proportion of polymorph-nuclear leukocytes (33 to 49%) compared with quarters that were culture negative on all three test occasions (17 to 25%).

Riollet et al. (2001) defined changes in peripheral blood and milk cell subpopulations during chronic \textit{S. aureus} infection. The expression of specific antigens on the surface of lymphocytes and neutrophils was studied by flow cytometry. Cytokines and cytokine transcripts elaborated by the milk-derived cells were also investigated, using ELISA and reverse transcription polymerase chain reaction, respectively. The results indicated that cell subpopulations in blood from infected cows were not modified. In contrast, changes occurred in infected milk: neutrophils were the main cell population, but they were not in a highly activated state; the CD8+ T-lymphocytes were mainly recruited compared with the CD4+ T-lymphocytes, suggesting that CD8+ T-lymphocytes play an important role in chronic \textit{S. aureus} infection. Also, the proportion of the B-lymphocytes among the total lymphocyte population was increased, suggesting that a humoral response developed, and no change was observed in the \(\gamma\delta\) subset.

Cows without mastitis should have SCC of less than 100,000. Cows infected with unimportant organisms have counts between 100,000 and 300,000 cells/ml. Cows with SCC of greater than 300,000 are most likely infected. However, most authorities have agreed that an appropriate threshold value to divide uninfected cows from infected cows would be approximately 200,000 cells/ml. It appears that as long as stage of lactation, age, season and series of counts have been considered, the level of 200,000 cells/ml produce a reasonable success rate in classification.

2.4 Factors affecting somatic cell counts

The factors affecting SCC are as follows
2.4.1 Udder infection

The most important factor affecting the SCC of an individual cow is the infection status of her udder. General agreement rests on the values of less than 100,000 cells/ml for uninfected cows and greater than 300,000 for cows infected with significant pathogens such as *S. aureus* or *S. agalactia*. Cows with SCC between these values may have recovered from an infection, sustained an injury or be infected with a less important organism such as *C. bovis*.

2.4.2 Teat or udder injury

Somatic cells consist primarily of leukocytes (white blood cells) that are present in the udder in response to infection and to repair damaged tissue. Somatic cells also include epithelial cells which make up the internal lining of the mammary gland tissue and are normally replaced during the events of lactation. When the udder or teat is severely injured there are large increases in SCC. Some elevation in counts in these cases is in response to the increased prevalence of mastitis with injury.

2.4.3 Number of quarters with mastitis

The dilution of high cell count milk from infected quarters with low cell count milk from uninfected quarters can be an important consideration in the interpretation of individual cow SCC. One cow has one-quarter infected combined with high milk production, while other has two-quarters infected and lower milk production, therefore higher somatic cell counts.

2.4.4 Age

Higher SCC has been found in the milk of older cows. This is primarily due to an increased prevalence of mastitis in older cows. It may also be the result of a greater cellular response to infection or of a greater amount of permanent udder damage after infection in older cows. For example, consider two cows from the same herd infected with *S. agalactiae* and treated in the same month. Cow A, a first lactation heifer, has a high SCC and a good response after treatment of the infection. Cow B, a fourth lactation cow with a very high SCC, has a slower reduction in somatic cell count after treatment.
2.4.5 Stage of lactation

Somatic cell counts are elevated immediately after calving and remain elevated for up to two weeks. Interpreting increases in SCC early in lactation as evidence of mastitis must be done with caution. The counts of cows late in lactation are higher than the average throughout lactation, but this is due to an increased prevalence of subclinical infections in late lactation. When cows are not infected, there is no change in SCC due to stage of lactation or daily milk yield. Some cows exhibited increased cell count at the end of lactation without having mastitis, but it only occurs immediately before drying off or after milk production has dropped below 4 kg/day. For example, cow A was sampled three days before going dry after a long lactation. Cow B was sampled seven days after calving. She had blood-tinged milk and considerable udder edema. There is no significance in the moderate increases in the SCCs of these cows.

2.4.6 Season

Somatic cell count results reflect seasonal variations. Counts are lowest during the winter and highest during the summer months of July and August. The reasons for these seasonal variations are, as yet, unknown and only speculated to be the effects of housing and temperature changes on infection status.

2.4.7 Stress

Changes such as isolation of an individual, mixing groups of cows or being chased by a dog have been shown to increase SCC in the absence of mastitis. However, it has been reported that there was no increase in SCC associated with cows being in heat.

2.4.8 Day to day variation

There can be considerable differences in SCC from individual cows from test day to test day and even if samples are taken on successive days. It has been suggested that this is a normal physiological variation. However, other suggestions are that these periodic large increases in cell counts are due to stress or injury infections that were
eliminated before being detected. Regardless of the reasons for the variations it is advantageous to study at least the last five counts and the lactation average in making an interpretation with respect to an individual cow. Some veterinarians request their clients to construct a chart that will show a cow’s SCC results for the entire lactation before making important management decisions. Cow A, a third lactation cow, has had a consistently low SCC and no problems with udder health. However, on one test day her SCC was moderately increased. Culture and sensitivity tests were negative for growth of mastitis-causing organisms so the elevated SCC was attributed to normal variation. No treatment was given and normal counts resumed.

### 2.4.9 Technical factors

The methods of transportation, storage, and electronic counting of the milk sample all can have an influence on the resultant values. Different labs use slightly different testing machines and may find differing values on the same milk sample, especially when the counts are very low. However, these minor differences are relatively unimportant provided there is consistency in the handling, and processing of samples.

### 2.4.10 Management factors

Mastitis control procedures such as teat dipping, dry cow treatment, milking machine maintenance, and the use of single service paper towels have all been useful in reducing SCC. These are secondary effects manifested through the elimination of existing cases of mastitis and the prevention of new udder infections. A control program that includes selection of lactating cows for treatment solely on the basis of high SCC would have several drawbacks. Depending on the level of cell count used to decide treatment was indicated, many uninfected cows might be treated. There would be many infections treated with antibiotics to which the organism is resistant.

### 2.5 Infectious organisms

#### 2.5.1 Bacterial mastitis

Mastitis can have an infectious or non-infectious etiology, and the infectious pathogenicity is the most important ones that frequently due to infection by one and/or the
other pathogens, such as bacteria, viruses, mycoplasma, yeasts and algae (Watts, 1988; Malinowski et al., 2006; Chaneton et al., 2008; Osumi et al., 2008; Wellenberg et al., 2002). Fortunately the vast majority of mastitis is of bacterial origin and just a few of species of bacteria account for most cases, such as *E. coli*, *S. aureus*, *S. uberis*, *S. dysgalactiae* and *S. agalactiae* (Dogan et al., 2006; Varella et al., 2007; Aouay et al., 2008; Chaneton et al., 2008; Kuang et al., 2009). The data Enterobacteriaceae were the commonest cause responsible for 40.9% of all mastitis, and *S. aureus*, *S. dysgalactiae*, *S. agalactiae* accounted for only 10% of clinical cases (Bradley, 2002).

Five bacterial species, *S. aureus*, *S. dysgalactiae*, *S. agalactiae*, *S. uberis*, and *E. coli* are responsible for the bulk of bovine mastitis cases. *S. aureus*, *S. dysgalactiae*, and *S. agalactiae* exhibit a contagious route of transmission, whereas *S. uberis* and *E. coli* are considered to be environmental agents. The biggest challenge facing the modern dairy industry is the pressure to reduce the incidence of mastitis, and the extensive investigation and research of mastitis etiology may be capable of helping to provide an important and optimistic approach to control this disease.

