mastitis in dairy animals
an update

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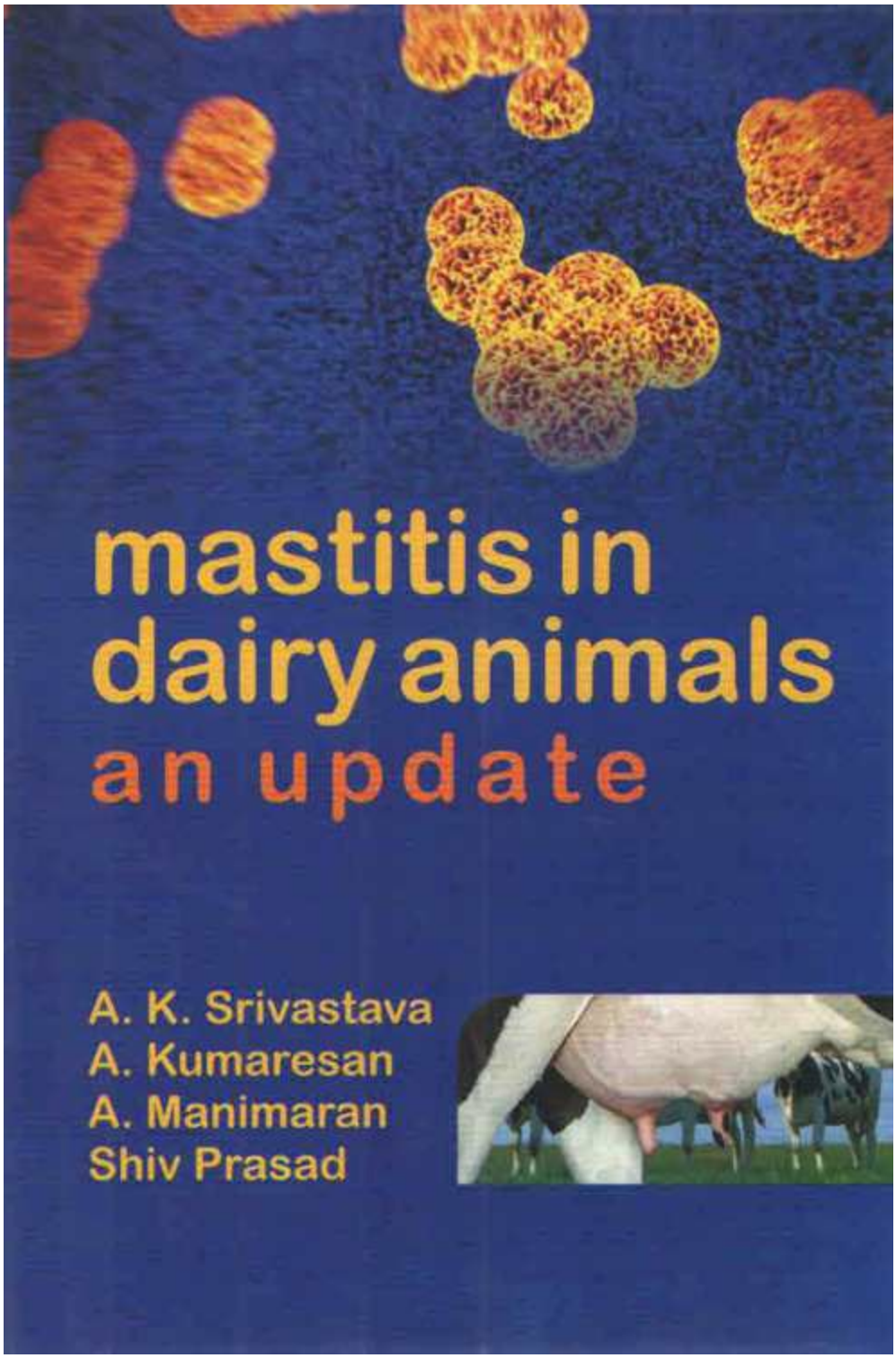
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Declaration

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Preface

Mastitis, the inflammation of parenchyma of mammary glands, is a complex multi-etiological disease affecting dairy cattle worldwide causing substantial loss to the dairy farmers. Bovine mastitis has huge effects on farm economics due to reduction in milk production and treatment costs. It has been estimated that the loss in milk yield due to mastitis range from 100 to 500 kg/cow per lactation. Over the decades, the prevalence of mastitis in dairy animals has increased at high rates. Recent trend of transformation from traditional dairying towards commercial dairying where hundreds to thousands of animals are reared together necessitates development of suitable measures to control mastitis. However, multiple causative agents, poor understanding of the early immune response and complexities associated with mammary epithelial cell damage by both the agents and the host factors makes the condition most complicated to decide a preventive and therapeutic strategy against mastitis. Since mastitis affects the milk quality, its consequences are not restricted only to the farm but expand beyond the dairy farm. Worries about the antimicrobial residues, antimicrobial resistance, milk quality and animal welfare all become a concern to the consumers and the society.

In view of the importance of mastitis in dairy animals, the scientists of National Dairy Research Institute, Karnal has brought out a status paper on "Mastitis in dairy animals" in the year 2012. Further, the National Academy of Agricultural Sciences has also organized a Brainstorming Session and brought out a policy paper on 'Mastitis management in dairy animals" in the year 2013. During the meeting at NAAS, a pressing need was felt collectively by all the experts to bring out an updated publication on bovine mastitis. As such, to update and strengthen the knowledge on mastitis particularly in the areas of diagnosis, management, development of bio-markers, vaccines and to define a strategy for effective prevention and control, experts were contacted to contribute chapters, which resulted in bringing out this publication. The very

purpose of bringing out this book is to update the readers with the facts, figures and recent developments in different aspects of bovine mastitis.

We hope that this book will aid students, researchers, planners and development agencies in updating their knowledge on bovine mastitis and in selecting those paths of research and strategies that will lead to control of mastitis in dairy animals.

A. K. Srivastava
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chapter 1
Mastitis in Dairy Animal: Current Concepts & Future Concerns

India ranks first in milk production by producing around 17% of total world's milk production. Although the consumption of milk is heterogeneous over the world and varies among the different countries, the European Union and North America continue to be the largest milk consumers. In developing countries also demand for the dairy products has grown considerably, especially in China, India and several Asian countries. Taken together the increasing demand for milk and milk products, across the globe and to fulfill the increasing domestic requirements, the milk production needs to be increased. In India, milk production has registered impressive growth during the post¬independence era, which has jumped from 17 million tonnes in 1950-51 to 132.4 million tonnes in 2012-13. Increase in milk production boosted the per capita availability of milk to the population of the country. In 1950-51, the per capita milk availability was only 130 gram per day and today the national average is above the ICMR recommended level of 280 gram per day and is expected to reach 290 gram per day in 2013. In 2011-12, the rate of growth in milk production was also substantially higher (5%) than the world average of 1.5 per cent. The milk production and per capita availability over last few decades are given in figure 1.1. However, the demand of milk and milk products in India is projected to increase to 142.9 million tonnes by 2015 and further to 191.3 million tonnes by 2020 [1]. At the existing rate of growth in milk production, in next ten years, supply is likely to fall short of the demand. Among the several barriers in achieving the milk production targets, mastitis continues to remain as a most challenging impediment, since the affected quarters show 30% less productivity and cow looses about 15% production.

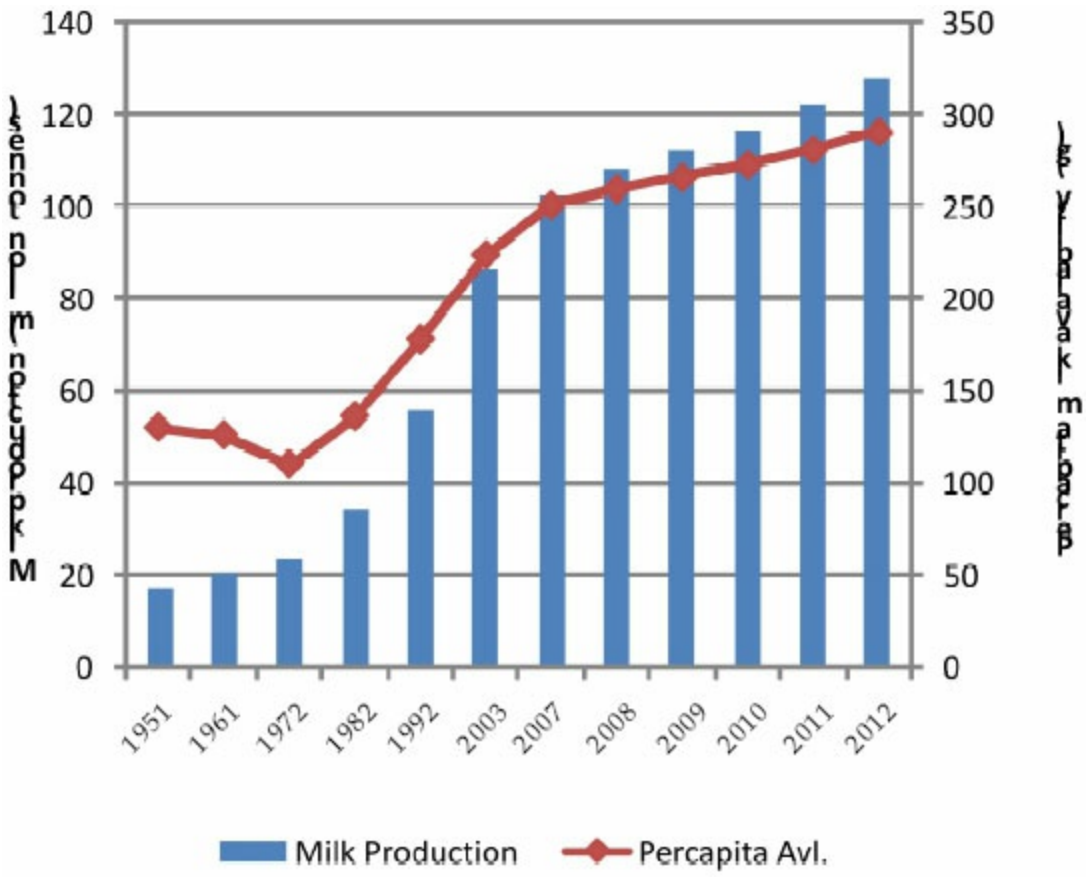

Fig. 1.1 : Milk production and percapita milk availability in India

What is Mastitis?

Mastitis, the inflammation of parenchyma of mammary glands, is a complex multi-etiological disease affecting dairy cattle worldwide. Based on the severity, the inflammation can be classified into sub- clinical, clinical and chronic forms, and its degree is dependent on the nature of causative pathogen and on the age, breed, immunological health and lactation state of the animal. Mastitis is characterized by physical, chemical and usually bacteriological changes in milk and pathological changes in glandular tissues affecting milk production and its quality.

The mammary gland, because of its anatomical position, is exposed to several external factors that influence the physiology and pathology of the mammary parenchyma. Several microbial species, when enter into the udder, have the ability to infect the mammary parenchyma resulting in mastitis.

Generally the udder defense mechanism clears off the infection, but when the animal is under immune-suppression (especially during immediate post-partum period), the organism gets upper hand resulting in mastitis. Thus, occurrence of mastitis is an outcome of the interplay between the infectious agents and management practices stressing the defense of udder. Poor milking hygiene, milking machine faults, teat injuries and populations of pathogens on the cow's skin and epithelia and in its environment predisposes the cow to mastitis. In spite of consistent efforts by the researchers on mastitis causing organisms and its various aspects of treatment and control, the problem of mastitis remains insurmountable to the dairy farmers and poses several challenges to modern veterinary practioners. A better understanding of the multiplicity of pathogens causing mastitis, knowledge of mammary gland immunology, bacterial virulence factors and mechanism of pathogens would help in developing effective preventive and therapeutic strategies, which would result in reduced severity of mastitis, increased production and profitability, and ensure supply of safe and nutritious milk and milk products to the consumers.

Present Status of Mastitis: Global *vis-a-vis* India

Mastitis, on account of causing serious wastage and undesirable milk quality, has emerged as a major challenge and continue to be one of the most common and costly diseases in dairy cattle across the globe accounting for around 40% of the total direct costs of the common production diseases [2].

The world's milk production has reached 711 million tonnes in 2010 from 547 million tonnes in 1996 and is expected to rise above 794 million tonnes in 2017. With an output of 257 million tonnes, Asia remains the region with both the largest production and the highest rate of annual growth in 2010. However, with the increase in milk production, the production diseases especially mastitis is also increased. In India, the economic losses due to mastitis have increased about 115 folds in last five decades [3]. Surveys on the prevalence of mastitis in most countries, irrespective of the cause, show a comparable figure of 50% among dairy cows. Subclinical mastitis is believed to be more prevalent than clinical mastitis in most countries. Globally, the prevalence of subclinical mastitis on farms could range from 19 to 78% as indicated in figure 1.2.

After analyzing the published reports, one can safely say that the average

prevalence of mastitis in 1960s to early 1990s, was not more than 30 per cent, however afterwards the prevalence increased to even more than 60 per cent (Fig. 1.3). Two decades ago the average incidence of clinical mastitis in India was 1-10% with subclinical mastitis ranging from 10-50% in cows and 5-20% in buffaloes, while recent studies reported high incidence of subclinical mastitis ranging from 20 to 83 per cent in cows and 45 per cent in buffaloes. Analyzing data reported by more than 100 recent studies spread over 21 states of India indicate that the overall prevalence of mastitis ranges from 25 to 97 per cent with an average prevalence of about 50 per cent [5]. This clearly indicates drastic increase in the prevalence of mastitis especially the subclinical form of the disease, which is an alarming situation to the dairy sector in the country.

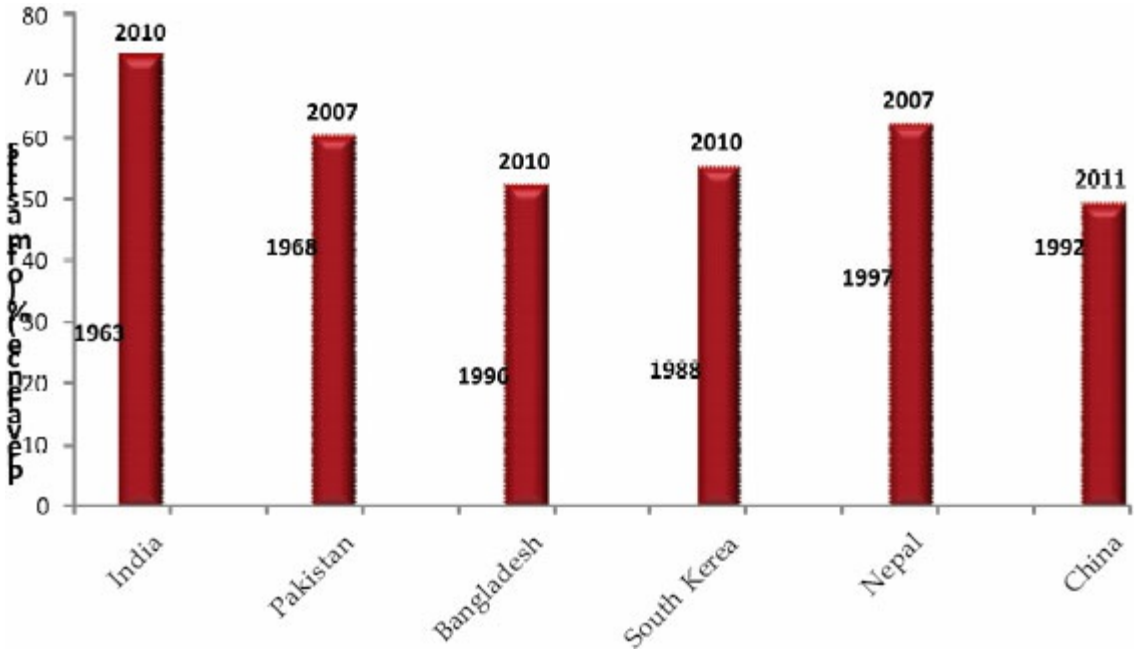

Fig. 1.2 : Prevalence of mastitis in Asian countries [3]

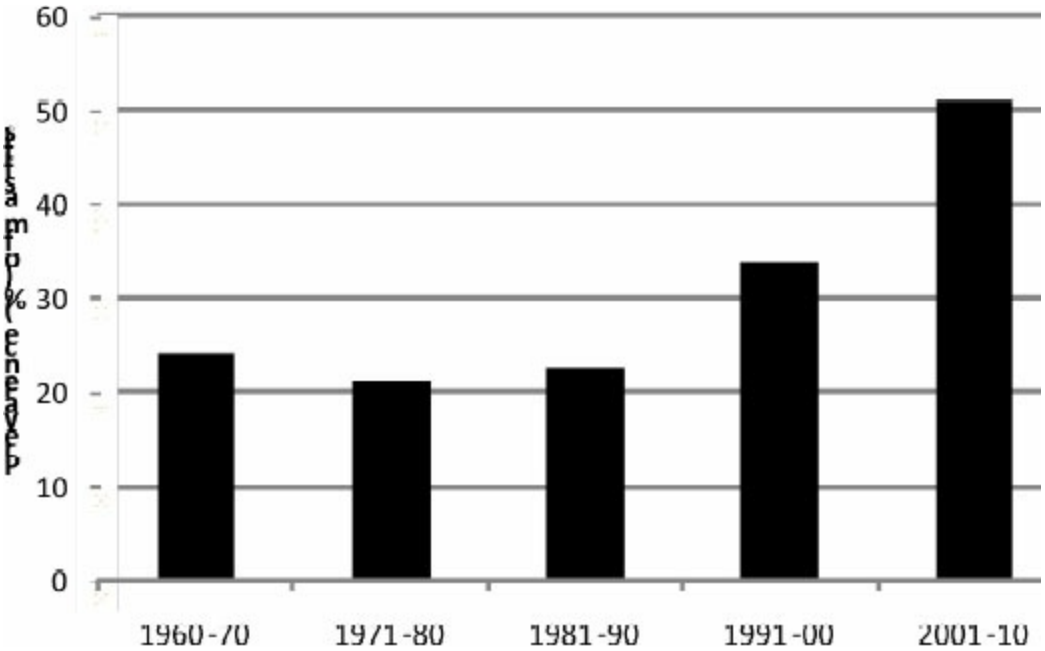

Fig. 1.3: Increasing trend of mastitis prevalence in dairy animals [3]

Economic Loss Due to Mastitis

In mastitis, the loss is due to temporary or permanent loss of milk production, poor milk quality, discarding of milk after antibiotic treatment and pre-mature culling or reduced productive life of animals. The loss due to subclinical mastitis overweighs the loss associated with clinical mastitis. In the affected animals, the milk yield is reduced considerably. Different workers estimates of milk yield loss range from 100 to 500 kg/cow per lactation. When Clinical Mastitis occurs, additional costs result from discard of abnormal milk, drugs, and veterinary services.

According to a study, the estimated loss following clinical mastitis was almost 700 kg for cows in first lactation and 1,200 kg for cows in second or higher lactation [6]. Each case of clinical mastitis has been estimated to cost between $100 and $200 per cow within the lactation. A Sweden based study reported the average cost of clinical and subclinical mastitis to be €275 and €60 per case, respectively [7]. Globally, losses due to mastitis are estimated at about US$ 35 billion annually. The disease accounts for an overall annual loss of about €1.55 billion in European Union [8]. In the United States of America, National Animal Health Monitoring Survey indicated that mastitis is the most

prevalent disease affecting adult dairy cows and costs the American dairy producers approximately $2 billion each year, which is approximately 10 percent of the total value of farm milk sales [9, 10]. Other major milk producing countries also suffer huge financial losses due to mastitis. As per the recently reviewed information, Australian and New Zealand dairy sector lost annually US$ 200 million and US$141.70 million, respectively due to poor udder health [11]. The Brazilian dairy farmers suffer an economic loss of approximately one billion reas (BRL) annually [12]. In India, annual economic losses incurred by dairy industry on account of udder infections have been estimated about Rs. 6053.21 crore. Out of this, loss of Rs. 4365.32 crore (70 - 80 %) has been attributed to sub-clinical version of udder infections [13]. In another report from India, the annual economic losses due to mastitis, has been calculated to be approximately Rs. 7165.51 crores; losses being almost same for cows (3649.56 crores) and buffaloes (3515.95 crores). Subclinical mastitis has been estimated to account for 57.93% (4151.16 crores) of the total economic loss due to mastitis [14]. In another report, it has been estimated thatthe estimated loss due to mastitis is around Rs 16,702 million per annum [15].

Almost all authors agree that at least 70% of economic losses come from reduction on milk production and discard of milk from sick animals. Other causes are the elimination of milk containing antibiotics used in treating sick animals, loss of genetic value by culling cows early and therefore more expensive replacement, veterinary fees, drug expenditures, payments of extra labour hours and the commercial value reduction of cows removed. The economic losses associated with mastitis has been estimated to be due to reduced milk yield (up to 70%), milk discard after treatment (9%), cost of veterinary services (7%) and premature culling (14%).

Changing Epidemiology: A Cause of Concern

Mastitis is the outcome of interaction of various factors associated with the host, pathogen(s) and the environment. Association of some host, managerial and housing determinants with mastitis is well established. The predominant causal organisms responsible for causing mastitis are cell-walled pathogens, although mycoplasma, yeast and algae have also been reported to cause mastitis [16, 17, 18]. Interestingly, 137 species and subspecies of potential

pathogens can be associated with infection of the mammary gland [19]. However, only 5 species of bacteria account for the bulk of bovine mastitis cases. Interestingly, dominant causal agents of mastitis may bear some geographical signatures, as the distribution of mastitis-causing bacteria display substantial geographic variation. For example, *Staphylococcus aureus* is most frequently involved in clinical mastitis, followed by *Streptococcus dysgalactiae* in Norway. In the midwestern United States, coliforms are the most frequently isolated bacteria, whereas in the United States, *Klebsiella* and E. coli mastitis are of equal importance. In Europe, clinical *Klebsiella* mastitis occurs less frequently than clinical *E. coli* mastitis. In contrast, coliforms are less important and *Streptococcus uberis* is the main concern in both clinical and subclinical mastitis in New Zealand.

In India, many workers have reported that Staphylococcus spp. is the chief etiological agent of mastitis in cattle and buffaloes. However, there are no studies on nationwide distribution of mastitis- causing bacteria in India. Apart from regional difference, cows in tie- stalls have higher incidence of *Staphylococcus aureus, Streptococcus uberis,* coagulase-negative staphylococci and other streptococcal infections compared with those in free-stalls, where *Klebsiella spp.* and *E. coli* are main concern. Collectively it suggests that distribution of organism may vary between region and husbandry systems and it is important to know the epidemiological pattern of mastitis pathogen before the implementation of control strategies.

In recent years, there have been changes in the relative and absolute importance of different pathogens. In UK, during 1960s, it was observed that *Staphylococcus aureus* was the most common organism in mastitis, but in 1980s *Escherichia coli* was most commonly isolated from the milk of mastitis affected cows and the same trend was continued in 1990s also. In several countries, *Staphylococcus aureus* continues to be the major cause of sub-clinical mastitis and the pathogens previously considered to be purely environmental may also be capable of causing persistent infection.

Mastitis Pathogenesis

Onset of mastitis comprises three stages- invasion, infection and inflammation. Most pathogens enter the mammary gland *via* teat canal. The stage of entering of organisms from the exterior of the teat to the milk and

inside teat canal is termed as invasion. Following invasion, pathogens establish in the teat canal, multiply in milk (infection) and achieve a concentration as high as 10^4 cfu per ml with no apparent host response. The initial response in udder is a breakdown of epithelial barrier separating the site of infection from humoral defense. When pre-existing defense systems in the normal udder fail to eliminate the infection during early stage, inflammation develops with a consequent influx of serum proteins and inflammatory cells. Sudden and intense influx of PMN within a few hours of infection can readily eliminate mammary pathogens from milk. If infection is cleared by the PMN, the infectious condition resolves, otherwise persistence of bacteria in the gland lead to chronic inflammation. More severe consequence can occur due to delay or absence of the inflammatory response leading to significant production of toxins and further increase in the severity of the disease

Stage of inflammation, following establishment of infection in mammary tissues, is apparent in the form of clinical mastitis. Itis characterized by increased SCC due to recruitment of PMN involving a sequence of events occurring at the site of infection, at vascular level involving cellular and humoral components of the host immune system. The pathological consequences of udder infection/injury may range from transient to persistent (duration), mild or sub-clinical to per acute and fatal (severity) mastitis. Per acute form of mastitis is marked by severe inflammation with swollen and hot painful udder quarter with systemic reaction. In acute mastitis, there is severe inflammation. Sub¬acute mastitis is characterized by mild inflammation with persistent abnormality of milk. Chronic mastitis develops following recurrent attacks of inflammation with little change in milk. Overall, there are three categories of mastitis clinically- abnormal milk (abnormality in secretion), abnormal gland (abnormal size, consistency and temperature of udder) and abnormal animal (systemic reaction). Mastitis can also occur in sub-clinical form when there is evidence of inflammation reflected by high SCC in milk without any visible abnormality in milk or udder.

Diagnosis of Mastitis

Correct and quick diagnosis is the most important aspect for effective control of mastitis. Current diagnostic methods to detect mastitis are based on abnormalities of milk and udder. Systemic reactions such as toxemia, fever,

anorexia and depression are important in deciding severity of infection. Recent advancement in diagnosis of mastitis includes application of modern molecular methods such as PCR for rapid and reliable classification of mammary pathogens. The clinical mastitis is easily recognized by abnormality of milk and udder. However, for regular monitoring of udder health and confirming the udder infections, particularly, sub-clinical mastitis, following tests are used.

Direct tests: Somatic cell count and cultural examination of milk are used as the direct indicators of udder health status and considered as gold standard tests for comparing sensitivity and specificity of other tests.

Somatic cell count in milk:It is suggested that high SCC in milk is indicative and correlated to response to microbes in mammary gland. A low total SCC with low proportion of neutrophils indicates minor chronic lesion. Elevated SCC in milk is associated with compositional changes specifically low potassium, reduced casein and calcium, increased pH, calcium, albumin, whey proteins, lactoferrin, immunoglobulins, sodium, chloride concentrations and activities of blood enzymes including lipase and plasmin that have negative impact on milk quality. According to the guidelines of International Dairy Federation, status of udder quarters is classified into following categories on the basis of SCC and culture of milk samples for three consecutive days.

Quarter health status	**Culturing of milk samples**	**SCC of milk samples (cells per ml)**
Healthy	2 times negative	All 3 times < 5,00,000
Specific mastitis	2 times positive	Minimum 1 time > 5,00,000
Non-Specific mastitis	2 times negative	Minimum 1 time > 5,00,000
Latent infection	2 times positive	All 3 times < 5,00,000

Bulk milk cell count (BMCC) and individual cow cell count (ICCC) are the two methods of counting SCC that can be performed by direct microscopic examination or using electronic cell counter. BMCC is used for continuous monitoring of udder health at farms, which have no current problem of mastitis and a cell count of >3x 10^5 cells per ml indicate necessity of ICCC. An ICCC threshold of >4x 10^5 cells per ml is used as an indication of infected cow. However, besides inflammation of udder, number of other factors such as stage

of lactation, number of quarters infected, kind of infection, and genetic variables also influence the SCC and these need to be considered while concluding the interpretation of SCC results.

Microbial examination: Culture of individual quarter milk is important to detect intra-mammary infection and the udder health. Teat is washed, preferably using 0.01% (1:1000) potassium permanganate solution and dried properly to collect milk samples for microbial examination. Teat end is cleaned with a 70% alcohol swab and teat sphincter is pressed to remove dirt or wax from the teat orifices. The first two or three streams are rejected because its cell count and bacterial load are likely to reflect the disease situation within the teat rather than the gland as a whole [20]. Both pre-milking and post¬milking samples can be collected for better accuracy.

Indirect mastitis tests: Mastitis induces several compositional changes in milk that can be monitored by various chemical, cytological, immunological and physical methods. Various indirect mastitis tests have been developed based on these changes such as to monitor SCC, concentration of sodium, potassium chloride, albumin, trypsin inhibitor factor and other acute phase proteins in milk.

California mastitis test (CMT): The test determines the quantity of DNA and thus the approximate number of leukocytes. It is a simple, rapid and efficient indirect test. The test reaction is graded as negative (N), trace (T), 1, 2 or 3 approximately equating mean cow cell counts of 100,000, 300,000, 900,000, 2,700,000 and 8,100,000 per ml in milk, respectively. Owing to its simplicity and rapid reactivity, this test has been used extensively used as a rapid cow side test in the field. The test has variable sensitivity (ability of test to differentiate infected and non-infected milk samples) and specificity (ability of test to predict infected samples correctly). In Indian conditions, the sensitivity of the test is reported to be >71 percent.

Electrical conductivity (EC): It is equally popular test for detection of mastitis. The test is based on measurement of increased conductivity of milk due to high ionic concentration of sodium, potassium and chloride in the infected milk. When measurements are taken accurately and due consideration is given to breed and age, the conductivity of milk can distinguish between infected and uninfected udder quarters [21]. EC can be performed in line while milking. Stage of lactation can influence EC and the test may give non-specific diagnosis.

NAGase: Measurement of activity ofN-acetyl-beta-D- glucosaminidase (NAGase), along with lymphocytes and polymorphs in milk, has been also used to detect mastitis. The test is a reliable indicator of high cell count in milk and is proved as a most accurate indirect test equivalent to SCC in sensitivity and specifity. It is considered a superior test for accurate diagnosis of subclinical mastitis. In general, NAGase test is rapid, can be done on fresh milk and if facilities for all tests are available, the best option to confirm mastitis is to carry out NAGase and SCC tests.

Other indirect tests: These include the estimation of lactose, chloride, pH and protein (albumin and c- reactive protein) in milk. Immunological test, a *direct capture ELISA* measures elevated PMN antigens and could provide estimation of as low as 100,000 cells per ml in milk. Antitrypsin test, based on trypsin inhibitor potency of milk was proposed as a good indirect test that could be easily automated. Detectable difference has been observed in the level of heptoglobin, fibrinogen, alfa-1 antitrypsin and ceruloplasmin. The elevated levels of these *acute phase proteins,* both in serum and milk, can be helpful in detecting bacterial infection as well as to assess the severity of the udder infection. In general, a range of direct and indirect tests is available for diagnosis of mastitis, but each of these tests has its own merits and demerits. As such, results of a single test should not be relied upon to make management decisions, which generally need routine sequential testing.

Mastitis Therapy

Treatment of mastitis has been the traditional procedure since the antibiotics were discovered and continues to be an important component of mastitis control in dairying. Despite improvement in understanding of patho-physiology of mastitis, successful treatment strategies for all organisms has not been achieved. Although the importance of bacteriological cure has been recognized, the main aim of the treatment of clinical mastitis has been to restore the milk yield with short duration of treatment rather than eliminating the infection. This could be the probable reason for unsuccessful in mastitis control or reoccurrence of mastitis despite the treatment, especially under Indian conditions. Our experience indicates that 20% of the mastitis affected animals relapsed 2.36 times in same lactation, suggesting failure to achieve bacteriological cure with the antimicrobials used or resistance of the

underlying organism to the antimicrobials used. Further, different group or species of organisms needs different treatment strategy and thus, treatment decision should be based on culture and sensitivity results. However, it is uncertain whether result of "time consuming" antimicrobial susceptibility test can accurately predict treatment success and most suitable drugs at *in vitro* level often fails to cure the animals.

So far, antimicrobial susceptibility testing is of no value in predicting duration of treatment or bacteriologic cure rate, thus the global adaptability of this test is very less. Since the *in vitro* assays do not account pharmacokinetic properties of the drugs, the most suitable drug at in vitro level fails to perform well at *in vivo*. Further, the kinetics of drugs may also vary in infected (mastitis) animals and lack of studies on pharmacokinetic and dynamic behaviour of the drugs under the influence of pathogen, animal and environment as a whole, rather than targeting against organism alone could be the possible reason for therapeutic failure. Moreover, the efficacy of therapy appears to be more influenced by the pharmacokinetic than minimum inhibitory concentration.

Since, antimicrobial treatment for gram negative or culture negative mastitis is not recommended; culture of milk sample will help to reduce the unnecessary antimicrobial usage. On other hand, it is useful in chronic and unresponsive mastitis cases. For instance, treatment of *S. aureus* induced mastitis is very challenging due to its intracellular locality and other virulence factors. Further, *S. aureus* mastitis is chronic in nature or non-responsive to drugs, thus culling of chronically infected animals would be very effective way to reduce mastitis incidence. Therefore, diagnostic facilities at regional laboratories or farm level would be very much useful to take treatment decision. In contrast to traditional time consuming culture tests, recent bacteriological methods like selective diagnostic media and rapid PCR- based test, which are available in many countries would allow rapid diagnosis and thus proper selection of the antimicrobial drugs. These methods should be encouraged in India.

Unlike clinical mastitis, treatment of subclinical mastitis remains controversial due to the efficacy of treatment. Although, the success of dry cow therapy is believed to be based on the pathogen involved, it is one of the potential ways to check the subclinical mastitis in dairy animals. For example, dry cow therapy is an established method of mastitis control in USA, where

100% of cattle on 73% of dairy farms practice this method. Information on the extent of such kind of practices in India is not available. Though, treating of all animals may not be economically feasible in India, selective dry cow therapy appears to be better option in animals with high risk at drying off and herd with high prevalence of infections. Further, non-antimicrobial alternatives to dry cow therapy such as internal teat sealants, combination of selective dry cow therapy and teat dipping at drying off has been shown to reduce risk of clinical mastitis. However, extensive studies involving large number of animals especially under field conditions are required to assess the efficacy before adapting such strategies.

Although antibiotic therapy is continue to be an important part of current mastitis control programme, alternate approaches are needed at present due to various reasons. Differential efficacy of intra¬mammary infusions during lactation period particularly against major mastitis pathogens, cost-effectiveness, antibiotics residues, emergence of antibiotic resistant human pathogens and public awareness of food safety issues are some valuable reasons for non-antibiotic approaches of mastitis therapy.

Why Therapy Fails in Mastitis?

Microbial Factors

Localization of microbes: Intracellular location and tissue invading properties of *S. aureus* makes very difficult to achieve effective concentration of antibiotics at target site even the antibiotics are sensitive. Further, scar formation in udder parenchyma prohibits the killing ability of antibiotics.

Mechanism to evade from antibiotics: Development of temporary a capsular or L-form (bacteria without cell wall) makes beta-lactam antibiotics ineffective. Further, dormant stage of bacteria is not susceptible to penicillin group of drugs. Bacterial mechanisms that evade antibacterial effect, like interference with phagocytosis, slime formation, etc., are favoring bacterial multiplication in udder.

Host Factors

Although mastitis is a management related disease, in any farm with similar management strategies, susceptibility of some animals to mastitis over other animals suggest that individual animal is a deciding factor. The recent

understanding of peri-partum immune status and immunobiology of udder further substantiate that immune compromised animals are most susceptible to mastitis.

Drug Factors

Low bioavailability of drugs and thus inadequate concentration at target site, low passage of drugs across blood-milk barrier, duration of therapy, antibacterial resistance, side effects and half-life of drugs are some important drug-related factors causing failure of mastitis therapy.

Management Factors

Inaccurate diagnosis, delayed initial treatment, aseptic intra¬mammary infusions, trauma in teats all leads to failure to achieve bacterial cure, relapse (mastitis within 21 days of treatment) and re¬occurrence of mastitis.

Mastitis Vaccines

An approach to reduce the impact of several important mastitis causing organisms has been the introduction of vaccines against those pathogens. The use of vaccination to control infectious diseases in dairy cattle is common and vaccination against mastitis pathogens is a control strategy used by some dairy farmers in developed countries. Currently, commercial vaccines against mastitis caused by *Staphylococcus aureus* and *E. coli* are available in USA. These vaccines can reduce the severity and longevity of clinical mastitis as well as provide a degree of protection against new intra mammary infection by these pathogens. The J-5 type of vaccine is used to protect against intra mammary infection caused by coliform *(E. coli, Klebsiellaspp., Citrobacter spp.,* and *Enterobacterspp.),* when given to adult cows during the dry period, have been shown to reduce the incidence of coliform mastitis. Since the coliform vaccine has been shown to reduce economic losses related to production, culling and death, majority of the mastitis consultants recommend their use in most of the dairy herds.

Several experimental and field trials have shown that commercially available *Staphylococcus aureus* vaccines increased cure rate of mastitis and thus reduce the development of chronic infections. However, it is well known fact that the successful control of *Staphylococcus aureus* mastitis depends on prevention of new infections rather than reducing development of chronic infections. Therefore, the failure to prevent new infections is probably the reason that this vaccine is being used on a limited basis in mastitis control programs. Though, there are no commercial vaccines available to protect against Streptococcus mastitis, the research for vaccines against *Streptococcus uberis* has been initiated in recent years.

Effective mastitis vaccines should reduce the severity and frequency of mastitis, prevent new infections and eliminate existing infections. Further, it is necessary to identify different bacterial phenotypes causing mastitis and characterization of their genotype in different agro- climatic area, in order to use those vaccines in other countries. Also, the vaccine-induced antibodies should have wide range of protection capacities against the organism with high affinity. At present, in India, no vaccine is available against any of the major mastitis causing organisms.

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chapter 2
Etiology and Epidemiology of Bovine Mastitis

The etiology of mastitis can be of an infectious, traumatic or toxic nature. During early days, the mastitis researchers associated mastitis condition with mostly the physical factors like cold and mechanical injuries only. It was Frank in the year 1876 proved the infectious nature of this disease and put forward an entirely new concept in the investigation of bovine mastitis. Consequently, an association between mastitis and pathogenic micro-organism was established in 1887 [1]. Of the several causes of mastitis, the microbes are the vital players in causing bovine mastitis. The cow udder is an ideal environment for microbial growth and under optimum udder conditions, such as temperature, nutrition and freedom from outside influence, pathogenic organisms multiply astronomically and it is this factor that causes udder damage and triggers the response that is recognized as mastitis [2]. Although bacteria, fungi, yeasts and possibly virus can cause udder infections, the main agents are bacteria. Most of the major pathogens causing mastitis were identified during 1940s [1], and the first known etiological agent of bovine mastitis identified belonged to the genus *Streptococcus*. Till date, more than 200 microbial species, subspecies and serovars have been isolated from the bovine mammary gland. The causative agents identified to be associated with bovine mastitis are enlisted in Table 2.1.

Bacteria	*Staphlococcus aureus, Streptococcus agalactiae, Escherichia coli, Streptococcus dysgalactiae, Streptococcus uberis, Streptococcus zooepidermidis, Streptococccus faecalis, Streptococcus pyogenes, Corynebacterium pyogens, Pseudomonas aeruginosa, Mycoplasma bovis, M. canadensis,*

	M.californicum, M. bovihirnis, M. dispar, M. bovigenitalium, M alkalescens, Campylobacter jejuni, Heamophilus somnus, Streptococcus pneumonia, Corynebacterium ulcerans, Klebsiella pneumonia, K. oxytocia, Enterobacter aerogenes, Mycobacterium bovis, M. tuberculosis, M. lacticola, M. fortuitum, Bacillus cereus, Pasteurella multocida, Bacteroides funduliformis, Serratia marcescens, Acholeplasma laidlawii, Yersenia pseudotuberculosis, Mannheimia haemolytica, Mannheimia granulomatis.
Facultative	*Peptococcus indolicus, Bacteroides melaninogenius, Eubacterium*
bacteria	*combestii, Clostridium sporogene, C. perfringes type* A *and Fusobacterium necrophorum, Citrobacter, Proteus sp.*
Fungi/Yeast	*Aspergillus fumigatus, A. nidulans, Trichosporon cutaneum, Trichosporon beigelii, Pichia spp, Geotrichum candidum, Nocardia asteroids, N. Brasiliensis, N. Farcinia, N. neocaledoniensis, Candida krusei, C.tropicalis, C. paratropicalis, C. quillermondii, C. rugosa, Cryptococcus neoformans, Saccharomyces spp, Torulopsis etc.*
Algae	*Prototheca zopfii, P. trispora, P. wickerhamii, P. blaschkeae etc.*
Viruses	Adeno virus, Herpes virus, Rota virus, Reo virus, Mammilitis virus, Pseudocowpox virus, Parainfluenza virus, Apthovirus, R.P. virus, Bovine immunodeficiency virus etc.