A research by DaRong and co workers (2010) confirmed the prevalence of the five vast major species of bacteria in part region of China, and also found *S. epidermidis* and *S. saprophyticus*, previously considered as naught pathogenic bacteria, were existed in the diseased mammary gland of the cows. It revealed that *E. coli* was the commonest organism in most mastitis cases, and *S. uberis* was continues to be a prevalent pathogen closely followed by *S. aureus*, *S. dysgalactiae* and *S. agalactiae*. In addition, *S. uberis* and *S. aureus* were more frequently associated with clinical mastitis than sub-clinical case, while the infection rates of *E. coli*, *S. dysgalactiae*, *S. agalactiae*, *S. epidermidis* and *S. saprophyticus* were similar (DaRong et al., 2010). Some published work has shown that 3 percent of all animals are infected with *S. aureus* (Schukken et al., 2009). However, *S. aureus* represents 10 to 12 percent of all clinical mastitis infections (Tenhagen et al., 2009). In other studies researchers found that Staphylococci are the most frequently isolated microorganisms from bovine mammary glands of cattle and buffaloes (Watts, 1988; Kupur et al., 1992). The genus Staphylococcus consists of 28 species (Birgerssen et al., 1992), 14 of which have been isolated from the bovine udder (Watts, 1988). In most countries, *S. aureus* is the predominant cause of subclinical
mastitis (Singh and Buxi, 1982) and is also frequently isolated from the clinical cases (Kupur et al., 1992). These isolates show haemolytic activity on sheep blood agar, Deoxyribo-nuclease activity and coagulase activity (Patrick et al., 2002). The frequent association of these organisms with increased SCC in milk with considerable production losses (Timms and Schultz, 1987; Rainard et al., 1990), as well as their occasional association with clinical mastitis, has spawned considerable interest in these organisms. Interestingly, cows infected with *S. aureus* do not necessarily have elevated SCC. During 1978-1980, nearly 27,000 milk samples from 28 herds were aseptically collected. Culture results showed 10% of cows were infected with *S. aureus* (Jones et al., 1984). Heifers are also a reservoir for *S. aureus* infections. In several research trials, 12 to 15% of first-lactation cows were found infected with *S. aureus* at calving (Boddie et al., 1987; Trinidad et al., 1990). Furthermore, infected heifers left untreated produce 10% less milk in early lactation when compared with those who received dry cow antibiotic treatment prior to calving (Owens et al., 1991).

In addition, multiple microbial infections (82%) were more prevalent in bovine mastitis in five dairy herds. Staphylococcus and Streptococcus, considered being the major contagious pathogens of bovine mastitis, frequently combined and mixed infection with *E. coli*. No single case infected with *S. epidermids* or *S. saprophyticus* was found. Furthermore, there are 7% cases were no *E. coli*, Staphylococcus and Streptococcus was identified, which may be caused by other pathogens, such as viruses, mycoplasma, yeasts and algae (DaRong et al., 2010).

Other pathogens such as mycoplasma, yeasts and moulds are difficult to cultivate. But these agents cannot be the explanation for all culture-negative milk samples from mastitis cows, because these agents are no common udder pathogens (Pfutzner, 1994; Wendt, 1994). Despite intensive aetiological research, still around 20–35% of clinical cases of bovine mastitis have an unknown aetiology (Miltenburg et al., 1996; Wedderkopp, 1997). Due to the high percentages of unknown causes of mastitis, it is obvious to study the role of viruses in the aetiology of bovine mastitis. This in spite of the fact that viruses are generally considered not to play an important role. Watts (1988), identified 137 microbial species as causative agents of bovine mastitis, including agents involved in its pathogenesis. However, viruses were not included.
The reasons for this negligence could be manifold. Historically, mastitis research has concentrated on bacterial pathogens. In case of viral infections, signs of mastitis may not have been recognized because other clinical signs were more prominent. Subclinical mastitis cases are often not diagnosed and consequently their aetiology is not investigated. This may cause an underestimation of virus infections involved in bovine subclinical mastitis. Bovine Herpes Virus (BHV)1 (Gourlay et al., 1974; Roberts et al., 1974), BHV4 (Wellenberg et al., 2000), Foot-and-Mouth Disease (FMD) virus (Burrows et al., 1971), and Para-Influenza 3 (PI3) virus (Kawakami et al., 1966a,b) have been detected in milk from cows with clinical mastitis. A few other viral infections have been associated with bovine mastitis. For example, mastitis, which may be secondary, has been attributed to a systemic virus disease such as malignant catarrhal fever (Beckman et al., 1960). This report suggests that severe lesions in the mammary gland may account for a decline in milk production and cracking of the epithelium of the teats. However, this is the only report on any possible relation between malignant catarrhal fever and mastitis. In addition to viruses that cause teat lesions, other viral infections may induce or enhance bovine mastitis due to their immunosuppressive effects. Although, so far there is no any clear evidence for this. However, the detection of virus in milk from cows with mastitis obviously does not prove that these agents are the cause of mastitis, or that they are involved in an indirect way.

2.5.2 Mycoplasma mastitis

Mycoplasmas are distinguished phenotypically from other bacteria by their minute size and total lack of a cell wall. Taxonomically, the lack of cell walls is used to separate mycoplasma from other bacteria in a class named Mollicutes (mollis, soft; cutis, skin, in Latin). Several species of mycoplasma have been associated with mastitis (M. bovis, M. californicum, M. canadense, M. bovigenitalium, M. alkalescens, M. arginini, M. bovihirnis and M. dispar) (Kumar and Garg, 1991). As the causative agent of mycoplasmal mastitis, Mycoplasma bovis causes considerable economic losses to the dairy industry (Brown et al., 1990; Gonzalez et al., 1992). One hundred colony-forming units can colonize the udder and cause disease (Bennett and Jasper, 1980), and the incubation period for M. bovis induced disease can last from 2 to 6 days, during which
time shedding may occur (Jasper, 1981). The highly virulent and pathogenic nature of *M. bovis* creates a demand for the rapid identification of infected animals, because identification during early stage of infection results in lower overall impact on the herd (Feenstra et al., 1991). The losses due to bovine mastitis caused by *M. bovis* may be higher than that for respiratory disease with estimates from the USA of up to $108 million per year (Rosengarten and Citti, 1999).

The prevalence of mammary infection due to *M. bovis* has been studied using epidemiological investigations of bulk tank milk in New Zealand (McDonald et al., 2009), Prince Edward Island (Olde Riekerink et al., 2006), Australia (Jelinek et al., 1993), and the USA (Kirk et al., 1997), with the prevalence varying from 0% (New Zealand, Australia) to 3% (California). In France, *M. bovis* is frequently associated with respiratory disease in unweaned (Arcangioli et al., 2008) and weaned calves, but has rarely been identified as the cause of mastitis in dairy cattle. The prevalence of *M. bovis* infection in the population of dairy cows in France is even less well-known. The only available data come from a regional serological survey carried out in 1990 on 90 herds from seven departments; only 10% of the cows sampled were seropositive for *M. bovis* (Poumarat et al., 1991).

### 2.5.3 Fungal mastitis

The incidence of mastitis due to fungi is usually very low in dairy herds, but sometimes it can occur in epizootic proportions (Gonzalez, 1996). Several species of yeast have been reported in many countries as causative of mastitis (Richard et al., 1980; Jensen and Aalback, 1995; Elad et al., 1995; Lagneau et al., 1996). Fungal infection of the mammary gland is predominantly caused by yeast of the genus Candida (Watts, 1988). Other genera like Cryptococcus, Pichia and Trichosporon, albeit found in low prevalence, have also been isolated from clinical cases with reports of 1-12% of prevalence (Krukowski and Saba, 2003). Fungi are not an unusual agent in bovine mastitis and are usually considered an environmental mastitis due to poor animal hygiene (Sneena and Siegler, 1995). Bovine mycotic mastitis was reported (Malinowaski and Krzyzhanwaski, 1982; Costa et al., 1993) to be responsible for 1–12% of all mastitis cases (in Poland about 2–9%). In a study conducted by Henryk and co-
workers (2000) shows an occurrence of 9.6% of yeast isolated from mammary secretions of cows with mastitis. Similar results were also obtained by Malinowski and Krzyzanowski in Poland (1982). In small-type farms in the Lublin region, the incidence of mastitis due to yeast sometimes occurred after intramammary infusion of antibiotics. It was found that fungal mastitis appeared mostly after antibiotic treatment (large doses), often without microbiological examinations of milk from the affected quarters or after infusion of antibiotics that were often homemade (Henryk et al., 2000). The large doses of antibiotics may cause a reduction in the vitamin A, leading to injury to the udder’s epithelium, thus facilitating the invasion of fungi (Kauker, 1955). Teat injuries may facilitate a yeast infection too (Gonzalez, 1996). Some intramammary yeast infections may result in death of affected animals (Tucker, 1954; Gonzalez, 1996).

The great majority of yeasts are considered saprobic, though in some cases they are considered as potentially pathogenic (Chengappa et al., 1984). In a study carried out using 2078 milk samples from healthy and infected animals, 10% (208) corresponded to yeasts, being 3.2% (66) of the genus Candida (Costa et al., 1993). In relation to outbreaks of bovine mastitis, some reports point a non-albicans yeast cause, represented by species such as Candida tropicalis, Candida guilliermondii, Candida lusitaniae, Candida kefyr, Candida rugosa, Candida catenulata, Candida zeylanoides, Candida lambica and Candida inconspicua (Crawshaw et al., 2005; Santos and Marin, 2005). Cryptococcus neoformans, which is considered the most dangerous species, was isolated in several cases of clinical and subclinical mastitis as well as from the milk of healthy cows (Ebrahimi and Nikookhah, 2002). Other authors reported the presence of Cryptococcus laurentii and Cryptococcus curvatus in cases of mastitis (Klimaite et al., 2003) and in tanks of milk storage (Swinne et al., 1997). Prototheca spp. has been also isolated from mixed infections with yeasts from affected quarters (Krukowski et al., 2006).

G. candidum is an opportunistic, keratinophilic yeast-like fungus that is widely distributed in nature, i.e. soil, fodder, etc. Samborski et al. (1983) isolated G. candidum from placenta of 2.8% aborted cows in Poland. However, there are only a very few reports from around the entire world regarding its incrimination with bovine mastitis. Mishra and Panda (1986) found only one case of G. candidum out of 135 cases of
mastitis in Orissa State, India. Similarly, Costa et al. (1993) detected only one mastitis milk sample positive for *G. candidum* among a total of 2078 samples screened in Sao Paulo, Brazil. It has been documented that association of *G. candidum* with mastitis is greater in those patients which have been subjected to prolonged irrational antibiotic therapy, as is evident in this case (Rajesh et al., 2001).

The presence of yeasts and yeast-like fungi may trigger alterations in the milk and dairy products due to the release of extracellular enzymes such as lipases and proteinases (Chen et al., 2003), which affect the quality and organoleptic characteristics influencing the shelf-life of the product. The yeasts found in bovine milk may be part of the normal microbiota or might cause damage to the mammary gland (Costa et al., 1993; Spanamberg et al., 2004). The diversity of yeasts and yeast like fungi might be influenced by the type of management system employed in the milk farms. Although it is not expected that these microorganisms can survive the thermal treatment, milk may be a carrier for a great diversity of agents that could be harmful to public health.

### 2.5.4 Algal mastitis

The genus *Prototheca* comprises a group of chlorophyll- lacking algae. These microorganisms are widespread throughout different environments, but are found most frequently in those with high humidity and organic matter, particularly in damp areas contaminated with manure (Pore et al., 1983). *Prototheca* spp. was first described as a cause of mastitis by Lerch in 1952. Since then *Prototheca zopfii* have been isolated from clinical and subclinical cases of mastitis in many countries (Janosi et al., 2001; Leslie et al., 2001; Pengov, 2001). In animals, protothecosis presents primarily as clinical and subclinical bovine mastitis (Aalbaek et al., 1994; Costa et al., 1996). In recent years, the number of reports of bovine mastitis caused by this agent has increased (Almeraya, 1994).

The occurrence of bovine mastitis due to *Prototheca* spp. has been described by several authors who studied different features: epidemiology (Costa et al., 1994) clinical aspects (Kirk, 1991) susceptibility to antimicrobials (Segal et al., 1976; Mcdonald et al., 1984) and histopathology (Furuoka et al., 1989). The detection of mastitis caused by *Prototheca* spp. indicates a serious problem which can affect an entire herd. Infected
cows usually have a marked decrease in milk production and granulomatous changes in mammary tissue may occur (Cheville et al., 1984; Mcdonald et al., 1984; Furuoka et al., 1989). These microorganisms do not respond to routine therapy, resulting in the elimination of the infected animals as the best method to control the disease (Kirk, 1991). The transmission of the infection caused by Prototheca spp. usually occurs by means of direct contact. The possibility of mechanical transmission by insects and fomites cannot be disregarded. The microorganism can be passed mainly through milk and faeces (Pore et al., 1983).

Mastitis in cows is mostly caused by P. zopfii and sometimes by Prototheca wickerhamii (Gonzalez, 1996). These algae are considered as the environmental pathogens of mastitis (Costa et al., 1998). Protothecal mastitis occurs worldwide and appears sporadically in a therapy-resistant form (Corbellini et al., 2001). P. zopfii infection usually results in a chronic subclinical or mild clinical inflammatory process in the udder and is followed by a dramatic loss in milk production and a permanent increase in somatic cell count (Janosi et al., 2001; Leslie et al., 2001). From literature it is known that algae can cause chronic mastitis, difficult to diagnose and treatment. It seems, that udder inflammations caused by P. zopfii occurred in the Bydgoszcz region earlier but they could be inadequately diagnosed as fungal mastitis or missed, because the colony morphology of Prototheca spp. is indistinguishable from that of yeasts (Pengov, 2001; Tenhagen et al., 2001). So far, no suitable serological test for the identification of infected animals is available for routine diagnosis (Roesler et al., 2001).

2.6 Metagenomics- Culture-independent insight

A variety of pathogens can establish chronic infection that only occasionally manifests clinical signs of mastitis. The primary focus of most subclinical mastitis programmes is to reduce the prevalence of contagious pathogens viz. S. agalactiae, S. aureus and other Gram-positive cocci, most notably S. dysgalactiae (which may be contagious or be environmentally acquired) and environmental pathogens including S. uberis, Enterococcus and numerous other coagulase-negative staphylococci, including S. hyicus, S. epidermidis, S. xylosus and S. intermedius. This list is not complete and organisms that cannot be cultured by standard culture conditions may escape
notification. Metagenomic study provides an opportunity to record such pathogens that can help in planning effective therapeutic and preventive measures.

The global microbial diversity presents an enormous, largely untapped genetic and biological pool (Cowan, 2000). In spite of their obvious importance, very little is known about environmental microbes or their diversity; for example, how many species are present in the environment and what their ecological functions are (Singh et al., 2008). Until recently, there were no appropriate techniques available to answer such questions because of the limitations encountered in the culturing of microbes; traditional methods of culturing micro-organisms only detect those organisms that grow under laboratory conditions (Hugenholtz et al., 1998; Rondon et al., 2000). It is widely accepted that up to 99% of the microbes in the environment cannot be readily cultivated (Kamagata and Tamaki, 2005; Sekiguchi, 2006). To overcome these difficulties and limitations associated with cultivation techniques, different DNA-based molecular methods have been developed for characterizing microbial species and assemblages, and these have significantly influenced our understanding of microbial diversity and ecology (Delong, 2005).

Massively parallel pyrosequencing- a next-generation sequencing technique- is a new molecular approach that allows for extensive sequencing of microbial populations in a high-throughput, cost-effective manner (Ronaghi et al., 1998; Von Bubnoff, 2008). This technique has been successfully applied to determine bacterial diversity within various environmental ecosystems, such as hydrothermal vents of a deep marine biosphere (Sogin et al., 2006; Huber et al., 2007) and soil (Roesch et al., 2007). Within the human body, vaginal microflora (Sundquist et al., 2007) and bacteria of chronic wounds (Dowd et al., 2008) have been assessed by this approach, but we are not aware of reports on the oral microbial population.

Although much of metagenomics focuses on bacteria (especially the 16S rRNA gene), the field is expanding rapidly to encompass the entire spectrum of organisms in an environmental sample that includes bacteria, archaea, viruses, small eukaryotes, plasmids and short RNAs (William, 2010). In general, methods based on 16S rRNA gene analysis provide extensive information about the taxa and species present in an environment; however, these data usually provide only little, if any, information about
the functional role of different microbes within the community and the genetic information they contain about microbial niches (Streit and Schmitz, 2004). Thus, Metagenomics is a rapidly growing field of research that aims to study uncultured organisms to understand the true diversity of microbes, their functions, cooperation and evolution in environments such as soil, water, ancient remains of animals or the digestive system of animals and humans (Ghazanfar and Azim, 2009), and therefore is capable of overcoming these difficulties.

A new “sequencing-by synthesis” strategy was published (Margulies et al., 2005; Zhang et al., 2006). This approach uses emulsion-based PCR amplification of a large number of DNA fragments and parallel pyrosequencing with high throughput. In a single sequencing run, >500 million base pairs of sequence can be generated, at a lower price per base than Sanger-based methods. The most important advantage of the new sequencing approach for metagenomics is that it does not require cloning of the target DNA fragments and therefore avoids cloning biases resulting from toxic sequences killing their cloning hosts.

Three platforms for massively parallel DNA sequencing read production are in reasonably widespread use at present: the Roche/454 FLX (Margulies et al., 2005) (http://www.454.com/enablingtechnology/the-system.asp), the Illumina/ Solexa Genome Analyzer (Bentley, 2006) (http://www.illumina.com/pages.ilmn?ID=203), and the Applied Biosystems SOLiDTM System (http://marketing.appliedbiosystems.com/images/Product/SolidKnowledge/flash/102207/solid.html). Recently, another two massively parallel systems were announced: the Helicos HeliscopeTM (www.helicosbio.com) and Pacific Biosciences SMRT (www. pacificbiosciences.com) instruments. The Helicos system only recently became commercially available, and the Pacific Biosciences instrument will likely launch commercially in early 2010. Each platform embodies a complex interplay of enzymology, chemistry, high-resolution optics, hardware, and software engineering. These instruments allow highly streamlined sample preparation steps prior to DNA sequencing, which provides a significant time savings and a minimal requirement for associated equipment in comparison to the highly automated, multistep pipelines necessary for clone-based high-throughput sequencing. By different approaches outlined below, each technology seeks to amplify single strands
of a fragment library and perform sequencing reactions on the amplified strands. The fragment libraries are obtained by annealing platform-specific linkers to blunt-ended fragments generated directly from a genome or DNA source of interest. Because the presence of adapter sequences means that the molecules then can be selectively amplified by PCR, no bacterial cloning step is required to amplify the genomic fragment in a bacterial intermediate as is done in traditional sequencing approaches.