Bacteria as Causative Agents

Amongst all the etiological agents of mastitis, majority of intramammary infections (IMI) are caused by bacteria. Although a vast array of bacteria are involved in causing bovine mastitis, *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis* and *Escherichia coli* are the predominant bacteria associated with mastitis. The other pathogens may cause occasional herd outbreaks [3]. Comprehensive data

on the distribution of bacteria causing clinical bovine mastitis are not available for any country. According to the results from individual studies, it appears that the bacterial etiology of clinical mastitis changes over time and there are clear differences between countries. The distribution of these common pathogens in the different countries is been depicted in table 2.2.

Table 2.2: Distribution of common mastitis organisms in different countries

Country	Prevalence (%) of common mastitis pathogens					Reference
	S. aureus	CNS	Strep. spp	*E. coli*	Others	
India	74.71	-	21.13	-	4.15	[30]
	42.10	-	5.26	18.95	3.16, 4.74, 5.26, 2.10	[31]
	60.32	-	31.98	-	-	[32]
	74.04	-	6.00	-	7.32, 2.93, 5.71	[33]
	39.01, 52.48	-	-	-	8.51	[34]
	18.99	-	15.50	-	17.05	[35]
	27.90	16.28	6.98	17.44	5.81,4.65, 5.81, 4.56, 3.49, 3.49, 3.49	[36]
	-	-	15.45	12.73	10	[5]
	34.38	25	9.38	21.87	6.25, 3.12	[37]
	39.53	-	20.98	9.30	16.27, 6.97, 6.97	[38]
	16.66	40.47	33.38	-	9.52	[13]
	59.37, 4.90	-	16,10.63, 1.1	2.9	2.2, 1.8, 1.1, 1.1, 1.1	[39]
	27.86	72.13	-	-	-	[11]
	27.27, 15.91	-	50,4.55	-	2.27	[40]
	57.27	-	15.45	12.73	10	[3]
	27.37	12.63	5.79	8.95	7.89,1.35	[41]
	33.83	97.8	-	-	-	[42]
	33.14	49. 5	11.63	14.04	-	[17]

Pakistan	12.06	-	7,3	10	3.5, 3	[43]
	33.99	-	-	27.09	35.46,1.97, 1.48	[44]
	13.42	-	9.39	46.98	14.77,4.02, 2.01, 1.34	[45]
	45	-	23	18	14	[46]
	49.63, 6.54	0.93	23.83, 8.88, 0.93	1.4	3.74,0.93, 0.47, 0.47	[47]
	28.32	-	7.51	16.18	13.29, 12.42, 7.22, 6.44, 5.2, 3.17	[48]
South Korea	51.40, 2.7	-	15.8, 2.1, 1.0	16.5	5.1, 0.3, 0.3	[4]
	27.4	25.04	-	-	-	[49]
	12.2	40.7	5.3	4.5, 19.5	-	[50]
Bangladesh	49.30	-	14	6	8, 4.7	[51]
	39.64	-	2.47	11.11	3.7	[52]
	31	-	3.1	11.3	4.7	[7]
	23.81	-	39.36	-	25.39	[53]
China	79.12, 6.59, 1.1	-	2.2	-	1.1, 1.1	[54]
	22.61	-	25.22	-	15.65, 16.52, 6.96	[55]
	41	-	53, 29, 27	82	-	[56]
	29.5	4.7, 19.7	7.6, 4.7, 2.9	25.70	16.2	[57]
Iran	2.89	-	22.11, 11.43	10.16	1.07, 1.76, 0.14, 0.21, 0.03, 0.03	[58]
	-	3.88	8.33	9.44	-	[59]
	68.80	-	9.8, 0.4	7.9	5.8	[60]
	14	36.18	-	-	8, 7	[61]
Isreal	62.50	-	21.88	12.50	-	[62]
Japan	-	-	-	75	13, 8, 4	[63]
Nepal	15.38, 57.69	-	17.31	1.92	-	[64]
US	-	26	28	13	6	[64]
	5	3	21	40	10	[65]
	2	3	26	18	9	[66]
Italy	5.3	88.5	-	-	5.4	[67]

Staphylococcus aureus

S. aureus has been reported as the chief etiological agent of mastitis in India and in most of the Asian countries by various researchers [3, 4, 5, 6, 7]. *S. aureus* is ubiquitous organism and is capable of causing peracute, acute,

subacute, chronic, gangrenous and sub-clinical types of mastitis [1]. The acute form of the disease usually occurs shortly after parturition and tends to produce gangrene of the affected quarters with high mortality.

Coagulase Negative Staphylococci (CNS)

Classically, CNS was classified as minor pathogens and their importance as an independent cause of subclinical or clinical mastitis was judged to be limited. However, the significance of CNS needs to be reconsidered as in many countries they have become the most common mastitis causing agents [8, 9]. In India, the prevalence of CNS in milk samples increased from 9.91% in 2003 to 72.13% in 2009 [10, 11]. Thus, CNS are now emerging as a major pathogen associated with subclinical mastitis in many countries [12, 13]. More than 50 species and subspecies are included in this group [14]. *Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus simulans, Staphylococcus saprophyticus, Staphylococcus hyicus, Staphylococcus warneri, Staphylococcus chromogenes, Staphylococcus scrimi* and *Staphylococcus xylosus* are the commonly encountered species of CNS in bovine mastitis [15, 16].

In a study to assess the prevalence of the mastitis pathogens, it was found that 74.04% of intramammary infections were due to *Staphylococcus* spp. including the coagulase negative staphylococci [17]. The most predominant species was *S. aureus* (33.1%) followed by *S. chromogenes* (24.3%), *S. epidermidis* (16.7%), *S. sciuri* (9.6%), *S. haemolyticus* (7.9%), and other staphylococcus (8.4%) (Fig. 2.1). Prevalence of the major mastitis associated pathogens reported by various researchers of India and elsewhere is depicted in table 2.2.

Streptococcus Species

Streptococcus agalactiae, S. dysgalatiae and *S. uberis* have been reported as the three most common etiological agents of mastitis 18]. *Streptococcus agalactiae* has been widely reported as an important pathogen causing contagious mastitis. Other Streptococcal species such

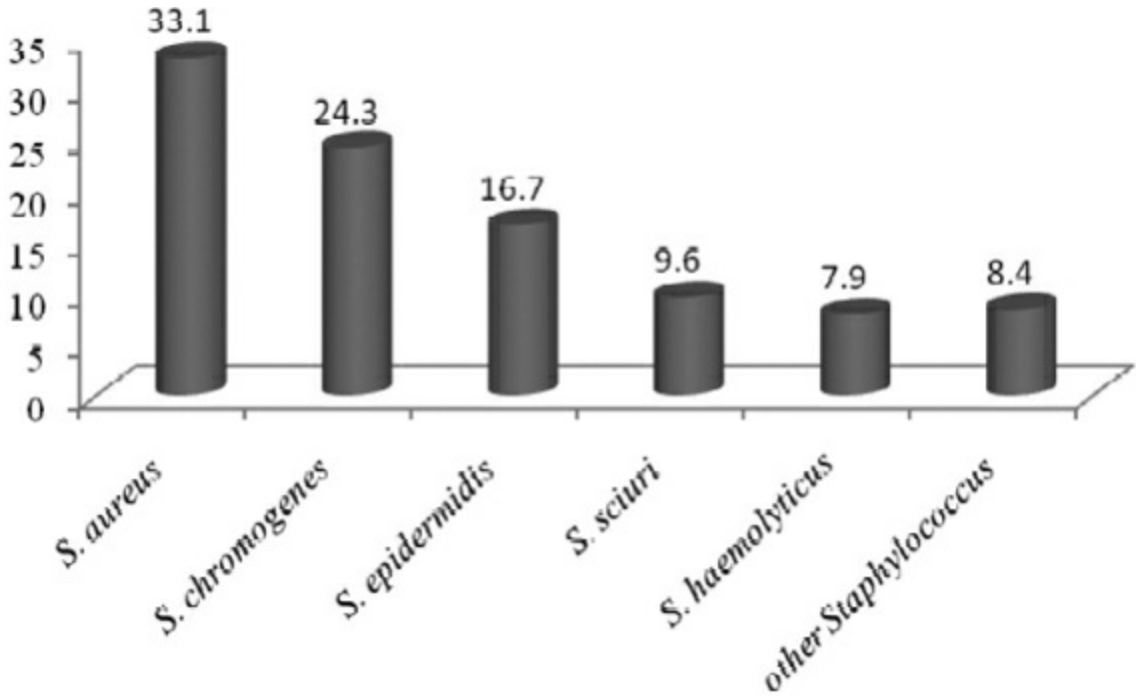

Fig. 2.1 : Prevalence of *Staphylococcus* spp. obtained from bovine milk samples [17]

as *S. bovis, S. acidominimus, S. alactolyticus, S. canis, S. equi, S. equinus* and *S. parauberis* have also been implicated in bovine mastitis, although they are relatively infrequent [18]. All of these organisms (other than *S. agalactiae)* may be contracted by the cow from the environment and not just from another cow, thus they are called environmental streptococci. These bacteria are found in bedding, soil, walkways or any surface the cow is in contact with. Environmental streptococcal mastitis may be apparent (clinical) or inapparent (subclinical).

Escherichia coli

Mastitis caused by *Escherichia coli* is common in high-producing cows with low milk somatic cell count. Environmental mastitis caused by *E. coli* has increased in many countries and herds [19]. The proportion of *E. coli* as a causative agent in bovine clinical mastitis varies between countries. In Finland, less than 20% and in Israel more than 60% of clinical mastitis is caused by coliforms [20, 21], In India,

E. coli is the second most common mastitis pathogen subsequent to

Staphylococcus spp. Different studies in India reports the prevalence of E. coli to be 14% forming the second major pathogen causing bovine mastitis [17,22]. In E. coli mastitis, severity of clinical signs is considered to depend mainly on the host response.

Mycoplasma

Mycoplasma spp. that have been associated with mastitis are considered contagious in nature, transmitted at time of milking from a reservoir, the infected udder; via fomites, hands of a milker, milking unit liners, or udder wash cloths. With respect to *Mycoplasma* mastitis, *M. bovis* is the predominant causative agent and *M. californicum* and *M. bovigenitalium* appear to the next most common. Other species that have been noted as causes of *Mycoplasma* mastitis include *M. arginini, M. bovirhinis, M. canadense* and *M. dispar*. In the European Union countries of Belgium, France, and Greece, the range in prevalence was less than 1% to 5.4% of herds [23, 24, 25]. Yet surveys done in Mexico, Iran and Australia indicate prevalence estimates as high as 55% to 100% of herds [26, 27, 28]. Survey conducted in 244 herds of New Zealand could not detect *Mycoplasma* spp. in any bulk tank samples [29], suggesting a very low prevalence. The wide variation in global prevalence may be a function of exposure to these agents.

Other Bacterial Pathogens

A large number of other bacteria such as *Brucella melitensis*, *Corynebacterium bovis, Enterobacter aerogenes, Klebsiella oxytoca, Klebsiella pneumoniae, Pasteurella* spp., *Proteus* spp., *Pseudomonas aeruginosa*, *Mannheimia granulomatis*, *Trueperella pyogenes* (previously *Arcanobacterium pyogenes*), etc., have been reported to cause mastitis.

Mastitis caused by *Pseudomonas aeruginosa* rarely develops in cattle, and occurs only as sporadic form following intramammary infusions of contaminated material. Mastitis caused by *Mannheimia haemolytica* and *Pasteurella* spp. are more common in sheep, but is rarely reported in cattle and is usually sporadic. Other mastitis that evolves sporadically in a herd, affecting one or two cows, can be caused by *Nocardia spp*. and *Serratia* spp.

Bacillus cereus and *Bacillus subtilis* are saprophytic organisms, occasionally causing acute hemorrhagic mastitis in cattle. Mastitis caused by *Listeria monocytogenes* is important because of the zoonotic risk due to the consumption of contaminated dairy products. Also, mastitis can be rarely caused by *Clostridium perfringens* type A, and has zoonotic risk as it can cause food poisoning in humans [68].

Generally, the microorganisms causing mastitis can be grouped into three categories: 1) Contagious *(S. aureus, S. agalactiae, C. bovis, Mycoplasma* spp.), 2) Environmental *(E. coli, K. pneumonia, K. oxytoca, Serratia* spp., *Citrobacter* spp., *S. uberis, S. bovis* and *S. dysgalactiae)* and 3) Others viz., Coagulase negative staphylococci (CNS), *Pseudomona aeruginosa, Nocardia asteroids, etc.* [69]. The contagious mastitis pathogens can be controlled effectively by procedures that prevent spread of bacteria at milking time including good udder hygiene, proper milking procedures, and post milking teat disinfection. Use of dry cow therapy can help eliminate existing infections and prevent new infections during the early dry period.

Fungi, Yeast and Algal Mastitis

Although bacteria are the predominant cause of bovine mastitis, individual cases and occasional outbreaks of the disease have been attributed to infection by fungi. Only a few of these have been substantiated by the experimental reproduction of the disease with the suspected causal organism, but it is most likely that a number of the species of fungi regularly isolated from mastitis milk samples and at present considered to be without pathogenic significance, will eventually be shown to infect the bovine udder under certain conditions.

A survey conducted in different countries on rate of isolation of fungi from milk revealed isolation rate of 6.1% in Egypt, 1.3% in South Korea and 25.4% in Brazil [70, 71, 72]. The most frequently encountered mycobiota are *Candida* spp., *Aspergillus* spp., *Trichosporon* spp., *Cryptococcus* spp., *Saccharomyces* spp., *Penicillium* spp., etc. The other species of fungi that may be involved in the pathology of mycotic mastitis in cows are: *Pichia* spp., *Torulopsis* spp., *Rhodotorula* spp., *Geotrichum candidum, Trichoderma koningii, Trichotecium roseum.* Fungi are widespread in nature, being noted inbedding and gear from the stables, on milking machines.

Yeasts are supposed to be the main fungal agents of mastitis and they are very often isolated with pathogenic bacteria. The frequency of isolation of potentially pathogenic yeast from milk samples has been shown to be about 7% in central and northern Europe and also in the USA [73]. Several species of yeast or yeast-like organisms have been reported to cause bovine mastitis. *Cryptococcus neoformans* and *Candida albicans* are by far the commonest causes but other *Candida* species have also been associated with bovine mastitis, and more rarely may be a cause of mycotic abortion in cows. *Candida* spp. infection is often *acute* and transient, the organism disappears in about three weeks. In the infections with *Aspergillus fumigatus* and *Aspergillus nidulans* numerous abscesses surrounded by granulation tissue are formed in the mammary quarters, but the lactipherous ducts are not generally affected.

The algae involved in the etiology of mastitis in cattle are mainly: *Prototheca zopfii, Prototheca wickerhamii, Prototheca trispora* and *Prototheca blaschkeae. Prototheca* spp. is considered opportunistic germ and may cause mastitis under the influence of some predisposing factors (immunodeficiency, abusive treatment with antibiotics). These algae generally cause sporadic infections and can cause approximately 0. 1% of mastitis. In cows, *Prototheca* spp. may cause clinical and subclinical mastitis, accompanied by mammary gland tissue necrosis, thrombosis of arteries, veins and lymphatic vessels, these algae can even diffuse in the body by lymphatic pathway and produce a systemic infection. In India, however, bacteria are the major causative agent of mastitis and prevalence of mycotic mastitis is very less. The use of antibiotics as the standard treatment for bovine mastitis makes the evaluation of fungi as a primary cause of the disease extremely difficult.

Viral Infections and Bovine Clinical Mastitis

Historically, mastitis research has been concentrated on bacterial pathogens. In case of viral infections, signs of mastitis may not have been recognised 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. But there are certain viral infections associated in a direct or indirect way with bovine mastitis. Thus, viruses may act as

predisposing agent as well as primary etiological agent in bovine mastitis cases. In natural cases of mastitis, BHV1, BHV4, FMD and PI3 viruses have been isolated from milk.

Bovine herpesvirus 2, vacciniavirus, cowpox, pseudocowpox, vesicular stomatitis, foot-and-mouth disease (FMD) and bovine papilloma viruses can induce teat lesions that decrease the natural defence mechanisms of the udder and thus may promote mastitis. Evidence for the replication of FMD virus in the mammary glands, as a result of a systemic infection, was found in cattle that were infected by (simulated field-type) contact exposure to FMD virus infected animals [74]. But, mastitis associated with FMD virus is assumed to be due to secondary bacterial infections. Bovine herpesvirus 1, bovine viral diarrhoea virus, bovine immunodeficiency virus and bovine leukemia virus infections cause immunosuppression which may indirectly lead to mastitis by contributing the bacterial pathogens. In experimental studies it has been shown that bovine herpesvirus 1, foot- and-mouth disease virus and parainfluenza 3 virus can cause clinical mastitis and viruses such as bovine herpesvirus 4 may lead to subclinical mastitis in cattle [75].

Geographical Variation in Distribution of Mastitis Pathogens

In most countries, the major mastitis pathogens are *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*, *Staphylococcus aureus*, and *Escherichia coli*. But, the predominance of a bacterial species may vary according to the geographical region under scrutiny. The main organisms associated with clinical mastitis in England and Wales are *E. coli* and *S. uberis* [19, 76]. In New Zealand, as in the UK, *S. uberis* is the most common pathogen associated with clinical mastitis, but in contrast to the situation in the UK, *E. coli* mastitis is rarely observed in New Zealand [77]. In Norway, neither *S. uberis* nor *E. coli* are commonly found, rather, *S. aureus* and *S. dysgalactiae* are the most common causes of clinical mastitis [78]. *S. aureus* is the main pathogen associated with clinical mastitis in The Netherlands, followed by *E. coli* and *S. dysgalactiae*. *Mycoplasma* and *Klebsiella* mastitis are major concerns in the USA [79, 80]. Of all the pathogens,

S. aureus remains to be the predominant and one of the significant pathogens causing mastitis in dairy ruminants in many countries, such as the Netherlands, Norway and Italy [73, 81]. The incidence of CNS mastitis is significantly increasing in many countries around the world. Results of eight studies from seven countries and three continents indicated that the most common CNS species isolated from bovine milk were *S. chromogenes* followed by *S. hyicus, S. simulans*, and *S. xylosus* [81]. Whereas, in the USA the trend was *S. chromogenes, S. epidermidis, S. hyicus* and *S. simulans* [82]. So far, 16 CNS species have been isolated from bovine mastitis, and despite some variations between herds and countries, *S. simulans*, *S. chromogenes*, *S. hyicus* and *S. epidermidis* have been reported to be the most common [83, 84]. In India, however, *S. chromogenes, S. epidermidis, S. sciuri* and *S. hemolyticus* are predominant CNS species [17]. The distribution of mastitis pathogens and the adaptation of these microbes thus vary with the agro-climatic factors of a particular geographic region.

Temporal Variation in Distribution of Mastitis Pathogens

Within a geographical region, there occurs temporal drift in the major bacterial pathogens associated with mastitis. Such changes in the trend of mastitis pathogens were encountered in some countries when the standard mastitis control programmes were introduced. For example, in the U.K, the implementation of 5-point mastitis control programmes formulated at the National Institute for Research in Dairying (NIRD) was successful in curtailing contagious mastitis but concurrently there was an emergence of environmental mastitis as a major concern. In well managed herds of U.K, now the contagious pathogens *(S. aureus, S. dysgalactiae, S. agalactiae)* accounts for only 10% of clinical cases. The main organisms associated with clinical mastitis in England and Wales are *E. coli* and *S. uberis* [19, 76, 85].

As more herds implement the standard control measures, pathogen populations at regional or national level start to shift. In some European countries, e.g. Belgium, Denmark, Norway and the UK, occurrence of *S. agalactiae* is now sporadic [86]. In other areas, *S. agalactiae* is still quite common, e.g. Germany, Brazil, Uruguay, USA have herds 29%, 60%, 11%, and 10% respectively positive to *S. agalactiae* [9, 87, 88, 89]. In the Netherlands,

the prevalence of CNS among bacterial isolates from milk samples increased from 16.2% in 1999 to 42.2% in 2004 for subclinical mastitis, and from 7.3% to 14.1% for clinical mastitis [90]. Mycoplasma [79, 91] and Klebsiella [80, 92]) mastitis have become a major concerns in the USA.

Genetic Diversity of Mastitis Pathogens

Knowledge on the etiological agent alone will not aid in designing and implementation of any control strategies, a detailed knowledge on the genetic diversity of these pathogens is equally essential. The application of molecular or DNA-based methods in mastitis research and diagnostics has contributed to an increased understanding of mastitis epidemiology and control options. Using these methods, isolates belonging to a bacterial species can be differentiated at the subspecies or strain level, allowing for improved recognition of sources and transmission routes of pathogens [93]. A variety of molecular methods have been used for typing and sub-typing of bacteria associated with bovine mastitis with different degrees of discrimination, including phage profiling, ribotyping - RAPD [94], restriction fragment length polymorphism - RFLP [95], Multilocus enzyme electrophoresis - MLEE [96], Multilocus sequence typing - MLST, Multiple Locus Variable Number Tandem Repeat - MLVA and Pulse field gel electrophoresis - PFGE. More recently, staphylococcal protein A typing *(spa* typing) was found to be effective, rapid and reproducible method among the various typing methods listed above for the typing of *Staphylococcus aureus,* a major mastitis pathogen [97, 98, 99].

A study on the major mastitis pathogens and their genetic diversity analysis revealed that *S. aureus* t267 was the predominant clone associated with the mastitis in India followed by t359 and t6877 [100]. The study also identified eleven new *spa* types amongst bovine *S. aureus* isolates in India. The *spa* type t267 and t359 were reported in bovine milk *S. aureus* isolates from Brazil, Canada and Japan [101, 102]. *S. aureus spa* type t106 is a common causative agent of bovine mastitis in Montana State of United States (http://cris.csrees.usda.gov/). The *spa* types detected in *S. aureus* isolated cases of bovine mastitis in different countries are summarized in the table 2.3., which clearly indicate differences in the predominance and distribution of bacterial strains between countries/ geographical regions. Likewise, the genetic diversity analysis of streptococci by multi-locus sequence typing

(MLST) revealed the presence of new clones of *S. agalactiae* (ST-483) and *S. uberis* (ST-439, ST-474 and ST-475) in India [103]. MLST has been widely used in describing genetic evolution and the population structure. Depicted below are the eBurst diagrams of *Streptococcus* spp. (Fig. 2.2 and Fig. 2.3) derived using the ST found in the study and the already available STs of the MLST database. This type of cluster analysis presents several advantages, such as the ease of interpretation and the creation of a hierarchical grouping of the isolates that can provide a global overview of the relatedness of the isolates under study and how the defined clusters are connected to each other.

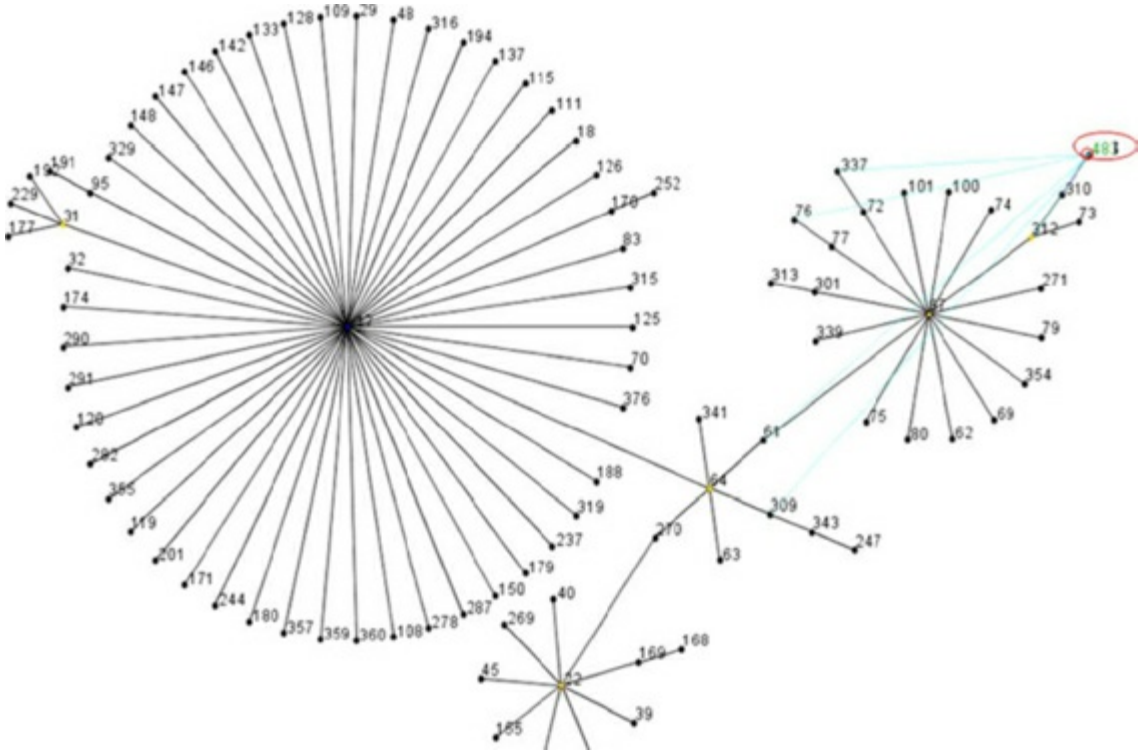

Fig. 2.2 : eBURST diagram derived by eBURSTv3 software using sequence types (STs) available in the *S. agalactiae* MLST database including new ST-483 submitted from the present study. ST-483 (encircled) was found to be a single locus variant of the predicted founder ST-310 and a double locus variant of ST-61, ST-76, ST-309, ST-312, and ST-337.

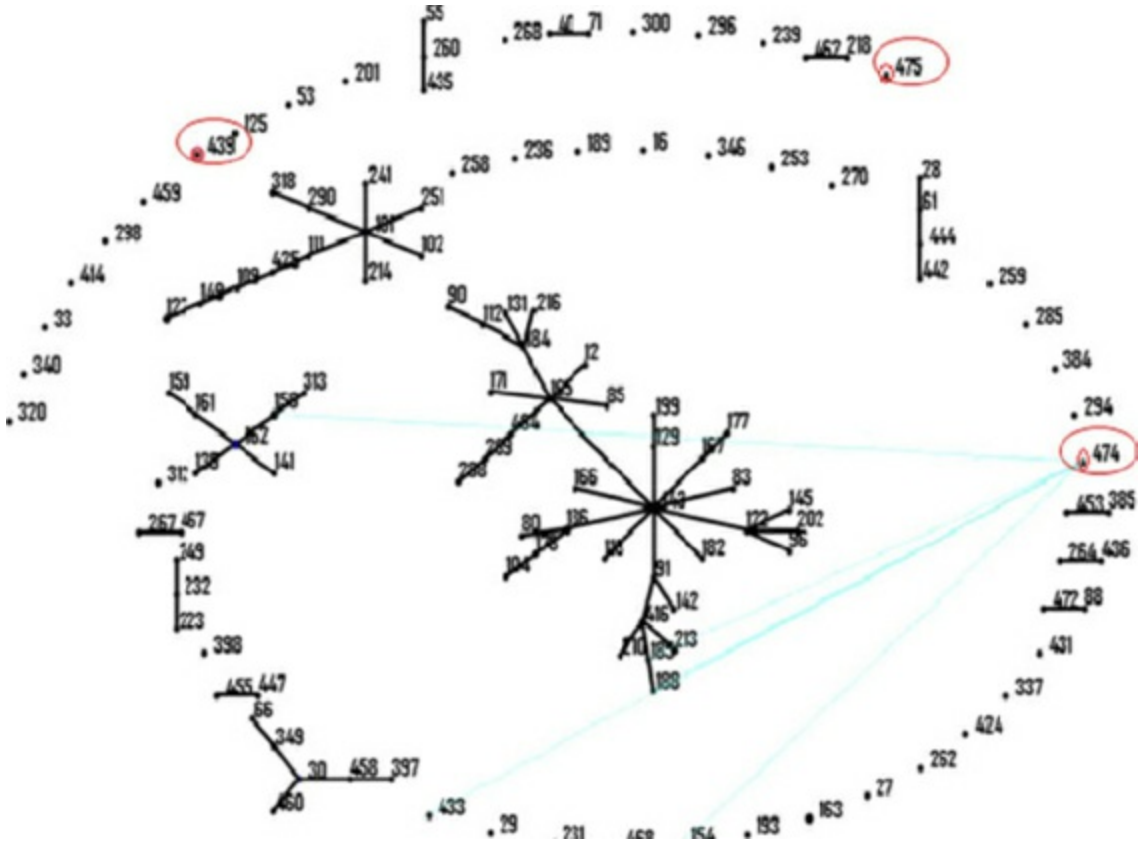

Fig. 2.3 : eBURST diagram derived by eBURSTv3 software using sequence types (STs) available in the *S. uberis* MLST database including new ST-439, ST-474, and ST-475 (encircled). Only ST-474 was found to be a double locus variant of ST-150, ST-185, ST-433 (of global clonal complex ST-143), ST-154, and ST-188. ST-439 and ST-475 were singletons.

The characteristics of these strains might differ in terms of virulence and host adaptability and hence an in depth knowledge of the strains predominant in a particular region is also essential to design effective control strategies.

Table 2.3: Distribution of *spa* types of S. *aureus* from bovine origin [100]

***spa* types**	***spa* type association**	**Country**
t359	Human, Bovine, Buffalo & Ostrich	Spa, Swe, Ger, Can, UAE, Nor, Neth, UK, Austria, Israel, Bel, Fra, Hung, Den, Tai, Bra, Jap, India
t267	Human,	Swe, Ger, SA, UK, Nor, Spa, Jor, UAE, NZ, Neth,

	Bovine & dog	Austria, Fra, Bel, Leb, Ita, Tai, Den, Bra, Jap, India
t6877	Bovine	Can, India
t7696*	Bovine	India
t3992	Pigs & Bovine	Ger, Den, Spa, India
t7286*	Bovine	India
t2770	Human & Bovine	Indonesia, India
t311	Human & Bovine	Austria, Nor, Arg, Spa, Ger, Swe, Gabon, Can, Den, Bel, Neth, SA, NZ, Fra, Czech Republic,Chi, India
t7287*	Bovine	India
t2478	Bovine	UK, India
t6861	Bovine	NZ, India
t7680*	Bovine	India
t2802	Human & Bovine	Spa, Swe, Iceland, India
t1200	Human & Bovine	Ger, Fra, Neth, Indonesia, India
t5109	Bovine	Neth, India
t2915	Bovine	US, India
t008	Human & Bovine	Swe, Ger, SA, UK, Nor, Spa, Jor, UAE, NZ, Neth, Austria, Fra, Bel, Leb, Ita, Den, Bul, Istonia, Crotia, Gab, US, Arg, Ice Land, Czech Republic, Swi, Fin, Pol, India
t7681*	Bovine	India
t7682*	Bovine	India

t7683*	Bovine	India
t7684*	Bovine	India
t7288*	Bovine	India
t7695*	Bovine	India
t2104	Bovine	India, Neth, Jap, Nor
t7867*	Bovine	India

Recent Advancement in Mastitis Etiological Research - Lessons from Metagenomic Approach

Routine bacteriological diagnosis of bovine mastitis usually fails to provide an index to the obligate anaerobic flora involved in the intramammary infections. The polymicrobial nature of udder infections shows that multiple anaerobic as well as facultative anaerobic species colonise and act together. Studies on the prevalence of strictly anaerobic bacteria in the secretions from untreated cases of mastitis in lactating dairy cows have been carried out from time to time. A study way back in 1983 revealed the involvement of a variety of anaerobic organisms concurrently with facultative bacteria in 5.3% and 58.8% of cases classified as subclinical and clinical, respectively [104]. *Peptococcus* spp. was associated with *Corynebacterium pyogenes* and *Bacteroides* spp. with *Staphylococcus aureus* and/or *Streptococcus agalactiae* in 80% anaerobic udder infections. The importance of *Clostridium* spp.: *C. sporogene, C. perfringens* type A, *Fusobacterium necrophorum, Bacteroides melaninogenius, Peptococcus indolicus, Eubacterium combesii* in the pathogenesis of mastitis is not well established as they are often unnoticed by the routine bacteriological studies of milk.

Apart from anaerobic bacteria there are various other species of organisms that cannot be cultured by standard culture conditions and thus may also escape notification. 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 [105, 106]. 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 [107]. Recently, a culture-independent PCR-single strand conformation polymorphism method to investigate bovine mastitis milk samples has been employed [108]. In addition to the known mastitis pathogens, the method was suitable for the detection of fastidious bacteria such as *Mycoplasma* spp., which are often missed by conventional culturing methods.

Recent studies based on genome sequencing data have begun to investigate the true diversity of the microbial world. An impending area of metagenomics, which is the genomic analysis of microorganisms provides an opportunity to record such non-cultivable pathogens associated with bovine mastitis that can help in planning effective therapeutic and preventive measures. The ongoing revolution in metagenomic sequencing technology has led to the production of sequencing machines with dramatically lower costs and higher throughput thus making the technology affordable.

Sequencing and analysis of hyper variable regions within the 16S rRNA gene can provide relatively rapid and cost-effective methods for assessing bacterial diversity and abundance and may be useful for pathogen discovery and identification [109]. Barcoded pyrosequencing on the Genome Sequencer FLX/454 Life Sciences platform, enable a dramatic increase in throughput via parallel in depth analysis of many samples with limited sample processing and lower costs. In a recent publication, barcoded pyrosequencing to characterize the diversity of bacterial communities in human milk samples has been used and this technique identified a much greater diversity of bacteria in milk than what has previously been reported in culture-independent studies [110].

The shotgun pyrosequencing has been employed to analyse the milk microbiome of Kankrej, Gir (*Bos indicus)* and crossbred (*Bos taurus* x *Bos indicus)* cattle with subclinical mastitis [111]. In the study, phylogenetic and metabolic profiles by the web-based tool MGRAST revealed that the members of *Enterobacteriales* were predominant in mastitic milk followed by *Pseudomonadales, Bacillales* and *Lactobacillales.* Around 56 different species with varying abundance were detected in the subclinically infected milk. *Escherichia coli* was found to be the most predominant species in Kankrej and Gir cattle followed by *Pseudomonas aeruginosa*, *Pseudomonas*

mendocina, *Shigella flexneri* and *Bacillus cereus*. In crossbred cattle, *Staphylococcus aureus* followed by *Klebsiella pneumoniae, Staphylococcus epidermidis* and *E. coli* were detected in descending order. Hence, by the use of pyrosequencing it was possible to reveal the true milk microbial flora in subclinical mastitis cattle.

A recent study used metagenomic pyrosequencing of bacterial 16S rRNA genes to investigate bacterial DNA diversity in milk samples of mastitic and healthy dairy cows and compare the results with those obtained by classical bacterial culture. It was observed that in samples that were aerobic culture negative, pyrosequencing identified DNA of bacteria that are known to cause mastitis, DNA of bacteria that are known pathogens but have so far not been associated with mastitis, and DNA of bacteria that are currently not known to be pathogens. Interestingly, pyrosequencing revealed that in samples characterized by culture as *Klebsiella* spp. or *E. coli,* DNA of *S. uberis* was also detected. *Helcococcus ovis* a pathogen associated with bovine endocarditis, was also prevalent in samples from culture negative, *E. coli, S. aureus, Staphylococcus* spp. and *S. uberis* mastitis cases. The presence of such mixed bacteria reemphasizes the hypothesis that subclinical mastitis is not merely caused by single pathogenic species of bacteria. Opportunistic anaerobic bacteria such as *Fusobacterium necrophorum* and *Porphyromonas levii* were highly prevalent in mastitic milk samples and this was not the case for the non-mastitic milk samples. Hence a possible role of anaerobic pathogens in bovine mastitis is also suggested [112]. In another study, culture independent pyrosequencing of amplicons of 16S ribosomal RNA genes (16S rDNA) was used to compare the microbial community composition of culture negative milk samples from mastitic quarters with that of non-mastitic quarters from the same animals. The relative abundances of seven genera were found to be significantly different between the clinical and healthy samples with greater abundances of *Brevundimonas, Burkholderia, Sphingomonas,* and *Stenotrophomonas* found in clinical samples and *Pseudomonas, Psychrobacter,* and *Ralstonia* in healthy samples [113].

This shows that the use of metagenomic pyrosequencing of the 16S rRNA should be considered as an important tool to advance our knowledge regarding the etiology of bovine mastitis and could pave the way to develope effective diagnostic tool.

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chapter3
Functional Anatomy of Bovine Udder

The mammary gland is a modified sweat gland and ectodermal in origin. In case of cow there are two large glands which are placed on either side of the median line in the inguinal region between the thighs. The two glands are separated medially by a double septum. The surface of the gland which exposed laterally is convex. Each gland is comprised of a body and two papillae or teats. Customarily each gland consists of two quarters (fore and rear) but there is no existence of physical barrier internally between the two quarters. However, externally a faint groove separates the two. Even experimentally it is proved that the cavities of two quarters do not communicate each other. The anatomical orientation of the quarters provide natural barrier in case of inter quarter transmission of microorganisms.

The udder is a complex organ made up of a series of systems, which includes

- Supportive system.
- Secretory system
- Duct system for storage and conveyance of milk.
- Teat
- Teat canal
- Blood, lymph, and nervous systems

Supportive System

Suspensory Apparatus: A well-developed suspensory apparatus gives the udder a firm attachment with the abdominal wall. It extends caudally and is

attached to the pelvic symphysis by means of the strong plate of tendinous tissue. This plate of tissue further attaches the prepubic tendon to the ventral part of the symphysis. Besides the external covering of skin, superficial fascia and coarse areolar tissue which give a very poor support, there are other four sheets of tissues which comprise the suspensory ligament [1]. Superficial fascia comprises a layer of areolar subcutaneous tissue which attaches the skin to the underlying udder tissue. It provides a soft padding between the skin and the udder tissue. A loose band of areolar connective tissue generally occur between the dorsal surface of the front quarters and abdominal wall for keeping the fore-quarters closely attach to the ventral wall of the abdominal cavity. It actually provides a minor support of the udder and if there is any minor damages the udder use to break away from abdominal wall.