Roche/454 FLX pyrosequencer was the first to achieve commercial introduction in 2004 and uses an alternative sequencing technology known as pyrosequencing. In pyrosequencing, each incorporation of a nucleotide by DNA polymerase results in the release of pyrophosphate, which initiates a series of downstream reactions that ultimately produce light by the firefly enzyme luciferase. The amount of light produced is proportional to the number of nucleotides incorporated (up to the point of detector saturation). This strategy allows the 454 base-calling software to calibrate the light emitted by single nucleotide incorporation. However, the calibrated base calling cannot properly interpret long stretches (>6) of the same nucleotide (homopolymer run), so these areas are prone to base insertion and deletion errors during base calling. By contrast, because each incorporation step is nucleotide specific, substitution errors are rarely encountered in Roche/454 sequence reads. The FLX instrument currently provides 200 flows of each nucleotide during an 9-h run, which produces an average read length of 450 nucleotides (an average of 2.5 bases per flow are incorporated). These raw reads are processed by the 454 analysis software and then screened by various quality filters to remove poor-quality sequences, mixed sequences (more than one initial DNA fragment per bead), and sequences without the initiating TCGA sequence. The resulting reads yield 500 Mb of quality data on average. Downstream of read processing, an assembly algorithm (Newbler) can assemble FLX reads. Although shorter than reads derived from capillary sequencers, FLX reads are of sufficient length to assemble small genomes such as bacterial and viral genomes to high quality and contiguity. As mentioned, the lack of a bacterial cloning step in the Roche/454 process means that sequences not typically sampled in a WGS approach owing to cloning bias will be more likely represented in a FLX data set, which contributes to more comprehensive genome coverage.
Regardless of the sequencing approach used to generate the data, the first steps in analysis of any metagenome involve comparing those sequences to known sequence databases. This computationally intensive task provides the basic data types for many subsequent analyses, including phylogenetic comparisons, functional annotations, binning of sequences, phylogenomic profiling, and metabolic reconstructions. A freely available, fully automated open source system for processing metagenome sequence data to generate these basic elements was generated by Meyer et al. (2008). A public implementation of this system has been provided for all researchers to analyze their metagenomes. The metagenomics RAST server (mg-RAST for short), is available over the web to all researchers, and access is not limited to specific groups or data types. More than 500 metagenomes have been processed through the beta version of the pipeline so far.

The MG-RAST server is an open source system based on the SEED framework for comparative genomics (Overbeek et al., 2005; McNeil et al., 2007). Users can upload raw sequence data in fasta format; the sequences will be normalized and processed and summaries automatically generated. Genome annotation systems are ever evolving; therefore, in order to accommodate new methods that may be developed, the pipeline was designed with a modular framework that allows the rapid addition of new analysis steps or comparative data at any stage of the analysis. The server provides several methods to access the different data types, including phylogenetic and metabolic reconstructions, and the ability to compare the metabolism and annotations of one or more metagenomes and genomes. In addition, the server offers a comprehensive search capability. Access to the data is password protected, and all data generated by the automated pipeline is available for download and analysis in variety of common formats (Meyer et al., 2008).

The abundance of comparative metagenomics tools is central to the utility of the MG-RAST platform. Various tools have been built into the framework, allowing users to compare their data against other metagenomes or complete genomes taken from the SEED (Overbeek et al., 2005) environment. The subsystems heat map and the taxonomic heat map provide comparative metagenomics summaries that encapsulate the differences between samples. The subsystem comparison tools identify the number of
pegs in each metagenome that are connected to a subsystem via protein level similarity. Based on these connections, each subsystem present in a sample is scored by counting the number of sequences that are similar to a protein in each subsystem. This score is divided by the total number of sequences from the sample that are similar to any protein in a subsystem, to give a fraction of sequences in subsystems that are in a given subsystem. This approach allows comparisons between samples that have different numbers of sequences.

The MG-RAST service handles both assembled and unassembled data. Each approach has advantages that should be considered when comparing metagenomes. For example, if one is carrying out comparative metagenomics or if statistics are being used to compare samples (Tringe et al., 2005; Rodriguez et al., 2006), the sequences cannot be assembled, since the assembly process loses the frequency information critical for determining differences between samples. In contrast, assembled sequences tend to be longer and therefore more likely to accurately identify gene function or phylogenetic source from binning (McHardy et al., 2007).

2.7 Bovine cytokines

The genetic nature of bovine cytokines has been investigated using heterologous genetic probes. Developing and using bovine probes may give a better understanding of the exact role of bovine cytokines at the gene level and make them important candidates from the veterinary point of view. Persson-Waller et al. (2003) examined cytokine kinetics in milk and in afferent and efferent lymph of the supra-mammary lymph node after intramammary infusion of endotoxin from *E. coli*. Cows were sampled 0, 2 and 4 h post infusion (p.i.). Neutrophils appeared in afferent lymph 2 h p.i., and in efferent lymph and milk 4 h p.i. The milk contained high concentrations of interleukin (IL)-8 at 2 and 4 h p.i. IL-8 was also found in lymph, but at lower concentrations. The tumor necrosis factor-α (TNF-α) concentration tended to increase in afferent lymph at 2 h p.i., and increased in milk at 4 h p.i. The level of IL-1β increased at 4 h p.i. in milk, but was not detected in lymph. Interferon-γ was not detected in any sample, at any time. The results indicated a primary role for IL-8 in the recruitment of neutrophils into the gland, and suggested that IL-1b and TNF-α were not necessary for IL-8 production and release.
in response to endotoxin. Rambeaud et al. (2003) conducted a study to determine leukocyte and cytokine dynamics during experimentally induced S. uberis mastitis. Five Jersey and five Holstein cows were challenged via intramammary inoculation of S. uberis into two uninfected mammary glands. Sixteen of 20 challenged mammary glands developed clinical mastitis with peak clinical signs observed at 144 h. The number of S. uberis in milk increased (p < 0.05) at 48 h after challenge, in spite of an increase in milk somatic cells that began at 18 h P < 0.001 and remained elevated throughout the study. Increased TNF-α, IL-1β and IL-8 in milk were detected 66 h after challenge P < 0.05. Peak TNF-α and IL-8 concentrations occurred 120 h after challenge and preceded peak clinical signs. Experimental S. uberis IMI induced local production of TNF-α, IL-1β and IL-8, which may play a role in the pathogenesis of S. uberis mastitis. Other mediators may be involved in initial leukocyte recruitment to the mammary gland, since increases in milk somatic cells occurred earlier than cytokine production. Mingala et al. (2006) studied comparative assessment of Th1 and Th2 cytokines of three bubaline breeds namely swamp buffalo, its crossbreed with riverine buffalo (CB), and the improved breed of Bulgarian Murrah buffalo (BMB), by molecular cloning, sequencing and phylogenetic analysis. The Th1 cytokines analyzed included IL-2, IL-12 and IFN-γ while Th2 cytokines included IL-4 and IL-10. Both groups showed strict conservation in the putative secondary structures and amino acid residues within the tribe Bovine, which indicated functional cross-reactivity. Nucleotide sequence homology ranged from 98.6 to 100.0% and was lowest for IL-12. With regard to amino acid sequence, the lowest homology was observed in IL-4 with 97.8%. This substitution was mainly due to differences in mRNA splicing. The phylogenetic relationship of the buffalo breeds was analyzed and showed them as a cluster comprised mainly of species belonging to the order Artiodactyla, including cattle and pigs.

2.8 Role of cytokines in pathogenesis of mastitis

Cytokines are immune-regulatory mediators that play a central role in the regulation of immune responses against different infections (Campos et al., 1994). Cytokines are one of the sensitive means in examining the immune responses of mammary glands and they could serve as a suitable tool for the udder health control or
in evaluating mastitis treatment or vaccine efficiency. Overwhelming use of bovine cytokines in immunotherapy of mastitis or as adjuvants in the immunopotentiation of the mammary gland has revealed their important role in the regulation of the mammary gland defenses (Godson et al., 1997). In recent years cytokines have been employed as adjuvant or as innovative therapeutic means in treatment and/or diagnosis of mastitis (Alluwaimi, 2004).

2.8.1 Tumour necrosis factor -α

Tumour necrosis factor-α (TNF-α) is an inflammatory cytokine which is locally released during the acute-phase of mastitis. The immunological significance of TNF-α has been studied in mastitis. TNF-α is considered to be important for accumulation of phagocytic leucocytes in the udder (Shuster et al., 1996; Persson-Waller et al., 1997). Intramammary infusions of TNF-α actually induce increased number of somatic cells, mainly consisting of neutrophil leucocytes in ruminant udder and teat cistern (Persson-Waller et al., 1996). TNF-α is also considered to be important for activation of bactericidal functions of neutrophil leucocytes in the udder (Paape et al., 1996). TNF-α regulates not only the functions of phagocytic leucocytes but also those of mammary epithelial cells. It was reported to inhibit casein (CN) secretion from bovine mammary explants. Decreased concentrations of milk-specific proteins such as CN are a feature of mastitic milk (Haenlein et al., 1973; Kitchen, 1981). Therefore, TNF-α is hypothesized to be implicated in mastitic changes in the protein composition of milk via functional regulation of mammary epithelial cells. In addition, TNF-α is possibly implicated in the influx of serum proteins into mammary secretion, because the cytokine increases endothelial permeability (Shuster et al., 1996).

Sordillo and Peel (1992) found elevated sera and milk concentrations of TNF-α in cows that had died from acute E. coli mastitis during the peri parturient period. Elevated tumor necrosis factor concentrations were especially evident in cows that developed severe clinical symptoms and eventually died from endotoxemia. These results indicated that both milk and sera tumor necrosis factor concentrations are associated closely with the manifestation of per acute signs of coliform mastitis and are important factors contributing to morbidity and mortality of endotoxic shock. Watanabe
et al. (2000) evaluated the effects of TNF-α on lactating bovine mammary function such as milk protein secretion and the integrity of the milk-blood barrier. They examined the effect of different concentrations of serum haptoglobin (Hp), a major inflammatory acute-phase protein, on the induction of the systemic inflammatory response as an index. One hundred micrograms per mammary gland of recombinant bovine (rBo) TNF-α or placebo saline was individually infused into a rear mammary gland of each of four lactating cows, and milk and blood samples were collected before and 4, 8, 24, 32, 48, 96 and 168 h after infusion. They reported increase in the concentrations of somatic cell counts at 4-48 h, lactoferrin at 4 h and Hp at 8-32 h. Although concentrations of total milk protein were not changed, compositions of milk proteins varied following rBoTNF-α infusion. Concentrations of caseins, α-lactalbumin and β-lactoglobulin were significantly decreased at 4 and 8 h. Significant infiltrations of serum albumin, immunoglobulin G1 (IgG1) and IgG2 were observed at 4 and 8 h. Their results showed that single rBoTNF-α infusion into the lactating mammary gland suppresses the lactogenic function of the gland and influences the function of the milk-blood barrier, with little effect on the generalized inflammatory response.

### 2.8.2 Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)

GM-CSF was first identified by its capacity to induce hematopoietic progenitor cells to develop into granulocytes and macrophages. Several recent studies of dairy cows have shown that GM-CSF is not only an important molecule for inducing growth, but also affects a variety of functions of mature granulocytes. Treatment of bovine peripheral blood and mammary gland neutrophils with rbGM-CSF significantly increased the chemotactic and bactericidal capabilities of these cells (Sordillo et al., 1997).

Stabel et al., (1991) studied the immunomodulatory effects of recombinant bovine granulocyte colony stimulating factor in periparturient dairy cows and reported enhanced lymphocyte blastogenesis and mitochondrial methylthiazoltetrazolium cleavage activity in unstimulated cultures of lymphocytes isolated from peripartueient cows from 5 wk to 7 wk before parturition, higher serum IgM, and increased in vitro IgM production by B lymphocytes. These data provide support for the use of
recombinant bovine granulocyte colony-stimulating factor to alleviate immunosuppression in periparturient cows.

*In vivo* administration of GM-CSF induces differentiation of hematopoietic progenitor cells to neutrophilic granulocytes (Welte et al., 1987). In addition, GM-CSF enhances effector functions of mature neutrophils by increasing phagocytic and cytotoxic activities as well the secretion of superoxide anion (Avalos et al., 1990). Hirai et al. (1999) reported that rboGM-CSF enhanced bactericidal activity of bovine neutrophils both *in vitro* and *in vivo*. Takahashi et al. (2004) reported that rboGM-CSF was useful for the treatment of subclinical mastitis in dairy cows caused by *S. aureus* infection. Reddy et al. (1990), Sordillo et al. (1997), and Hirai et al. (1999), demonstrated that rboGM-CSF activated the bactericidal activities of blood neutrophils in *in vitro* and *in vivo* experiments, suggesting that rboGM-CSF could be a good candidate as a therapeutic agent in cattle. Sordillo et al. (1997), using cultured mammary gland neutrophils, indicated that rboGM-CSF enhanced chemotactic and bactericidal activities of milk neutrophils.

Takahashi *et al.* (2004) studied the effect of intramammary injection of recombinant bovine granulocyte-macrophage colony-stimulating factor (rboGM-CSF, 400 µg/10 mL) on quarter milk levels of chemiluminescence (CL) activity, and SCC and shedding pattern of *S. aureus* was investigated in ten Holstein cows, naturally infected with *S. aureus*, with either early-stage or late-stage subclinical mastitis. Injection of rboGM-CSF caused a remarkable increase in milk CL activity with a peak at 6 h after the cytokine injection in the early and late-stage groups. In the early-stage group, milk SCC stayed around preinjection level at 6 h, rose significantly on days 1 and 2, and was followed by a smooth and significant decline to an under preinjection level (below 2,00,000 cells/ml) on day 7 postinjection. Alternatively, in the late-stage group, milk SCC rose significantly at 6 h after the cytokine injection and maintained high levels thereafter. The results suggested that the rboGM-CSF has a potential as a therapeutic agent for *S. aureus* infection causing subclinical mastitis of dairy cows, if the cytokine is applied at the initial stage of infection.
2.8.3 Interferon (IFN)-γ

Interferon-γ is a cytokine derived from T lymphocytes that is often produced in response to stimulation by antigens or mitogens. IFN-γ could elicit functional changes in phagocytic cells in the mammary gland that could make it effective in the control of bovine mastitis. Sordillo and Babiuk (1991) reported that the in vitro treatment of bovine mammary gland neutrophils with IFN-γ could reverse the suppressive effects of mammary gland secretions and significantly increase the functional capabilities of these cells against S. aureus. They examined the influence of recombinant bovine IFN-γ treatment on the establishment and severity of experimentally induced E. coli mastitis in postpartum dairy cows. Dairy cows that were treated intra-mammarily with IFN-γ 24 h before E. coli challenge had fewer infected quarters, infections of shorter duration, and lower clinical scores than cows treated with a placebo. All cows that had been treated with IFN-γ survived the experimental E. coli challenge, but the group receiving the placebo had a 42% mortality rate, which was attributed to coliform mastitis within 3 d of the experimental challenge.

2.8.4 Interleukins

Interleukin (IL)-6 is one of the pro-inflammatory cytokines incriminated in the development of signs of acute septic shock in coliform mastitis (Shuster et al., 1993; Shuster et al., 1997; Riollet et al., 2000). LPS-stimulation of a mammary gland epithelial cell line induces IL-6 in a dose-dependent manner (Okada et al., 1999). In naturally occurring coliform mastitis, IL-6 has been detected in serum and milk, higher in the latter except in severe coliform mastitis (Nakajima et al., 1997). Shuster et al. (1993) detected IL-6 expression in mammary gland infected with E. coli as early as 14 h pi and earlier in endotoxin-infused mammary gland.

Kaplanski et al. (2003) postulated that IL-6 facilitates the transition of the inflammatory process from influx of neutrophils to monocytes. A shift from neutrophils to monocytes is essential for suitable immune responses and to decrease the noxious effect of neutrophils.

In response to bacterial invasion, endothelial and epithelial cells release the chemokine interleukin-8 (IL-8). IL-8 mediates neutrophil function, allowing neutrophils
to resolve bacterial infections by migrating through blood vessel walls and to the site of infection (Kehrli and Harp, 2001). IL-8 also impacts neutrophil killing and survival ability during the inflammatory response (Chertov et al., 2000).