1. **Median Suspensory Ligament -** It is two in number and placed medially. The two laminae detach from the abdominal tunic descend on either side of the median line, having a layer of areolar tissue between them and form the double septum separating the two glands. It is composed of heavy yellow elastic sheets of tissue. It acts as a shock absorber and accommodates changes in size and weight of the udder that occur with milk production and age. The median suspensory ligament has great tensile strength and can withstand the overextension of the gland when it fills with milk. Usually a strong median suspensory ligament is essential for udder conformation. It is located at the center of gravity of the udder to give balanced suspension of the quarters. The equilibrium of the udder is greatly affected if there is any damage to the ligament. Rupture of the suspensory ligaments of the udder (usually median ligaments) occurs gradually in some older cows and leads to a dropping of the udder floor resulting in lateral deviation of the teats. Occasionally, acute rupture can occur at or just after parturition. Animals with this condition are at high risk for developing mastitis. A weak median suspensory ligament results in a lowering of the floor of the udder, sometimes below the hock which makes it more difficult for the calf to nurse and the teats may drag in the mud when the cow walks, and the teats may be suspended inward or outward when filled with milk instead of straight down.
2. **Symphysial or Subpelvic tendon** - It is composed of white fibrous tissue and arises from pelvic symphysis. Fibers of this ligament do not contribute directly in the formation of suspensory apparatus. The lateral

ligaments arise from sub-pelvic or symphysial tendon caudal to the udder and after descending down at level of the floor of the abdominal cavity they diverge and pass laterally to the superficial inguinal ring. They reflect ventrally over the udder and divide into superficial and deep layers.

3. **Superficial layers of lateral suspensory ligament** - The superficial sheets are mostly composed of fibrous tissue (with some elastic tissue) extend ventrally over the udder. According to its position, this ligament is reflected between the external udder surface beneath the skin and attaching to the areolar tissue to the medial face of the thigh.
4. **Deep lateral suspensory ligament** -It is thicker than the superficial ligament and extends over the convex lateral surface of the udder by numerous lamellae which pass into the gland and become continuous with the interstitial framework of the udder. The large mammary lymph node is situated caudal to the ligament. There is a presence of a bulk fatty deposition keeping the rear quarters closely attach to the ventral wall of the abdominal cavity. It actually provides a minor support to the udder and if there is any minor damage, the udder may break away from the abdominal wall.

The deep and superficial components of lateral suspensory ligaments give substantial support to the udder due to inadequacy of elastic fiber. The left and right divisions of lateral suspensory ligaments never join each other at the bottom of the udder which limit the overall holding capacity of milk when the gland is filled with milk.

Classification of Udder

According to the shape the udder is classified, as follows [2].

1. **Bowl shaped udder**: In this group of udder the mammary parenchyma is compact and the udder is continuous ventro¬laterally.
2. **Globular udder**: They are mostly globular in appearance and the attachment with abdominal wall is very narrow as compared to the bowl shaped udder.
3. **Pendulous udder:** The mammary parenchyma is loosely attached and

there are loosening of fatty tissue which usually gives support to the rear and front quarters. The median suspensory ligament is absent resulting in a loose and pendulous attachment of the udder. The quarters are not leveled and teats are not perpendicular to the ground when filled with milk. Due to the proximity of the udder to the ground they are more prone to mastitis.

4. **Trough shaped udder:** This kind of udder shows a proportionate distribution of parenchyma along the center of gravity of mammary gland. The trough udder is tight to the body cavity. The floor is leveled. As the median suspensory ligament becomes less pronounced, the udder floor becomes more rounded. The cows with trough shaped udder had the lowest risk of subclinical mastitis [3].

According to the attachment of the udder with the abdominal wall there are three types of attachment like loose, tight and intermediate. Anatomically the suspensory apparatus is responsible for different types of attachments. The median suspensory ligament is vague resulting in loose attachment of the udder. The tighter udder attachment indicates lesser chances of mastitis [4, 5].

According to the shape of udder cleft it is classified as deep, shallow and intermediate. Pronounced medial ligament creates deep udder cleft. External demarcation indicates the looseness and tightness of the udder parenchyma. The deeper udder cleft showed lesser chances of mastitis [4].

According to the quarter symmetry or balancing, udder can be classified as follows.

1. Equal quarters
2. Unbalanced quarters
3. Heavy front quarters
4. Heavy rear quarters.

Udder type traits are important for prevention of mastitis in dairy cows. Udder balance has negative genetic correlation with clinical mastitis [5]. The trabeculae which extended from the capsule divided the gland parenchyma into numerous lobes and lobules.

Secretory Unit of the Udder

The mammary parenchyma is composed of connective tissue, areolar tissue and secretory epithelial cells. The gland is covered by a tuff connective tissue covering. The relative amount of connective and secretory tissue varies from animal to animal. In some cases amount of interstitial tissues may be considerable and that of the secretory tissue very small. Such udder looks large but will have very less milk production capacity. The trabeculae which extend from the capsule divide the gland parenchyma into numerous lobes and lobules

Lobe - Groups of lobules are surrounded by a connective tissue sheath and constitute a lobe. Each mammary gland is made up of numerous lobes.

Lobules - Clusters of 160-195 alveoli are encapsulated by a connective tissue sheath and are organized as a lobule (111.92±3.04 µ alveolus diameter).

Alveoli - An alveolus is the discrete milk producing unit. The shape is variable. The shape of mammary gland alveoli is generally ovoid, but pyriform and irregular contour are also seen. At times, alveoli appear fused to form flattened sac like alveoli [6]. The lumen of the alveolus is lined by a single layer of simple squamous to low cuboidal epithelial cells. Increased number and diameter of the alveoli indicate the maximum production space and milk holding capacity [6].

Thickness of the inter alveolar barrier among the adjacent alveolus is about 12 µ in desi cows and 6 µ in crossbred cows [7].The inter- alveolar space of both the breeds of cows contains a good number of myoepithelial cells and connective tissue cells such as fibroblast, plasma cells, lymphocytes, neutrophils and macrophages. The population of aforesaid connective tissue cell is more in the mammary gland of desi cows when compared with crossbred cows. The predominance of neutrophils amongst the connective tissue cells of the inter-alveolar space of mammary gland is found in both the groups. More than 90% of the total mammary gland leukocytes in the mammary tissue of cow are neutrophils and are predominant cell type [8]. The neutrophils are the source of small antibacterial peptides, the defensin, which are able to kill a number of mastitis causing pathogens [9].

The immunocomponent cells are found between the alveolar epithelial cells and represented by monocytes, small and medium lymphocytes including some granule containing lymphocytes. The quantity of immunocomponent cells are found to be increased in during pregnancy and to reach its maximum on day - 3 of lactation. This is accompanied by appearance of macrophages and plasma cells. Similar changes are noticed in stromal immunocomponent cells [10].The

epithelial lining of the alveolus is surrounded by contractile myoepithelial cells. Myoepithelial cells contract in response to the hormone oxytocin, resulting in milk being squeezed out of the alveolar lumen and into the small ducts. Outside of the myoepithelial cells the alveolus is surrounded by a connective tissue basement membrane. The capillary bed on the outside of the alveolus is part of the stromal tissue (connective tissue) between alveoli. Many cells occur in the lumina of the gland. These cells include sloughed cells, macrophages and leucocytes [11]. The IgA and IgM are produced locally by plasma cells locate near the secreting epithelium of mammary alveoli [12].

Ducts and Cisterns: While the cow's udder contains a large amount of secretory tissue responsible for synthesis of milk, it also contains a large proportion of ducts that are the tubing by which milk moves from the alveoli to the teat for milk removal. In addition, between the teat and the large ducts are open areas called cisterns. A cistern is a large cavity where milk is collected between milkings. Milk is synthesized in the microscopic alveoli. As it accumulates in the alveolar lumen, some milk oozes down into the smaller ducts and eventually into the large cisterns. This allows the cow to accumulate more milk in her udder between milkings or suckling by the calf.

Teat: Four well developed teats (two front and two rear) are present in cattle and buffaloes. It is covered externally by the skin devoid of hairs and glands. Glands are found at the tip of the teat which secretes a sebaceous lubricating substance, which protects the teats during the suckling of the young and prevents the coagulation of milk at the orifice [13]. Usually, only one teat drains one quarter. Depending on the shape, the teats are categorized as conical, cylindrical, funnel shaped and bottle shaped [2]. Cows with cylindrical teats exhibit significantly higher incidence of mastitis [3, 14,15]. The risk of mastitis for cows with funnel shaped teat is lower [3, 16].

The teat end shape is mostly round, pointed, flat and inverted. The length and diameter of teat varies from animal to animal. The teat end shape and mastitis prevalence has a significant relation [17]. The frequency of occurrence of mastitis in cows with round teat is high [18,19]. The flat and inverted teat end shape has lower chances of mastitis [20]. The factors for milk leakage include high peak milk flow rates, short teats, and inverted teat ends. Occasionally, cows are observed to leak milk continuously. These cows usually have sustained a severe teat injury or have an abnormal streak canal. In general, little can be done to correct this condition, and most of these cows

will develop mastitis.

The placement of teat varies from udder to udder. In well balanced udder the placement of front and rear teats are closed and centrally placed. The closer teat placement indicated lesser chances of mastitis [4] .Some cows have extra teats, referred to as supernumerary teats. Some of these extra teats open into a "normal" gland, but many do not. A pseudo-teat has no streak canal, and therefore, no connection to the internal structures of the gland. Many animals of other species have supernumerary teats, including humans and pigs. The prevalence of these teats is low in humans, while many sows have extra, non¬functional teats.

Streak canal - Each teat has a single lactiferous duct which proceeds from the lactiferous sinus of the gland passes straight through the teat and terminates at its apex. Functionally the canal communicates between internal milk secretory system and the external environment. The lower portion of the canal is narrow and is closed by a sphincter muscle. The teat wall musculature is composed of inner circular and outer longitudinal muscle layers. Inner circular muscle layer is thick and accommodated vessels, nerves and lymphatics in between the muscular bundles [21]. The circular muscle layer is divided into inner and outer zones due to presence of some irregular fibres and connective tissue elements in it.

In between the luminal epithelial layer and inner circular muscle layer, little amount of loose sub mucosal tissue is present, which contained vessels, nerves and connective tissue elements. Few well demarcated tubular glands are present in the sub mucosal area towards the apex of the teat. The tubules of these glands are lined by single layer of cuboidal epithelium [22].

The submucosa of the teat contains finger like projections that are (Furstenburg's rosette) created by invagination of luminal epithelium. The number of the projections varies from 10-14. The teat lumen is engorged with these projections, leaving the lumen into an irregular narrow space and it looks like a rosette. It may be a major point of entry for leukocytes leaving the teat lining and entering into the teat cistern. These foldings provide more space for functioning of immunogenic cells. A progressive increase in number of infiltrating cells from the distal teat cistern (sinus papillaries) to the junction of Furstenberg's Rosette (distal termination and convergens of mucosal fold lining the teat cistern) and the streak canal (ductus papillaries) are observed. Plasma cells contribute to cellular increases in sub- epithelial connective tissue and

are the most prevalent infiltrating cell type. Plasma cells also penetrate the basal epithelial lining of the rosette and occasionally migrate to the luminal surface near the squamo- columnar junction. Neutrophils and monocytes contributed to the increase in cells infiltrating the epithelial lining. Few infiltrating cells are observed in epithelium and underlying stroma of the streak canal [23]. Region at the proximal end of the teat cistern that marks the boundary between the teat cistern and the gland cistern is distributed by some annular folds.

These are not always recognizable in the dissected gland.The mucosal covering of the projected portion is composed of 4 to 5 layers of squamous cells which are arranged parallel to the surface with a considerable amount of ground substance in between them. The cells of the deeper layer are arranged in oblique or vertical manner with fewer amounts of intercellular substances. The epithelial layer is thicker at the invaginated part and thinner at the projected part. The streak canal is the main barrier against intra-mammary infection. It is lined with a skin-like epidermis that forms the keratin material that has antibacterial properties. The fissures in the keratin layer of streak canal are one of the factors responsible for intra-mammary infection in cows. It also avows that the larger surface area provided by connective tissue folds of Furstenberg's rosette provides more surface area for infiltrating leucocytes for phagocytosis of bacteria [23].The keratin layer of the streak canal in susceptible quarter of cow is thinner, less dense and detaches easily from epithelium compared with those of resistance quarters against mastitis [24].

The musculature maintains the tight closure between the milking and hindered bacterial penetration through teat canal [25] .The streak canal is the main barrier against intra-mammary infection. Canal patency decreases and streak canal length increases with increasing lactation number.

When a cow is milked, the sphincter muscles relax allowing the orifice to open. The streak canal remains open for an hour or more after milking. This provides ready access of bacteria to the inside of the gland. Post-milking germicidal teat dips are designed to help minimize the chance of bacteria gaining access to the gland after milking. Keeping cows standing for a time after milking, such as providing access to fresh feed, also helps minimize teat end contamination before the streak canal closes again. During the dry period (non-lactating period), the epidermal tissue lining the streak canal forms a keratin plug, that effectively seals off the canal.

Teat cistern - The lactiferous duct or streak canal widens dorsally into a smaller teat sinus popularly known as teat cistern. It is continuous with the gland cistern. The teat cistern is lined with bistratified squamous epithelium. The numerous longitudinal and circular folds in the mucosa, which form pockets on the inner lining of the teat, allow storage of milk. During milk letdown, the teat cistern fills with milk.Some of the milk in the gland cistern just above the teat cistern is removed with each sucking action of the calf.

A number of large ducts (tubes) branch off from the gland cistern. These ducts branch and re-branch into smaller and smaller ducts (similar to roots of a tree) and finally into the small ductules that drain each alveolus.Ducts are the tubes by which milk drains from the alveoli down to the gland cistern.

Gland Cisterns - (sinus lactiferus): Also called the udder cistern.The resulting large ducts or interlobar ducts converge to the centre of the gland and open into a large lactiferous sinus or gland cistern. It opens directly into the teat cistern. Occasionally a septum forms between teat and gland cisterns and the quarter may be blind. This can be corrected surgically. The gland cistern varies greatly in size and shape. There are often pockets formed in the cistern at the end of the larger ducts. Where the milk ducts branch-off from a larger duct, a narrowing of the tube occurs. As the lobules (milk secreting glands) fill up with milk, their weight causes them to sag downwards This causes the narrow branch of each duct to be pinched and close. Consequently, milk does not drain from the udder until the cow is stimulated to let-down her milk.

Neural system - Nervous innervation inside of the udder is sparse compared with other tissues.

1. The inguinal nerve innervates the gland.
2. Caudal mesenteric plexus of the sympathetic nerve is present in the tissue. These are the nerves that associate with the arteries. They do not innervate the alveoli.
3. Sensory nerves are found in the teats and skin. These are critical for initiating the afferent pathway (neural pathway) of the milk ejection reflex.
4. There is no parasympathetic innervation to the gland. This is similar to other skin glands.
5. There is no innervation of the secretory system. Myoepithelial cells are not innervated. Myoepithelial cells do not contract in response to direct

innervation, but rather contract in response to the blood-borne hormone, oxytocin.

Blood Vascular System

The blood supply to the mammary gland is extremely important for mammary function. All of the milk precursors come from blood. On avg. 400 - 500 units of blood passes through the udder for each unit of milk synthesized by a high producing dairy cow; that is ~280 ml per sec.

Total udder blood volume for lactating cows is about 8% of total body blood volume, while for a non-lactating cow it is about 7.4%. There is a 2-6 fold increase in blood flow in the mammary gland starting 2-3 days prepartum. The decrease in production with advancing lactation is not due to decreased blood flow; rather it is due to the loss of secretory epithelial cells through a process programmed cell death (apoptosis).

Arterial system - Aorta arises from the heart and divided into anterior and posterior aorta. Posterior aorta having two parts thoracic and abdominal. Blood flows towards the rear of the cow by the abdominal aorta. Abdominal aorta after giving several branches terminates into the internal and external iliac arteries. They are left and right and total four terminal branches of abdominal aorta is called iliac quadrification. The external iliac arises below the body of the fifth or sixth lumber vertebra and descends downwards. It continues as the femoral artery. A branch off the femoral divides into the posterior abdominal artery and the external pudic artery. In case of female this artery then passes through the inguinal canal and out of the body cavity. The inguinal canal is the orifice in the body cavity in the inguinal region where blood vessels, lymph vessels and nerves enter and leave the body cavity to supply the skin in the posterior part of the animal. As the external pudic artery passes out of the body cavity it divides into the anterior and the posterior branches. The posterior branch is popularly known as mammary artery (~1cm diameter). Once the mammary artery enters the gland, then at the supero-posterior part of the mammary gland it divides into the anterior and posterior mammary arteries which again branch and are distributed to the anterior and posterior quarters of the gland.

Perineal artery- One of the branches of the mammary artery may be found

passing backwards and upwards between the thighs and gives of number of cutaneous branches. A small amount of blood also reaches the mammary gland by the perineal artery, but this only supplies the upper rear portion of gland.

Sigmoid flexure - Just below the inguinal canal, the pudic artery forms an S-shaped flexure. This allows for downward distention of the udder as it fills with milk, without stressing the blood vessels. There is essentially no crossover of blood supply between udder halves.

Venous system- There are three veins on each side that carry blood away from the mammary gland.

1. External pudendal vein leaves the udder parallel to the external pudendal artery and anastomoses with the subcutaneous abdominal vein at the posterior and anterior part of the udder.
2. Subcutaneous abdominal vein (milk vein): These are anterior extension of the large mammary veins and run forward along the ventral abdominal wall just under the skin. They penetrate the thoracic cavity at the xiphoid process via milk well and eventually join the anterior vena cava. This is the large vein (1-2.5 cm dia.) that is visible under the skin on the belly of the cow.
3. Perineal vein leaves the rear of the gland parallel to the perineal artery (0.5 cm dia.) carries less than 10% of blood leaving udder.

Venous circle - Formed by anastomoses between anterior and posterior mammary veins.Prevents pinching off of areas of venous outflow when the cow is lying down.

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chapter4
Defense Mechanism in Bovine Mammary Gland

Understanding natural udder defenses is the foremost has led to further advances and may provide practical implications toward preventing Intra Mammary Infection (IMI). This chapter provides an overview of udder defenses, providing resistance to IMI, and recent developments in understanding the nature of protective mechanisms.

A.Teat Canal: Bacteria enter the udder through the teat canal thus; this structure and associated tissues provide the first barrier to pathogens. The duct system is divided into three parts viz., streak canal, Furstenberg's rosette and teat cistern as shown in Fig. 4.1&4.2.

Streak canal: The streak canal is lined by stratified squamous keratinized epithelium. Buffaloes have thicker and compact epithelium which provides an extra resistance against penetration of pathogens through epithelium (Fig. 4.3) [1]. Keratin a mesh like substance derived from the teat canal lining partially occludes the lumen and inhibits bacterial penetration (Fig 4.4). The keratin lining of teat canals in susceptible quarters is thinner, less dense, and detached from the epithelium compared with those in resistant quarters [2]. Buffaloes have thicker keratin (av. value 154.70µ) compared with cow (av. value 101.30µ) which constitutes an important anatomical factor responsible for lesser IMI in buffaloes (Fig 4.5) [3].

Electron microscopy of teat has shown the presence of fissures in keratin layer. These fissures were considered to be one of the factors responsible for IMI in cows [4]. In buffaloes, the fissures of keratin layer were smaller and narrower than those observed in cows [1] (Fig.

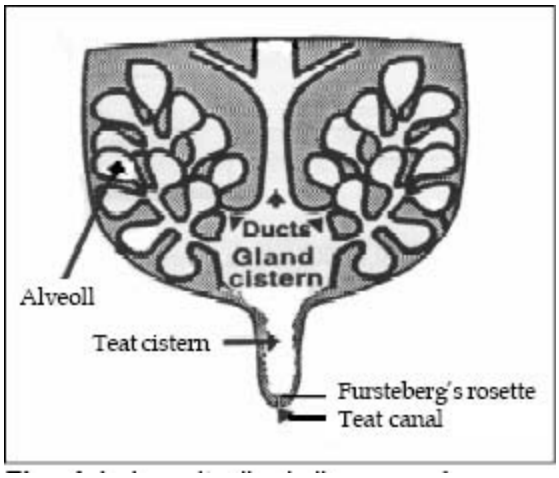

Fig. 4.1: Longitudinal diagram of a quarter of mammary gland

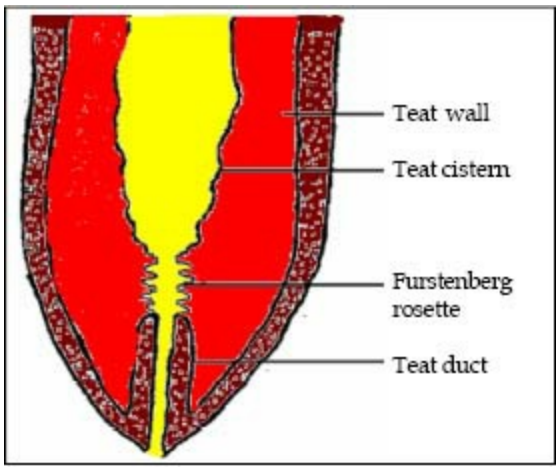

Fig. 4.2: Line diagram of distal part of teat in longitudinal section.

Fig. 4.3: Cross section of buffalo teat at streak canal region showing

epithelium (Ep), keratin (K). Masson's trichome stain x 17.5

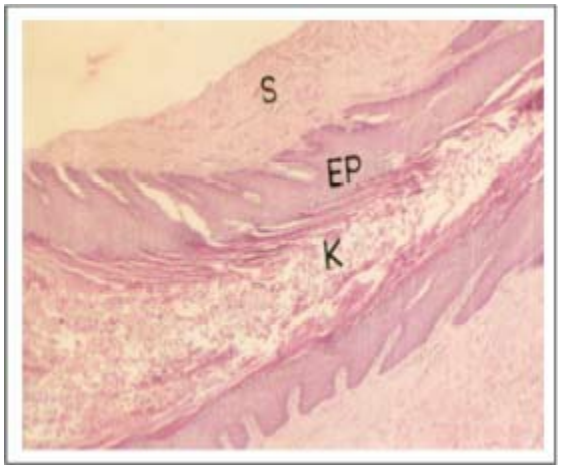

Fig. 4.4: Longitudinal section of buffalo teat at streak canal region showing keratin (K), epithelium (Ep) and sub-epithelial stroma (S). H&E stain x 17.5

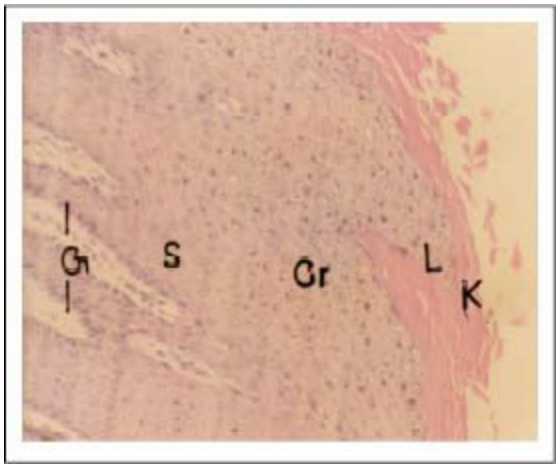

Fig. 4.5: Cross section of cow teat at streak canal region showing stratum germinativum (G), spinosum (S), granulosum (Gr), lucidium (L) and corneum (K). H&E stain x 140.

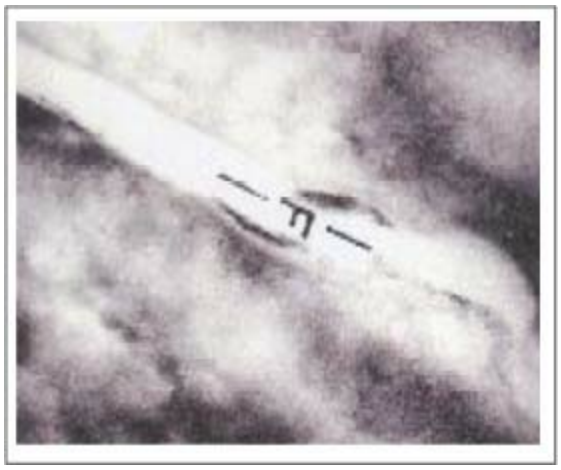

Fig. 4.6: Electron micrograph from cow teat at streak canal region showing longitudinally oriented fissures (F) x 21,500.

stearic, linoleic, and oleic acids are increased in susceptible quarters [5] . The heritability of fatty acid composition in keratin is fairly high and selection for resistant cows through genetic transmission may be advantageous. Cationic proteins such as ubiquitin isolated from keratin inhibit growth of *Streptococcus agalactiae* and *Staphylococcus aureus.* These proteins bind to and inactivate negatively charged bacterial cells by inducing alterations in osmoregulatory mechanisms leading to swelling and lysis.

The smooth muscle sphincter surrounding the teat canal inhibits bacterial penetration by maintaining tight closure and limiting organisms to the orifice. The muscle sphincter present around the streak canal has higher thickness in buffaloes (Fig. 4.8) than in cows (Fig 4.7).This helps in maintaining the tight closure of the duct in buffaloes, thus limiting IMI. Loss of muscle tonus increases susceptibility to IMI: a greater rate of infection occurs in quarters with leaky teat canals. Therefore, thicker and better organized muscle sphincter around the buffalo streak canal is another anatomical factor responsible for lesser IMI in buffalo than in cow. Streak canal diameter is slightly lesser in buffaloes (average value 1.70+ 0.070 mm) when compared with cow (average value 1.83+ 0.11 mm), which might be preventing movement of organisms through streak canal [3]. The frequent flushing action of fluid during milking helps to wash out bacteria colonizing the ductal lining and teat orifice. Because the teat canal lumen remains dilated for up to 2 hours after milking, feeding cows at this time to keep them on their feet allows time for teat canal constriction and avoid bacterial contamination [6]. Dipping teats in a germicidal solution also reduces the number of udder pathogens at the teat end.

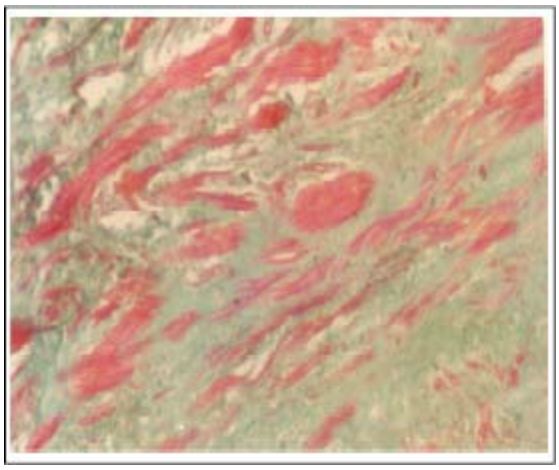

Fig. 4.7 : Cross section of cow teat at streak canal region showing disorganized stain x 70.

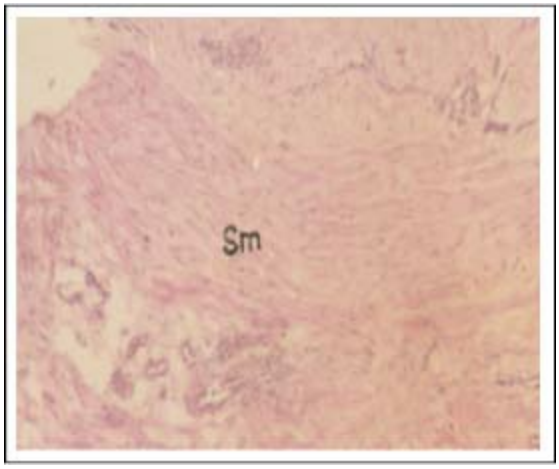

Fig. 4.8 : Cross section of buffalo teat at streak canal region showing thick and well organized smooth muscle sphincter (Sm).H&E stain x 70.

Furstenberg's rosette:The mucosal epithelium lining the Furstenberg's rosette has either stratified cuboidal or columnar cells. Sub-epithelial connective tissue is thrown into folds rich in blood vessels and nerve fibers (Fig 4.9). The larger surface area provided by connective tissue folds of rosette provide more surface area for infiltrating leucocyte [4,7]. Rosette region of buffaloes teat have more folds (range 10 to 14 average 13.40) compared with cattle (10 to 1 average 12.75) [3]. The intraepithelial infiltration of lymphocyte and monocyte, and sub-epithelial infiltration of poly morpho nuclear (PMN) lymphocytes, monocyte, plasma cell and mast cell were observed [1].

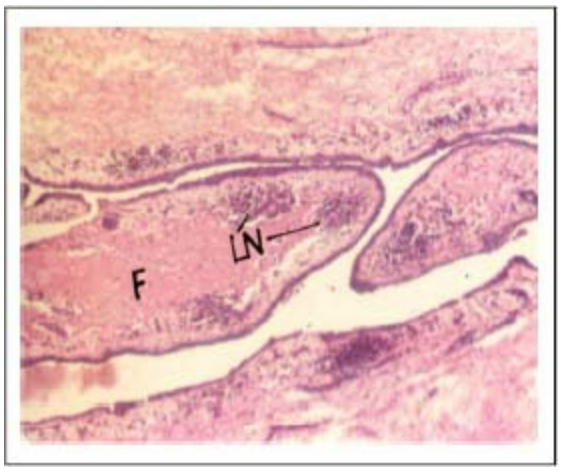

Fig. 4.9: Cross section of buffalo teat

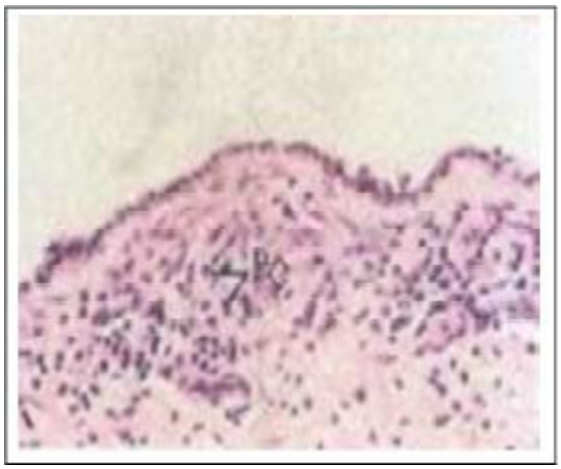

Fig. 4.10: Cross section of buffalo teat at rosette region showing folds (F) and at cistern region showing plasma cell lymph nodes (LN). H&E stain x 70 (Pc). H&E stain x 280.

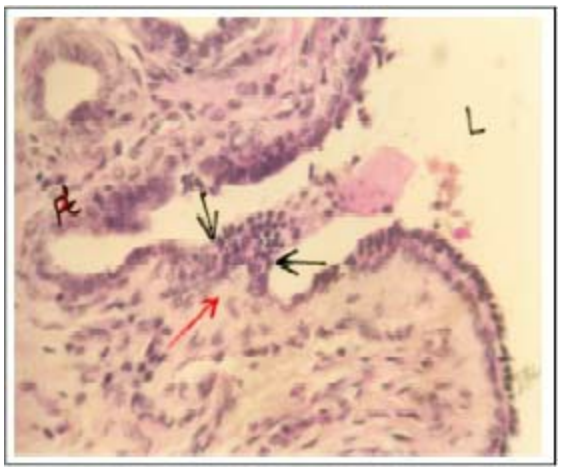

Fig. 4.11 : Cross section of cow teat at rosette region showing sloughing of buffalo streak canal showing plasma epithelial cell. Lymphocyte migrating cell (Pc). x 2650 towards lumen (L) and plasma cell (Pc).H&E stain x 280.

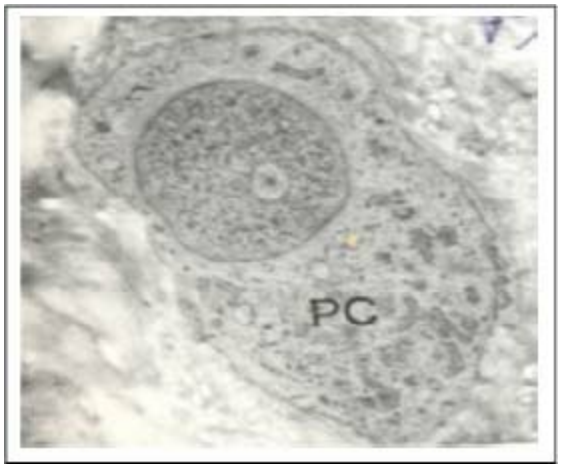

Fig. 4.12 : Electron micrograph of buffalo streak canal showing plasma cell (Pc). x 2650

Aggregations of lymphoreticular tissue in the form of lymphoid nodules are observed in rosette region (Fig. 4.9) and are the sites for antibody production. The lymphocytes may also interact with macrophages and neutrophils to stimulate phagocytosis of bacteria. The occurrence of PMN cells is also considered as primary defense after the bacteria had penetrated the teat duct. Thus rosette region helps to prevent bacterial infection.

Teat cistern: Compared with Furstenberg's rosette, the mucosa of teat cistern has fewer folds. The region has stratified cuboidal to columnar epithelium. The cellular infiltration in the epithelium by PMN cells, mast cell,

monocyte, macrophage, plasma cell and lymphocytes are observed (Fig. 4.10 - 4.12). These cells are thought to be responsible for checking bacterial infection progressing upward to mammary tissue and thus act as primary defense against the infection at the level of teat cistern.

B.**Cellular Defense Mechanisms in Udder Secretions:** Bacteria breach the teat canal by multiplication, physical passage, and propulsion during milking. Milk leukocytes and the small percentage of epithelial cells collectively termed somatic cells and constitute a second barrier to infection [8]. Somatic cells count in milk from normal, uninfected glands varies from 1 x 10^5 to 3 X 10^5cells/ ml. Migration of leukocytes from blood to milk increases when infection is established.

Poly morphonuclear neutrophilic leukocytes- Most leukocytes (90%) present during inflammatory stages are poly morphonuclear neutrophilic leukocytes (PMN) that accumulate in response to bacterial components, enzymes released from damaged secretary cells and leucocytes and lymphokines. These leucocytes function by phagocytosing and killing bacteria. Increased concentrations are required because of a decreased capacity of PMN function in milk due to lack of an energy source, decrease in opsonins and interference by casein and fat [8]. An increase in strippings or foremilk PMN concentration was attempted by inserting plastic intramammary devices (lMD), into gland cisterns to produce mechanically a sterile inflammation and induce leukocytosis near the point of bacterial entry [9]. Quarters with IMD have intensified and more rapid response to intra-mammary injection of *E. coli* endotoxin and increased resistance to challenge exposure to *S. aureus* than do quarters without devices. However, these IMD are not effective in reducing natural occurrence of new IMI due to an inability to increase SCC to protective amounts.

Macrophages- Most milk leukocytes from uninfected, lactating udders are macrophages (35-79%); the first bacteria-cell interaction may involve these cells [10]. Macrophages phagocytose and kill bacteria and function in cell-mediated immunity (CMI) by processing bacterial antigens to lymphocytes and regulating the magnitude of lymphocyte response. However, indiscriminate ingestion of fat, casein, and other milk components, leads to less efficiency of PMN cells & macrophages [7]. Phagocytic and bactericidal activities of these cells are especially diminished during the peri-parturient period.

Lymphocytes- Approximately 45% T cells and 20% B cells are in the lymphocyte population of normal milk during lactation [11]. The T cells produce lymphokines that act as chemoattractants for PMN and macrophages and stimulate leukocyte microbicidal activity. After intramammary injection of heat-killed staphylococci, contact of cell- wall antigens with immune-competent lymphoid cells may result in sensitization [12]. Subsequent exposure would lead to a CMI response including lymphokine release, resulting in enhanced recruitment, activation, and immobilization of phagocytic cells. The B cells associated with the mammary epithelium have access to bacterial antigens, and contact may lead to multiplication of sensitized clones and maturation into plasma cells. Cytotoxic cells may act as scavengers, removing old or damaged secretary cells, the presence of which increase the susceptibility of the mammary gland to infections. Thus, T and B cells become sensitized to antigens, maintain this sensitivity, and initiate a heightened response to subsequent exposure.

Soluble Defenses

Milk antibodies- Most immunoglobulin (Ig) G is serum derived, whereas IgA and IgM are synthesized in the udder and pass into milk through the mammary epithelium with IgG. Antibody concentration in normal milk is low (1 mg/ml) and is dependent on the degree of vascular permeability of udder tissues. When this permeability barrier is broken during inflammation, antibody concentrations approach 50mg/ml in colostrum and secretions from infected glands. Immunoglobulin G_1 is selectively transferred into mammary secretions, thus, it is the major Ig class in milk (0.4 mg/ ml). Serum- derived IgG_2, as well as that produced locally, is present at 0.03mg/ml, whereas IgA and IgM concentrations are 0.15 and 0.05 mg/ ml, respectively [13]. A major function of antibodies is opsonization of microorganisms for phagocytosis. Antibody binding to bacteria alone or with the C3b component of complement is necessary for opsonization and classes include IgG_K IgG_2 and IgM. Immune complexes of antibody-bacteria and antibody-C3b-bacteria bind to receptors on phagocytes to initiate phagocytosis. Immunoglobulin A does not opsonize but may prevent bacterial adherence to epithelial membranes, inhibit multiplication, neutralize toxins, and agglutinate bacteria.

Vaccination has been attempted to increase the antibody titer in blood and milk to a specific organism, thereby providing immunity to that organism. To be effective, a vaccine should inhibit bacterial growth and toxin production. *S. aureus* responds poorly to antibiotics, therefore vaccines against this organism have been studied extensively. Immunization against *S aureus* mastitis revealed that new IMI are not prevented, but occurrence and severity of clinical mastitis are reduced. Serum antibody concentrations increase after systemic immunization, but total antibodies in milk increases only after the vascular endothelium and glandular epithelium become more permeable, a phenomenon that occurs during inflammation after bacterial invasion (Fig. 11). The increase in milk antibodies may be effective in reducing severity of mastitis, but ineffective in preventing IMI.

Other Soluble Factors

The **lactoperoxidase/ thiocyanate/ H_2O_2 system** in milk inhibits growth of *S. aureus* and most streptococci and coliforms. Lactoperoxidase (LP) is synthesized by mammary epithelium at concentrations of 2 to 35 mg/ ml. Thiocyanate is derived from green feed stuffs containing thiocyanate precursors (1 to 10 ppm) and H_2O_2 is produced by streptococci (2 to 4 ppm). This system is active when LP, in the presence of H_2O_2 oxidizes thiocyanate to hypothiocyanite that damages the inner bacterial membrane of lactic acid-producing streptococci, leading to leakage of cell contents and interference with nutrient uptake. *Escherichia coli* and *S. aureus* are killed when an exogenous supply of H_2O_2 is available, thus, IMI may be delayed by injecting glucose oxidase as an H_2O_2 source. Concentrations of LP and thiocyanate are increased in infected quarters and may be protective if supplied with a source of H_2O_2.

Lysozyme- Locally synthesized or derived from blood, it destroys bacteria by lysing cell wall peptidoglycan. The concentration of this protein in milk is low (0.13 µg/100 ml) although it increases during IMI. Animals with low milk lysozyme titers are more susceptible to mastitis, indicating that a deficiency predisposes the udder to infection.

Lactoferin (LF) is bacteriostatic because of iron chelating properties in the

presence of bicarbonate making iron unavailable for bacteria that require it for growth. Lactoferrin is in small amounts in milk from normal, non-infected glands (0.1 to 0.5 mg/ml). It is derived from epithelial cells and leukocytes. Streptococci have low iron requirements so the effect of LF is minimal however, LF is effective against staphylococci and coliforms. Lactoferrin also may be active in modulation and control of macrophage, lymphocyte, and PMN functions [14].

Cytokines- Cytokines are proteins produced by both immune and non-immune cells under diverse circumstances that play an important role in essentially all aspects of host defense by regulating the activity of cells that participate in specific and nonspecific immunity. To date, >30 cytokines have been identified. The major groups of cytokines studied include interleukin (IL), colony-stimulating factor (CSF), IFN, and TNF. Recombinant cytokines have immunotherapeutic potential for the control of bovine mastitis.

IL-2 is the most extensively characterized of all the bovine cytokines. Originally described as T cell growth factor, IL-2 is primarily produced by T lymphocytes of the helper phenotype and is responsible for clonal expansion of the initial T lymphocyte immune response and establishment of immune memory following mitogenic or antigenic stimulation. This cytokine also plays a role in B lymphocyte growth and differentiation, enhancing thymocyte proliferation, activating NK cells, and inducing cytotoxic T-cell activation [15]. Colostrum samples obtained during the final week of gestation have low IL-2 activity, which correlates with diminished immune cell function and increased susceptibility to mastitis during this period [16]. *In vitro* and *in vivo* studies indicate that recombinant bovine (rb) IL-2 may enhance functional capabilities of populations of mononuclear cells within the mammary gland [17,18,19]. Exposure to rbIL-2 markedly enhanced the proliferation of mononuclear cells isolated from milk to suboptimal concentrations of mitogens that normally do not elicit a proliferative response [18]. Lymphocyte populations isolated from mammary tissues had increased cytotoxic and bactericidal activities following *in vitro* culture with IL-2 [19,20]. When administered as an intra-mammary infusion, IL-2 can induce an infiltration of neutrophils into the milk in dose related manner [21]. Other changes in milk composition included a reduction in lactose concentration and a concomitant increase in BSA, pH, and total SCC. The range is narrow between therapeutic and toxic doses of IL-2 in the bovine mammary gland [21].

The CSF are a group of cytokines required for the proliferation and differentiation of a variety of hematopoietic stem cells. These growth factors are distinct glycoproteins that bind to cells by a common receptor and are produced by a variety of cells, including fibroblasts, endothelial cells, macrophages and T cells. Each CSF tends to target a specific lineage to expand or activate its function. Granulocyte (G)- CSF is required for the growth, survival, and differentiation of granulocytes phagocytic cells. The pronounced influence of G-CSF on phagocytic cell populations suggests possible clinical applications in the prevention of infectious bacterial diseases, such as mastitis. Both recombinant human G-CSF [22] and recombinant bovine G-CSF [23] have been administered subcutaneously to cows in doses ranging from 1 to 5 μg/kg per day. All studies demonstrated a two to five fold increase in peripheral blood neutrophils after 3 to 5 d of injection.

Granulocyte-macrophage (GM)-CSF induces hematopoietic progenitor cells to develop into granulocytes and macrophages. GM- CSF is not only an important molecule for inducing growth, but also, affects a variety of functions of mature granulocytes. Intra-mammary infusion of rbGM-CSF at doses up to 5 mg did not significantly affect total milk SCC but increased the ability of neutrophils to produce superoxide and also increased the percentage of phagocytic cells [17,24]).

Interferon-g is a cytokine derived from T-lymphocytes that is often produced in response to stimulation by antigens or mitogens and has immune-modulatory properties. It enhances NK cell activity and cytotoxic T cell activity. This cytokine also enhances cytotoxicity mediated by macrophages against tumor cells, induces membrane bound Fc receptors for IgG on macrophages, and stimulates the synthesis and release of reactive oxygen species from both macrophages and neutrophils [25].

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chapter 5
Genetic Deteminants of Mastitis in Cattle

Man has always remained busy in improvement of his domestic animals, and particularly since the turn of second half of the previous century this practice is in vogue to strengthen the dairy industry. Creation (crossbreeding or assisted reproductive technologies) and propagation of animals with high milk yield and better reproductive efficiency are key objectives of breeders and the process is dynamic, scales are being raised to higher and higher with success. However, the dairy cows with high milk yield and reproductively regular cycling are usually highly susceptible to several diseases, for instance mastitis. The biggest challenge in selection of animals for disease resistance is to identify accurately the phenotype and/or to have reliable genetic markers with high predictive values. Identification of molecular markers in host discrimination loci, which reflect the resistance/ susceptibility of mastitis at early stage, could be the pragmatic solution of this disease [1].

In a wide range of situations development and utilization of host genetic resistance to disease is an attractive tool for its control in livestock. Recent trends in molecular diagnostic tools have led to accumulation of information and better understanding of mastitis at an early stage of infection as well as at the time of birth of calves. Molecular markers, large-insert libraries and radiation hybrid panels have been used to build the genetic linkage, physical and comparative maps in different farm animal species. Moreover, expressed sequence tags, genome sequencing and SNPs maps along with DNA microarray technologies and proteomic methods are providing insight to understand how genomes function in various organisms [2]. As most of the economically important traits in farm animals are polygenic and are controlled by multiple genes and their interactions with environmental conditions. The concept of molecular marker started in 1970s [3] and there is enough literature available

on these aspects.

Thus the major goal of genome research is to map and characterize genes determining quantitative trait loci (QTLs) and their application as marker-assisted selection in breeding programmes. In 1980s and 1990s, molecular markers were adopting as tool for breed improvement programmes. Now information on QTLs is available and such approach to propagate animals with resistance to diseases is being adopted across the dairy developed countries [1,3]. Besides QTLs, a large number of polymorphisms within the genes or in causal regions or genetic markers associated with disease resistance/susceptibility traits have been identified [4].

The containment of mastitis with genetic change would supplement treatment and preventive measures. This approach requires a single, relatively inexpensive input at mating to produce resistant replacement females [5]. Major loci or multiple alleles for disease, viz. DRB3.2, TLRs, etc. have been detected with potential roles in mastitis, which are currently are used for controlling this disease. Presently several molecular marker methods are available, which are briefly described in this chapter along with genetic determinants or related genes of animals.

Mastitis Resistant Genetic Determinants in Animals

In the last few decades candidate gene approach is in practice to find the relationship between the attribute of interest and the responsible allele underlying the trait. These candidate genes associated with economic traits in farm animals are being used in marker assisted selection. In many experiments different QTL have been identified in cattle affecting functional expressions such as mastitis [6]. High throughput technologies such as microarray analysis offer the possibility to study changes in expression profiles of thousands of genes simultaneously, as a response to infection of the pathogen. In addition, the newly discovered miRNA and epigenetic mechanisms have been associated with mastitis resistance or susceptibility [7].

A variety of mechanisms make cows resistant to mastitis and a large number of genes operate in this array of defences. Although, mastitis resistance is polygenic in nature, single genes with potentially large effects have also been discovered [8]. A recent study has shown 934 loci involved in mammary gland

development, milk production traits and resistance or susceptibility to mastitis in cattle [4]. In these 22 genes (Table - 5.1) have been reported associated with mastitis. Among the candidate genes four (BoLA-DRB3, FEZF2, LTF, and TLR4) have been shown associated with mastitis resistance or susceptibility. Eleven genes (IL6, IL8, CD14, TLR-4, IL1B, LBP, TLR2, C5AR1, TNF, IFNG, and SAA) have been observed differentially expressed during mastitis. Furthermore eighteen genes have been shown in association studies or expression experiments located in the regions overlapping with QTL (Table 5.1). Brief description on some of the important mastitis related genes has been reviewed and is presented in this chapter.

Drb3

The major histocompatibility complex (MHC) is the region on the specific chromosome/ s, which controls humoral and cellular immune response to a variety of antigens. The major histocompatibility complex of cattle (also termed as bovine leukocyte antigen or BoLA) plays a significant role in host immunity. The bovine MHC has been mapped on chromosome 23 and consists of three classes: I, II and III [9]. The BoLA class II molecules are important for antigen presentation and for development of the number and type of T-cells in the immune system. MHC class II molecules have at least two isotypic forms designated DQ and DR. Isotypic form DR of bovine (DBR) classified into three genes: DRB1, DRB2 and DRB3. However, only the one designated DRB3 is expressed at a high level in peripheral blood lymphocytes [10]. The DRB3 gene in bovine codes for the beta-chain of the MHC class II molecule and is composed of six exons and five introns. The exon I codes for the leader sequence to the polypeptide and the exon 2 codes for the first domain of the beta-chain, which provides the peptide-binding site of the class II molecules. In dairy animals, the DRB3 gene of the MHC class II has been extensively investigated due to its high level of expression and polymorphism [11]. It has been evidenced that few alleles of the DRB3 exon 2 (DRB3.2) have significant association with mammary gland disorders especially in context to causative microbial pathogens [12,13].

Polymorphic patterns in bovine DQ and DR genes and their association with mastitis have been extensively investigated using four restriction enzymes viz. *BamHI,* EcoRI, *PvuII HaeIII, BstYI, RsaI* and *TaqI* [14,15,16]. The

alleles revealed by HaeIII, BstYI and RsaI are being accepted by the BoLA nomenclature committee (http:// www.projects.roslin.ac.uk/bola/bolahome.html) and used as standard in the investigation of DRB3.2. In cattle, 90 alleles have been detected in DRB3 locus [17] by different techniques, viz. PCR-RFLP [16], PCR-SSCP [18], heteroduplex analysis [19], and direct sequence analysis of the second exon of DRB3 gene [20], The example of allelic variation in DRB3 gene is shown in Fig. 5.1.

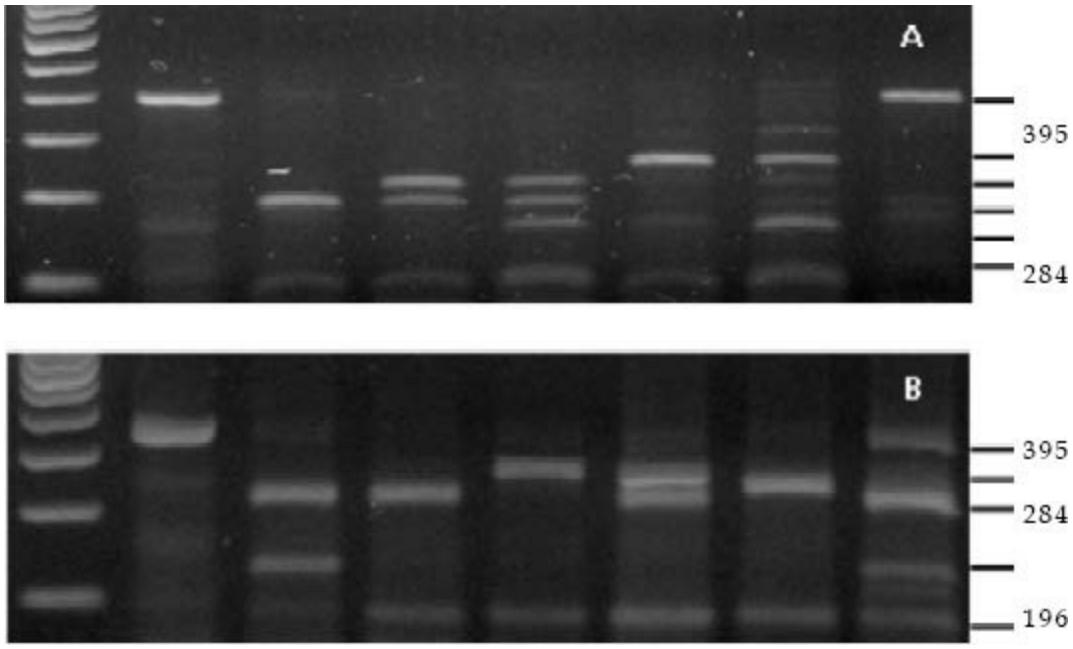

Fig. 5.1: Examples (A&B) of polymorphic patterns in DRB3.2 gene using RFLP

Further allelic variations by different methods (PCR, PCR-RFLP and SSCP) were reported by several researchers [13,15,18,19,21] in different breeds namely, Swedish Red and White American Holstein, American Angus, Norwegian Red cattle, Kankrej, Ongole, Karan Fries, Karan Swiss, etc.

Association of allelic variation of DRB3 with various infections is also well documented [13,18,22,23,24]. Association between BoLA class II genes and bull breeding value for various diseases including clinical mastitis, ketosis, retained placenta and milk fever has been evaluated [25]. Genotyping of BoLA class II revealed that a RFLP banding pattern of DRB was associated with resistance to *S. aureus* mastitis [22], Cows with particular BoLA-DRB3.2 alleles were found to be associated with elevated Somatic Cell

Scores (SCS) [23]. Furthermore, cows with BoLA- DRB3.2 (allelic form 11 and 23) were more resistant to clinical mastitis by major mastitis pathogens. Significant genetic correlation of allelic form of DRB3.2 with increased and decreased clinical mastitis has been identified [24]. Numerous other investigations also have indicated the importance of this genetic component in mastitis resistance/ susceptibility [12,13,18,24]. Hence, DBR3 is ideal genetic component/ molecular marker for selection of infected and resistance animal at early stage.

CXCR2 gene

Similar to DBR 3, genes (CXCR) associated with neutrophil function are also potential genetic markers for mastitis, as neutrophil migration from blood to the site of infection is essential for resolution of most mastitis pathogens [26]. The ability of neutrophils to migrate into infected tissues is dependent upon recognition of inflammatory mediators by cytokines, chemokines and complement receptors [27]. The two chemokine receptors present on neutrophil surfaces, CXCR1 and CXCR2, are required for maximum neutrophil function during infection [28]. Recognition of chemokines by CXCR1 and CXCR2 induces neutrophil activation, chemotaxis, and eventual phagocytosis of pathogens [29].

Additionally, the CXC receptors are members of the large family of serpentine receptors with seven transmembrane domains that couple to *Bordetella pertussis* toxin-sensitive heterotrimeric G-proteins for signal transduction. G-protein coupling to the receptor results in activation of phospholipase-C, formation of the second messenger inositol 1,4,5-trisphosphate, and the subsequent increase in cytosolic free calcium concentration, as well as activation of phosphatidylinositol 3'-kinase and subsequent generation of phosphatidylinositol-3-phosphate [30,31,32]. All these mechanisms mediate many of the events necessary for proper neutrophil activation and migration to eliminate invading pathogens.

Both CXCR1 and CXCR2 are potential candidate genes for mastitis resistance; however, only the sequence for bovine CXCR2 mRNA is publicly available (GenBank Accession No. U19947). In *Bos taurus* (bovine) CXCR2 gene has been mapped to autosome (BTA) 2, approximately 90.3 cM from the centromere. The genomic structure of CXCR2 (IL-8RB) consists of three exons

interrupted by two introns of 3 and 5.4 kb. The 1065 bp open reading frame is encoded entirely in the third exon [33]. Thus, bovine CXCR2 gene is an excellent potential candidate marker for mastitis, as CXCR2 is a critical component of neutrophil migration to the mammary gland during mastitis [34].

In an effort to expand the pool of genetic markers that could potentially be associated with disease in cattle, the presence of polymorphisms within a variety of immune-related genes, including the coding region of CXCR2 present within the third exon, have been evaluated [35], Within this region, 4 SNPs have been identified that vary only in certain parents, and their alleles segregate according to Mendelian inheritance patterns in their progeny. One additional SNP within a 311-bp segment of the coding region of CXCR2 sequence in Holstein and Jersey dairy cattle, which was not present in the other beef cattle population has also been, identified [34]. All 5 identified polymorphisms exhibited strong linkage disequilibrium and were located at positions +612, +684, +777, +858, and +861 relative to the published sequence, show a significant association with subclinical mastitis. Later, in a study of Holstein cows reported that cows expressing the CXCR2+777 CC genotypes, shown a higher prevalence of intra-mammary infections [36], This study provides a potential mechanism to explain the genetic differences in mastitis susceptibility observed in cattle with different CXCR2 +777 genotypes. An example of CXCR2 polymorphism is shown in Fig:5.2. These investigations show the CXCR2 as potential candidate marker for mastitis resistance and

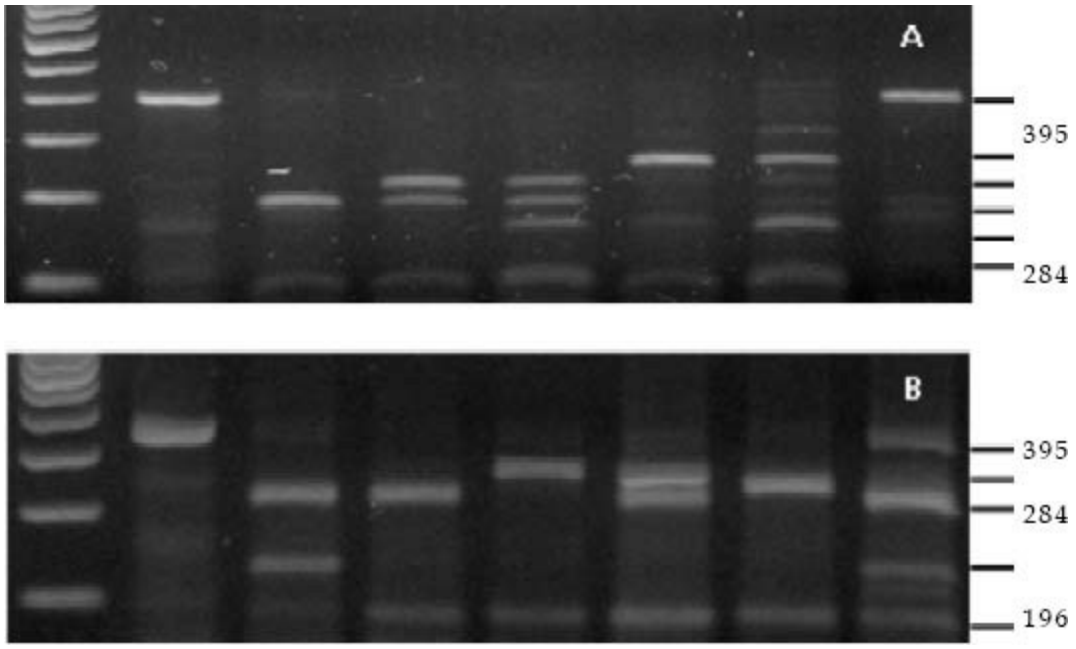

Fig. 5.2: Example of polymorphic band patterns of CXCR2 exon 2 using SSCP

Table 5.1: List of most promising candidate genes associated with mastitis.

Genetic determinants	Gene	Reference
Associated with mastitis resistance		
Actin, beta, cytoplasmic	*ACTB*	[4]
Complement component 5a receptor 1	*C5AR1*	[4]
CD14 antigen	*CD14*	[4,37]
E26 avian leukaemia oncogene 2, 3' domain	*ETS2*	[4]
Fez family zinc finger 2	*FEZF2*	[4,38]
Interferon gamma	*IFNG*	[39]
Interleukin 1 beta	*IL1B*	[37,40]
Interleukin-6	*IL6*	[37,39]
Interleukin-8	*IL8*	[37,41]
Interleukin-8 receptor, alpha	*IL8RA*	[36, 42]

Lipopolysaccharide binding protein	*LBP*	[4]
Prostaglandin-endoperoxide synthase 1	*PTGS1*	[4]
Serum amyloid A3	*SAA3*	[4]
Toll-like receptor 2	*TLR-2*	[43]
Toll-like receptor 4	*TLR-4*	[43,44, 45]
Tumor necrosis factor	*TNF*	[37,39]
Associated with milk production and mastitis resistance		
ATP citrate lyase	*ACLY*	[4]
MHC class II, DRB3	*DRB3*	[12,13]
Chemokine ligand 20	*CCL2*	[42]
Potassium channel, subfamily K, member 1	*KCNK1*	[4]
Lactoferrin	*LTF*	[46,47]
RAR-related orphan receptor alpha	*RORA*	[4]
Transformation related protein 53	*TP53*	[4]

Beta-Defensins

Beta-defensins are multi-functional peptides with antimicrobial activity against Gram- positive and Gram-negative bacteria, viruses, and fungi [48]. Apart from their antimicrobial activities, they have a function as signal molecules in the immune system and are chemo- attractants for T-lymphocytes, immature dendritic cells and monocytes [49,50].

Defensins can be divided into three distinct subfamilies: α-, β-, and θ-defensins. Beta-defensins are cationic, amphipathic molecules consisting of 38-42 amino acids [51]. They contain six cysteins that form characteristic disulfide bridges at the C1-C5, C2-C4, C3-C6 residues. The genomic structure

of bovine β-defensins consists of two exons and one intron of approximately 1.5 kb and they are located in the same cluster in the synteny group U25 on chromosome 27 [52,53]. The first mammalian β-defensin, tracheal antimicrobial peptide (TAP) gene was identified [54] from bovine tracheal mucosa. Isolation and characterization of 13 antimicrobial peptides (BNBD1-13) has been done from bovine neutrophils [51].

Bovine β-defensins exhibit antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Candida ssp.* [54], which belongs to causal agents of bovine mastitis. Six novel β-defensin genes have been identified by the PCR screening of two different bovine bacterial artificial chromosome (BAC) libraries [48]. Analysis of cDNA revealed the expression of several β-defensin genes in the bovine udder. All the identified genes show the conserved six cysteine residues and the two exon-intron structure typical of β-defensin genes. Among these genes, LAP (lingual antimicrobial peptide) was expressed constitutively in healthy and affected tissue and an isoform of tracheal antimicrobial peptide was expressed in healthy lactating udder. Whereas, the gene of β-defensin designated DEFB401 gets expressed in the lactating bovine udder during purulent mastitis caused by *Actinomycoces pyogenes.* The example of variation in β-defensins is shown in Fig.5.3.

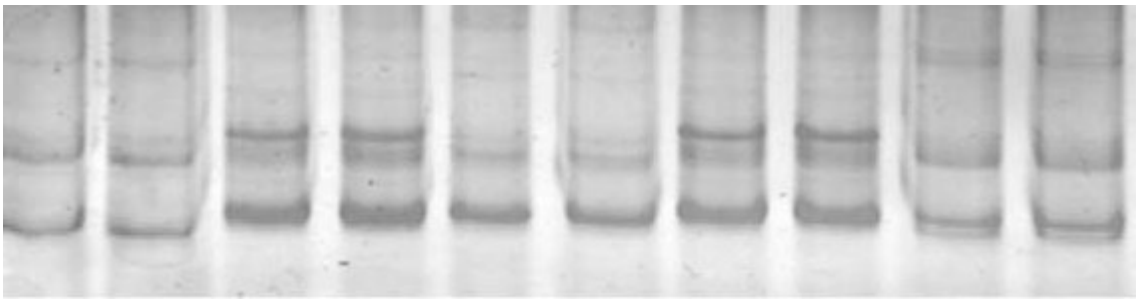

Fig. 5.3: Example of polymorphic band patterns of β - defensin using SSCP

Polymorphism in the β4-defensin gene and its observed its association with production and somatic cell count in Holstein-Friesian cows [55]. The activity of β-defensins against causal agents of mastitis and performed investigation confirmed that they may be a factor of inborn resistance and may be considered as candidate genes.

Toll Like Receptor

Toll-like receptor (TLR) family plays a fundamental role in pathogen recognition and activation of innate immunity. TLRs are a multigene family of pattern recognition receptors that are members of the TLR-interleukin-1 superfamily. These receptors recognize a great variety of pathogen associated molecular patterns (PAMP), and therefore play a central role in the initiation of inflammatory response and subsequent adaptive immune response to microbial pathogens [56]. Currently, approximately 13 members of the TLR family have been identified in mammals; genes encoding 10 of these receptors have been mapped in the bovine genome [57]. Polymorphisms in genes encoding receptors associated with the innate immune system are likely to contribute to the overall variation in the resistance or susceptibility to mastitis in dairy cattle [36], Among the various TLR gene families, TLR 4 is prominent marker for mastitis. The whole genomic length of bovine TLR4 is estimated to be around 11 kb, of which the first intron comprises about 5 kb and the second, 3 kb [58], TLR4 is known to reside on the distal tip of bovine chromosome 8 [58], In buffaloes, the sequence of TLR4 is available in NCBI Gene Bank with accession number EU 386358 [59],

Polymorphisms (example shown in Fig. 5.4) in the coding and promoter regions of TLR4 can also determine different host resistance or susceptibility patterns to various infectious diseases [60,61]. TLR4 gene might be a potential candidate for use in marker-assisted selection to enhance mastitis resistance in dairy cattle owing to its involvement in PAMP recognition and association with IMI [58].

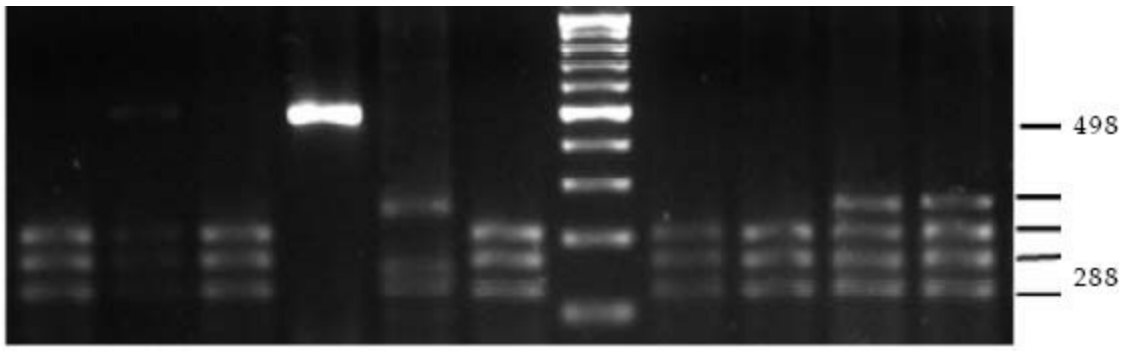

Fig. 5.4: Examples of polymorphic patterns inTLR4 gene using RFLP

It has been reported that the TLR4 gene expression of one SNP within the

5<C UTR region at position 226 (c.-226C>G) to be associated with health related traits in the Canadian Holstein population [44],

The broad range of pathogens and potential pathogens that produce TLR4 ligands indicate that the gene could be considered a candidate gene for resistance to mastitis.

Immunoglobulin Gene

In bovine immunoglobulins, the gamma (Gm) system has been reported to show the greatest degree of polymorphism [62,63]. The characterization of cattle immunoglobulins at the protein level was pursued by a number of investigators through the late 1960s, 1970s and 1980s; however, work led to a limited understanding [64]. Afterward, the molecular characterization of immunoglobulins revealed their functions and potential role in bovine [62,65,66,67,68].

The immunoglobulins, namely IgG1, IgG2, IgA and IgM are known to combat against mastitis causing microorganisms in the mammary gland defence system [67]. The Ig allotypic marker has been shown to be located in the constant regions of heavy chain of these antibodies. The availability of detailed information on the constant regions of bovine Ig (IgG1 and IgG2) heavy chains has been found to be associated with some diseases that might be helpful in cytokine profiling in bacterial infections [62,65,66]. Some of the bovine heavy chain constant regions have such character [62].

The concentration of each immunoglobulins class in mammary secretion varies depending on the stage of lactation and infection status of the mammary gland. In healthy glands, the concentration of immunoglobulins is low during lactation; however, slowly increases in the non-lactating period and reaches to peak at some point in colostrogenesis [67]. High concentrations of immunoglobulins also occur in the mammary gland during inflammation. Although IgG1 is the predominant isotype in healthy bovine lacteal secretion, neutrophils can transport IgG2 to the mammary gland as they immigrate to the site of infection. IgG1, IgG2, IgG3 and IgM act as bacterial opsonins that enhance phagocytosis of neutrophils and macrophages [67]. Apart from IgG1, IgG2, IgG3 is also a potential immunogen (Rabbani *et al.,* 1997) [62]. Recently, genetic variants of IgG3 gene (Fig. 5.5) and their association with

mastitic infection have been reported [68].

The investigation has described the association between IgG3 genotypes and staphylococcal mastitis in Karan Fries, Sahiwal and Murrah, respectively [68]. Genotypes revealed by RFLP were significantly associated with mastitis susceptibility and resistance. Such

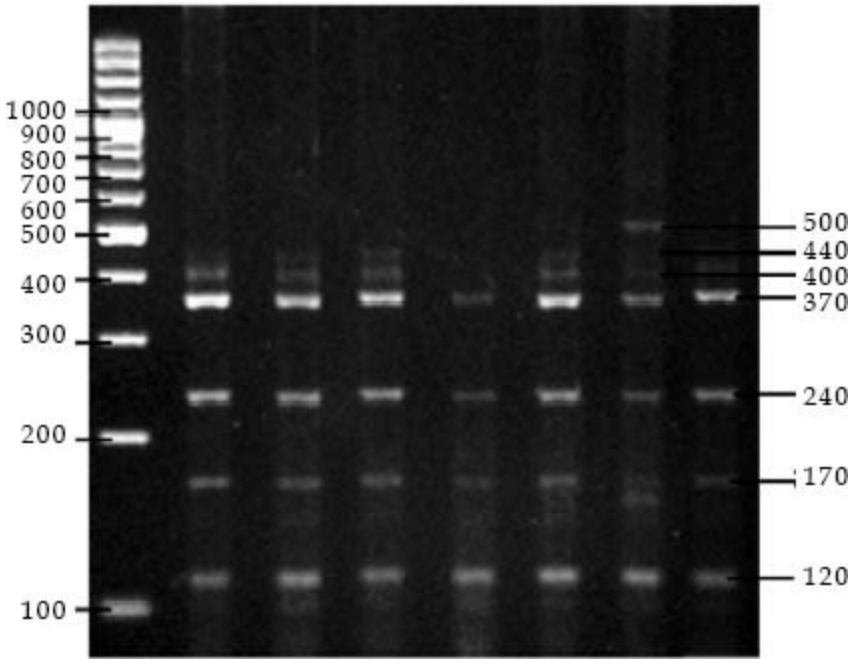

Fig.5.5: Example of polymorphic patterns of lgG3 gene using RFLP

kind of molecular analysis revealed that IgG3 gene could be used for selection of animals against mastitis.

Lactoferrin

Lactoferrin is known for its strong iron binding properties and has several biological functions including host defence against microbial infections and anti-inflammatory activities [69]. The lactoferin (LTF) gene has been found to involve with mastitis expression experiments and a strong functional candidate marker for mastitis resistance [46,47]. The AB allele of LTF has been reported in animal having the highest SCC, whereas AA with lowest one [47],

In addition, the genes namely prostaglandin-endoperoxide synthase 1, cytoplasmic beta actin, transformation related protein 53 and E26 avian

leukaemia oncogene 2, 3' domain were also found in mastitis expression studies that resulted in increased tumorigenesis of mammary gland and abnormal lactation [4],

Molecular Approaches as Diagnostic Tools

The availability of large number genetic markers in almost all species of human interest comes in existence with the development of molecular techniques. These techniques are capable of detecting variation at both DNA and protein sequence level among individuals in the population.

These polymorphisms are used to build up genetic maps and to evaluate in the expression of particular traits in a family that might indicate a direct effect in terms of genetic determination. The almost unlimited numbers of polymorphisms at DNA sequence level in both coding and non-coding regions of DNA have provided a number of markers, viz. restriction fragment length polymorphisms, microsatellites, single nucleotide polymorphisms, minisatellites or VNTRs, randomly amplified polymorphic DNA (RAPDs), etc. Some of major techniques used for analyses of genetic variations in farm animals are explained in this section, rest are listed in Table - 5.2 along with their principle.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is an extensively used research technique since its inception. PCR was invented and named by Mullis of the Cetus Corporation, USA, in the year 1985. For PCR, DNA sample (template), oligonucleotide primers, deoxyribonucleotide triphosphates (dNTPs), *Taq* DNA polymerase are needed in a suitable buffer [70]. The working of PCR involves the steps of denaturation, annealing and extension. First steps heat involve the denaturation of the double-stranded DNA, followed by annealing of primers by cooling the mixture and then primer extension by DNA polymerase at a temperature suitable for the enzyme reaction. This results in the synthesis of new DNA strands complementary to the template strands. These strands exist at this stage as double-stranded DNA molecules. The entire cycle is then repeated many times. Since the products of one cycle serve as

templates for the next, each successive cycle essentially doubles the amount of the desired DNA sequence. PCR is in use since the three decades for various purposes of molecular characterization of animals including mastitis. Additionally, modifications of PCR such as multiplex PCR, Nested PCR and real time PCR have been used for characterization of genes (e.g. DRB3) of mastitic animals [71].

Rflp

Chromosomal DNA restriction enzyme digestion analysis was the first of the genotyping schemes. The banding patterns that result after cutting and separation of the DNA fragments by electrophoresis are referred to as DNA fingerprinting. As of the high specificity of restriction enzymes and the stability of chromosomal DNA, reproducible patterns of fragments are obtained after the complete digestion. These variations in the banding patterns between strains are ascribed as basic differences in the DNA base composition of the organism examined. One general criticism about this method is the complexity of banding patterns. Nevertheless, there are some researchers who believe that using the right enzyme and specified conditions RFLP could still be a relatively rapid and reliable technique. PCR-RFLP has been successfully used to identify the variations in genes (BoLA-DRB3, IgG3 and TLR4) related with mastitis [72]. 22 BoLA- BRB 3.2 alleles were reported in Holstein cows using PCR-RFLP [23]. Similarly, by RFLP using *RsaI, HaeIII* and *BstYI* restriction enzymes, association between parameters of innate immunity and measures of mastitis in Holstein cattle has been reported [24]. A number of investigations have indicated the utility of RFLP as diagnostic method in mastitic animals [23,24,72].

Single Strand Conformation Polymorphisms

Single Strand Conformation Polymorphisms (SSCPs) first described by Orita *et al.* [73] have proved to be quite useful tool for the detection of SNPs. It has been used successfully for screening and detecting unknown point mutations [34,35,36,74,]. SSCP is ideal for screening relatively short fragments (< 400bp) because the mobility shift is less apparent with larger

size. In PCR-SSCP analysis, an amplified product of DNA is denatured and prevented from re-annealing to its complement by dilution, chilling, or adding denaturing agent. These force each strand from re-annealing in a stable conformer. When these conformers are electrophoresed under native (non-denaturing) conditions, the mobility of each strand is affected by its conformational structure. This structure depends on the sequence of nucleotides within the strand. Sequence variation within the PCR product, such as a point mutation, produces a different conformer, resulting in a change in electrophoretic mobility. Genomic variation can be identified comparing the strand mobilities between samples. This method was utilized for analysis of second exon of CXCR2 gene in mastitic animals [34]. Similarly, identified 4 SNPs within the third exon of CXCR2 has been identified with the help of SSCP that varied only in certain parents, and their alleles separated according to Mendelian inheritance in their progeny [35]. In the present scenario, SSCP is less preferred for molecular characterization due to its some limitation. Still this method is in use in many laboratories for characterization of mastitis associated genetic components.

RAPD Fingerprinting

The RAPD technique is a PCR-based discrimination method in which short arbitrary primers anneal to multiple random target sequences, resulting in patterns of diagnostic value. In RAPD analysis, the target sequence/ s to be amplified is unknown and a primer with an arbitrary sequence (a 10-base pair sequence or a 10-bp sequence randomly generated by computer) is designed and synthesized. After these sequences have been synthesized they are used in PCR reactions with low-stringency annealing conditions, which results in the amplification of randomly sized DNA fragments. As the reproducibility of RAPD patterns is occasionally poor; this method needs to be performed under carefully controlled conditions. Various groups have adopted the use of RAPD to identify and characterize the mastitis.

Amplified Fragment Length Polymorphism

Amplified fragment length polymorphisms (AFLP) are PCR based markers

for quick analysis of molecular variations. This method has the ability to give numerous highly replicable markers from DNA of individual. AFLP markers have emerged as a major new type of genetic marker with broad application in systematics, pathotyping, population genetics, DNA fingerprinting and quantitative trait loci (QTL) mapping. In AFLP procedure, restriction enzymes are used to digest genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. Afterward, a subset of the restriction fragments is then selected to be amplified. This is achieved using complementary primers to the adaptor sequence, the restriction site sequence and a few nucleotides inside the restriction site fragments. The amplified fragments are separated and visualized on denaturing polyacrylamide gels, either through fluorescence methodologies or autoradiography.

Genome screening for QTL is costly and highly laborious, however, a new cheap and easy QTL mapping method has been developed by implementation of AFLP markers, DNA pooling and bioinformatics tools [75]. Similarly, use of AFLP as diagnostic method in mastitic animals is also being explored [76]. This method provides high-resolution genotyping of fingerprinting quality with less time consuming and cost, which makes it superior over other markers (e.g. RAPD, RFLP, microsatellites) in identification of mastitic animals.

Real Time PCR

Real time PCR modifies the technique in a way that reduces (99.9%) the chance of false positives as observed in traditional PCR. Even a single copy of target DNA can be detected owing to a high dynamic range. Real time PCR is based on detection and quantification of a fluorescent reporter whose signal increases in direct proportion to the amount of PCR product in a reaction. General methods for the quantitative detection of the amplicon, DNA-binding agents and fluorescent probes including TaqMan technology, molecular beacons and fluorescence resonance energy transfer probes have been used. The procedure of real time PCR has similar general principle (denaturation, annealing and extension) of traditional PCR. There are two common methods for the detection of products in real-time PCR: (1) non-specific fluorescent dyes, which intercalate with any double¬stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter, which permits detection only after hybridization of the

probe with its complementary sequence to quantify messenger RNA and non-coding RNA in cells or tissues. Various investigators have reported diagnosis of mastitis using 'Real Time PCR' [77,78].

The use of real time PCR has been explored by quantifying the bovine cytokines (IL)-2, IL-6, IL-8, IL-12 p40, TNF-α, IFN-g and growth factors contributing to immunity against bacterial infections of the mammary gland in cattle [77]. Differences in cytokine profiles was shown successfully. The utility of real time PCR to analyse the infections of the mammary glands has been proved by quantifying mRNA of TLR9, TLR2, and TLR4 with β-defensin in healthy and infected mammary glands [78]. The approach was successful to differentiate expression of these genes in healthy, subclinical and clinical infections. The application of this approach is increasing day by day in monitoring animal udder health.

DNA Microarrays

DNA chips or DNA arrays, which are well-organized and immobilized oligonucleotides on an organic substrate, provide new opportunities for assessing genetic diversity among organisms. These arrays rely on the hybridization of isolated DNA to large sets of oligonucleotides or DNA fragments present at a precise location on a miniaturized inorganic substrate (e.g. a glass slide). DNA chips are now being used by various research groups for characterization of

Table 5.2: Major methods used for molecular typing in cattle

Typing Method	Principle	Analysis procedure
Gene specific PCR	Analysis of conserved domains of genes in different species	Amplification of species specific sequence for identification of strains
PCR-RFLP	Amplification of targeted gene followed by digestion with restriction enzymes	Evaluation of amplified or targeted gene

SSCP	Single Strand Conformational Polymorphism corresponding to differences at a single nucleotide position, useful tool for the detection of SNPs	Electrophoretic separation of single-stranded nucleic acids based on subtle differences, which results in different secondary structures and measurable differences in mobility through a gel
AFLP	Total genomic DNA digestion with restriction enzymes followed amplification of subset of the fragments	Restriction fragment length polymorphism evaluation of selective amplified fragments
RAPD	Evaluation of random segments of genomic DNA with single primer of arbitrary nucleotide sequence	Random amplification of polymorphic regions of genomic DNA for uniqueness
Multiplex PCR	Investigation of more than one gene in single reaction	Amplification and detection of multiple genetic determinants
Nested PCR	Second round of amplification formed with primers that hybridize within the target DNA amplified by the first primer set	Greatly increases the sensitivity and specificity of the PCR
Real Time PCR	Amplify and simultaneously quantification of a targeted DNA molecule	Detection and quantification of copies or relative amount when normalized to DNA input or additional normalizing «'lies
DNA	Array of oligonucleotides immobilized on an	Genetic patterns of strains on the basis of gene

microarrays	organic substrate and hybridization between two DNA strands due to complementary nucleic acid sequences to specifically pair with each other	presence and absence

mammary glands. The number of oligonucleotides spotted on a chip can vary from several hundreds to almost a million. Oligonucleotides can be derived from every region of the genome and point mutations (single nucleotide polymorphisms) are targeted as well. Recently, the affected genes of mastitis were analysed using microarrays and their different expression profiling collectively to reveal the biological functions and pathways, which are intrinsic to the general disease response [79].