McClenahan et al. (2006) used in situ hybridization with an IL-8 riboprobe to determine IL-8 mRNA expression by mammary gland epithelial and endothelial cells in cows with experimental *E. coli* mastitis. Epithelial cells of the gland, especially surrounding the alveoli, had increased IL-8 mRNA levels at all time points at which tissue samples were collected (8, 12, and 24 h) after *E. coli* challenge. Levels of IL-8 expression in the epithelial cells decreased at 24 h post infection. IL-8 expression by mammary gland endothelial cells was low, but did increase slightly at 24 h post-infection. Both epithelial and endothelial cells of the mammary gland can contribute to the production of IL-8 that was typically seen in coliform mastitis.

Interleukin-8 is potent neutrophils-chemoattractant factor (Barber and Yang, 1998). Milk somatic cells consist of several cell types, including neutrophils, macrophages, lymphocytes and a small number of epithelial and natural killer (NK) cells. The presence of these cells at certain localization involves the migration upon a chemotactic signal. Migration of neutrophils and to a certain extent T-lymphocytes from the bloodstream to the site of recruitment is IL-8 dependent (Ribeiro et al., 1991; Wang et al., 1996). In addition, IL-8 is also an important factor for activating neutrophils during inflammatory processes (Galligan and Coomber, 2000). An earlier report described the lack of IL-8 mediated chemotaxis in non-mastitic milk. The absence of IL-8 mediated chemotaxis can be explained by a lack of IL-8 expression or by the existence of a low molecular weight inhibitor. Studies based on both human and mouse have demonstrated the central role of Th1 biased cell mediated immunity in a number of antimicrobial immune responses in which IFN $\gamma$, IL-12 and IL-18 are recognized as pivotal components (Kaufmann and Ladel, 1994). IL-12 is a critical factor in the development of Th1 biased cell-mediated immune responses, largely through the stimulation of IFN $\gamma$ secretion (Macatonia et al., 1995). The production of IL-12 is augmented by other cytokines, notably IFN $\gamma$, which is in turn largely dependent upon IL-12 for its induction (Macatonia et al., 1993; Flesch et al., 1995).
Although IFN-γ is not an absolute requirement for the production of IL-12, it does provide a powerful positive feedback mechanism for maximal production. Trinchieri (1995) suggested that IL-12 is potent cytokine that enhances the pro Th1 cytokines production and results in considerable mobilization of innate and humoral immunity. Alluwaimi and Cullor (2002) studied the cytokines profile in late and mid lactation in Holstein cattle and reported that the cytokines interaction in late lactation was more co-ordinated and their transcriptional levels were significantly correlated among each other, whereas, in mid-lactation significant correlation of the cytokines transcription was only seen with the TNF-α, GM-CSF, and IFN-γ. Cytokine mRNA profiles between mid- and late lactation showed significant differences, which can be attributed to the dramatic changes that the mammary gland is subjected to during late lactation.

Riollet et al. (2006) used the rapid amplification of cDNA ends (RACE) method to obtain a cDNA of bovine IL-17 (BoIL-17) containing a 462-bp open reading frame (ORF) encoding a protein of 153 amino acids (aa) with a molecular mass of 17.2 kDa, a 23-residue NH2-terminal signal peptide, a single potential N-linked glycosylation site, and 6 cysteine residues. BoIL-17 protein shared 73.5% identity with the human protein and 67% with the mouse and rat proteins. Sf9 insect cells were transfected with BoIL-17 cDNA, and supernatant was tested for biologic activity on a primary culture of bovine mammary epithelial cells (MECs). mRNA synthesis of IL-6, IL-8, and growth-related oncogene α (Gro-α) was induced, suggesting a functional role for IL-17 in mammary immunity.

### 2.9 Cytokines expression in mammary gland

The immunoregulatory role of cytokines in mobilizing the innate and specific immunity of the bovine mammary gland is well documented (Sordillo et al., 1997). Cytokines of bovine mammary gland have been considered useful markers in defining the mammary gland defenses (Godson et al., 1997). There are a number of studies on the application of cytokines to modulate the immune response of the mammary gland. Pro-inflammation and regulatory cytokines have been demonstrated to play a fundamental role in mammary immune mechanisms. Understanding the regulation of the immune
response during infections of the mammary gland is important for the design of prophylactic vaccines and for the optimization of therapeutic protocols.

Covert and Splitter (1995) developed a reverse transcriptase polymerase chain reaction (RT-PCR) assay to monitor gene expression of bovine T lymphocyte cytokines. Bovine cDNA was reverse transcribed from total RNA and subsequently amplified using primers designed from bovine or murine and human consensus sequences. Cytokine transcription of \( \beta \)-actin, IL-1, IL-2, IL-4, IL-6, IL-10, TNF and IFN-\( \gamma \) was detected from concanavalin A activated peripheral blood mononuclear cells and CD4+ purified T lymphocytes. The assay allows detection of many cytokine mRNAs in a species where research has been hindered by lack of commercially available reagents and sequence information.

Leutennegger et al. (1999) designed feline-specific Taq Man probes to encompass an intron, thus allowing differentiation of complementary DNA versus genomic DNA amplification products. Quantitative analysis of cytokine cDNA concentrations was performed in comparison to feline GAPDH. Messenger RNA (mRNA) from the universally expressed housekeeping gene GAPDH proved to be useful as an amplification control and allowed for correction of variations in the efficiencies of RNA extraction and reverse transcription. GAPDH mRNAs were readily detectable in cDNA prepared from un-stimulated feline peripheral blood mononuclear cells (PBMCs) and from frozen cell pellets, while cytokines (IL-4, IL-10, IL-12 p35, IL-12 p40, IFN-\( \gamma \), IL-16) were expressed at variable amounts. IFN-\( \gamma \) transcription was found to be up regulated in stimulated PBMCs and feline cell lines.

Alluwaimi and Cullor (2002) examined the gene expression of cytokines, IL-1\( \alpha \), IL-1\( \beta \), IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFN-\( \gamma \) and TNF-\( \alpha \) in milk cells from cattle two weeks before their parturition and cattle at their mid-lactation with RTPCR. All cytokines were detected in milk cells from periparturient period except IL-12, whereas in milk cells from mid-lactation, cytokines IL-2, IL-4, and IL-12 cDNA failed to be detected. The results indicated the versatility of this approach in providing flexible tool to reveal the status of the mammary glands at this period. Leutenneger et al. (2000) used real-time TaqMan PCR systems were to assess cytokine profiles in cells extracted from milk from healthy Holstein cattle in mid lactation. All cows showed high levels of
transcription for TNF-α, GM-CSF, IL-12 p40 and IFN-γ. Lower but consistent transcription was detected for IL-8. IL-6 transcription was detected in two of seven and IL-2 was not detected in any of the samples. TNF-α was transcribed to highest levels. It was higher than IL-12 p40, IFN-γ and IL-8. No significant differences were observed between TNF-α and GM-CSF and IFN-γ expression or between GM-CSF and IFN-γ and IL-12 p40. However, GMCSF was significantly higher transcribed than IL-8.

In contrast, IL-1α, IL-1β, IL-6 and TNF-α proinflammatory cytokine and IL-10 and IL-12 regulatory cytokine mRNA were synthesized in cells derived from infected mammary glands, whereas no IL-2 nor IL-4 mRNA were found. Therefore, cells present in milk during chronic *S. aureus* infection were activated, but did not reveal any polarization of the immune response. Alluwaimi and Cullor (2002) compared the level of normal transcription of cytokines, IL-2, IL-6, IL-12 subunit p40 (IL-12), TNF-α and GM-CSF in the milk cells of bovine mammary gland at the late stage of lactation period with that of mid lactation. They reported that transcripts for TNF-α, GM-CSF, and IFN-γ were detected in all samples of both stages. However, IL-12 was only detected in 80 and 58% of late- and mid-lactation samples, respectively. IL-12 expression was up-regulated in late lactation in comparison with the corresponding level in mid-lactation. The cytokines interaction in late lactation was more co-ordinated and their transcriptional levels were significantly correlated among each other, whereas, in midlactation significant correlation of the cytokines transcription was only seen with the TNF-α, GM-CSF, and IFN-γ.