Similarly, a genome-wide association mapping was carried out in cattle with transcriptomic data using microarray investigations on various mastitis pathogens and host species *in vitro* and *in vivo* [80]. This investigation revealed for several candidate genes including IL- 17 and IL-8 signalling pathways, responsible for the recruitment and migration of inflammatory cells into tissues during inflammation and infection. Such observations are making DNA chips popular for large- scale testing in cattle. The only difficulty of this method faced is the cost of a chip, which can only be used once, and the need to purchase expensive equipment for hybridization and analysis. Nevertheless, this technique will be the method of choice to identify dairy cattle in the near future because of its high degree of reliability in terms of specificity and sensitivity.

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chapter 6
Machine Milking and Mastitis Risk

The advent of intensification in concert with mechanization in farm management has tremendously revolutionized dairy production operations over the last few decades and undoubtedly, machine milking has emerged as one of the most utilized technological interventions in that process. Many factors have eventually paved the way for extensive adaptation of milking machine in modern day dairying. It simplified the most labour intensive part of dairying, improved milking performance (speed/completeness of milking) and subsequently increased the milk quality. But inadvertently, it has also resulted in udder disorders in many cases; as evident from the inter herd variation in udder health (16-45%) which has been primarily attributed to defective milking machine and faulty milking management [1]. Improper handling of machine, over use, machine malfunction and inadequate training of the personnel may accrue chances for increased incidence of mastitis and other teat related disorders. The present chapter will give a brief account on the various risk factors in machine milking, their evaluation and optimum management to minimize the chances of mastitis in dairy animals.

A glance on functional anatomy of teat will prove much useful to begin with, for providing meaningful insights, in realizing the condition more vividly. Bovine teat canal is highly specialized in its unique function of preventing both leakage of milk and entry of bacteria [2]. This keeps the internal environment of the mammary gland sterile most of the time, despite frequent soiling and contamination of the outer teat surface. Considering the relatively short length of the teat canal, the specialized epithelial structure is extremely effective in preventing pathogens from penetrating into the gland [3]. Vacuum and pulsations applied in the milking procedures lead to certain changes of teat tissue appearance, such as teat end congestion, changes in teat dimensions,

colour, texture and formation of callus ring on top of the teats. Some of these are short term changes and the udder usually recovers between milking but some changes can be long term which may lead to chronic changes resulting in greater manifestations of mastitis.

Milking machine may increase the incidence of mastitis by

- Facilitating the transfer of bacteria between cows or quarters on the same cow.
- Aiding in the multiplication of bacteria at the teat end.
- Increasing the bacterial penetration of the teat during milking.
- Modifying the teat/ intra-mammary environment to enhance bacterial infection or impair host defenses.

Transferring Bacteria to Udder

Using the same milking unit used for an infected cow without disinfection may easily transfer the infection to an uninfected one. Contamination from the milking environment can also pass through milking machine if the unit gets contaminated between milking. Bacteria can also move from one quarter to another during the milking process when the unit is attached to a cow. Using improvised claw design that has adequate volume and the ability to remove milk quickly from the claw into the milk hose away from the cow could be employed to reduce this movement.

Multiplication of Bacteria

The milking machine influences the overall teat end and teat skin condition. Smooth teat ends and teat skin reduce the ability of bacteria to multiply and develop on these surfaces. Issues like teat end hyperkeratosis and chapped skin are commonly observed with machine milking and both of these will favour bacterial growth on the teat skin. Milking machines may also cause small hemorrhages which predisposes bacterial multiplication there after.

Increased Bacterial Penetration

The action of the milking machine can cause bacteria to be propelled directly at the teat end and, in some cases, directly into the teat sinus during milking. Cyclic or irregular fluctuation of vacuum level in milking machine can lead to increased bacterial penetration. The penetration of bacteria into the teats occur predominantly towards the end of milking; as there is little or no milk coming out through the teats and therefore the possibility of flushing the bacteria out get reduced. Moreover, during cluster removal there are chances of new infection due to persistence of partial vacuum relative to the atmosphere. The stress associated with over-milking and improper vacuum levels and pulsation ratios reduces the effectiveness of the canal as a barrier to infection.

Modification of Intra Mammary Environment

If the massage action of pulsation ceases when vacuum is continually applied, excessive congestion and edema will result as blood and interstitial fluid collect in the large veins and lymphatics, respectively [4]. Recovery of teat end thickness may take from 6 to 8 hours in conventional milking. Milking at shorter intervals may lead to incomplete recovery of the teats which further worsen the teat injury. Machine milking has a greater effect than calf suckling on teat swelling and teat end callosity [5].

Several factors related to milking machine predispose the development of mastitis.

- Liner slips cause reverse flow of milk and hence responsible for a large number of new mastitis cases. It occurs when the mouthpiece of the liner slips-down from the teat, thereby allowing air to get sucked into the milking unit. Reasons may be low claw vacuum, heavy milking units and/ or poor unit alignment.
- Closure of the liner around the teat with excessive values of compressive load results in formation of micro-fissures around the teat orifice. (Compressive load is the vacuum level within the pulsation chamber at which milk flow just begins). The *stratum corneum* responds to the micro-fissures by increasing keratinization, which renders the animal prone to mastitis development.
- Pulsator setting is another important factor as improper milking and

massage phase may lead to increased teat thickness and hyperkeratosis.
- Liner thickness, liner hardness, resistance to collapse and improper handling of milking machine can inflict teat injury, leading to mastitis.
- Vacuum fluctuation within the claw is responsible for fluctuation of pressure at the teat end and it can also lead to teat end injury.

Evaluation of Milking Machine Performance

Proper functioning of the several parts of the milking machine is a prerequisite for hassle free and smooth operation of milking machine. In order to avoid machine generated factors leading to mastitis regular appraisal of machine performance at various phases is required. Milking machine can be evaluated in several phase starting for simple checking of the machine to critical checking by an engineer in detail.

Table 6.1: Evaluation of milking machine performance

Phase 1 testing. Simple machine checks

- Teat cup liners
- Claws
- Milk line
- Vacuum level
- Regulator Response
- Fall off Test
- Pulsation

Phase 2 testing. Pulsator performance and milking vacuum stability

- Pulsator testing
- Milk line vacuum stability
- Receiver vacuum stability
- Average claw vacuum

Phase 3 testing: Complete professional machine evaluation

- Rate and ratio of all pulsators
- Operating vacuum tests

- 'Fall off' test
- System leakage test
- V acuum pump capacity test

Phase 1 Testing: Simple Machine Checks

This level is intended to identify possible machine or milking management causes of mastitis, teat condition problems, or slow or incomplete milking. The equipment required for this entry level testing includes a stopwatch, a level, and a vacuum gauge of known accuracy. A simple mercury column is recommended for regular calibration of the test vacuum gauge.

Following observations should be made when the machine is not running.

1. **Teat cup liners:** Teat cup liners should be in good condition without cracks and there should be no bulging out in mouthpiece lip or inner barrel. Distortion or cracking of the liners is an indication of overuse beyond prescribed usage time. The cup should not distort the mouthpiece and the liner should be held firmly enough not to twist easily in the shell. The length of the shell must match the design length of the liner so that it is mounted under the correct tension.

They should have a barrel diameter about 1 or 2 mm less than the average diameter of the teats after milk letdown *(i.e.* a barrel diameter around 21-22 mm for typical Holstein cow). They should be long enough to collapse below the teat. The effective length of any liner is measured from the mouthpiece lip to the bottom of the location on liner barrel that the walls touch when milking vacuum is applied to the liner while mounted in the teat cup shell. The effective lengths of typical liners made of natural rubber or synthetic rubber, should be 130 mm for liners up to 20 mm bore; 135 mm for 21-22 mm bore; 140 mm for liners of 23-24 mm bore.

1. **Claws:** The air vent in the claw should be clear and unblocked. Blocked air vents will reduce milking vacuum. The air vent in claws range from about 0.8 mm to 1.2 mm in diameter allowing 7 to 12 L/min of air.
2. **Milk line**: The milk line should slope towards the receiver with a minimum fall of 1% and preferably 1.5 to 2.0 %. It is especially

important to maintain adequate slope of the milk line near the receiver and in areas of bends and fittings. The milk line should be mounted as low as possible above the cows and never more than 2.0 m above the cow standing level.

Following parameters should be recorded when machine is running but milking is not done.

- **Vacuum level**: Farm gauges are often damaged and inaccurate. Sometimes the indicator needle sticks and it will not move above the operating vacuum level to indicate a high operating vacuum if the regulator fails. The face of the gage should be tapped to check for a sticking needle. If the farm vacuum gauge is not functional or inaccurate a functional gauge should be installed.
- **Regulator Response:** Sound of air entering the regulator should be listened when the vacuum pump is running with all other units shut off. Milking units should be opened enough so that the air admitted reduces the receiver vacuum by about 3 kPa (about 1" Hg). This should cause the regulator to close resulting in a noticeable reduction in the sound caused by air entering the regulator. If the regulator does not appear to close, regulator filter should be checked and cleaned if necessary.
- **Fall off Test:** A unit fall off test can be performed to determine the reserve capacity of milking machine. The average vacuum in the receiver should be measured with all units in operation and all teat cups plugged. Then one milking unit should be opened to admit air through all four teat cups and average vacuum in the receiver should be measured again. If fall in average vacuum level is less than 2 kPa (0.6" Hg), then the system has sufficient reserve capacity to cope with unit fall off.
- **Pulsation:** Each pulsator should be listened closely as a first check for uniformity between units. The sound of air entering the pulsator should be regular and intermittent. This simple check is made more sensitive by partially covering the air inlet with a finger. A continuous sound of air entering indicates a leak (usually grit or dirt) under the pulsator valve seat. In that case, pulsator air filter or air inlet should be checked for any obstruction and each liner should be checked to ensure that they are not twisted in their shells.

Phase 2 Testing: Pulsator Performance and Milking Vacuum Stability

Proper pulsator functioning is critical to the success of the milking process. Milking-time tests are the most direct method to determine

the adequacy of vacuum production and vacuum regulation in any milking system.

- **Pulsator testing:**These tests are done with milking units connected, pulsators operating and liners fitted with teat cup plugs. The objective of these tests is to determine if the pulsation system and all pulsators are operating according to the manufacturer's specifications. Pulsation testers are used to determine the pulsation rate and the duration of the four phases of pulsation. The main parameters to be checked are as follows:
    - Pulsation rate should be repeatable from day to day and should not deviate more than 3 cycles per minute from one unit to the next.
    - Pulsator ratio should not differ more than 5 percentage units from manufacturer's specifications or from one pulsator to another.
    - The B phase (milking or liner-open phase) of pulsation should be at least 30% of the cycle.
    - The D phase (massage, rest or liner-closed phase) of the pulsation cycle should not be less than 15% and not less than 150 mS.
- **Milk line vacuum stability:** ISO recommends that the performance criteria for a vacuum stability in a milking machine is that the vacuum drop in or near the receiver should not exceed 2 kPa (0.6" Hg) for at least 95% of the normal milking. Normal milking is considered to be the time that milking units are attached to cows including events such as teat cup attachment and removal, liner slips and cluster falloff. The most useful sites of measurement are in the milk line, in or near the receiver and in the claw. Vacuum at these sites should be recorded while the system is under full milk and air flow conditions.
- **Receiver vacuum stability:** If the system passes the milk line vacuum stability test it is not necessary to record vacuum stability in the receiver. Additional measurements should be made in the receiver to determine if vacuum fluctuations in excess of 2 kPa (0.6" Hg) in the milk line are caused by milk line slugging or by inadequate vacuum production.

- **Average claw vacuum:** A suitable vacuum recorder should be connected to the claw. The vacuum at the regulator should be set so that the average claw vacuum during peak milk flow is between 35 kPa and 42 kPa (10.5 and 12.5" Hg).

Phase 3 Testing: Complete Professional Machine Evaluation

These tests require the use of a vacuum recorder that can display the average; minimum and maximum vacuum recorded over a known or pre-set measurement period and an air flow meter. A flow simulator, as described by Stewart *et al.* [6], can be constructed or purchased. A milking machine technician should perform a complete system evaluation after each 500-1000 hours of operation as part of a regular service, or whenever modifications are made to the milking machine, or when milking time tests indicate that there may be a problem with the milking machine. A complete evaluation includes the following measurements:

- Rate and ratio of all pulsators.
- Operating vacuum in the receiver and vacuum difference between the receiver and the vacuum pump, regulator and pulsator air line.
- 'Fall off' test.
- Effective reserve - An airflow measurement of the reserve pump capacity actually available to maintain receiver vacuum stable within 0.6" Hg when extra air enters the system during milking.
- Manual reserve - A measurement of the airflow capacity potentially available to maintain the receiver vacuum stable within 0.6" Hg if the regulator could close completely.
- Air used by components: Pulsation system, clusters, regulator, and other ancillary equipment.
- System leakage.
- Vacuum pump capacity.

There are several other specialized dry tests are available apart from the above mentioned which are used regularly for evaluation of milking machine functionality.

- Vacuum in the mouthpiece chamber of the liner (MPC vacuum) during peak milk flow can be used to determine if this is a cause of rings at the base of the teat and/ or discolored or swollen teats after milking. The Average MPC vacuum should be at least 10 kPa (3" Hg) less than the average claw vacuum during peak milk flow [7].
- Average claw vacuum and vacuum drop through the long milk tube and through ancillary equipment using a milk flow simulator [6] can also serve as good measure of milking machine functionality. The vacuum at the regulator should be set so that the average claw vacuum during peak milk flow is between 35 kPa and 42 kPa (10.5 and 12.5" Hg). Flow rates of 3.5 L/min to 5.5 L/ min cover the range of expected peak flow rates for high producing cows. Flow simulators can be used as a reasonable estimate of the average vacuum in the claw and peak milk flow rate of individual cows, but do not provide a reliable estimate of vacuum fluctuations in the claw or short milk tube during milking.

Milking Management to Reduce Mastitis Incidence

Milking management has a greater influence on the success of the milking process than milking machine related factors. A thorough and systematic review of milking procedures in a particular farm would be the most important part of determining the source of milking associated problems.

- **Cow cleanliness:** Cow cleanliness is a major determinant of both milking efficiency and the rate of intra-mammary infection [8]. Management practices that reduce teat-end exposure to the pathogenic organisms will reduce the risk of developing mastitis. Bedding material should be clean, dry and comfortable to minimize pathogen growth. For example, inorganic bedding such as sand can be used, which will reduce the number of pathogens if groomed daily. Further improvements in cow cleanliness can be made through routine removal of udder hair twice yearly and keeping tails trimmed or docked.
- **Cow handling:** Human-animal interactions have a greater influence on the milking process [9]. The release of adrenaline within 30 minutes of milking can interfere with milk letdown, thus prolonging the duration of milking. Cow handling techniques should be re-examined and improvised

if dairy cows shows hesitation in entering the milking area or defecating frequently during milking.

- **Cow grouping:** Uninfected cows should be grouped and milked in an order to minimizing exposure to cows known to be infected with sub-clinical mastitis. It is safe to assume that cows with somatic cell counts (SCC) greater than 250,000 are chronically infected. Fresh heifers are generally put in the uninfected group until their first SCC is obtained. Fresh mature cows should be classified based upon their previous SCC status or cultures obtained at calving. The uninfected cows should be milked first, followed by cows of unknown infection status and the infected cows should be milked at last. Alternatively, few milking units can always be used on infected cows.
- **Pre-milking cow preparation:** Pre-milking preparation includes cleaning teats before unit attachment and examination for clinical mastitis and abnormal milk. The combination of effective teat cleaning and fore-stripping will provide sufficient stimulation for milk letdown.

Proper teat-end disinfection can reduce teat surface bacteria by 75% [10]. The lowest milk bacterial counts have been shown to be produced with methods that wet and clean only teats and not the whole udder. If cows are clean, teats can be adequately disinfected by the use of pre-dipping without additional washing. Pre-dipping is most effective in the control of environmental pathogens *(E. coli* and environmental *streptococci)* [11,12]. A minimum contact time of 20-30 seconds is needed for effective disinfection. If washing is required to remove excess manure, then: 1) only teats should be washed, 2) minimal water should be used and 3) teats should be thoroughly dried. The most important portion of the teat disinfection process is thorough drying of teat ends; as water on teats aids in transporting bacteria and concentrating them at the opening of the teat canal. A cloth towel is a better absorbent than paper [13]. When cloth towels are used they should be disinfected by washing with bleach or very hot water and drying at high temperature in an automatic dryer. Effectiveness of teat cleaning can be checked using a clean swab rubbed across the end of the teat prior to unit attachment. A dirty swab indicates that teat preparation methods should be improved. Fore-stripping prevents the buildup of microorganisms; hence should be done prior to strip cup test and milking. Cows in stall barns should never be fore-stripped into the bedding.

- **Unit attachment:** In order to maximize milking efficiency, units should be attached from 45 to 90 seconds from the beginning of stimulation. Prep-lag times greater than 3 minutes result in more residual milk and lower milk yields. Effective support should be provided for the long milk tube and units should be adjusted so that cluster weight is evenly distributed on the four teats. Proper unit adjustment and long milk tube support results in fewer liner slips and unit falloff.
- **Unit removal:** Milking units should be removed promptly when the milk flow rate from the udder drops below 0.5 kg/ min. Early unit removal may result in reduced milk yield and promote the development of sub-clinical mastitis to the clinical stage. Over¬milking poses highest risk for developing new mastitis infections and teat damage. Machine stripping should not be routinely practiced.
- **Post milking management:** Post-milking teat antisepsis reduce the transmission of contagious mastitis pathogens. Spray applicators are preferred by some operators because of convenience and to prevent teat dip from becoming tainted with contaminated milk. The last step in an effective milking routine is to ensure that the cows remain standing for at least 30 minutes after milking is completed. Providing fresh feed/fodder after milking will encourage this behavior.
- **Milking time and average milk flow rate:** Average milk flow rate is a good indicator of the efficiency of milking and can be calculated as the total milk yield divided by the total machine-on time. Low average milk flow rates or longer milking times can result from interference with the letdown response due to uneasiness of the cows, inadequate cow stimulation, improper timing of unit attachment in relation to milk letdown, milking machine problems or over milking because of improper detachment procedures.
- **Completeness of milking:** The completeness of milking can be assessed by hand stripping each quarter immediately after the milking machine is removed. If milking units are being removed at the proper time the majority of quarters will have little or no milk present after unit removal. As a thump rule, not more than 20% of quarters should yield 50 mL or more milk when hand stripped immediately after unit removal. It is common for the slowest milking quarter to have some residual milk left after unit removal.
- **Teat condition:** Severity of teat injuries and hyperkeratosis should be

recorded immediately after units are removed. The number of teats with good condition (no ring or small ring with smooth skin, no roughness at the teat end, normal color after milking) and the number of teats with poor conditions (raised ring that is with rough and cracked giving the teat-end a "flowered" appearance or teats that have a blue color after milking) should be recorded. Not more than 20% of cows should have problematic teat condition [14]. Of the various milking management factors, the total time per day when milk flow rate is less than about 1 kg/ min appears to have a major effect on teat-end condition. Pre-milking udder preparation practices, degree of over-milking, as well as milking equipment settings such as high milking vacuum or liners with stiff mouthpieces may also influence the teat condition.

- **Frequency of slipping or falling teat cups:** Note the number of times the units must be adjusted by operators because of slipping or falloff. Operators' goal should be to minimize the slipping or falloffs to less than 5% of cow milkings [15]. Heavy clusters, uneven weight distribution within the cluster, blocked air admission holes or poor liner design are other common causes of slips and falloffs.
- **Use of back flushers:** Back flushers have been developed to sanitize the liners and claws between milking, which comprises of a first cycle water rinse, followed by a sanitizer rinse, a clear¬water rinse and finally a positive air-dry cycle. Researches have demonstrated that back flushers do reduce the number of bacteria on the liners between cows but do not reduce the number of bacteria on teats. But this also can be accomplished at a much lower cost by teat dipping. Moreover, back flushing has no effect on environmental pathogens that are encountered between milking.

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Chapter 7
Mastitis and its Public Health Significance

The predominant causal organisms responsible for causing mastitis are cell-walled pathogens, although mycoplasma, yeast and algae have also been reported to cause mastitis. *Str. agalactiae* and *S. aureus* were more frequently associated with clinical mastitis than sub-clinical case.

Enterotoxigenic Staphylococcus aureus

Milk and dairy products are frequently infected with *S. aureus* and milk of infected animals is the main source of heat-resistant enterotoxins, which are responsible for staphylococcal food poisoning outbreaks. Thus, bovine mammary gland is a significant reservoir of enterotoxigenic strains of *S. aureus*. It can produce two different types of toxin with super-antigen activity: enterotoxins and toxic shock syndrome toxin (TSST-1). Staphylococcal food poisoning occurs when food is consumed that contains endotoxins produced by *S. aureus*. There is also a significant correlation between the staphylococcal enterotoxin (SE) and TSST-1 in *S. aureus* from mastitis milk. The strains producing the staphylococcal enterotoxin type C (SEC) have been widely isolated from mastitisafflicted cows.

Food handlers carrying enterotoxin producing *S. aureus* in their noses or on their hands are regarded as the main source of food contamination via direct contact or through respiratory secretions [1]. The udders and teats of cows are known sources of enterotoxigenic *S. aureus,* and the occurrence of *S. aureus* in unpasteurised milk and cheese is common. *S. aureus* is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance. This bacterium is a significant cause of nosocomial infections, as

well as community-acquired diseases.

The spectrum of staphylococcal infections ranges from pimples and furuncles to toxic shock syndrome and sepsis, most of which depend on numerous virulence factors. On the other hand, some infections, such as staphylococcal food poisoning, rely on one single type of virulence factor: the SEs. The symptoms of staphylococcal food poisoning are abdominal cramps, nausea, vomiting, sometimes followed by diarrhea. The onset of symptoms is rapid (from 30 min to 8 h) and usually spontaneous remission is observed after 24 h. These enterotoxins are produced due to improper cooling of milk, during cheese manufacture from raw milk and also due to post-processing contamination. These toxins cannot be destroyed by heating or drying [2]. In a study regarding enterotoxigenic *S. aureus* in bulk milk, it was detected in 165 (75%) bovine and 205 (96.2%) caprine bulk milk samples and in 31 (37.8%) raw milk product samples. Enterotoxin production was observed in 22.1% and 57.3% of *S. aureus* isolates from bovine and caprine bulk milk, respectively. While SE genes were detected in 52.5% of the bovine and 55.8% of the caprine bulk milk isolates.

Shiga Like Toxin Producing *E. coli*

Shiga like Toxin producing *E. coli* (STEC) are of immense economic and public health significance and characterized by low infectious doses i.e. 1-100 cfu. STEC are highly pathogenic in humans, where they cause serious acute illness and long-term sequelae. Shiga toxin- producing *E. coli* (STEC) strains are considered to be the most important pathogens between recently emerged groups of food borne strains. STEC are linked to the production of Shiga toxins and are responsible for non-bloody diarrhea, diarrhea-associated hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura.

E. coli O157:H7 produce verotoxins (VT) which affect Vero cells, hence they are known as vero-cytotoxigenic *E. Coli* (VTEC) to reflect their biological activity. VTEC are also referred to as Shiga-toxin (ST) producing *E. coli* (STEC) or shiga-like toxin (SLT) producing *E. coli* (SLTEC). STEC strains produce two potent phage-encoded cytotoxins called Shiga toxins (Stx1 and Stx2) or verotoxins (VT1 and VT2). In addition to toxin production, another virulence-associated factor expressed by STEC is a protein called

intimin. This protein is responsible for intimate attachment of STEC to the intestinal epithelial cells, causing attaching and effacing lesions in the intestinal mucosa.

Enterohemorrhagic *E.coli*

Escherichia coli is considered an environmental pathogen and one of the most significant causes of bovine clinical mastitis, which is mostly seen in the early lactation period and in high-producing cows with low somatic cell counts. *E. coli* is a usual inhabitant of the gastrointestinal tract of animals and humans and some particular strains of *E. coli* cause intestinal or a variety of extra-intestinal infections. Enterohemorrhagic *E.coli* (EHEC) strains constitute a subtype of STEC serotypes that have been associated with bloody diarrhea and HUS.EHEC are generally more pathogenic than other STEC because they possess a fuller complement of virulence determinants that encode production of intimin and enterohemolysin [3]. Serotypes O26, O111, O103 and especially O157, are the predominantly isolated EHEC serotypes.

Streptococcus agalactiae

Str. agalactiae is a major cause of elevated somatic cell count (SCC). As SCC increases because of mastitis, milk quality decreases due to the drop in lactose and casein. Milk yield of infected quarter may drop by 40% without showing any apparent clinical signs of mastitis. Higher the SCC, the greater is the risk of raw milk contamination with pathogens and antibiotic residues. There is also reduced suitability of the raw milk for manufacturing and processing.

Str. agalactiae causes mastitis in dairy cattle. When infected, the vast majority of cows show no signs of clinical mastitis, though some will show intermittent mild clinical signs of mastitis including clots and/ or flakes in the milk and occasional mild swelling of the affected quarters. Infected quarters can run extremely high bacteria counts, as high as 500,000,000 CFU/ml of milk. As such, these infected quarters can produce enough bacteria to cause illegal elevations in the SPC. In humans, *Str. agalactiae* has been described as one of the most common factors of invasive infections in neonates, but it also

causes invasive and non-invasive infections in adults [4]. These infections have major consequences for public health as they may cause neurological problems in newborn humans and endometritis and sterility in the mothers. Humans also act as a significant reservoir of *Str. agalactiae,* since these bacteria may be carried in the vaginas of women without apparent clinical signs.

Str. agalactiae survives a very short time in the environment, but it can persist indefinitely within the mammary gland. Infected heifers and cows are the reservoir of *Str. agalactiae.* The number of herds infected by *Str. agalactiae* has been reduced by modern mastitis control programs. *Str. agalactiae* can be eradicated from dairy farms; however it remains a biosecurity threat for dairies that purchase cattle. Cows' with *Str. agalactiae* mastitis usually have elevated somatic cell counts but normal milk. Occasionally the cow may progress from subclinical to clinical mastitis. During episodes of clinical mastitis the signs are usually limited to abnormal milk and udder swelling. Cows affected by *S. agalactiae* infections can shed very high levels of the bacteria into the bulk tank and cause elevated plate counts.

Campylobacter jejuni

C. jejuniis the most frequently identified cause of acute infectious diarrhea in developed countries and its prevalence in bulk tank milk has been reported to range from < 1% to > 13%. *C. jejunican* cause mastitis in cows and it can be shed in milk of asymptomatic carrier. Humans get infected through ingestion of contaminated, non- pasteurized and improperly pasteurized milk. Direct milk excretion of *C. jejuni/coli* by clinically healthy cows has been reported & implicated in the etiology of human enteritis [5]. *Campylobacter* spp. causes sporadic cases of chronic gastritis, enterocolitis and septicemia. Milk borne infections by *Campylobacter* spp can also result in Campylobacter-associated Guillian-Barré Syndrome.

Listeria monocytogenes

Listeria monocytogenes is a pathogenic bacterium that can cause listeriosis

in human and various animal species. In human, foodborne *L. monocytogenes* causes large outbreaks of listeriosis, with a mortality rate of 9 % to 44 % [6]. *L. monocytogenes* can cause mastitis in cows and it can be shed in milk of carrier asymptomatic cows. Pasteurized milk is known to be one of the vehicles through which *L. monocytogenes* can be transferred to humans [7]. It causes septicemia and meningitis in humans. Pregnant women are particularly susceptible to *L. monocytogenes* infection and it may result in spontaneous abortions or stillbirth of the fetus. So many countries have initiated a zero tolerance policy prohibiting the sale of processed ready to eat food contaminated with *L. monocytogenes.* This bacterium can grow in refrigerated raw milk and can also survive at the minimum HTST pasteurization temperature. Humans get infected through the consumption of raw milk or products manufactured with raw milk or through ingestion of processed food, cross-contaminated with pathogens present in the food processing plant environment. The disease manifestations include septicaemia, meningitis (or meningoencephalitis) and encephalitis, usually preceded by influenza-like symptoms including fever. In pregnant women, intrauterine or cervical infections may result in spontaneous abortion or stillbirths.

Mycobacterium spp

Tuberculous mastitis in dairy cows and buffaloes is commonly due to *Mycobacterium bovis,* but *Mycobacterium tuberculosis* is also involved. Some saprophytic acid-fast bacilli, apparently of soil origin, such as *Mycobacterium lacticola* and *Mycobaterium fortuitum* and related organisms have also been reported to cause chronic destructive mastitis resembling tuberculosis of the udder. Tuberculous mastitis has public heath significance. *Mycobacterium bovis* is the causative agent of tuberculosis in a range of animal species and man, which causes great economic loss. *M. bovis* is the agent responsible for bovine tuberculosis, however it can also cause the disease in humans if there is consumption of infected materials. Pasteurization of milk has been a major preventative factor in stopping transmision of bovine tuberculosis in humans; however in many under developed countries, where pasteurization is not practiced, there is still a concern with infection by *M. bovis*.

Salmonella spp

Human salmonellosis occurs through the consumption of raw milk or dairy products manufactured with raw milk. *Salmonella enterica* serotype *typhimurium* definitive type 104 (DT 104) is of particular concern to animal and public health agencies because of its multiple antibiotic resistance. Since 1990, epidemic spread of multi-resistant *Salmonella typhimurium* definitive type 104 has occurred with resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline. Symptoms in humans consist of diarrhea, fever, headache, nausea, abdominal pain, vomiting, and, less frequently, blood in the stool.

Salmonellosis is the most common food-borne bacterial disease worldwide. There have been increased outbreaks of human salmonellosis in most parts of the world resulting from animal infections. The prevention and control of animal salmonellosis has become a global issue, as this has been established as the main source of outbreaks in humans. Raw milk and milk products are increasingly becoming important sources of human infection with *Salmonella.* The microbial contamination of milk is multi-factorial originating from sources like the air, feed, soil, faeces, grasses and the milking cow itself.

Coxiella burnetii

C. burnetii is the cause of Q fever, which is recognized as an occupational disease of workers in abattoirs in Australia and as a tick- transmitted disease in the United States. Milk is the most significant source of these organisms among the products of animal origin. Personnel in dairies and their families with the greatest use of raw milk can get infected with Q fever. This opinion on foodborne transmission was based on a survey in the United States where 10·7% of people consuming raw milk had a positive serological test, compared to 0-7% among non-exposed people [8].

While *C. burnetii* is shed for extended periods in the milk of dairy cattle, and has been revealed to be immunogenic in dairy cattle, probable links with clinical or subclinical mastitis have only rarely been examined. In reviews of mastitis etiologies, most authors identify the potential public health implication of *C. burnetii* excretion in milk without describing the organism as a cause of

mastitis. Some reviews have included *C. burnetii* among "the lesser known organisms that may cause mastitis" [9].

Pasteurization and its Effect on Milk Quality

The presence of different bacteria in the milk renders it unsuitable for human consumption and helps in the spread of disease like tuberculosis, brucellosis, Staphylococcal toxemia, Streptococcal sore throat, scarlet fever, gastroenteritis, etc. With severe clinical mastitis, abnormalities of milk are easily observed. Such milk normally would not enter the food chain. But fraudulent practices can pose a severe threat by mixing mastitic milk with normal milk. Consumption of faulty pasteurized milk, or consumption of dairy products adulterated with contaminated raw milk has been linked directly to the cases of human foodborne disease [10,11,12].

Unlike sterilization, pasteurization is not intended to kill all micro-organisms (pathogenic) in the food. Pasteurization aims to achieve a five-log reduction, killing 90% of the number of micro-organisms in milk, so unlikely to cause disease (assuming the pasteurized product is refrigerated and consumed before its expiration date). Pasteurization kill certain (but not all) bacteria and disable certain enzymes & in return minimize the effects on taste. It destroys almost all yeast, molds and spoilage bacteria and common pathogenic, heat-resistant organisms (including *M. tuberculosis),* but not *Coxiella burnettii*

Various outbreaks of disease in humans have been traced to the consumption of unpasteurized milk. Unpasteurized milk is consumed directly by dairy producers, farm employees, and their families and neighbors. It is consumed directly by a large segment of the population via consumption of several types of cheeses manufactured from unpasteurized milk. Inadequate/faulty pasteurization will not destroy all pathogens and pathogens like *Listeria monocytogenes* can survive and thrive in post-pasteurization processing environments, leading to recontamination of dairy products. Entry of pathogens *via.,* contaminated raw milk into dairy food processing plants can lead to persistence of these pathogens in biofilms, and subsequent contamination of processed milk products and exposure of consumers to pathogenic bacteria. These pathways pose a risk to the consumer from direct exposure to foodborne pathogens present in unpasteurized dairy products as well as dairy products that become re-contaminated after pasteurization [13].

Effect of Mastitis on Milk Composition

Mastitis decreases the milk production by causing tissue damage, reduced lactose production and scar tissue formation in the udder. Effect of mastitis on milk quality and composition are:

- Increase in somatic cell count
- Decrease in lactose, casein and fat production
- Increase in blood components such as Na, K, Cl, bicarbonate, IgG and serum albumin
- Proteolysis
- Lipolysis and globule breakdown
- Off flavors

Table 7.1: Relation between California Mastitis Test (CMT) and Somatic Cell Count in milk of individual cows

CMT Score	Average somatic cell count in milk
Negative (No mastitis)	1,00,000
Trace (Of mastitis)	3,00,000
Class 1 (Light mastitis)	9,00,000
Class 2 (Severe mastitis)	2,700,000
Class 3 (Very severe mastitis)	8,100,000

Impact of Somatic Cell Count on Quality of Milk

Milk with a high somatic cell concentration can be harmful to human health and contains less casein and yields lower cheese. It contains an increased amount of enzymes, which have effect on the quality of the protein and the fat in milk. The presence of these enzymes in milk increases the potential for off-flavours and odours. Thus, the impact of somatic cell count of raw milk is important for the shelf-life, flavour and the yields (particularly of cheese).

Table 7.2: Effect of Somatic cell on Milk Composition

Measurements	Normal	High cell count	% of normal
Total solids	13.1	12.0	92
Lactose	4.7	4.0	85
Fat	4.2	3.7	88
Chlorides	0.091	0.147	161
Total protein	3.6	3.6	100
Caseins	2.8	2.3	82
Whey proteins	0.8	1.3	162

Antibiotic Residues & Emergence of Antibiotic Resistance

Antibiotics ranging from narrow to broad spectrum have been used extensively over the past 40 years in the control of bovine mastitis. However, because of the emerging antibiotic resistance believed to be probably due to their overuse and the induction of prolonged persistent antibiotic resistance in biofilms by many mastitis-causing pathogens, as demonstrated recently for *S aureus* isolated from cases of bovine mastitis, effectiveness of antibiotic therapy has been compromised. There are four major use patterns of antimicrobial agents in livestock, as described in table 7.3. It is important to note that these four patterns have been in use for many decades [14,15,16]. Antimicrobials are commonly used in food producing animals for treatment, prophylaxis and growth promotion. However, such use can also lead to the development of drug-resistant bacteria, which may be transmitted to humans through the food supply.

Antibiotic resistance development is another human health concern. Factors such as pharmacokinetic problems, and phagocytosis depressing effect of certain antibiotics and appearance of residue in milk restricts the success of antibiotic therapy. Use of antibiotics in mastitis may be partly responsible for emergence of antibiotic resistant bacteria, which in turn may decrease effectiveness of similar antibiotics used in human medicine and transfer this

resistance to other pathogens capable of causing human illness, thereby decreasing therapeutic effectiveness of antibiotics viz., *Salmonella typhimurium* DT 104 and methicillin-resistant S. *aureus,* vancomycin-resistant *S. aureus*.

Antibiotic resistance can spread through direct and indirect path. By direct path, a resistant pathogen becomes established in dairy cows, either because competing non-resistant bacteria in the cow are decimated by repeated antibiotic use or because R-factors are passed from non-pathogenic resistant bacteria in the cow. The pathogen could then be present in milk, which could produce human exposure to infection that was resistant to antibiotics. While in indirect path, non- pathogenic bacteria with an R factor which are present in milk transfer the R-factor-containing plasmid from non-pathogenic bacteria to pathogenic ones in the human gut, creating the potential for antibiotic resistance which is difficult to treat.

Table 7.3: Major use patterns of antimicrobial agents in livestock

Use pattern	**Definition**
Antimicrobial growth promotion	Administration of an antimicrobial, usually as a feed additive, over a period of time to growing animals that results in improved physiological performance (i.e. weight gain, feed conversion)
Prophylaxis	Administration of an antimicrobial to exposed healthy animals considered to be at risk, but prior to onset of disease and for which no etiologic agent has yet been confirmed by culture or other detection method(s)
Metaphylaxis	The mass treatment of animal populations currently experiencing any level of disease before the onset of

	blatant
	illness
Treatment	Administration of an antimicrobial to an animal, or group of animals, which exhibits frank clinical disease

Table 7.4: Categorization of antimicrobials used in veterinary medicine according to their importance in treatment of disease

Veterinary critically important antimicrobials	**Veterinary highly important antimicrobials**	**Veterinary important antimicrobials**
Aminoglycosides	Rifamycins	Bicyclomycin
Cephalosporins	Fosfomycin	Fusidic Acid
Macrolides	Ionophores	Novobiocin
Penicillins	Lincosamides	Orthosomycins
Phenicols	Pleuromutilins	Quinoxalines
Quinolones	Polypeptides	Streptogramins
Sulfonamides		

Table 7.5: Antimicrobial drugs and drug classes approved for use in food-producing animals

Aminocoumarins	**Macrolides**
Novobiocin	Carbomycin
Aminoglycosides	Erythromycin
Apramycin	Oleandomycin
Dihydostreptomycin	Tilmicosin
Efrotomycin	Tulathromycin
Gentamicin	Tylosin

Hygromycin B

Neomycin

Spectinomycin

Streptomycin

Amphenicols

Florfenicol

Cephalosporins

Ceftiofur

Cephapirin

Diaminopyrimidines

Ormetoprim

Fluoroquinolones

Danofloxacin

Enrofloxacin

Glycolipids

Bambermycin

Ionophores

Laidlomycin

Lasalocid

Monensin

Narasin

Salinomycin

Semduramicin

Penicillins

Amoxicillin

Ampicillin

Cloxacillin

Hetacillin

Penicillin

Pleuromutilins

Tiamulin

Polypeptides

Bacitracin

Polymixin B

Quinoxalines

Carbadox

Streptogramins

Virginiamycin

Sulfas

Sulfachlorpyridazine

Sulfadimethoxine

Sulfamerazine

Sulfamethazine

Sulfaquinoxaline

Sulfathiazole

Tetracyclines

Lincosamides	Chlortetracycline
Lincomycin	Oxytetracycline
Pirlimycin	

* 2009 SUMMARY REPORT on *Antimicrobials Sold or Distributed for Use in Food Producing Animals,* Food and Drug Administration Department of Health and Human Services.

Out of 40 animal drugs approved 11 approved drugs to treat mastitis and respiratory infections are as follows:

- Penicillin, Ampicillin, Cephapirin, Hetacillin, Amoxicillin
- Ceftiofur
- Oxytetracycline, Chlortetracycline
- Novobiocin
- Erythromycin
- Sulfadimethoxine

Antibiotic Residues

Improper use of drugs for the control of mastitis is the major source of residues found in the milk supply. Antibiotic residues in foods of animal origin are one of the sources of concern among the public and medical health professionals. There are various reasons for the occurrence of antibiotic residues in milk like poor record management, extra label usage, multiple dosing, not following the recommended withdrawal times, etc. Antimicrobials used in veterinary medicine have been categorised according to their importance in treatment of disease is described in the table 7.4.

Drug resistance, allergic reactions, photosensitization, aplastic anemia, effects on bones and teeth, anaphylactic shock, etc. are some of the public health hazards associated with antibiotic residues. Antibiotic residues in milk can lead to severe reactions in people allergic to antibiotics and can cause sensitization of normal individuals and development of antibiotic-resistant strains of bacteria.

Antibiotics Residues may have Side Effects Like

- Soft stools or diarrhea and mild stomach upset

- Vomiting
- Severe watery diarrhea and abdominal cramps
- Vaginal itching or discharge
- White patches on the tongue, etc.

Allergic Reactions Commonly have the Following Symptoms

- Rashes and hives
- Itching and swelling of the lips, face or tongue
- Shortness of breath, fainting, etc.

With severe clinical mastitis, abnormalities of milk are easily observed and milk is discarded by the producer. Such milk normally would not enter the food chain. But when milk of cows with sub- clinical mastitis, *i.e.* with no visible changes, is accidentally mixed into bulk milk, it enters food chain and can be dangerous to humans. Although pasteurization is likely to destroy all human pathogens, there is concern when raw milk is consumed or when pasteurization is incomplete or faulty. Milk and other dairy products are frequently infected with *S. aureus*.

Management of Mastitis

- Maintain good hygiene during milking.
- Cut off any long hair on the udder. Wipe the teats with dry disposable paper.
- Wash dirty teats under running water and dry them well with disposable paper.
- Use a test beaker at every milking to see whether the milk contains mucus, pus or blood.
- Teat liners of milking machines must be washed and disinfected after every milking.
- Dip teats in a suitable teat remedy after every milking.
- Hands must be clean when teat ointment is applied or while milking by hand.
- The people milking the cows must not have any sores on their hands.

Management of Antibiotic Residues and Antibiotic Resistance

- Following label directions
- Extra-label usage of antibiotics to be done only under veterinary guidance
- Identification of treated animals
- Establishment of herd health management program
- Establishment of effective record keeping
- Discarding milk from all 4 quarters of animals under treatment
- Education of farm personnel
- Paying attention to withdrawal times
- Mastitis treatment should be monitored
- Development of vaccines and phage therapy.

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Chapteer 8
Economic Implication of Bovine Mastitis-? Conceptual Framework

With the increasing intensification and commercialization of livestock and poultry industries, the economic implications of livestock diseases are becoming more important both at farm and national levels [1], as animal diseases symbolize avoidable waste of scarce resources. From an economic point of view, a disease destroys the basic resources, reduces the attainable output and/or dwindles the resource use efficiency [2]. As a result, a disease is considered to be a "negative resource" in animal production.

In response to the increasing concern over the enormous economic losses associated with the animal diseases, estimation of diseases losses and assessment of the relationship between the disease and the animal environment were considered to be important so as to draw attention to the adverse effects of the diseases on livestock farm and to determine livestock research priorities. In recognition of this, there arose a need for an interaction of the disciplines such as veterinary epidemiology and livestock economics, which could help veterinarians on the economic importance of animal diseases. This interaction gave birth to a new discipline viz. Animal Health Economics to assess the economic implications of animal diseases.

Animal health economics is described as the discipline that aims to provide a framework of concepts, procedures and data to support the decision making process in optimising animal health management [3] and in turn farm management. In fact, the scientific foundation for this new discipline was laid by Morris [4] in Australia about four decades back by introducing economic principles for making veterinary decisions. The Netherlands is one of the first few countries to adopt a newer approach in this field. Since then, lots of

conceptual refinements were made and now the discipline has come to remain as a multidisciplinary field encompassing the fields of animal science, epidemiology, statistics and economics. Though there have been a number of studies undertaken in this field in the UK (University of Reading), the Netherlands (Wageningan Agricultural University), the New Zealand (Massey University), Australia and many developed countries, this field is yet to be amply exploited in developing countries like India.

Setting

Diseases associated with dairy production cause large economic effects. In fact, the most expensive disease on dairy farms is mastitis, because the economic damage is spread out over the year a longer period. Mastitis is of considerable interest because of its high incidence and the extensive costs associated with the disease. It results in severe economic losses from reduced milk production, treatment cost, increased labor, milk withheld following treatment and premature culling [5,6].

Mastitis has been known to cause a great deal of loss or reduction of productivity by influencing the quality and quantity of milk yield, and culling of animals at an unacceptable age [7]. It has been considered to be detrimental to a dairy farm's profitability, not only in terms of production loss and treatment costs, but also because of the loss of the cows themselves [8,9,10,11,12]. Most estimates had shown a considerable reduction in productivity per affected quarter and a further reduction in production per cow/lactation, making the disease one of the most costly and serious problems affecting the dairy industry [13,14].

Economic Framework of Mastitis

The following figure shows the economic implications of mastitis on dairy production. The effect of mastitis in a given dairy production system is to reduce the efficiency with which inputs are converted into outputs, i.e. they decrease productivity of dairy animals. The effects of mastitis can be classified as direct or indirect.

Direct Effects Include

1. Destruction of the basic resource of the dairy production process through mortality of productive stock;
2. Lowering the efficiency of the dairy production process and the productivity of resources employed (for e.g. reduced feed conversion); and
3. Reduction in the quantity and or the quality of milk produced.

Indirect Effects Include

4. Additional costs incurred to avoid or reduce the incidence of mastitis (e.g. cleaning and sanitation, quarantine) or to treat sick cases, etc.; and
5. Posing a threat to human health through spreading infections and passing antibiotics.

Measuring the Economic Impact of Mastitis

Measuring the economic impact of any animal disease, including mastitis, is not a simple task, because the effects of disease [15]:

- Are not always obvious and well pronounced;
- Are influenced by other factors such as management and environment;
- Have a temporal dimension which adds to the complexity of evaluating their impacts at different stages of time; and
- Often manifest themselves in a complex condition with other diseases.

Estimation of losses due to mastitis in dairy herd is not just a problem of measurement by itself; it is a problem of disease making. Conversely, quantification of economic losses due to mastitis is not very important in itself, but it can help to provide a better overall view of the impact of disease and can contribute in estimating the extent of the losses to be avoided. The data on losses are required as information to guide decisions about allocating resources to mastitis control. Quantification of losses is not only important for describing the actual situation but also to answer two inter-related questions:

1. How to limit the loss by veterinary intervention?
2. What efforts are required to avoid the losses and at what costs?

The defects introduced by the presence of mastitis to dairy production

system are manifested by the extra resource uses (expenditures) incurred in offsetting the effects of the disease and the output losses represented by lowered production [16].

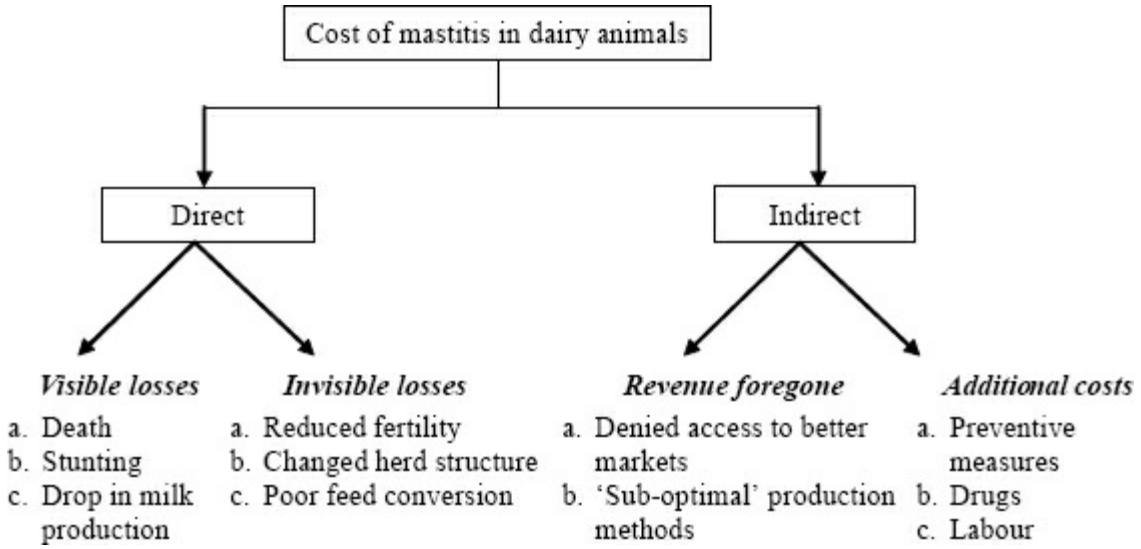

Fig. 8.1: Direct and indirect losses due to mastitis in dairy animals

Estimating the Cost of Mastitis

Quantification of losses due to mastitis in a dairy herd usually follows the disease investigation/diagnosis work undertaken. Once the prevalence or incidence and the nature and magnitude of the losses experienced have been defined, the economic portion of the analysis proceeds to:

- Classify and present the information on mastitis losses;
- Quantify losses, due to mastitis, in monetary terms;
- Identify and quantify the indirect losses attributable to mastitis.

The majority of the effects are most conveniently calculated in terms of lowered output. In some cases, the loss may be more easily evaluated in terms of wasted inputs. A more sophisticated estimate would include the time value of the delay in reaching the peak yield calculated by discounting to obtain the present value of the costs and receipts involved. Losses in the final output can be evaluated on a lactation basis and then adjusted for changes in animal numbers. Quantifying indirect effects of mastitis can be complex, but it is

possible.

a) Losses as Constraint to Milk Production

Besides causing *direct* losses, mastitis can act as constraint on production by determining the producer's efforts to avoid as far as possible the risks of mastitis in his animals. If a mastitis prevention strategy removes the constraint, the benefits resulting from such changes are called *indirect benefits.* The losses thus avoided are called *indirect losses.* Quantifying such effects principally involves the estimation of *changes* in the income of the producers involved, which would arise if mastitis threat were removed and the producers were able to improve existing systems of production or adopt new ones. These *income changes* can then be related to the effects of the mastitis control policy.

b) *Other losses*

* **Trade effects:** Mastitis in larger herds will have a major effect on the availability of export markets to a country. An estimate of costs can be made by assuming that after an initial loss of exports, an alternative market offering lower prices can be found.

c) *Secondary Effects, Externalities and Intangible Effects*

Secondary effects are effects arising upstream (e.g. in the feed industry) or downstream (e.g. in processing and marketing) of the affected production process, as the dependent industries also suffer. These effects are seldom evaluated. Intangible of disease are effects that exist but are very difficult to quantify. An example is the effect of a disease risk to people and animals on the quality of human life. Some aspects of this could perhaps be quantified, but generally it is acceptable to state that such effects exist and that they should be taken into consideration. This approach may also be the most practical way of dealing with some externalities.

Costs of Mastitis Control

The costs of mastitis control will obviously vary not only with the source of infection and the type of control policy adopted, but also with the domain in which the programme is being implemented. The reasons for this are easy to identify: different institutional frameworks, different salaries of those

involved, different terrain and different production systems leading to very different transport costs. Nevertheless, it is possible to make some generalizations about the types of cost incurred and the components of these costs.

Components of Mastitis Control Costs

As in any costing exercise, in costing mastitis control measures that is adopted on a large scale, it is essential to distinguish clearly between variable and fixed costs.

Variable Costs Include the Cost of:

- Drugs for treatments, vaccinations, sanitizers, etc.;
- Syringes, needles and other small equipment; and
- Staff travel and subsistence allowances.

Fixed costs or overheads in disease control include:

- Vehicle expenses;
- Permanent staff salaries;
- Office and administration;
- Depreciation on vehicles, equipment and buildings; and
- Office rents, rates, water and electricity, etc.

Medicinal Measures and the Minimization of Mastitis Incidence

The direct actions taken against mastitis may include:

- Identification of a source of infection through diagnosis and surveys.
- Treatment of the disease, which usually entails diagnosis, treatment and follow-up. Treatments continue to be necessary for as long as the disease remains in the animal.
- Prophylaxis or vaccination, which is repeated at specified intervals in the population to be protected, determined as a result of an epidemiological.
- Vector (indirect agent) control, which may be repeated at determined intervals.
- Use of mastitis-resistant animals (probably more of indigenous blood), a

form of disease control policy requiring experimentation, surveys and follow-up.

Eradication normally involves an intensification of one or more of the methods outlined above. It always involves intensive surveillance and investigative work. The initial costs of eradication are high but should be substantially reduced once the objective has been achieved. In examining and comparing different mastitis control policies, two aspects should be emphasized:

- The overall level of costs and their relation to the funds available.
- The *timing* of expenditures over the years. Treatments and prophylaxis typically involve costs over a number of years, while eradication demands a much higher level of expenditure but for a much shorter period. In all cases the present values of the costs, i.e. the sum of the discounted costs, need to be compared, not the simple sum of costs.

Non-medicinal Prevention

This covers preventive care within the daily routine of an animal production system. The cost is the producer's time spent observing the animals, ensuring that they have a clean environment etc. Non- medicinal prevention can include attempts to contain mastitis by maintaining livestock environments, clean milking, etc.

Assessing Epidemiologic Measures of Association

Epidemiologic Measures of Association

Statistical significance is a function of the magnitude of difference, the variability of the difference and the sample size. Once the decision is made the sampling variation (chiefly a function of sample size) is not a probable explanation for the difference observed, epidemiologic measures of association can be applied which are independent of sample size and include the strength of association, the effect of factor in exposed individuals and the importance of the factor in the population. Formulas for these measures are as

below [17]:

a) Relative Risk (RR)

The strength of association between a factor and mastitis is known as relative risk (RR).

$$RR = \frac{\text{Rate of disease in the exposed group}}{\text{Rate of disease in the unexposed group}}$$

$$= \frac{\frac{a}{a+b}}{\frac{c}{c+d}}$$

Where,

a - number affected in the exposed group;

b - number unaffected in the exposed group;

c - number affected in the unexposed group; and

d - number unaffected in the unexposed group

b) Population Relative Risk (RR_{pop})

Population relative risk indicates the relative impact of the factor in the population.

$$RR_{pop} = \frac{\text{Rate of disease in the population}}{\text{Rate of disease in the unexposed group}}$$

$$= \frac{\frac{a+c}{n}}{\frac{c}{c+d}}$$

Where, 'n' is the population size.

c) Odds Ratio (OR)

Odds ratio is useful when mastitis is relatively infrequent and is approximately equivalent RR in such occasions.

$$OR = \frac{ab}{bc}$$

d) Population Odds Ratio (OR_{pop})

Population odds ratio, as RR_{pop}/ indicates the relative impact of the factor in the population.

$$OR_{pop} = \frac{d(a+c)}{c(b+d)}$$

e) Attributable Rate (AR)

Attributable rate is the rate of mastitis in the exposed group minus the rate in the unexposed group.

$$AR = \left[\frac{a}{(a+b)}\right] - \left[\frac{c}{(c+d)}\right]$$

f) Attributable Fraction (AF)

Attributable fraction is used to know what proportion of mastitis in the exposed group is due to the factor.

$$AF = \frac{(RR-1)}{RR}$$

g) Population Attributable Rate (PAR)

Population attributable rate gives the importance of a casual factor in the population and is determined by multiplying its effect (AR) by the prevalence of the factor.

$$PAR = \frac{a+b}{n} \times AR$$

h) Population attributable fraction (PAF) or etiologic fraction

Population attributable fraction is the proportion of mastitis in the population that is attributable to the factor.

$PAF = (RR_{pop}\text{-}1)RR_{pop}$

E.g., Let us have an example of assessing the influence of breed factor in mastitis:

Measures of Association	Breed
Strength of the factor	
Relative risk (RR)	2.969
Effect of the factor	
Attributable rate (AR)	0.106
Attributable fraction (AF)	0.663

Relative risk (RR) with respect to breed factor for the disease - mastitis was found to be 2.969. If there was no association between the factor and the disease, the RR will be 1. The greater the departure of RR from (either larger or smaller), stronger is the association between the factor and the disease. Hence, since the RR was 2.969 here, it can be concluded that the rate of mastitis in exotic purebred/crossbred cows and Murrah/ graded buffaloes was 2.969 times greater than the rate of mastitis in native pure / non-descript cows and buffaloes.

The larger the attributable rate (AR) is, the greater the effect of the factor, *i.e.,* exotic purebred/crossbred/Murrah/graded group, in causing mastitis in dairy animals. The calculated AR for the breed factor was 0.106, which implied that the rate of mastitis in dairy animals that might be attributed to exotic / crossbred / upgraded germplasm was 10.60 per cent. The attributable fraction (AF) calculated of 0.663 indicates that 66.30 per cent of mastitis in superior / upgraded breeds could be attributable to the exotic / crossbred / graded breed factor.

Quantitative Techniques in Assessing Losses Due to Mastitis

The models used in evaluation of economic impact of any animal disease, particularly mastitis, on production can be grouped under two headings namely statistical / epidemiological models and economic models [15]. Statistical /

epidemiological models are used to identify the factors that contribute to the development of mastitis, the magnitude and direction of the contribution, and relationships between other diseases. Common models in this category include Regression analysis, Path analysis, Discriminant analysis and Analysis of variance. On identifying, disaggregating and quantifying the causes of mastitis, the next step is to attach monetary value to the quantified impacts. Economic models used for this purpose are equi-marginal principle, partial budgeting, cost-benefit analysis, decision analysis, linear programming, Markov chains, systems simulation and dynamic programming [15]. Economic quantitative techniques like mathematical programming, network analysis, decision analysis, simulation and cost benefit analysis helped the decision makers to choose appropriate control strategies and the value of information from these quantitative techniques would depend upon skill and judgment of analysts [18].

a) Choice and Opportunity Costs

In the traditional area of the economics of mastitis loss and control, many methods and techniques have been used for analysis. The idea of choice is central to any economic analysis. The basic premise in analysis is to compare a single control strategy with the consequences of doing nothing.

In allocating resources to one activity (say, activity 'A'), rather than to other activities (ranging from 'B' to 'Z'), a cost is incurred. Economists call this the opportunity cost of a decision, i.e. the benefit that one foregoes by not selecting the best alternative course of action among the choices available (B). The benefit obtained from performing 'A' can then be compared with the associated economic cost of carrying out 'B', i.e. the opportunity cost. As long as this benefit exceeds the cost of 'B', project 'A' is worth undertaking. Using this methodology, one can rank different activities on the basis of net benefit value, in descending order of preference. Although the opportunity cost is real, it is convenient, when analysing the costs of economic activities, to value such activities in common monetary accounting units [19].

b) *The Marginal Principle*

When shifting resources from one activity to another, one should examine what happens at the margin by comparing the extra benefit that will result from this shift analyses, comparing the situation without the project to the situation with the project (the 'with and without project methodology') is preferable to comparing the situation before the project with the situation after the project

(the 'before and after methodology'). By using the 'with and without project methodology', one can correctly attribute the net benefit to the control strategy in question [19,20]. This 'with and without' methodology is closer to experimental methodology than the 'before and after' methodology. The basic structure of the analysis compares (marginal) changes in costs and benefits which result from undertaking the project. This is called the partial analysis approach [20].

c) *Decision Analysis*

Decision analysis techniques are helpful when making choices under conditions of uncertainty, especially with complex but poorly structured economic problems. Risk analysis is incorporated in the technique. Data on risk probabilities (empirical or imputed) are essential. The technique can be used at the producer level or at higher levels of aggregation.

d) *Simulation*

A number of mathematical and 'simulation' models have been developed in an attempt to try to isolate the effect of mastitis on livestock and the livestock industry. Useful models are those that treat mastitis as an economic phenomenon, and identify all the ways in which the disease impinges on economic values. Such models start from the animal herd, develop links that encompass the entire livestock sector and the wider economic system. This entails linking epidemiological models and adding data to the economic models. The economic model then indicates what type of data and what level of aggregation are required. The data, in turn, are obtained via socio¬economic surveys and epidemiological investigations. Hence, economic analysis demands the integration of both epidemiology and economics [21].

Simulation attempts to mimic real-life occurrences of variables (states and quantities) over time. The link between the epidemiological model/input and the economic model is demonstrated in Fig. 2. Epidemiological simulation can take one of two forms. Either individual animal within a herd are 'observed' over time, their states changing stochastically or a herd of animals is moved through time, with these animals changing states deterministically using fixed transition coefficients.

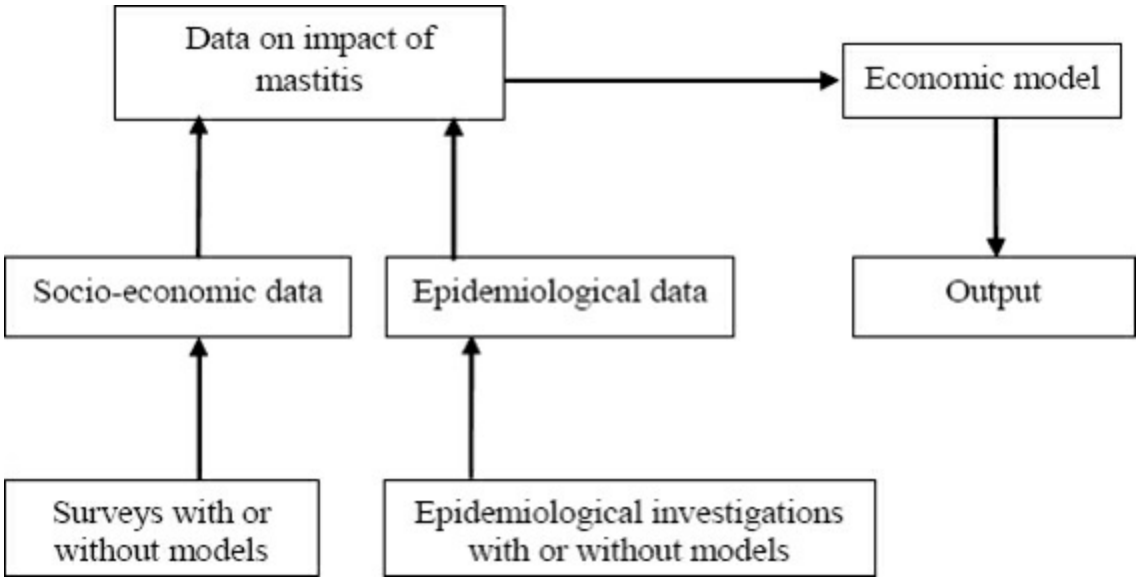

Fig. 8.2: Linking epidemiological models and data to economic models in economics of mastitis ***[22]***

e) *Optimising Mathematical Models*

Optimising mathematical models makes use of linear programming and variants which, although purely mathematical procedures, are useful aids in resource allocation work, where constraints abound [15,18,23],

f) *Budgeting Techniques and Cost Analysis*

Budgets are a planning tool in economics of mastitis, especially when alternative control strategies are evolved. Partial budgeting technique takes into account the changes in the dairy farm that result from the implementation of the new measures and does not consider the total budget of the farm. Partial budgeting is considered with four basic items i.e. New Revenue (R), cost Saved (S), revenue Forgone (F) and New cost (N).

The 'R' and 'S' elements take into account all additional revenue and reductions of costs that are a consequence of the control strategy or programme and on the other hand, element 'F' consider the revenue that will no longer be received and 'N', the costs that are related to the implementation of the control programme. Therefore, the sum of 'F + N' is considered to be an estimation of the negative effect that the programme will have. The difference between the positive and negative effects equals the net effect of the changes made to control the mastitis in the herd. For each of the four items, a group of elements were selected and every one of them received a monetary value so that the

necessary calculations could be done.

The description of each element from the four items is as follows:

- **New Revenue (R)** or **Additional Returns** (returns that will not be received unless the change is undertaken) includes the additional milk yield during and after control measures followed in the absence of mastitis e.g. cost of milk yield loss during the period of mastitis and afterwards.
- **Cost Saved (S)** or **Reduced Costs** (costs present in the initial situation that will be avoided if the change is made) include avoidable total economic losses e.g. reduced milk receipt, treatment cost and premature disposal cost.
- **Revenue Forgone (F)** or **Returns foregone** (returns received in the initial situation that will not be received if the change is made) include cost of sale for culling animals.
- **New cost (N)** or **Extra costs** (costs associated with the change that are not present in the initial situation) include cost of control measures e.g. udder washing, animal washing, animal shed washing, inter cow hand washing and post milking teat dipping.

Net benefit = (R+S) - (F+N)

If the sum of new revenues and reduced costs is greater than that of revenues foregone and new costs, the change can be economically justified.

g) Cost-benefit Analysis

In a cost-benefit model, economic effects for any given scenario are calculated as total revenues weighed against total costs. In this model, the expected cost or benefit of a specific plan of action calculated. An expected cost or benefit was associated with each of these outcomes. For example, correctly identifying a true positive result and acting on it would have certain benefits, such as a reduction in the infection rate and a reduction in the decline of milk yield and milk quality. It also has costs associated, such as the cost of treatment and the cost of additional labour. Similarly, incorrectly identifying a true negative result as positive would have costs associated, such as an increase in the loss of milk, an increase in antibiotic residues present in milk and in the bulk tank due to unnecessary treatment, and the cost of treatment. Partial-budget analysis is used to calculate the relative costs and benefits. In

partial-budget analysis, alternatives are compared without calculating a complete budget for each scenario. Instead, only the revenues and costs that are affected by the specific scenario are considered. Total returns were calculated as extra returns plus reduced costs. Total costs were calculated as reduced returns plus extra costs. Both direct and indirect effects of plan were taken into account.

h) *Functional Analysis*

i) Multiple Regression Analysis

A linear regression function of the following form can be fitted to study the factors influencing the economic losses incurred in lactation due to mastitis.

$EL_l = a + ß_1X_1 + ß_2X_2 + β_3X_3 + ß_4X_4 + μ$

Where,

El_l - Economic losses due to mastitis per lactation in rupees;

$x1$ - Average daily milk yield in litres in the preceding week before infection;

$x2$ - Number of infected quarters;

X - Number of days of treatment;

$x4$ - Reduction in lactation length in days (calculated by subtracting current lactation length from average / previous lactation length);

a, ßj - Coefficients to be estimated; and

u - Error term

ii) Logistic Regression Analysis

Predicting whether an animal will pick up mastitis infection or not, as well as identifying the variables in making the prediction, is most important in dairy farming. A variety of multivariate statistical techniques can be used to predict a binary dependent variable (yes/ no) from a set of independent variables. The

most popular technique, multiple regression analysis, poses difficulties when the dependent variable can have two values - mastitis will be occurring or not occurring.

When the dependent variable can have only two values (occur and not), the assumptions necessary for hypothesis testing in regression analysis are necessarily violated. For example, it is unreasonable to assume that the distribution of errors is normal in such occasions. Further, with multiple regression analysis, the predicted values cannot be interpreted as probabilities. They are not constrained to fall in the interval between 0 and 1. However, the logistic regression model, multivariate technique, can estimate the probability of occurrence of mastitis. The advantage of this technique is that it requires far fewer assumptions, besides performing better than other tools [24].

The logistic regression model is the technique of choice for analyzing binary response of occurrence [25]. The logistic regression model would estimate the probability of a particular milch animal to pick up mastitis. The purpose of this model is to determine the probability that an individual animal with a given set of attributes will pick up the infection or not. In logistic regression, we directly estimate the probability of an event occurring. The logistic regression model can be written as

$$\text{Prob(event) or Pi} = E\left(\gamma = \frac{1}{V_i}\right)$$

$$= \frac{e^{\delta+\gamma_1 V_1+\gamma_2 V_2+\ldots\ldots+\gamma_i V_i}}{1+e^{\delta+\gamma_1 V_1+\gamma_2 V_2+\ldots\ldots+\gamma_i V_i}} \quad i = 1,2,3,\ldots\ldots,14$$

Or, equivalently,

$$\text{Pi} = \frac{1}{1+e^{-(\delta+\gamma_1 V_1+\gamma_2 V_2+\ldots\ldots+\gamma_i V_i)}}$$

i.e.,

$$\text{Pi} = \frac{1}{1+e^{-z}}$$

Where,

δ and yi - the coeeficients to be estimated from the data;

v1- Species indicator (= 1 if cow; =0 if otherwise);

v2- Breed indicator 1 (=1 if exotic purebred cow; =0 if otherwise);

v3 - Breed indicator 2 (=1 if crossbred cow/murrah or graded buffalo); =0 if otherwise);

V4 - Average daily milk yield in litres;

v5 - Lactation number;

v6 - Stage of lactation in days;

v7- Season dummy 1 (=1 if rainy; =0 if otherwise);

v8 - Season dummy 2 (=1 if summer; =0 if otherwise);

v9- Stall hygiene indicator (=1 if less hygienic; =0 if otherwise);

V -v 10 Udder hygiene indicator (=1 if less hygienic; =0 if otherwise);

V -11 Grazing dummy (=1 if allowed for grazing; =0 if otherwise);

V -12 Milking method (=1 if manual; =0 if machine);

V -13 Weaning dummy (=1 if day old weaning; =0 if otherwise);

v14- Udder morphology (=1 if pendulous/abdominal udder or teat (s); =0 if otherwise);

e - the base of the natural logarithms, approximately 2.718 and

Z - the linear combination such that

$$Z = \delta + \gamma_1 V_1 + \gamma_2 V_2 + + \gamma j Vj$$

The probability of the event not occurring is estimated as Prob (no event) = 1-Prob (event)

The probability estimates will always be between 0 and 1, regardless of the value of Z. In linear regression, parameters of the model are estimated using the least-squares method, in which the regression coefficients are, selected that result in the smallest sums of squared distances between the observed and the predicted values of the dependent variable. However, when estimated through ordinary least squares (OLS) method, the logistic regression model suffers from the following problems:

1. The variance of the disturbance term will not be homoskedastic which is against one of the assumptions of OLS.
2. The assumption of normality is no longer tenable because like dependent variable, error term also takes only 2 values.
3. Estimated probabilities lie outside the range of 0 and 1.
4. Estimation of probability by OLS assumes that the probability increases linearly with the explanatory variable, i.e., the marginal effect of the explanatory variable remains constant throughout, which may not happen in reality.

Given the limitations of OLS, the logistic coefficients are estimated using the maximum likelihood technique, where the coefficients that make the observed results most 'likely' are selected. To test the hypotheses about the coefficients, Wald statistic which has a chi-square distribution is used in the logistic models. Wald statistic is the square of the ratio of the coefficient to its standard error.

$$\text{That is, Wald} = \left(\frac{\gamma_i}{SE_i}\right)^2$$

As in the case with multiple regression, the contribution of individual variable in logistic regression is difficult to determine, because the contribution of each variable depends on the other variables in the model. A statistic that is used to look at the partial correlation between the dependent variable and each of the independent variables is the 'R' statistic, which can range from -1 to +1. A positive 'R' value indicates that the likelihood of mastitis occurring in an animal increases as the variable increases in value. If it is negative, opposite is true. Small 'R' values indicate small partial contribution to the model.

The equation for the statistic is

$$R = \pm\sqrt{\left(\frac{\text{Wald Statistic} - 2K}{-2LL(0)}\right)}$$

Where,

'K' is the degrees of freedom for the variable and -2LL(0) is -2 times the log likelihood of a base model that contains only the intercept. If the Wald statistic is less than 2K, R is set to 0.

For interpreting the logistic coefficient, the logistic model is rewritten in terms of the odds of mastitis occurrence. The odds of mastitis incidence are defined as the ratio of the probability that it will occur to the probability that it will not. The logistic model rewritten in terms of the log of the odds, called as a logit, is as follows:

$$\log\left(\frac{P_i}{1-P_i}\right) = \delta + \gamma_1 V_1 + \gamma_2 V + \ldots\ldots\ldots + \gamma_i V_i$$

Where the logistic coefficient (y)can be interpreted as the change in the log odds associated with one unit change in the independent variable. The logistic equation can also be written in terms of odds, instead of log odds, for easier interpretation, as below:

$$\frac{P_i}{1\text{-}P_i} = e^{\delta+\gamma_1 V_1+\gamma_2 V_2+\ldots\ldots\ldots+\gamma_i V_i}$$

$$e^{\delta} e^{\gamma_1 V_1} e^{\gamma_2 V_2} \ldots\ldots\ldots e^{\gamma_i V_i}$$

Where, e raised to the power y.is the factor by which the odds change when the 1^{th} independent variable increases by one unit.

iii) Discriminant Function

Discriminant analysis begins with the desire to distinguish statistically between two or more groups of cases, through a collection of discriminating variables that measure characteristics on which the groups are expected to differ. Discriminant function is a multivariate technique for studying the extent

to which different individuals diverge from one another. The mathematical objective of discriminant analysis is to weigh and linearly combine the discriminating variables in some fashion so that the groups are made to be as distinct as possible. The method attempts to 'discriminate' between groups in the sense of being able to tell them apart [15]. The use of this statistical technique has the advantage of parsimony of description and clarity of interpretation [26].

In discriminant analysis, a linear combination of the independent variables is formed and serves as the basis for assigning cases to groups. Thus information contained in multiple independent variables is summarized in a single index. In this analysis, the weights are estimated so that they result in the 'best' separation between the groups.

The linear discriminant function of the following form would find the factors that discriminate mastitic and non-mastitic milch animals:

$Z = a + bjMj + b_2M_2 + b_3M_3 + + bM, i = 1,2,3, ..., 11$

Where,

Z - total discriminant score for mastitic and non-mastitic milch animals

m1- standardized species indicator (=1 if cow; =0 if otherwise)

m2- standardized breed indicator (=1 if non-descript cow /buffalo); =2 if graded/murrah buffalo; =3 if crossbred cow; =4 if exotic purebred cow)

M3 - standardized average daily milk yield in litres

M4 - standardized lactation number

M5- standardized stage of lactation in days

M6- standardized season indicator (=1 if winter; =2 if summer; =3 if rainy)

m7- standardized stall hygiene score (=1 if cleaning daily with

disinfectants; =2 if cleaning only with water; =3 if occasional cleaning; =4 if no cleaning at all)

M8- standardized udder hygiene score (=1 if cleaning daily with disinfectants; =2 if cleaning daily only with water; =3 if occasional cleaning; =4 if no cleaning at all)

M9- standardized method of milking indicator (=1 if machine; =2 if manual)

m10 standardized weaning indicator (= 1 if day old weaning; =2 if weaning at first week; =3 if weaning at third month; =4 if weaning at sixth month; =5 if no weaning)

m11 standardized udder morphology score (= 1 if no udder/teat abnormalities; = 2 if udder/teat abnormalities)

If a linear discriminant function is to distinguish the milch animals which pick up udder infection from those which do not, the groups must differ in their Z values. Therefore, the b_i's are chosen so that the values of the discriminant function differ as much as possible between the groups, or so that for the discriminant scores, the ratio, called as eigen value is a maximum.

The discriminant score for a particular observation is obtained by multiplying the standardized co-efficients by the values of the variables and summing these products. Wilk's lambda statistic, sometimes called as the U statistic, is the test for univariate equality of group means. When variables are considered individually, lambda is the ratio of the within-groups sum of squares to the total-sum of squares. A lambda of 1 occurs when all observed group means are equal. Values close to 0 occur when within-groups variability is small compared to the total variability - that is, when most of the total variability is attributable to differences between the means of the groups. Thus, large values of lambda indicate that group means do not appear to be different, while small values indicate that group means do appear to be different.

The discriminant function should be tested for significance to examine

whether or not the variables considered together were significantly powerful in discriminating between two groups. A case is classified based on discriminant score, in the group for which the posterior probability is the largest. That is, it is assigned to the most likely group based on its discriminant score. The discriminant function is used to measure the net effect of the variables. It also helps to know the relative importance of the variables to discriminate between mastitic and non-mastitic milch animals. The method seeks to obtain co¬efficients such that the squared difference between the mean Z score for one group and mean Z score for the other group is as large as possible in relation to the variation of the Z scores within the groups.

iv) Step Wise Variable Selection in Discriminant Analysis

Instead of using a large number of potential variables to separate the groups without knowing which are important for group separation, it is better to identify the 'good' predictor variables using the algorithm for variable selection - step wise selection. Two criteria are usually used for variable selection, viz. minimization of Wilk's lambda and maximization of Mahalanobis distance (D^2).

In step wise method, the first variable included in the analysis has the largest acceptable value for the selection criterion. After the first variable is entered, the value of criterion is reevaluated for all variables not in the model and the variable with the largest acceptable criterion value is entered next. Variable selection terminates when no more variables meet the entry or removal criterion.

The two criteria - minimization of Wilk's lambda and maximization of Mahalanobis distance were tried to select the key variables of group separation. Each entry or removal of a variable is considered as a step. The maximum number of steps permitted in an analysis is twice the number of independent variables. The function should also be tested for multicollinearity before a variable is entered into the model by checking the tolerance of a variable, a measure of degree of linear association between variables.

Studies on Estimating Losses Due to Mastitis

a) Risk Factors

The risk of developing clinical mastitis was the highest in early lactation [27,28]. The multiparous cows were, generally, at higher risk of developing clinical mastitis [29,30], where the risk of developing clinical mastitis increased with increasing parity [27]. Mastitic cows tended to have higher milk yield than non-mastitic cows before they develop clinical mastitis [31,32,29], indicating that high milk yield was a risk factor for clinical mastitis. Previous occurrences of mastitis, clinical mastitis or high somatic cell count (SCC), substantially increased the risk of a cow developing a new case of clinical mastitis [27]. Other disorders, such as dystocia, milk fever, retained placenta, metritis, ketosis and lameness were also known to increase the risk of clinical mastitis [30,33]. Another study identified that cows with many calves had about *13-times* greater risk (62.9%) of developing an udder infection than those with fewer calves (11.3%) [34]. The influence of an irregular interval between morning and evening milking (<12 or >12 h/ day) on the prevalence of mastitis might have been the consequence of an enhanced chance for bacteria to colonize teat ends and streak canals during the longer milking intervals and also the knuckling and stripping method of milking could induce damage to the teat tissue increasing the risk for intra-mammary infections [35]. The infection rate of mastitis in cows with pendulous udder was higher than the non-pendulous udder [36] and udder hygiene significantly associated with the risk of environmental pathogen intra-mammary infection in cows [37]. Changes to teat tissue, particularly teat injury might favor penetration of bacteria into the udder and increase the risk of new mastitis infections [38]. During suckling the pathogens might get entry into the teat and often damage the udder to develop the mastitis [39].

b) Economic Losses

The total economic cost of diseases is categorized into two distinct components; production loss and control expenditures [40]. Losses included benefits that were taken away and benefits that were not realized. The former could be exemplified by milk that must be discarded following treatment with antibiotics and the latter by milk that was never produced as a result of disease. Expenditures were 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 was a great economic importance to milk producers, because the disease had negative impact on several important

aspects of cow and herd performance. Incurred costs were of both direct and indirect nature [41].