Schmitz et al. (2004) investigated the mRNA expression of various soluble immune components and of the major milk proteins during the acute phase of mammary inflammation by infusing five healthy lactating cows with 100g *E. coli* endotoxin (lipo polysaccharide, LPS) in one quarter and saline (9 g/l) serving as control in contralateral quarter. Mammary biopsy samples of both quarters were taken immediately before and at 3, 6, 9 and 12 h after infusion and mRNA expression of various factors was quantified via real-time RT–PCR. Blood samples for determination of leukocyte number were taken simultaneously with the biopsy samples and rectal temperature was measured at 1h intervals. Rectal temperature increased until 5 h (*P*<0.05) after LPS administration and remained elevated until 9 h after LPS inoculation. Blood leukocyte number
decreased \((P<0.05)\) from 0 to 3 hours from \(7.7-1.1 \times 10^9\) to \(5.7-1.0 \times 10^9\) and thereafter recovered to pre-treatment levels until 12 hours after LPS challenge. In LPS-treated quarters, tumor necrosis factor-\(\alpha\) and cyclooxygenase-2-mRNA expression increased \((P<0.05)\) to highest values at 3 hours after LPS challenge. Lactoferrin, lysozyme, inducible nitric oxide synthase increased \((P<0.05)\) and peaked at 6 hours after challenge, and platelet-activating factor acetylhydrolase-mRNA expression tended to increase \((P = 0.07)\). mRNA expression of insulin-like growth factor-I and of S1-casein (CN), S2-CN, CN and \(\beta\) lactoglobulin did not change significantly, whereas mRNA expression of 5-lipoxygenase and \(\beta\) lactalbumin decreased \((P<0.05)\) in both quarters and that of CN only in the LPS quarter mRNA expression of some investigated factors (TNF-\(\alpha\), lysozyme, 5-lipoxygenase, \(\beta\)-lactalbumin) changed in control quarters, however in all respective factors less than in the LPS quarters \((P<0.05)\). In conclusion, mRNA expression of most inflammatory factors increased within hours, whereas that of most milk proteins remained unchanged.

Bannerman et al. (2004) characterized several elements of the bovine innate immune response to intramammary infection with *Klebsiella pneumoniae*. The inflammatory cytokine response and changes in the levels of soluble CD14 (sCD14) and lipo polysaccharide (LPS) binding protein (LBP), 2 proteins that contribute to host recognition of gram-negative bacteria, were studied. The contralateral quarters of 7 late-lactating Holstein cows were challenged with either saline or *K. pneumoniae*, and milk and blood samples were collected. Initial increases in the chemoattractants C5a and IL-8, as well as TNF-\(\alpha\), were evident in infected quarters within 16 hours of challenge and were temporally coincident with increases in milk somatic cells.

Augmented levels of TNF-\(\alpha\) and IL-8 were observed in infected quarters until >48 hours post-challenge, respectively. Elevated levels of IL-12, IFN-\(\gamma\), and the anti-inflammatory cytokine, IL-10, which were first detected between 12 and 20 hours post infection, persisted in infected quarters throughout the study (>96 hours). Together, these data demonstrate that intramammary infection with *K. pneumoniae* elicits a host response characterized by the induction of pro-inflammatory cytokines and elevation of accessory molecules involved in LPS recognition. Lee et al. (2006) characterized the
expression of inflammatory cytokines, including IL-6, IL-8, IL-12, GM-CSF, TNF-α and IFN-γ, by milk somatic cells by real-time polymerase chain reaction in dairy cows

Experimentally challenged with either *E. coli* (*n* = 8) or *S. aureus* (*n* = 8). The mRNA abundance of a target gene was calibrated with that of a reference gene (β-actin) and expressed as fold of induction over the control quarter at each time point. At no single time point did all eight quarters challenged with the same type of bacteria demonstrated increased expression of a target gene and there was large variation among animals at each given time. As a consequence, most tested comparisons were not statistically significant except the peak time points of IL-8 expression. However, the average fold induction of all targeted cytokines was increased in response to both bacterial challenges with the exception of IFN-γ. The expression of IFN-γ was only increased in milk somatic cells isolated from *E. coli*, but not *S. aureus*, challenged mammary glands. Moreover, up regulated expression of cytokine genes had higher magnitudes and/or faster responses in glands challenged with *E. coli* in comparison with those challenged with *S. aureus* compromised up regulation of inflammatory cytokines in *S. aureus* infected glands may, at least partially, contribute to the chronic course of infection caused by this pathogen. Further research on identifying factors responsible for the differentially expressed cytokine profiles may be fundamental to developing strategies that mitigate the outcome of bovine mastitis.

Strandberg et al. (2005) characterized the innate immune responses induced by *in vitro* stimulation of bovine primary mammary epithelial cells (bMEC) using gram-negative lipopolysaccharide (LPS) and gram-positive lipoteichoic acid (LTA) bacterial cell wall components. Quantitative real-time PCR (qRT-PCR) was employed to examine the mRNA expression of a panel of 22 cytokines, chemokines, β-defensin and components of the Toll-Like Receptor signaling pathway. Stimulation of bMEC with LPS for 24 h elicited a marked increase in mRNA expression for IL-1b, IL-8, TNF-α, CXCL6 and β-defensin while members of the Toll-Like Receptor pathway, although present, were largely unaffected. The stimulation of these cells with LTA for 24 h did not significantly alter the expression of these genes. A time course of the expression of IL-1b, IL-8, TNF-α, CXCL6 and β-defensin was subsequently performed. The mRNA levels of all genes increased rapidly after stimulation for 2-4 h with both LPS and LTA
but only the former treatment resulted in sustained responses. In contrast, the increased gene expression for LTA stimulated cells returned to resting levels after 8-16 h with the exception of β-defensin, which remained up-regulated. The limited and unsustained cytokine response to LTA may explain why mastitis caused by gram-positive bacteria has greater potential for chronic intra-mammary infection than gram-negative infection. They concluded that bovine mammary epithelial cells have a strong but differential capacity to mount innate immune responses to bacterial cell wall components.

### 2.10 Cytokines and bovine mastitis immunotherapy

Milk ejection is a continuous process during the course of milking (Bruckmaier et al., 1994), it can be hypothesized that there are also changes in cell populations in different milk fractions. Immunomediators support the defense mechanism of the mammary gland by exerting potent chemotactic effects on leukocytes; they also enhance phagocytotic activity (Persson et al., 1993; Sanchez et al., 1994).

Cytokines are the signals that dictate immune responses in normal and mastitic udders. Hence, subtle changes in the cytokine network of mammary gland in health and disease could help in detecting early infection and in monitoring the effectiveness of the treatment. The study of the bovine cytokine network has been made possible through the production of a wide range of bovine recombinant cytokines and these have been used as a new avenue in mastitis therapy and prophylaxis. Emerging strains of bacteria that swiftly develop antibiotic resistance and the highly complex evasion mechanisms of pathogens incriminated in bovine mastitis have prompted study of the immunotherapeutic efficacy of recombinant cytokines in the treatment of bovine mastitis.

Research into the pathobiological role of cytokines in coliform and Staphylococcus aureus mastitis has been undertaken (Sordillo et al., 1997), and recombinant cytokines have been used in an attempt to raise the resistance or potentiate the immune system of bovine mammary gland infected with *E. coli* or *S. aureus* (Pighetti and Sanchez 1994; Sordillo, 1996; Erskine et al., 1998).

There are few reports of cytokine immunotherapy of coliform mastitis. The dominant role of the pro-inflammatory cytokines in driving the pathogenicity and
outcome of the infection has biased the investigation of cytokine’s immunoregulatory intervention rather than an immunotherapeutic regimen. The immunoregulatory approaches are primarily based on down-regulating the pro-inflammatory cytokines through use of receptor antagonists or anti-inflammatory medications. In essence, in coliform mastitis, mammary gland immune responses are exhaustively stimulated and manipulated in a way to enhance the infection. Hence, the ground for effective strategies that abort the pathogen-initiated inflammatory process is wide open.

In *S. aureus* mastitis, mammary gland immune responses are evidently suppressed or markedly down-regulated. Although cytokine immunotherapy, particularly with IL-2 and IFN-γ, showed promise of prophylactic activity in normal mammary gland, the resistance to *S. aureus* infection is not enhanced. Enhancement of the bactericidal effect of certain antibiotics by cytokines, such as IL-2, IFN-γ or GM-CSF, can be attributed to the substantial mobilization of innate and adaptive immunity. Although the studies on the adjuvant activity of cytokines, especially IL-2 and IFN-γ, have generated promising results on enhancing the immunity of normal glands, the stimulated immunity failed to show any protective effect in experimental or natural mastitis (Alluwaimi, 2004).

### 2.11 Treatment

Many animals remain infected throughout the first lactation and act as reservoirs for infecting other cows in the herd. Antibiotic treatment will not control this disease but it may, in certain cases, shorten the duration of the infection. Treatment effectiveness decreases as the cow becomes older and even as the first lactation progresses. Cure rates were 34 percent when 89 cows in 10 Dutch herds were treated for subclinical *S. aureus* mastitis (Sol et al. 1997). The results showed that the probability of cure was lower in older cows with high SCC and in cows infected in hind quarters during early and mid lactation. *S. aureus* infections were found in 36 percent of clinical mastitis cases in Finnish herds (Pyorala and Pyorala, 1997). Of these, 39 percent responded to treatment. Cows with an SCC of less than 1 million were more likely to cure an infection compared with those over the cut-off point. Successful treatment during lactation is greater if
detected and treated early, whereas the response is lower when treating chronic infections.