The first report on mastitis estimated an annual loss of about Rs.52.9 crore in India [42], which subsequently got revised to Rs.6053.21 crore annually [43]. Mastitis, the most important deadly disease of dairy animals was responsible for heavy economic losses due to reduced milk yield (up to 70%), discarded milk after treatment (9%), cost of veterinary services (7%) and premature culling (14%) [6]. Economic losses due to mastitis had traditionally been associated with reduced milk production, reduced milk quality with increases in SCC, costs with treatment and discarded milk, and increased risk for culling [44].

Economic loss due to mastitis was earlier reported in India by Singh and Singh [7], here certain modified formulae were used in the estimation of economic loss due to clinical mastitis. Loss due to Clinical Mastitis = (Production loss + Treatment loss + Depreciation loss). Thirunavukkarasu and Prabaharan [45] estimated the economic losses due to mastitis in bovines. Exotic cows were founded to suffer a greater loss (Rs.546.69 per lactation) due to mastitis as compared to cross bred cows (Rs.519.69). Similarly, Murrah buffaloes suffered greater loss (Rs.425.14 per lactation) than that of graded buffaloes (Rs.393.74). Cha *et al.,* [46] estimated that the average costs per case (US$) of gram¬positive, gram-negative, and other CM were $133.73, $211.03, and $95.31, respectively.

Economic Losses Due to Mastitis in Smallholders' Dairy Herd: A Case Study

Although efforts have been made to quantify the effects of mastitis, they were all skewed towards large farms. However, the livestock production systems across the Indian continent are characterized by low input and low productivity, with the system of production by and large being 'extensive'. Majority of livestock owners are only marginal farmers with an average herd size of 3.7 cattle and buffaloes.

Tamil Nadu, one of the leading states in milk production in India, produces 5.38 per cent of country's milk production with a daily milk production of 145.88 lakh litres. The major part of milk production in the State is from cows,

maintained under small holder production system. In this context, a study was conducted to assess the economic losses associated with mastitis under small holder farming conditions, in order to provide estimates that can support decisions regarding mastitis control in individual herds and facilitate derivation of appropriate economic weight of mastitis in the planning process.

a) Economic Losses Due to Milk Production Loss

Reduced milk receipt included the milk production loss and the loss due to discarded milk. Milk production loss during mastitis was calculated by subtracting milk yield at the time of mastitis from the milk yield of just before infection. Discarded milk was calculated as the milk produced and discarded during mastitis (Table 8.1). Total loss due to reduction in the attainable milk yield was Rs. 640.24 per mastitic case (with 35.57 litres of average quantity of milk loss).

Table 8.1: Milk production loss per clinical mastitis case (per lactation)

Reduced milk receipts	**Average quantity of milk lost for affected period (in litres)**	**Value (Rs)**
Milk production loss	24.18	435.20 (67.97)
Discarded milk loss	11.39	205.04 (32.03)
Total	**35.57**	**640.24 (100)**

(Figures in parentheses indicate percentages of each cost to overall cost)

b) Loss Due to Treatment of Mastitis Infection

Cost of treatment included veterinary fees (amount spent to veterinarian for treating the mastitic cows), drug expenses (amount spent by respondents to purchase drugs like intra mammary tube, ointment for external udder application and bolus for oral administration), diagnostic cost (incurred for antibiotic sensitive test and relevant tests for identification pathogens) and extra labour cost. Overall average cost of treatment of a mastitic case was Rs.1005.50 (Table 8.2).

Table 8.2: Cost of treatment of a mastitis case

Cost components	**Average cost (Rs.)**

Veterinary fees	813.75 (80.92)
Drug expenses	160.00 (15.92)
Diagnosis cost	12.00 (1.19)
Labour cost	19.75 (1.97)
Overall	**1005.50 (100.00)**

(Figures in parentheses indicate percentages of each cost to overall cost)

c) Total Economic Loss Per Clinical Mastitis Case

Overall figures indicated that the total economic loss due to mastitis was Rs. 3745.74 per case (Table 8.3). Loss due to early disposal or culling was more vis-a-vis other components.

Table 8.3: Total economic losses in a clinical mastitis case

Particulars	**Losses**	**Loss during infection (in Rs.)**	**Total loss in a lactation (Rs)**
Milk production loss	Reduced milk production	435.20 (67.97)	640.24 (17.09)
	Discarded milk	205.04 (32.03)	
	Total	640.24 (100.00)	
Treatment cost	Veterinarian's fee	813.75 (80.93)	1,005.50 (26.84)
	Drug expenses	160.00 (15.92)	
	Cost of diagnosis	12.00	

		(1.19)	
	Labour cost	19.75	
		(1.96)	
	Total	1005.50	
		(100.00)	
Loss due to culling	Cost for replacement	2,100.00	2,100.00
		(100.00)	(56.06)
Grand total losses			**3,745.74**
			(100)

d) Breed Wise Details of Economic Losses Due to Mastitis

Breed-wise economic losses due to mastitis is presented in Table 9.4. Crossbred Holstein Friesian cows suffered a greater loss (Rs.4808.19 per infection) compared to Crossbred Jersey (Rs.3544.59) and Non- Descriptive cows (Rs.2884.45).

Table 8.4: Breed-wise details of economic losses due to mastitis

Breed of cows	**Economic loss per mastitis case in Rs.**
Crossbred HF	4,808.19
Crossbred Jersey	3,544.59
Non-Descript	2,884.45
Overall	**3,745.74**

e) Economic Losses in Different Stages of Lactation

Economic losses due to mastitis was calculated during different stages of lactation and presented in Table 8.5. Occurrence of mastitis in different stages of lactation appeared to cause varying losses in cows. The economic loss due

to mastitis was more in the cases infected during early stage of lactation (Rs.5782.5) than mid (Rs.3132.52) and late (Rs.2886.13) stages. The losses of mastitis incurred continued to decrease as the stage of lactation advanced.

Table 8.5: Economic losses in different stages of lactation

Stages of lactation	Economic loss per mastitis case in Rs.
Early	5,782.50
Mid	3,132.52
Late	2,886.13
Overall	**3,745.74**

Conclusions

The economic losses arising out of mastitis indicate that the mastitis remains as a serious economic problem telling upon the profitability of dairy farming. Hence, to ensure economic viability of dairy units, steps are to be taken to bring down the losses due to mastitis by preventing or controlling mastitis. It can be seen that the rate of incidence of mastitis appeared to be large enough to bring down the profitability of dairy farming business. Incidence of mastitis, having been associated with a variety of factors inherent in animals and factors resulting from improper farming practices, appeared to decrease when the management practices are proper and scientific. This indicates that though some breeds of milch cattle especially the high producers, are found to be more susceptible to udder infection, correction of management practices ensure drops in mastitis incidences. Thus, controlling environment factors to mask the innate characteristics of this susceptible class is the only appropriate way to prevent or control mastitis for the reason that animal factors can seldom be manipulated. It was further identified that paying full attention to the maintenance of stall hygiene through disinfection and udder hygiene by teat dipping is the most important aspect of mastitis control.

Policy Implications

In pursuance of the results obtained and conclusions drawn from various studies, the following policy implications for controlling mastitis are elucidated:

1. As there is a genetic resistance to mastitis in local cattle, breeding for this factor may help to decrease the problem. It means that advocating rearing of cross breeds instead of exotic pure breeds is lightly to reduce incidence of mastitis to some extent.
2. Mastitis control must begin with calves. Feeding of calves with pasteurized milk will avoid transmission of organism to the udder of heifers through suckling.
3. Heifers calving for the first time need to be placed separately from the other older calved animals. It is also better to milk heifers before the older cows to reduce the possibility of bringing mastitis germs to the young udder.
4. The focus of mastitis control efforts should center on sanitizing the general environment and preventing the contamination of teat end during the period in which streak canal remains open after milking. Hence control programmes may aim at forcing the animal to stand for atleast 30 minutes after milking during which time the streak canal remains open. This can be achieved by feeding the animals after milking, instead of before milking.
5. Following precise milking procedures will help to reduce the infection rate, strictly avoiding 'careless' milking. Milking infected cows last will also protect non-infected group from picking up mastitis.
6. Segregation of mastitis cows from the shed will prevent transmission of infections within the herd. If necessary culling of chromic cases may also attempted to.
7. Strict housing management practices through providing adequate drainage, frequent and appropriate manure removal and disposal, adequate stall space for each animal and stall sanitization should be enforced.
8. Well knit extension programme is the need of the hour to effectively communicate the forming group the importance f mastitis control strategies in dairy farming.
9. Selection of milch stock should involve looking for udder/teat morphological abnormalities as these make the animals more prone to udder infection.

10. Breeding of animals to avoid calving in rainy season may also be looked into.
11. The accuracy of the outcome of any animal health economics study highly depends on the availability and usefulness of the underlying data. But even with enough data available, it is not a simple task to quantity the losses [15], because the effects of the diseases are not always obvious and pronounced, are influenced by other factors such as nutrition and housing and often manifest themselves in a complex form with other diseases.

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Chapter 9
Impact of Mastitis on Reproduction Efficiency

Reproductive efficiency is an important determinant for farm profitability. Milk production and reproductive efficiency shares a negative correlation, therefore reproductive performance of high yielding dairy cows have declined over the last few decades with increase in milk production [1]. Although reproductive diseases directly shows their effect on reproductive performance, diseases other than the reproductive diseases like metabolic diseases and mastitis are also have negative impact on fertility of dairy cows. Mastitis is also a critical factor affecting the reproductive success of the herd. Earlier, it was thought that the effect of mastitis was restricted to udder only, but now it has been proved that mastitis affects the reproduction efficiency of the animals also, especially during early lactation period. Extensive research has reported that both clinical and sub-clinical mastitis alter the reproductive process at several levels. Thus now-a-days more stress is given to study the relationship between metabolic disorders, mastitis and reproduction. This chapter discusses the effects of mastitis on reproduction and the possible mechanism by which mastitis affects reproduction.

Mastitis and Reproduction Efficiency

One of the first studies to demonstrate a correlation between occurrence of mastitis and altered reproductive pattern of dairy cows was performed [2]. There after several researchers reported the association of mastitis with reproduction efficiency. Although, controlled clinical studies in this regard are limited, decreasing pregnancy rates in dairy cattle over the last 30 years [3]

and increasing trend of mastitis during same period [4] suggested the possible influence of mastitis on reproduction. The highest incidence of clinical mastitis is observed during early lactation and has a negative impact on the reproductive performance of dairy cows [5, 6, 7] and increases involuntary culling rates [8]. The clinical mastitis had a greater negative impact on reproductive performance in primiparous cows than multiparous cows. It has been reported that heifers, which have an increased number of somatic cells measured between 5 and 14 days in milk were at an increased risk of being culled during the first lactation [9].

Onset of clinical mastitis before first AI shows its negative effect on reproduction performance by increasing days to first service, days open and services per conception [5]. However, onset of mastitis within the first 45 days of pregnancy in cows increases risk of abortion and pregnancy loss within the next 90 days compared to uninfected cows [10, 11], which may be due to premature luteolysis by release of inflammatory mediators (PGF_2á, nitric oxide) leading to embryonic mortality. Therefore, these reports indicated that mastitis, either prior to or after first postpartum AI, increases culling rate and decreases reproduction performance in dairy cows. Similarly, the effect of clinical mastitis and (or) other diseases on reproductive performance in lactating Holstein cows suggested that clinical mastitis alone affected reproductive performance by significantly increasing service period and services per conception [12].

From the existing literature one can safely infer that mastitis delays the postpartum ovarian function and alters some of the key reproductive functions like ovulation, fertilization, implantation, and pregnancy maintenance. Acute mastitis delays the calving to first service interval, calving to conception interval and increase the number of services per conception. When clinical mastitis occurs before the first AI, calving to first service interval was significantly increased, compared to when it occurs after the first AI. The probability of conception decreased by 44% when mastitis occurred a week before insemination, by 73% when mastitis occurred on the week of insemination, and by 52% when mastitis occurred a week after insemination. The effect of mastitis is not only limited to the affected animals but also continue on the developing fetus, since the daughters born to the cows that suffered mastitis during gestation have reduced reproductive efficiency. Mastitis in pregnant cows could decrease the number of healthy follicles in the

developing fetus and compromise future fertility. Anti-Mullerian hormone, a reliable biomarker for potential fertility, is severely decreased in the developing fetus as the

number of mastitis events during gestation of their dams increases. It has been estimated that the economic loss due to mastitis associated impairment in reproduction could range between $50 and $100 per cow.

Almost all the studies that estimated the relationship of mastitis with reproduction efficiency have been conducted elsewhere in the world and studies in this aspect are very limited in India. To obtain first-hand information on the effect of mastitis on reproduction in Indian dairy animals, the authors evaluated the effect of clinical mastitis during different time of post partum period on days open in zebu, crossbred cattle and Murrah buffaloes. In Murrah buffaloes, it was observed that when mastitis occurs before first service *(i.e.,* during voluntary waiting period- VWP), the days open is prolonged compared to unaffected animals. When mastitis occur after VWP (during breeding period), it also increase the days open in affected buffaloes. The same trend was observed in indigenous and crossbred cattle also. During the study period, a majority of the apparently healthy animals with no clinical mastitis had optimum days

open (i.e. < 85 days) than those mastitis before first service. $^{da}V^{s}$ ◦?θη in However, this effect was very noticeable in Murrah buffaloes and crossbred cows than in Sahiwal cows when the animals suffered with mastitis before first service. This suggest that the adverse effect of clinical mastitis on reproduction is more in Murrah buffaloes when compared to indigenous and crossbred cattle. The following figures indicate the effect of clinical mastitis on days open in Murrah buffaloes, Sahiwal cows and Crossbred cows.

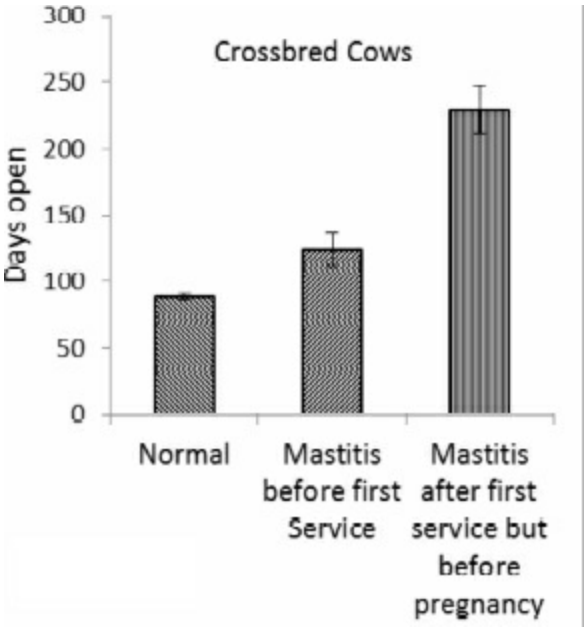


Fig.9.1

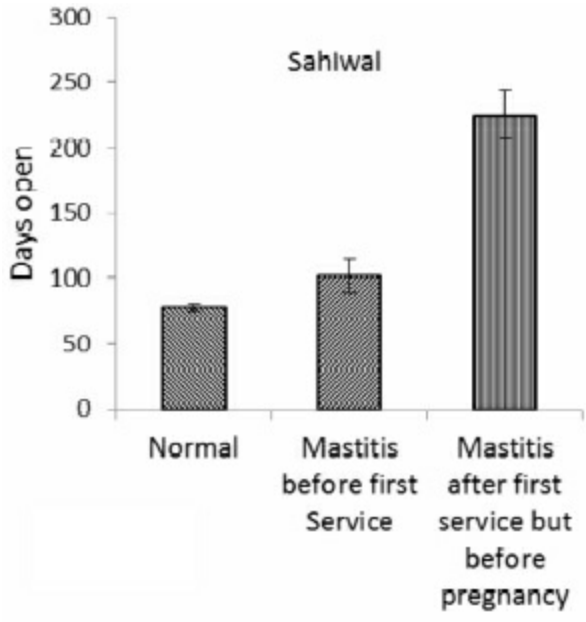


Fig.9.1

Fig. 9.1 & Fig 9.2: Effect of clinical animals that had clinical mastitis during post-partum period on crossbred cattle and

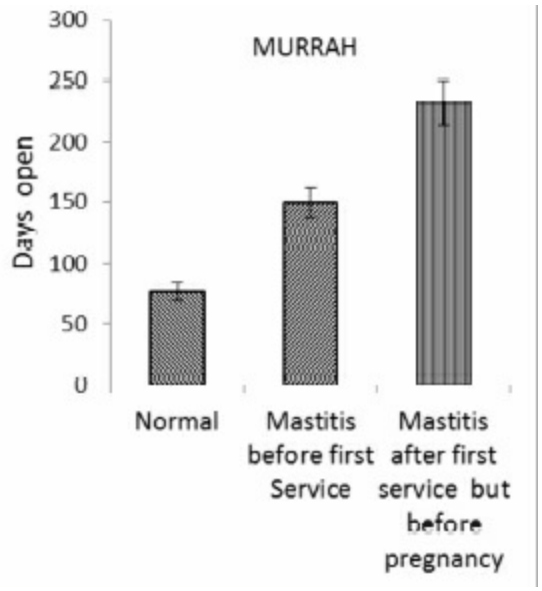


Fig. 9.3: Effect of clinical mastitis during post-partum period on days open in Murrah buffaloes Zebu cattla

Based on the literature survey, and from field level observations made by the authors, it is clear that clinical mastitis negatively affects reproductive efficiency and lead to increase in culling rate if they experience clinical mastitis because they may not become pregnant in a timely manner. Furthermore, the negative effects on reproduction are exacerbated when cows experienced with clinical mastitis and other metabolic diseases, low body condition score and lameness [11, 13],

Table 9.1: Effect of mastitis on reproductive efficiency of dairy cows

Reproductive parameters	**Alteration**
Effect on estrous cycle	Infected cows are 1.6 times more likely to have irregular estrous cycles
Service period (Days Open) and services per conception	Increases the service period and Services per conception
Loss of pregnancy	Infected cows within the first 45 days of gestation were at 2.7 times more risk of abortion

Conception Rate (CR)	Decreases CR (15-19%)

Possible Mechanisms by which Mastitis affect Reproduction in Dairy Animals

Although, exact mechanism of mastitis affecting reproductive performance is not very clear, it is well proved that the concentrations of reproductive hormaones and ovulation is altered in cows affected with mastitis. It has been documented that mastitis alters inter-estrus intervals, shorten or extend the luteal phase and extend the interval between estrus and ovulation (delayed ovulation) up to 56 hrs [2, 14]. The effect of experimentally induced mastitis before ovulation using *Streptococcus uberis,* on endocrine function, follicular growth, and ovulation in cows, revealed a significant reduction in luteinizing hormone (LH) pulsatility and estradiol-17ß production [15]. Subclinical long-term mastitis and short-term clinical mastitis in early lactation, both delay ovulation in about 30% of the cows [16]. Furthermore, the cows with high somatic cell count (from 200,000 to 500,000 cells / ml) had a significantly higher incidence of a prolonged luteal phase than cows with a somatic cell count of 50,000 to 100,000 cells/ml [17]. The possible mechanism(s) by which mastitis affects reproduction process in dairy animals, reported by different authors, are discussed here.

Mammary epithelial cells are the first line of defense against microbes through innate immune response [18]. Microbial infection of cells of mammary gland leads to release of endotoxins (LPS), proteoglycans and other molecules of bacterial origin that activate inflammatory and immune responses. The innate immune system relies on pattern recognition receptors (PRR) on host mammalian cells to detect microbe or pathogen-associated molecular patterns (PAMP). Upon PAMP recognition, PRRs initiate a series of signaling programs that execute host defensive responses necessary for killing infectious microbes. TLRs were the first PRRs identified. Each TLR recognize specific patterns of microbial components that are conserved among pathogens such as bacteria, viruses, mycobacteria, fungi and parasites [19]. The interaction of PRRs and PAMPs mediated the inflammatory response characterized by the production of inflammatory mediators such as cytokines, chemokines, typically IL-1ß, IL-6 and IL-8, TNF-a and acute phase proteins (APP) including serum amyloid A

(SAA), haptoglobin and other neuro transmitters and hormones including nitric oxide, PGF_2a [20]. These immune modulators are responsible for recruitment of leucocytes from blood to mammary gland, consequently milk SCC increases. The marked increase in milk SCC during infection is mainly due to influx of neutrophils from blood to the mammary gland, which can represent over 90% of leukocyte population in milk from infected udder quarters in contrast to low numbers of this cell population in uninfected ones [21, 22, 23]. The recruitment of leukocytes into the mammary gland is crucial to eliminate invaded bacteria by phagocytosis.

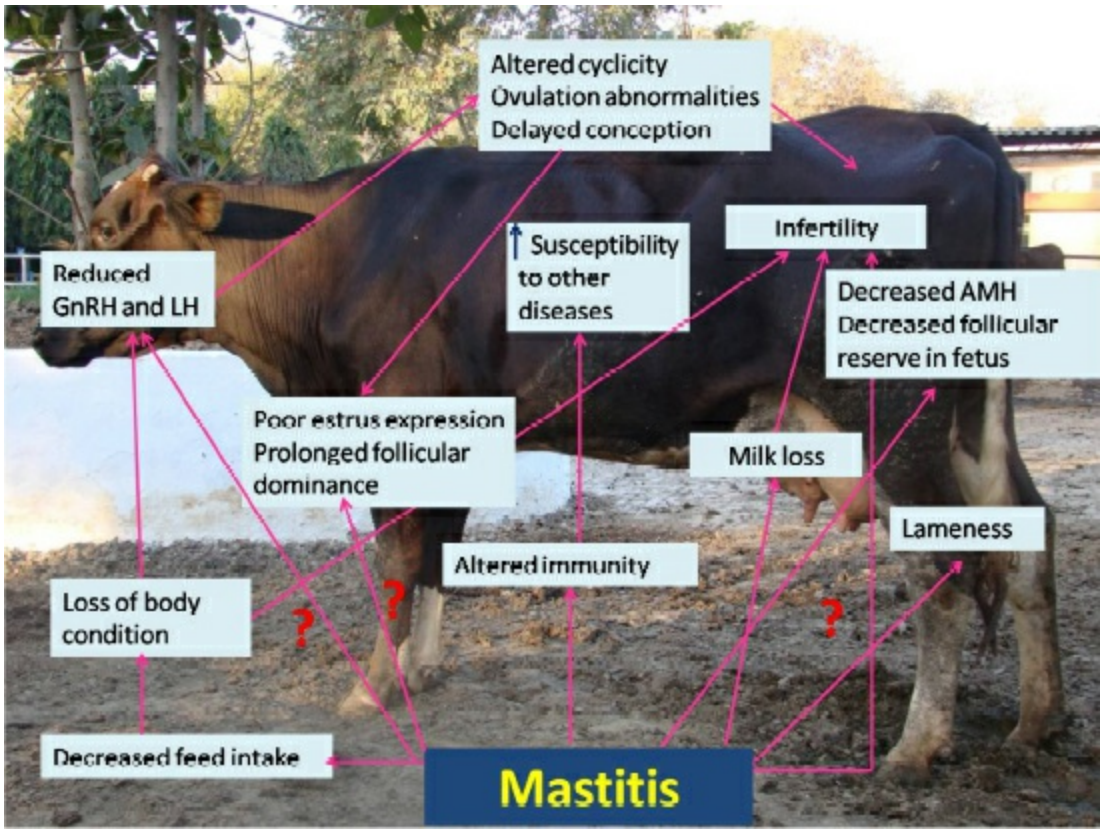
The release of inflammatory mediators is responsible for local and systemic response that can affect reproductive performance. Increased PGF_2a and nitric oxide may induce premature luteal regression and also have detrimental effects on embryonic development and quality, causing increased embryonic loss, and consequently increasing calving to conception interval. Furthermore, it has been also reported that LPS induces a TLR4-dependent inflammatory response on bovine granulosa cells of antral follicles lead to perturbation in oocyte development [24]. Release of inflammatory mediators into blood circulation may also have role in compromising oocyte and embryo development. TNF-a affects the maturation of bovine oocytes and reduced proportion of fertilized oocytes developing to the blastocyst stage [25]. Furthermore, TNF-a and nitric oxide have negative effect of on early embryonic development [25, 26]. Release of inflammatory mediators in mastitis results in elevation of body temperature, which may have detrimental effect on oocytes and embryos development as it has been reported that under heat stress smaller proportion of oocytes and embryos develop to the blastocyst stage cultured *in vitro* [27]. It has also been reported that exposure of lactating dairy cows and dairy heifers to heat stress, reduce quality of embryos and fertilization rate [28]. Besides the direct effect of elevated body temperature on oocyte and embryo quality and development, fever can also indirectly affect reproductive performance by decreasing feed intake and body condition leading to negative energy balance which could delay resumption of ovarian cyclicity.

Mastitis could influence reproductive function by altering follicular activity in two ways. In one side, elevated levels of cytokines produced from interaction of PRR and PAMP may block follicle stimulating hormone (FSH) action and the pulsatile secretion of luteinizing hormone (LH) and decrease LH

receptor expression on preovulatory follicle [14] leading to alteration in oocyte maturation, ovulation, and steroidgenesis by granulosa and luteal cells compromising embryonic development. Thus, onset of mastitis during the first month of gestation would likely result in decreased conception rates or increased pregnancy losses in lactating dairy cows [1].

Mastitis not only affect the fertility of the affected animal, but also shows negative effect on fecundity of animal and future fertility of unborn female calf by compromising the primordial follicular pool in the ovary [29]. *In vitro* exposure of bovine ovarian cortex to LPS reduces primordial follicle pool in the ovary of cattle by increasing follicle activation [30]. They also showed negative effect of LPS on primordial follicle pool, associated with increased follicle atresia of mice *in vivo.* As infection of mammary gland with pathogenic organisms lead to release of bacterial endotoxin, lipopolysaccharide (LPS), therefore, one paradigm is that mastitis might impact primordial and preantral follicle development, which would reduce the likelihood of conception rate and affecting the fecundity of dairy animals. As primordial follicles, containing an oocyte surrounded by a single layer of pre-granulosa cells, are formed during fetal life in ruminants [31] and LPS induces inappropriate premature activation of primordial follicles which would likely deplete ovarian follicle reserve and, thus, compromise subsequent fertility [30]. Therefore, mastitis not only shows negative effect on fertility of dairy animals at the time of infection, but also shows its negative effect even after resolution of disease [29].

The following figure shows the possible ways in which mastitis affects the reproduction process in dairy cattle



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Chapter 10
Mastitis Detection: Traditional and Advanced Diagnostic Techniques

In countries like India, where small holder farmers are high (70 %), mastitis is generally detected based on visual observation of milk, when flakes are present in milk or any other abnormal deviation in colour or consistency of milk. The most common method of detection of mastitis is the observation of flakes in milk and visual inflammatory changes in udder. These methods are grossly inadequate because by the time detection the severity of the disease very high and the animal is at an advanced stage of infection. It requires treatment with strong broad spectrum antibiotics. At times the mastitis becomes chronic that leads to fibrosis of individual quarters. Milk production drops drastically which eventually leads to the culling of animal.

Early diagnosis is extremely important due to the high costs of mastitis. If the lactating animals can be detected quite early before observation of any visual abnormality in milk, this will help the dairy farmers for taking adequate measures to prevent the animals from clinical mastitis. Therefore, various approaches to prevent the disease depends on the early and accurate detection of mastitis (sub-clinical) and application of the best suitable method to curb the disease. In this chapter various conventional and advanced diagnostic techniques for detection of mastitis and current areas of research to discover biomarkers of mastitis are discussed.

Diagnostics for Mastitis

The most common observation of the occurrence of mastitis is the deterioration of milk quality. A farmer's or herdsman's interest is in the

diagnosis of mastitis so that suitable preventive and curative methods could be adopted. While, from industrial standpoint, milk quality must be tested for bulk tank somatic cell count or unwanted chemical changes arising from mastitis so that suitable pricing could be fixed for the milk. Irrespective of the targeted application, mastitis diagnostics have been developed in past and are continuously being explored. Although large number of diagnostic tests has been developed for early detection of mastitis, every test has its own advantage and disadvantage in terms of its sensitivity and specificity.

Sensitivity (%) = true positive count / (true positive count + False negative count) x 100

Specificity (%) = True negative count/ (true negative count + False positive count) x 100

Sensitivity of a test refers to the probability that the smallest or minutest amount or number of positive cases will be identified as positive by the test. And the specificity of a test refers to the probability that when there is no disease the test should also declare the sample as negative. Sensitivity and specificity are interdependent. With the change in threshold both varies inversely.

Conventional Diagnostic Techniques

Somatic Cell Counts (SCC)

Somatic cells are predominately composed of white blood cells along with few sloughed off epithelial cells. The cells normally found in an uninfected mammary gland constitute neutrophils (1-11%), macrophages (66-88%), lymphocytes (10-27%), and epithelial cells (2-5%). The macrophages play an important role in the protection of mammary gland by performing the function of surveillance. When there is invasion of the mammary gland by bacteria, the macrophages respond by initiating inflammatory response which attract PMN cells and accumulate in milk. The PMNs engulf the bacteria and destroy them. In uninfected cows the SCC is normally less that $2 \boldsymbol{x} 10^5$cells / ml. SCC greater than $2 \boldsymbol{x} 10^5$ cells / ml is a strong indicator of mastitis. In somatic cell count of mastitic sample, neutrophil contributes more than 90% of total cells. SCC thresholds are often used to predict IMI at either the quarter or cow level. This is a very quick method to immediately screen animals or to identify the

affected quarter of udder. In dairy farm management, SCC aims at quick on farm problem solving by prediction of prevalence and calculation of likelihood ratios to predict IMI infections. Bulk tank somatic cell count (BTSCC) is adopted by big dairy farms and large scale purchasers. It varies regionally, seasonally and with herd size. This method is able to verify the existence of mastitis problem but unable to specify the origin of herd or cow.

Milk somatic cells can be counted by conventional haemocyto- meters (Fig. 10.1) or automated cell counters. This is a device which was invented by Louis-Charles Malassezand was originally used for counting of blood cells. Its use has subsequently been extended for counting of other cell types including milk somatic cells. In current practice, the haemocytometer is composed of nine equally sized bigger squares. The central chamber is different and divided into 25 smaller squares, while the corner chambers are divided into 16 smaller squares. The rest of squares are not used. The smaller squares inside the central square are subdivided into 16 even smaller squares each. This allows counting of very tiny cells.

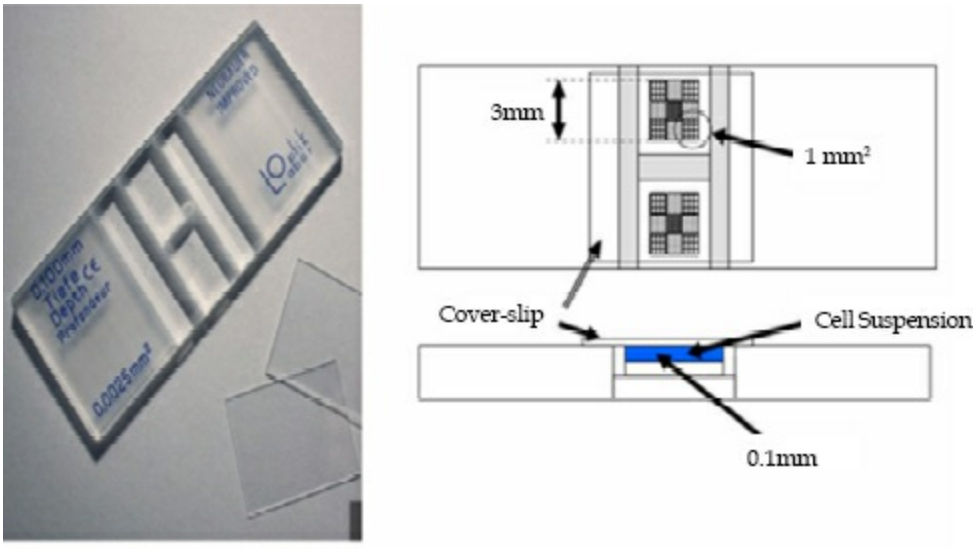

Fig. 10.1: Haemocytometer

Calculation:

Since the volume of 1 big square is: 0.1 cm χ 0.1 cm = 0.01 cm^2 of area counted the depth of the chamber is 0.1mm

0.1 mm = 0.01 cm

0.01 cm^2*0.01 cm = 0.0001 cm^3 = 0.0001ml = 0.1 μ?

So, for the Neubauer chamber, the formula used when counting in the big squares Concentration (cells / ml) = (Number of cells χ 10,000)/ Number of squares.

The ideal goal at any farm to rule out the incidence of subclinical mastitis has been specified; 85% of cows should be with somatic cell counts <250,000 per ml milk and less than 5% cows should develop new subclinical mastitis infections per month. Many other statistical programmes have been developed on the basis of data collected for SCC which includes Scatter graphs using linear score data.

Limitation: It is done on the raw milk only. Overall SCC of milk obtained from al the quarters of udder doesn't reflect the individual quarter characteristics. It is because; the contribution of affected quarter to SCC is diluted by the milk from healthy quarter. Therefore, an apparent lower SCC tends to overlook the affected quarter which results in misdiagnosis. The sensitivity of this method is only around 73-89% at corresponding specificity of 75-85%.

California Mastitis test (CMT)

This test is the most reliable test till date which can be used as a cowside test (Fig: 10.2). The principle of this technique is to detect change in the viscosity of milk when detergent and Bromocresol purple is added. The detergent breaks down the cell membrane of somatic cells and the nucleic acid forms a gel like matrix with cellular debris. Indirectly, this is also the measurement of total somatic cell count (SCC) because the degree of reaction is dependent on the number of somatic cells in milk. The importance of this test lies in its association with incidences of contagious pathogens mediated mastitis. The classification of cases based on SCC method and CMT score don't bear very precise relationship. It is mainly because each CMT score (negative, trace, 1, 2, 3) has a very wide and overlapping range of corresponding SCC score (0-2,00,000, 1,50,000-5,00,000, 4,00,000-15,00,000, 8,00,000- 50,00,000 and >50,00,000) respectively.

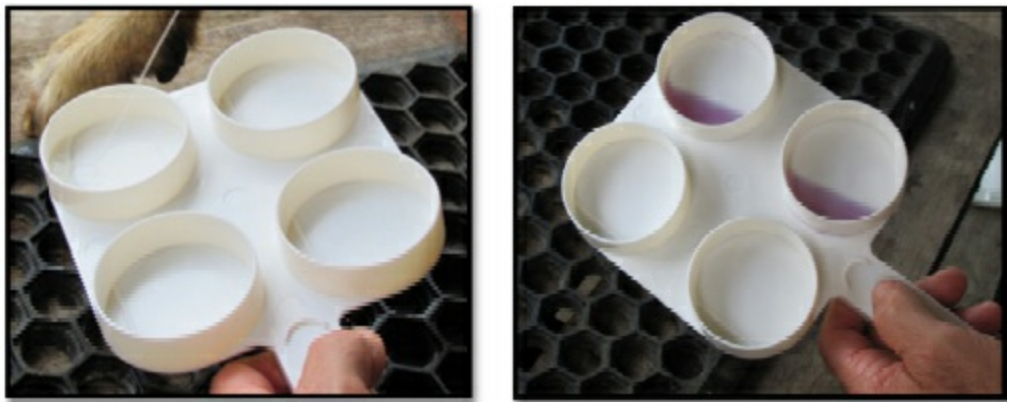

Fig. 10.2: First three strips are discarded and around 1/2 teaspoon of milk is stripped in strip cup each quarter in each separate cup. Reagent (Bromocresol-purple-containing detergent) is mixed in equal ratio to the milk. The paddle is rotated to mix the contents. In approximately 10 seconds, score is read while continuing to rotate the paddle.

How to read the results: The CMT reagent reacts with the neutrophils and the mixture thickens or gels in proportion to the amount of cells that are present. High levels of neutrophils indicate infection (Table 10.1). To obtain accurate and consistent results practice needs to be done on known SCC.

Table 10.1: Interpretation of CMT

CMT score	Interpretation	Visible reaction	Total cell count (/ml)
0	Negative	Milk fluid and normal	0-200,000 0-25% neutrophils
T	Trace	Slight precipitation	150,000-500,000 30-40% neutrophils
1	Weak positive	Distinct precipitation but no gel formation	400,000-1,500,000 40-60% neutrophils
2	Distinct positive	Mixture thickens with a gel formation	800,000-5,000,000 60-70% neutrophils

3	Strong positive	Viscosity greatly increased. Strong gel that is cohesive with a convex surface.	>5,000,000 70-80% neutrophils

Limitation: the test although indicative of mastitis at prima facie, it suffers with poor specificity and sensitivity. The measured sensitivity of CMT varies with pathogen involved which is best (84%) in Streptococcus infection. The interpretation of result is very subjective.

Fossomatic SCC

In this test also SCC is the indicator of mastitis. Ethidium Bromide is used to stain the nuclei of somatic cells. The fluorescence can be measured which is directly proportional to SCC.

How Fossomatic™ FC works: The Fossomatic FC counts somatic cells (Fig. 10.3) based on recognition of DNA from the cells. A mixture of milk and staining solution (Ethidium Bromide) is surrounded by a sheath liquid and passed through a flow cell. In the flow cell, the stained somatic cells are exposed to light of a specific wavelength. The cells then emit fluorescent light pulses at a different wavelength. The pulses are counted and displayed. The design of the flow cell ensures that only one somatic cell is detected at a time. Higher precision can be obtained at grading limits using precision set-up feature.

Fig. 10.3: Fossomatic™

DeLaval Cell Counter

The method was developed by Delaval Company (Fig. 10.4). They use propidium iodide to stain nuclear DNA of somatic cells present in milk. The intensity of color is measured by optical fluorescence readers which is directly proportional to SCC in milk.

Fig. 10.4: DeLaval Cell counter (DCC) and Online Cell Counter (OCC)

The test is rapid and the device is easily transportable. The major limitation of this system is that it is relatively expensive.

Electrical Conductivity (EC) Test

The effort for an on line system to monitor the quality of milk has become possible because of progress made in many engineering and instrumentation technologies. Many of the parameters like temperature, animal activity, daily milk yield and milk electrical conductivity (EC) can be recorded automatically during the milking. Alteration in EC is of critical importance to predict the quality of milk. Electrical conductivity of milk depends on the concentration of cations (mainly Na+, K+) and anions (mainly Cl) in milk. In mastitis, milk

concentration of lactose and K+ decreases and concentration of Na+ and Cl^- ions increases because of increased blood capillary permeability, the destruction of tight junctions and the destruction of ion transport systems. Typical EC of normal milk from a healthy cow varies between 4.0 to 5.5 mS/cm at 25°C. Differential EC can be determined by comparing the EC of milk from all the four quarters. If the sample from any quarter is = 16% above the lowest quarter value, it indicates mastitis. Similarly, animal should be considered positive for mastitis if the EC value is above the threshold value keeping in mind that other conditions are same for all the animals. The best qualitative application of this test is to determine the relative EC among the quarter of the same animal. This way the sensitivity is around 68% and the specificity is around 96%.

Commercially available Mas-D-Tec a portable hand held EC measuring device from Wescor Logan, Utah, US claims to detect subclinical mastitis. It suggests that =5 reading of absolute EC indicates the presence of subclinical mastitis. However, an independent microbiological test to compare the claim of Mas-D-Tec found the later not to be sufficiently accurate to be recommended as screening test. Similarly other available devices such as Dramnisk mastitis tester and Digital mastitis detector (Fig. 10.5) follow same principle.