Treatment of a cow acutely sick from mastitis must be directed towards saving the cow’s life. All clinical cases should be treated as they occur, otherwise a permanent loss could commence. Before any attempt made to treat mastitis, selection of the most likely effective antibiotic for the treatment is essential. Antibiotics are selected according to the identified pathogen and sensitivity of the organism cultured from a milk sample. Sensitivity testing has advantages over blind treatment, in that, it helps to cure animals within short period of time and return to production, reduce further disease spread and serving as a source of infection, avoids the risk of bacteria developing resistance and is more of economical. Treatment of sick animals without sensitivity testing and indiscriminate drug usage by many non-professionals leads to the development of drugs resistance. Conventional antibiotics like, penicillin, cloxacillin, erythromycin, and cephalosporins have excellent successes against mastitis caused by \textit{S. agalactiae} and \textit{S. dysgalactiae}. Before treating \textit{S. aureus} cases susceptibility testing is recommended. Systemic treatment with penicillin, ceftiofur or pirlimycin result greater cure when combined with local intramammary infusion containing cloxacillin and cephalosporin. Drugs like gentamycin, amikacin, trimethoprim-sulfa, and ticarcillin-clavulanic acid work against most coliforms, polymyxin B and cephalotin and tetracycline, ampicillin, neomycine and kanamycin work against 60-80\% and 40-60\% \textit{in vitro}, respectively (Rebhun, 1995).

Treatment of \textit{Pseudomonas} spp. with conventional antibiotics is rarely successful, while \textit{Serratia} spp. treatment can be done based on culture and sensitivity. Yeast infections can be cured spontaneously if all antibiotic therapy is stopped and the affected quarters are milked out four or more times per day. Miconazole, nystatin, and iodine have been used for the treatment of mastitis caused by yeasts. Antifungal drugs like miconazole, clotrimazole and ketoconazole can be used for the treatment of fungal mastitis. Alagal species have no successful treatments (Rebhun, 1995).

Due to one or other reasons bacterial agents that cause mastitis develop resistance of variable degree to different antibiotics. The emergence of bacteria resistance to antimicrobial agents within animal population or during therapy is a matter
of great concern (Fraster, 1986). Drug resistance isolated from domestic animals is important in limiting the use of antimicrobial agents in animals and potentially in humans (Prescott and Baggot, 1988). Among the main pathogenic organisms causing mastitis, some *Streptococcus* spp. and *S. aureus* develop resistance to antibiotics like penicillin, streptomycin and oxytetracyclines (Ak, 2002). Some of the bacterial agents isolated from a case of mastitis that develop resistance for *in vitro* trial in different places are summarized in Table 2.1.

**Table 2.1: Bacterial isolates that develop resistance to some antibiotics**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Type of drug</th>
<th>% of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus dysgalactiae</em></td>
<td>penicillin, oxacillin, chloramphenicol</td>
<td>3.7</td>
</tr>
<tr>
<td><em>Streptococcus dysgalactiae</em></td>
<td>erythromycin and oxytetracycline</td>
<td>7.4</td>
</tr>
<tr>
<td><em>Streptococcus uberis</em></td>
<td>penicillin, oxacillin, oxytetracycline</td>
<td>2.6</td>
</tr>
<tr>
<td><em>Beta haemolytic streptococcus</em></td>
<td>oxytetracycline</td>
<td>1.9</td>
</tr>
<tr>
<td><em>Beta haemolytic streptococcus</em></td>
<td>penicillin, oxacillin, chloramphenicol,</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>erythromycin</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>penicillin</td>
<td>31.3</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>oxytetracycline</td>
<td>26.1</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>oxytetracycline</td>
<td>30.4</td>
</tr>
<tr>
<td><em>Corynebacterium isolates</em></td>
<td>penicillin, erythromycin</td>
<td>15</td>
</tr>
<tr>
<td><em>Corynebacterium isolates</em></td>
<td>chloramphenicol</td>
<td>20</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>penicillin</td>
<td>83</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>streptomycin</td>
<td>60</td>
</tr>
</tbody>
</table>

Source: (Woods, 1986; Mallikarjunaswmy et al., 1997; Heras et al., 1999; Ak, 2002; Kerro, 2003).

The conventional antimicrobial agents have been the mainstay of mastitis therapy over the last many decades and have potential high cure rate when the treatment is well targeted. However, use of antibiotics is associated with cost, the possibility of development of acquired drug resistance, drug residues in the milk, and disruption of symbiotic gut flora of the host when systemic administration is used (Paape et al., 1990; Paape et al., 1992).
Sandholm and Pyorala, 1995). Given the seriousness of such problems, researchers and clinicians have been trying to find effective therapeutic agents using alternative medicine. Traditional healthcare of animals (ethnoveterinary medicine) includes the use of medicinal plants/herbs, surgical techniques and management practices to prevent and treat a range of diseases and problems encountered by livestock keepers. Research has shown that many of the plants used to prepare indigenous medicines do contain valuable active ingredients, however, much research remains to be done in this area (MacCorckle and Mathias, 1996).

A study has revealed efficacy of 21 different species of medicinal plants in the treatment of bovine mastitis in Ethiopia (Sahle, 2002). Similarly, in another study, the evaluation of therapeutic potential of a topical herbal gel has indicated elimination of bacteria and significant reduction in SCC in around 80% cases of subclinical mastitis treated with the drug (Verma and Nauriyal, 2009).

The role of cytokines in the pathophysiology of bovine mastitis has been the subject of many studies. Cytokines play a central role in the regulation of immune responses against different infections (Campos et al., 1994) and variations in their expression are often associated with disease activity in immune-mediated or inflammatory disorders (Mosmann and Sad, 1996; Hansen et al., 2004). Modulation of cytokine secretion may offer novel approaches in the treatment of a variety of diseases. One strategy in the modulation of cytokine expression may be through the use of herbal medicines. A class of herbal medicines, known as immunomodulators, alters the activity of immune function through the dynamic regulation of inflammatory molecules such as cytokines.

Ethnoveterinary practice of the Borana rangeland pastoral system indicated that *Acacia busei* (bark, burnt, powdered and mixed with butter to make paste, topical), *Carissa edulis* (leaf/root, paste, topical), *Rossa abyssinica* (root, paste/poultice, topical) and *Sasbania sasban* (root/bark/leaf, decoction, topical) have been used for the treatment of mastitis (Sory, 1999). Similarly, Chekol (2002) in his survey on ethnoveterinary knowledge and practices showed that farmers in North Gondar reported the use of *Thalicrum rynchocarpum* (root, juice) for the treatment of bovine mastitis.
In Kenya, a handful of *Sasbania sesban* leaves mixed with 125gm of cream or butter for 5 minutes, and rub the mixture onto the affected area until the swelling disappears for the treatment of mastitis. This practice is similar with the practice of the Borana pastoralists (Hyato, 2003). A handful of *Ajuga remota* leaves and stems were chewed and 2 mouthfuls of the juice and saliva directly spat onto the swollen udder once a day for 7 days for treatment of bovine mastitis (ITDG and IIRR, 1996).

In China injection of medicinal herb preparations containing the Honey suckle flower, *Chrysanthemum indicum*, *Voila yedoensis* and *Citrus reticulata* was effective for preventing clinical mastitis during the dry period (Jaing et al., 1994).

Sahle (2002) carried out test on *Persicaria senegalensis*, *Cyphostemma adenocaule* and *Cucumis ficifolius* against their effects on most pathogenic bacterial causative agents of mastitis. *E. coli*, *A. pyogenes*, *K. pneumoniaeae*, *S. aureus*, *S. agalactiae* and *S. dysagalactiae* were sensitive to *P. senegalensis* at different concentrations, and has shown a very good inhibitory effect in all concentrations against *S. aureus*, *S. agalactiae* and *S. dysagalactiae*. The *in vitro* test of *P. senegalensis* showed that isolates of *S. aureus*, *C. albicans* and *C. bovis* from subclinical cases and isolates of *P. aeruginosa* from clinical cases of mastitis were all inhibited. *In vivo* trial of 0.77 kg of leaf powder (equivalent to 3 kg of wet leaf) fed daily for 5 days resulted in an apparent cure rate of 92.8%, in contrast to 80% of in positive control group treated with intramammary antibiotic preparations (Dagne and Abdicho, 1999).