Limitations: EC of milk can change because of many factors other than mastitis. For example, milk temperature, stage of lactation, fat content, milking interval and breed etc. can change EC of milk. International Dairy Federation (IDF) study reveals that EC method is incapable to screen samples for clinical or subclinical mastitis. They concluded "the published information is too varied to justify a claim that mastitis, especially subclinical mastitis can be detected by means of electrical conductivity measurements in milk." Many other EC measuring devices have been tested and compared in UK and Australia. None of them found EC a good test in comparison to

Fig. 10.5: Draminski mastitis Tester and Digital mastitis

individual SCC values, CMT score and individual culture of microl from milk.

pH Based Test

Change in pH of milk can be detected by colorimetric metl: using Bromothymol blue dye. Although these methods are easy perform and cost effective, determination of pH is very indirect metl: to detect mastitis. pH changes can happen in milk because of m£ reasons (metabolic disorders, dehydration etc.) other than mastiti

Temperature Sensor Based Diagnostics

Temperature changes in the body of animal suffering with mast can be monitored and recorded by thermal camera. These came can sense the skin temperature of animals using infra red wave seni Strong correlation has been found between skin temperature and S' count [1]. Similarly, milk temperature can be measured using th thermo sensitive cameras which can measure changes of 1 to l.i [2], Independently, it seems that temperature based tests are subjective to interpret the udder health because temperature chan can be associated with a large number of factors other than masti For example ambient temperature fluctuation is the biggest trouble the application of this

technique. However, their importance becoi: local immunity in mammary gland like N- Acetyl glucosmainidase (NAGase) and lactate dehydrogenase (LDH) are increased. Colorimetric and fluorometric assays have been designed to detect these enzymes both qualitatively and quantitatively. Hiss and coworkers [3], have utilized LDH activity in milk for identification of subclinical mastitis in bovine.

Enzymatic Methods

These methods are based on detection of elevated enzymes in milk. In mastitis many of the metabolic enzymes or the enzymes involved in local immunity in mammary gland like N- Acetyl glucosmainidase (NAGase) and lactate dehydrogenase (LDH) are increased. Colorimetric and fluorometric assays have been designed to detect these enzymes both qualitatively and quantitatively. Hiss and coworkers [3], have utilized LDH activity in milk for identification of subclinical mastitis in bovine.

Esterase enzyme is secreted by somatic cells. It is assumed that if the activity of esterase is more in milk probably it is due to high number of somatic cells in milk which is indicative of mastitis. The assay called "Portacheck" is based on the measurement of esterase catalyzed enzymatic reaction product. This is the measurement of total SCC indirectly. It is user friendly, cost effective and a rapid test. Application of this test is limited by the low sensitivity at low SCC. Elevated ATP concentration from somatic cell can be measured by bioluminescence based assays [4]. Figure 10.6 indicate application of different methods to detect mastitis.

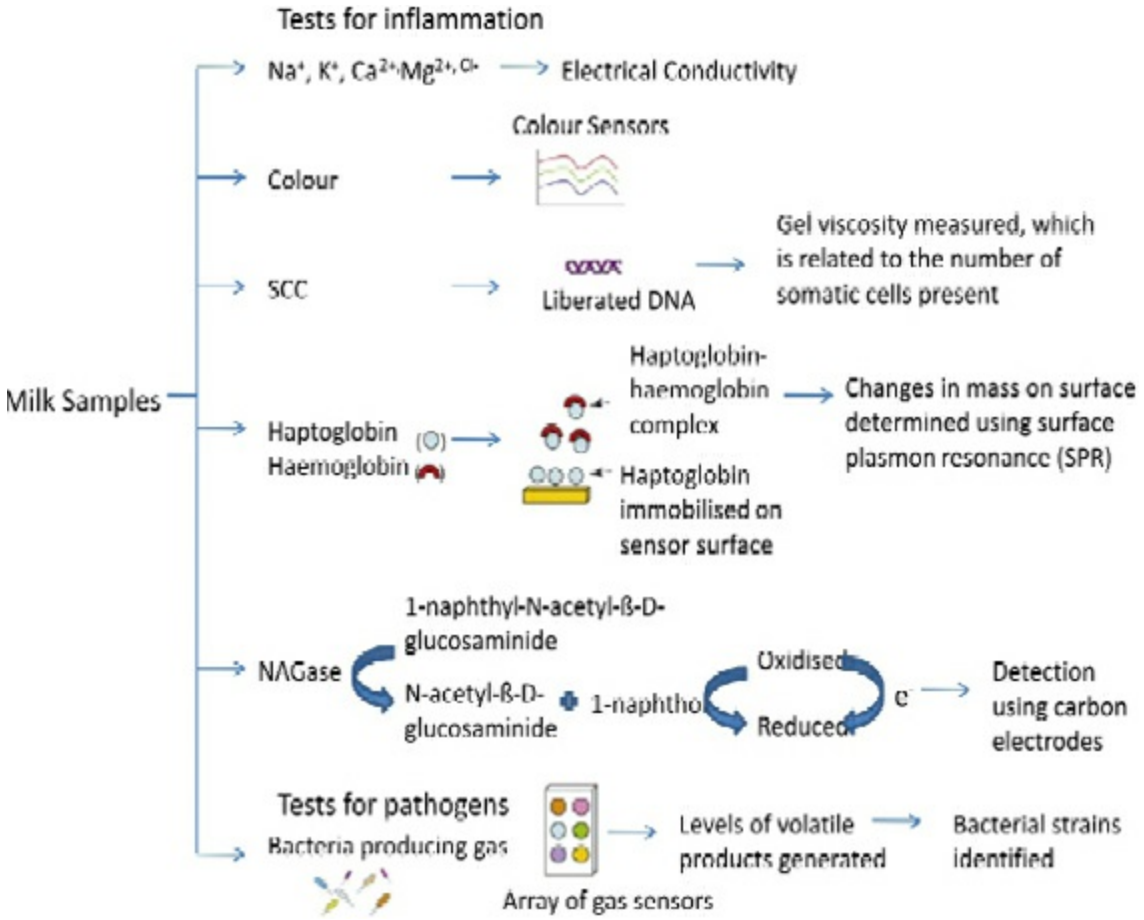

Fig. 10.6: A composite chart to show different methods of mastitis diagnosis based on physicochemical characteristics of milk [5]

Bacterial Culture and Biochemical Test

The techniques for identification and characterization of pathogenic bacteria isolated from mastitis vary from traditional to very recent advanced methodologies. All techniques have inherent difficulties from batch to batch, lab to lab and reagents to reagents variations. The results may sometimes be interpreted in different ways. Therefore, good laboratory practices should be put into place as much effectively as possible.

Culture of microbes in laboratory: It is still the gold standard test and accepted method of diagnosis where microbes present in milk are grown on selective agar petri dishes. However, specificity is relatively low which results in many false positive diagnoses. This test is based on the principle of

identifying the live causative organism in milk sample (Fig. 10.7). Different organisms involved in causing mastitis are cultured under different nutritional and physiological conditions and the methodology is adjusted according to the type of organism, sampling methodology and laboratory procedures.

Fig. 10.7: Culturing of mastitis causing organisms

The sensitivity of bacterial culture test varies with the type of bacteria involved and whether the sample is pre milked or post milked. For *S. aureus* the sensitivity is 91% in pre-milk sample and 81% in post milk sample. Similarly, for coagulase negative Staph (CNS) the sensitivity is 91% and 45%, for environmental Streptococcus 97% and 58% respectively for pre and post miliked sample. Similarly, colony forming unit is high for S. *aureus* in pre-milked sample. To enhance the sensitivity of detection of S. *aureus* in pre-milked sample, 2- 3 consecutive sampling is recommended. Culture tests are good particularly, to decide the appropriate antibiotic for therapy. Now with reduction in the cost of bacteriological nutrient medium it is very much possible. Sedimentation of milk components by centrifugation at 2000 g for 15 minutes and then plating the sediments dissolved in 0.05 ml of saline on blood agar is considered good to improve the frequency of detection. There is high degree of variability and limited sensitivity in detection of *S. aureus*. Therefore, conditions need to be optimized whether to take pre milk sample or post milk sample, or quarter versus composite milk or different inoculum volume. The sensitivity of detection of *S. agalactiae* varies in the range of 95% to 100% and specificity is around 95%. *S. agalactiae* detection is not affected by sampling conditions.

Genetic diversity among the mastitis pathogens has come a long way. With changes in environment and introduction of antibiotics to treat mastitis new strains with unique genotype have emerged. This continuous shift in genetic makeup has led to threat of persistent bacterial infection which are resistant to known therapeutic measures. Since, therapy is targeted to specific pathogenicity of organism; it is desirable that individual species and strains should be identified, characterized and studied to know its pathogenesis. Unless the correct causative organism is identified for each case, a best treatment cannot be employed. Therefore, at the route of correct diagnosis is the ideal approach towards sampling procedure and sample handling. For example, freezing of milk has been found to alter the native state of microbiotic composition of milk.

Limitation: The time period required for bacterial culture cannot be reduced below 12 hours. It requires very stringent and sterile laboratory condition so that only the pathogenic bacteria from milk could grow on culture plates and petri dishes. Sample collection should be done carefully so that environmental contamination must not come in milk.

Antibiotic Susceptibility Test

Recently, because of increasing concerns about the injudicious use of antimicrobials in dairy animals, there is much pressure on the very selective and minimal use of antibiotics in dairy animals. Guidelines have been made that indiscriminate use of antibiotics in food grade animals should be stopped. To achieve this, antibiotic sensitivity tests are mandatory to be performed before use of antibiotics. It is based on inhibition of bacterial growth (bacteriostatic) not killing of bacteria.

Both qualitative and quantitative observation is made which includes classification as sensitive, intermediate or resistant strain or in terms of minimal inhibitory concentration (MIC). The standard test in this line which is routinely used in veterinary diagnostic laboratory is Kirby- Bauer disk diffusion (KBDD) test. It works on the principle that antibiotics impregnated on small filter paper disk can diffuse into the nutrient medium and hence can inhibit the growth of bacteria in that plate. The extent of inhibition can be measured in term of the distance (radius / diameter) from the center of antibiotic disk which is inversely proportional to the MIC of the antibiotic.

Critical to the test is the use of standard inoculum which is approximately, 1 x 10^8cfu. The differences in the result of antibiotic sensitivity test arise because of its inability to meet the standard quantum of inoculums. Inoculum size of 1 x 10^8cfuis usually achieved by growing the cells till the turbidity shows 0.5 McFarland standards. "Prompt" is a commercial system from Becton-Dickson to obtain the standard inoculums size.

To help in the determination of exact dosage of the antibiotics, quantitative susceptibility testing is performed. Broth microdilution (MD) method is used to obtain the serial dilution (2 fold) of antibiotic and bacteria are grown in it. The highest dilution at which there is complete inhibition of bacterial growth is recorded as MIC. Although, actual serum concentration of the antibiotics is usually different than that of MIC value because of differences in pharmacokinetics of different drug, it serves as suggestive guidelines for the optimization of drug dosage. Serum concentration of beta lactam drugs should continuously be higher than MIC. Peak serum concentration should be 8-10 times greater than MIC for amino glycosides. It is reported that against enterococci only ceftiofur and enrofloxacin are effective. The MIC of various antibiotics for bacteria isolated from bovine mastitis has been determined [6]. The invitro testing is a good predictor of therapy for IMI caused by Staph spp. new *S. aureus* infection, *S. uberis, S. dysagalactia* and *S. agalactiae* but not for IMI caused by chronic *S. aurues.*

On similar lines MASTiK test is available from ImmunCel, Portland ME. It contains antibiotic coated microtiter plates. When raw milk from infected animals is added to it lactose fermenting bacteria can either grow or can be inhibited. The resistant bacteria grow which produces lactic acid that results in change in the color from purple to yellow. Absence of color change indicates that the bacteria are sensitive to the drug. The MASTiK result fits in agreement to KBDD test up to the extent of 80%.

Advanced Diagnostic Techniques

Molecular Diagnostics

Nucleic Acid Based Diagnosis of Mastitis

The complete genome sequence of many of the bacteria involved in bovine

mastitis is now known. The samples can directly be tested for the presence of pathogen specific nucleic acid (RNA or DNA). Polymerase chain reaction (PCR) can be used to detect very low level of infection where the bacterial count is very less. In spite of sophisticated technological and skill involvedin these molecular diagnostics tests which involve high throughput and intensive sample screening, automated methods may result in economic and effective diagnosis of mastitis. PCR has emerged as a robust technique which can detect pathogen in milk sample in matter of few hours which otherwise is difficult in culture method. Therefore, PCR like modern techniques can either replace the classical method or culture based diagnosis or can complement the conventional techniques. Modified version of PCR called multiplex PCR [7,8,9] and Real time PCR [10,11,12] detects closely related microbes in single reaction. Many commercial agencies provide standard kits for detection of pathogens in milk and they are in use in a number of countries. The robustness of nucleic acid based diagnostics can be understood from the fact that 11 of the major mastitis associated bacteria can be detected at species level [13].Molecular diagnostic methods are not only useful for the diagnosis of mastitis organisms, they may also help to identify particularly virulent strains of an organism or distinguish between clonal and non-clonal infection outbreaks. A clonal infection spread from a common source point in the environment and is indicative of contagious transmission of the microbe in the herd. Therefore, such information is helpful in decision making process for improving animal management or adopting clinical measures. Since, microbes possess unique genotype and phenotype with regard to its virulence, persistency and resistance to antibiotics, molecular diagnostic approach can pin-pointedly identify and differentiate the status of mastitis. Some strains of *S. aureus* are involved in persistent IMI which can be readily be identified based on their genetic makeup.

Another technological variation of nucleic acid based diagnostic is NASBA (Nucleic acid sequence based amplification). The advantage of this method is that it can discriminate between dead and living organism. Real Time molecular beacon NASBA has been used to detect *Bacillus cereus* in milk [14].

Research Status in Protein Based Molecular

Diagnostics of Mastitis

Sub clinical mastitis is a serious problem in dairy farm because it cannot be diagnosed accurately using commonly available methods. Effort for identification of different biomarkers that can be used to assess the health of udder is evolving continuously. Protein based diagnosis possess more advantages over DNA/RNA based diagnosis as the former is indirectly involved with patho-physiology of animal. Although nucleic acids are the fundamental molecule behind protein expression, there is often a weak correlation between RNA and protein. Therefore, a protein based biomarker is considered areal indicator of pathophysiology. Initial attempts to study milk protein go back to 1980s. Till recently much research was focused on only major proteins in milk such as ß-lactoglobulin, a-lactalbumin, a-casein, ß-casein, and κ casein.Because of the limited development in instrumentation technologies like 2D gel electrophoresis, Liquid chromatography (LC) and Mass Spectrometry, majority of the studies were carried out on these commonly occurring proteins. Moreover, sample was usually the whole bovine milk [15,16,17,18]. Recently more progress has been made in bovine milk proteomics where the possibility of studying low abundant proteins and peptides has increased tremendously.

Antibody Based Detection of Mastitis

Historically, protein identification has relied on the use of antibodies specific to a particular protein. The presence of the protein in the infected samples is detected by suitable methods like Western blot or ELISA which uses chromogenic or chemiluminiscence assays. The limitation with these techniques is the unavailability of antibody. It is not always possible to find specific antibody against the protein of interest. Moreover, cross reactivity sometimes lead to false positive result. When the unknown protein is the target of study, the problem becomes more serious. During mastitis, various acute phase proteins (APP) are expressed in milk. A few of them are Serum Amyloid A, haptoglobin (Hp), and lipopolysaccharide-binding protein against which antibody based ELISA kits have been developed [3,19, 20].

Detection of Pathogenic Bacteria in Milk Using ELISA

ProStaph is a commercially available ELISA kit which detects *S. aureus* specific antibody in milk. Literature evidences prove that the results are comparable to microbiological tests. The reproducibility of the test is up to 99.2%. However, the applicability of test is limited by the fact that an animal in early phase of infection which is positive in culture method may not be ELISA positive. Or, equally in chronic cases of *S. aureus* infected sample can be ELISA positive but culture negative because of intermittent shedding of pathogen pattern of cows. Additionally, it is also said that the test is not accurate for cows that are <30 days in milk or producing <30 lbs of milk per day. The test sensitivity is 69- 90%. The rate of false negative under rigorous gold standard conditions was around 10-25%. For surveillance purpose, ProStaph is good. However, additional tests are required to confirm the prevalence of mastitis in a herd.

An ELISA test has been developed to determine the antibodies level in milk against *Listeria monocytogenes* [21]. Magnetic bead based ELISA has been developed for the detection of *S. aureus* in milk. This method uses monoclonal antibody coated beads which requires shorter incubation time, and less handling [22]. A few other research reports include use of ELISA to detect pathogens in milk [23,24,25]. However, the results are limited to laboratory scale only. More application oriented conclusions need to be drawn from these works.

ELISA kits have been developed to measure few inflammatory marker molecules which have great potential to serve as biomarker for subclinical mastitis. Hp could be detected quantitatively in the plasma and milk sample of mastitc animals [3,26]. SAA could be detected in mastitic milk which is an acute phase protein and can be used as biomarker for mastitis [27,28,29]. SAA concentration in mastitis milk increases up to 322.26 ug/ ml in comparison to normal milk concentration of 11.67 ug/ml [30]. A sandwich ELISA kit is commercially available from Tridelta Development Ltd, Ireland) to detect SAA in milk. The ELISA kits have been developed for quantitation of Hp and SAA from serum and other biological fluids. However the protocols need further standardization for their assay in milk. These tests have limitations due to their expression in any pathological conditions. Non-specific inflammatory conditions may also cause expression of these molecules in biological fluids

which limits their applicability as confirmatory tests for mastitis. These tests are costly and needs specialized equipments like ELISA. Therefore their applicability is more suitable for organized herds.

Proteomics and Mass-spectrometry based approaches for mastitis biomarker discovery

Recent development in laboratory instrumentation like mass spectrometry and associated techniques (MALDI, ESI, MALDI-TOF/ MS, LC-MS and LC-MS/MS etc.) has promised crucial application in diagnosis of mastitis. Milk contains large number of proteins which are inherent to its composition. In bacterial infection, the overall protein profile changes which includes some bacterial origin proteins and some endogenous proteins from animal body whose normal expression level is altered. With Mass-spectrometry, now it is possible to identify a large number of proteins in a complex matrix like milk. It has removed the dependence on antibody based detection and identification of proteins thus making the process relatively faster, specific and more sensitive. With regard to mastitis, diagnosis interactive interface between host and pathogen is important. Either mammary gland associated changes can be monitored to know the state of udder health or bacteria can be studied with regard to the presence of specific species or strains, its virulence associated factors, cell wall components, antigens or endotoxin produced by bacteria. The latter has specially contributed in the development of vaccine and understanding mechanism of host specific protection from pathogen attack [31,32,33].

Significant research has been conducted in the last 10 years to understand the proteome of milk (lactome) from animals suffering from mastitis. This will help to identify potential biomarkers for the diagnosis of mastitis. Around 80 proteins related to the host response to IMI have been identified in bovine milk using proteomic investigations [34]. Strategies used in investigation of mastitic bovine milk include 2-dimensional gel electrophoresis (2D-GE) to MALDI-time-of-flight (TOF)/MS, or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The changes in milk protein as a result of natural mastitis [35,36,37] and under artificial induction of mastitis using *E. coli* or LPS][38] have been studied. Proteolysis in bovine milk following infusion with lipoteichoic acid isolated from *Staphylococcus aureus* has been studied [39]. Comparisons have been drawn between host defense proteins detected in both human and bovine milk fractions [40]. Various quantification strategies

have likewise been used to assess modulation in the bovine milk proteome during mastitis including densitometry, spectral counting, and incorporation of stable isotopes [35,41,42].

For the first time in the year 2002, Baeker and his colleagues conducted a study on clinical milk samples of mastitis from seven cows using 2D-GE and MALDI-TOF-MS and found that Lipocalin-type prostaglandin D synthase in milk can serve as a new biomarker for bovine mastitis [37], This study despite being limited to the simple sample preparation strategy and the discovery being restricted to only one potential biomarker of inflammation heralded a new age of application of Mass-spectrometry in the diagnosis of bovine mastitis. A lot of effort was made afterwards on sample preparation strategies [43] and optimization of methods for better protein separation [35], because high abundant proteins like casein, ß-lactoglobulin, and a- lactalbumin masked the low abundant proteins. These results revealed that in mastitis blood-milk barrier is affected because of which, serum albumin, transferrin, microsomal triglyceride protein, ß-lactoglobulin, and a-lactalbumin are present in excess in mastitic milk.

Fig. 10.8: The figure shows the workflow for2D-GE.

The protein profile of peak yield milk, colostrum and mastitic milk can be compared using direct LC-MS/MS, as well as 2D-GE followed by MALDI-TOF-MS or LC-MS/MS [36]. This research was the first evidence of the presence of a large number of host response proteins such as apolipoprotein A-1, cathelicidin-1, heat shock protein 70kD protein, peptidoglycan recognition receptor protein (PGRP), calgranulin B and C, and serum amyloid A (SAA) in mastitic milk. Boehmer and coworkers determined the differential expression of host response protein in *E. coli* mediated artificially induced mastitic milk using 2D-GE and peptide sequencing by MALDI-TOF PSD [38]. Many low abundance proteins transthyretin, lactadherin, ß-2-microglobulin precursor, a-1-acid-glycoprotein (A1AG), and complement C3 precursor were identified in whey samples from healthy cows. In mastitic milk, serum albumin, transthyretin and complement C3 precursor levels were found elevated. This study came out with many more new molecules belonging to antimicrobial peptides (AMPs) cathelicidin -1, -2, -3, and -4, and other proteins ß-fibrinogen, a-2-HS- glycoprotein, S100-A12, and a-1-antiproteinase. The presence of cathelicidin cationic AMPs, and the identification of the acute phase protein (APP) A1AG in both normal and mastitic whey samples was a new finding.

A few proteomics studies have been done on mammary gland tissue for understanding the metabolism of milk synthesis [44,45]. Yang and colleagues compared the protein pattern of mammary tissue from healthy and mastitis infected animals [46]. They found that k- casein is upregulated and cytochrome c oxidase and annexin V are down regulated in mastitis.

Proteomics based mastitis diagnosis is still in its infancy where vast amount of background information needs to be gathered. Milk contains large repertoire of proteins with wide dynamic range, variable physiochemical and hydrophobic characteristics and differential post- translational modification in normal and mastitis sample. Moreover, milk composition itself shows a wide range of dynamicity. Therefore, the pursuit for a single, specific, unique, universal methodology or molecule will go a long way. An intensive research approach towards bovine milk proteomics should include optimization of bovine milk protein sample preparation, depletion of high abundant proteins, enrichment of low abundant proteins and availability of high throughput bovine milk database. Keeping in mind that no single method can be sufficient in

biomarker discovery for mastitis diagnostics, a comprehensive and combined approach to include all suitable methods and techniques is the need of time. Concerted efforts to identify biomarkers of mastitis in bovine milk will lead to the development of sensitive, specific and accurate diagnostic kits.

Many natural proteins are either over expressed or down regulated during mastitis. Therefore, a quantitative measurement of proteins in normal and mastitic samples which could be categorically linked to the state of udder health is very much required. Efforts are needed to arrive at a threshold concentration of differentially expressed proteins below or above which the protein can be significantly associated with normal or mastitis sample. Few attempts in this direction have been made in the last six years where relative and absolute quantification of changes in protein abundance was studied [47,48,49,50]. Isobaric tagging for relative and absolute quantification (iTRAQ) has emerged as potential quantitative proteomics tool which can be applied with LC-MS/MS in the study of bovine mastitis. Mastitis pathogens [33], milk fat globular membrane [51] and bovine milk under challenge with LPS [42] have been studied using this technique. Other methods in use for the labeling of proteins and its quantification include metabolic labeling commonly known as stable isotope labeling by amino acids in cell culture or SILAC [52], proteolytic labeling with ^{18}O [53], or isotope incorporation by several means including chemical derivatization also known as isotope coded affinity tags or ICAT [54] and global internal standard technology [55]. In addition to the aforementioned techniques label free methods of quantification like Extracted ion chromatography (XIC) and spectral countof peptides using Mass Spectrometer are also available. Boehmer and colleagues used an ultra-pressure LC instrument coupled to a quadrupole TOF- MS for label free quantitative proteomics on experimentally induced *E. coli* mastitic milk [41]. A select number of lower abundance markers of inflammation including lactoferrin, transferrin, apolipoprotein A-I, fibrinogen, Glycam-1, PGRP, and cathelicidin-1 were identified. In their study they concluded that the accuracy of LC-MS/MS-based label¬free quantification strategies was comparable to antibody-based detection, and therefore, the LC-MS/MS can serve as a viable means of tracking changes in relative protein abundance in milk during disease. Recently conducted iTRAQ based quantitative proteomics has resulted in identification of ITIH4, kininogen, and clusterin as novel biomarkers of mastitis.

Biosensor for the Detection of Mastitis

With increase in automation of milking and many electronic devices capable of detecting analytes (DNA, chemical, proteins, hormones, and metabolites) and changes in physicochemical characteristics of milk, it is now possible to develop integrated biosensors which can determine the quality of milk during milking or immediately after milking. On-line mastitis detection program in some of the robotic milking farms now measures electrical conductivity, SCC, color of milk during milking. Although individually, these parameters may not be true indicator of mastitis but given a robust algorithm which includes all these results at a time and can interpret the data, it will serve as very highly reliable and desirable method of mastitis diagnosis at dairy farm.

Biosensor Against Altered Milk Constituents

An electronic tongue has been prepared which is a sort of chemical array based sensor [56], It can detect and measure CI", K^+, and Na^+ and various other organic and inorganic cations and anions in milk. The device could differentiate mastitic sample from normal milk with a specificity and sensitivity of 96% and 93% respectively. Mas-D-Tec is a product from USA which is used as electronic mastitis indicator (Fig. 10.9). Mas-D-Tec measures the levels of sodium and chloride in the milk. These are increased by bacterial infections. A small sample of quarter milk is squirted into the funnel top. Within two seconds the milk conductivity is analyzed and indicated on the graphic scale. The milk drains through the Mas-D-Tec as the readings are taken. There is no need to tip the milk out. There is no need to wash the Mas-D-Tec between quarters and cows. Washing at the end of milking is all that is required. Mas-D-Tec is so fast to use that it is possible to test the whole herd during milking. Mas-D-Tec should be used after herd tests to determine the quarter giving a cow's sample a high cell count, or used strategically when bulk milk cell counts are rising. Badly infected quarters may give milk with perfectly good appearance, but have cell counts in the millions. Often the only way to find these infected quarters is by conductivity testing of the whole herd. It operates on electricity or battery. Mas-D-Tec uses a scale marked Normal and Abnormal. These classifications are entirely arbitrary. Any milk

conductivity can be judged only on its relationship to adjacent quarters.

Fig. 10.9: Mas-De-Tec instrument to measure Sodium and Chloride ions in milk

Lactate concentration in milk increases due to high somatic cell activity in the absence of sufficient oxygen in mastitic milk. The biosensor containing lactate oxidase enzyme at the analyte interactive surface reduces lactate and produces electrons. The resulting current is measured using potentiostat which in turn can be correlated to the level of lactate in milk. The result of this method bears high correlation with SCC data [57].

Pemberton and coworkers developed an electro biochemical biosensor to detect NAGase level in milk. It contains the substrate 1- naphthyl N acetyl beta- D- glucosaminidine [58]. Enzyme present in the milk acts on the substrate printed on the sensor's interactive surface and converts the substrate to 1-naphthol. The detector electrode then measures the signal. The limit of detection of this system was found to be 10mU/ml.

A competitive biosensor was developed to diagnose subclinical mastitis [59]. It uses the principle of interaction between hemoglobin (Hb) and Hp. Hence, if the sample is mixed with known concentration Hp, the Hb present in milk binds to it and is sequestered. Remaining Hb in milk binds to the Hp immobilized on to the chip surface and results in signals that can be correlated to the concentration of Hp present in milk. It is suitable to detect subclinical mastitis because ideally Hb is almost nil in this state of mastitis.

The principle that has been used in CMT of detecting mastitis has also been developed in the form biosensor. Wu and colleagues developed a method where DNA and protein liberated from cell lysis is incubated with PicoGreen and the resulting fluorescence is measured using an optical sensor [60]. This assay showed good correlation with Fossomatic determination of SCC

(R^2=0.918).

Biosensors Against Pathogens

Similarly, electronic nose have been prepared which can distinguish mastitic milk from normal milk by sensing volatile substance (Sulphides, ketones, amines and acids) present in milk [61]. Hettinga and colleagues measured volatile bacterial metabolites in milk and identified different mastitis specific pathogens [40].

Chip Based Diagnostics

Recently, the knowledge in microfludics is growing at a faster rate. It has been possible to make biochip withthin capillaries which can carry nano and pico liters of sample. Thus, miniaturized devices can be made which can handle large number of samples at a time. Moon and colleagues, developed a disposable microchip to measure SCC in milk. In this technique, first the sample is mixed with lysis solution and a fluorescent dye is added which binds to released DNA [62]. When loaded on microchip it is evenly distributed and fluorescence is read.

Another method based on counting of leukocytes on microfludic chamber under fluorescence microscope is also developed [63], Milk is mixed on the chamber slide with metachromatic substance which stains the leukocytes. Stained leukocytes are distributed in micro capillaries and can be counted. A comprehensive chip based diagnostic was developed by Choi and coworkers which could simultaneously detect and measure pathogen, somatic cells and pH of raw milk [64]. Pathogen and somatic cells detection was based on antibody-antigen interaction and pH could be monitored by fluorescence based hydrogel entrapped pH indicator.

A PCR based DNA chip (Fig. 10.10) was developed by Lee and colleagues, which can detect around seven known mastitis causing pathogens [65], It can detect *Corynebacteriumbovis, Mycoplasma bovis, Staphylococcus aureus* and the Streptococcus *spp. S agalactiae, S. bovis, S. disagalactiae*and *S. uberis* within 6 hours with a detection limit of 10^3-

10^4cfu/ml. It basically includes four steps: DNA extraction from bacteria, PCR, DNA hybridization, and colorimetric reaction. A similar microfluidic device that integrates solid phase extraction and NASBA has recently been reported for the identification of low numbers of E. *coli* [66],

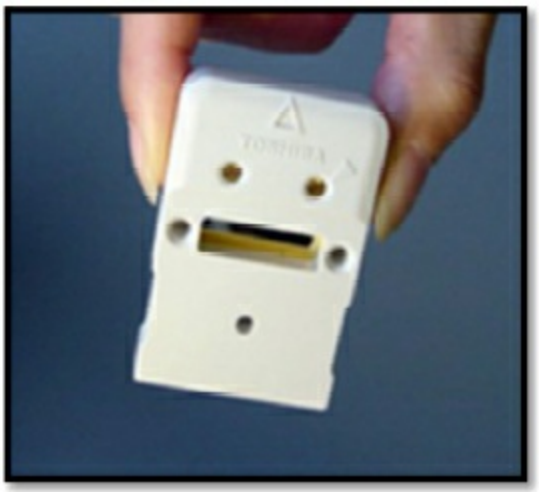

Fig. 10.10: DNA based chip.

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Chapter 11
Pharmacological Concerns for Treatment of Mastitis

Effective and economic way to check the mastitis is based on prevention strategies; however, treatment of mastitis during lactation is an inevitable tool to control the mastitis in dairy farms. The practices of mastitis treatment have been initiated long back with antiseptic solution and it became popular after antibiotic discovery. Currently, mastitis is one of the major reasons for antibiotic usages in dairy farms and more than 50% of total drug usage in a farm is for mastitis treatment. Besides, it was also found to be major reason for antibiotic residue in milk and milk products [1]. However, consensus about the most efficient, safe, and economical treatment is still lacking against mastitis. The complex nature of mastitis triangle i.e. cow, pathogen and environment factors pose difficult in therapeutic management. Readers are suggested to refer the figure in chapter 1 for details of factors contributing to development of mastitis in dairy animals. Further, lack of suitable clinical studies, improper assessment of treatment outcome, and geographical variation in prevalence of major mastitis pathogens makes mastitis treatment as a challenging program for dairy farmers and veterinarians. Further, unlike production related data, health data (e.g. mastitis treatment record) were not exploited as much for health monitoring purposes in farm. On other hand, understanding of mastitis treatment related data would be very helpful to improve the existing treatment strategies in future. Further, understanding of current mastitis treatment strategies and various reasons for its failure are important pharmacological concerns to improve the future treatment protocol. Despite the significant contribution of antibiotics in improving animal health over last few decades, use of antibiotics in food-producing animals has raised concern from public health, food safety and regulatory view due to development of anti-microbial

resistance from veterinary use of antibiotics [2], On other hand, how far the veterinary practices with antibiotics causes the antibiotic resistance is not clear. Therefore, understanding of the trend of antibiotic usage and its impact on the animals and public health are important to take policy decision by regulatory authorities. For instance, Norway and Sweden developed comprehensive policies and guidelines on drug therapy in mastitis to prevent prudent use of antibiotics in mastitis and to preserve its future usefulness. As a consequence of these policies, attention has been drawn to the need for reliable data on the use of antibacterial drugs in mastitis therapy [3], Therefore, understanding the current status of mastitis treatment practices, recent perception in pharmacodynamic as well as pharmacokinetic of drugs with reference to mastitis therapy, recent advances in evaluation of treatment efficacy and concerns related to antibiotic resistance are considered as important pharmacological concerns.

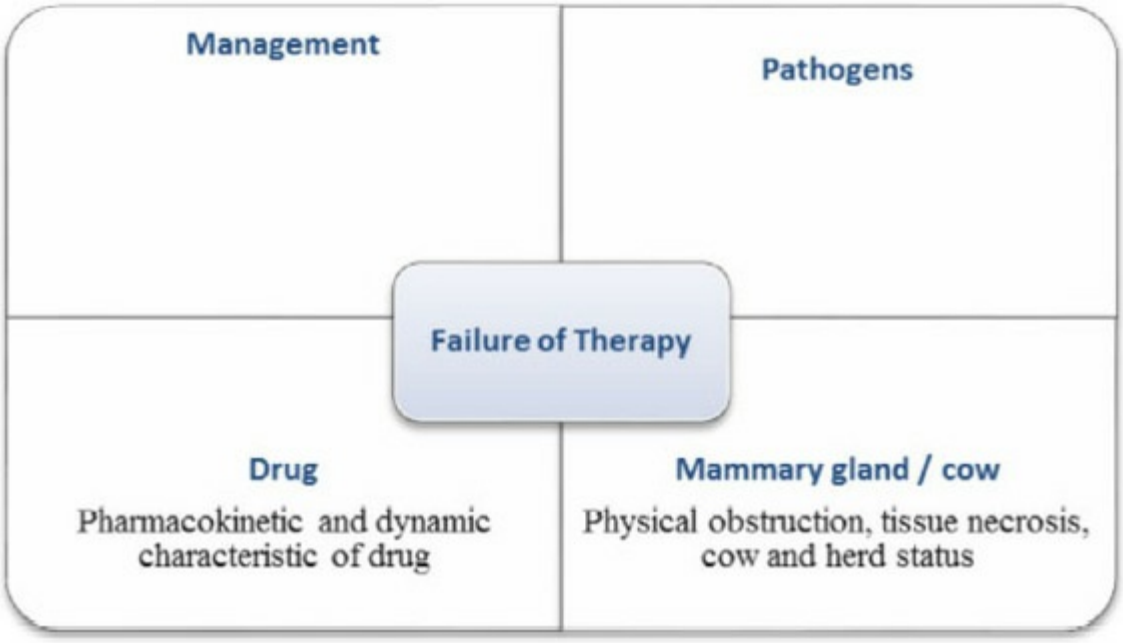

Fig. 11.1 : Factors associated with treatment failure in mastitis [4],

Current status of Mastitis Therapy During Lactation

Although the understanding on etiology, pathogenesis and therapeutic targets has been improved significantly, the treatment of mastitis has been mostly

based on personal experiences. The recent concept of mastitis therapy is based on severity of infection and culture or pathogen-directed decision [5], The drawback in blanket therapy (treating all mastitis animals) is believed as an inappropriate and needless use of antibiotics. Since, the parts of the clinical mastitis samples were shown to be "no growth", mild in severity and resistant to selected drug, it has been suggested that culture based therapy would be very useful to decrease the unjustified antibiotic usage in mastitis [6]. Further, on farm culture methods are useful in determination of gram status of the pathogens, most sensitive drugs, treatment outcome and thus higher bacterial cure with less antibiotic residues problems. However, culture-based therapy is also not fool-proof. Limitations in diagnostic facility and treatment efficacy (success) of most sensitive drug (at *in vitro)* could be the reasons for poor adoption of culture methods. Despite of differential opinion, several studies have demonstrated the benefits and feasibility of on farm culture based therapy [6, 7].

Determination of severity of clinical mastitis is believed to be first step in therapeutic management and it has been classified as mild, moderate and severe [8]. Only changes in milk is defined as mild cases while, abnormality in milk and udder parenchyma is considered as moderate and clinical mastitis with systemic involvement is defined as severe mastitis which is mostly associated with coliform organisms. However, severe mastitis occurs also due to other organisms such as environmental Streptococci, *Arcanobacterium pyogenes* and yeast. Although the indicator or definition of severity varies with different researchers, it has been suggested that mild cases can be treated after culture results. However, moderate cases should be evaluated cautiously for its increasing severity. On the other hand, severe clinical mastitis should be treated immediately as 10-20% of affected cows may die within 24 hours. In general, increased duration of treatment or number of drugs was suggested with increasing mastitis severity.

Although, antibiotic therapy is considered as secondary importance, it remains an integral part of therapeutic management for severe coliform mastitis and immediate treatment with fluids, non¬steroidal anti-inflammatory drugs (NSAIDs) and other supportive care for endotoxic shock is important. On other hand, the existing reports of NSAIDs (phenybutazone, flunixin melamine and melaxicam) efficacy are differential [9, 10]) and many of these studies were conducted in experimentally induced mastitis cases and needs to be evaluated in naturally occurring severe or moderate mastitis cases. Further, beneficial

effects of steroidal drugs such as dexamethasone and isoflupredone on experimentally induced mastitis cases have been reported [11, 12]. They suggested that maximum beneficial effects of glucocorticoides in endotoxin-induced coliform mastitis particularly during early course of the disease. Further, dosage appears to be an important determinant of treatment outcome in endotoxic mastitis, as administration of lower dose (30mg) of dexamethasone effectively reduced udder swelling, improved rumen motility and milk yield than higher dose (0.44mg/kg). Further, the beneficial effects of dexamethasone and isoflupredone were observed in experimentally induced mastitis, when they administered before appearance of systemic signs. On other hand, the situation in clinical cases is different and no data is available on effects of glucocorticoides in natural clinical conditions.Further, the glucocorticoides - induced immunosuppression and - pregnancy loss are the major concern for its usage. However, over all benefits and absence of much clear contraindication of these drugs outweigh the minimal risk by one-time treatment at early course of mastitis. Further, the effects ofglucocorticoides via intra-mammary administration, with or without antibiotic to be explored as local route appear to be more attractive than systemic action. The data related to beneficial effects of ancillary treatment such as frequent stripping of milk, oxytocin treatment, vitamin and mineral supplementation, calcium therapy, anti-fibrinolytic preparations are limited in global and Indian dairy animals and hence need to be evaluated.

Why Treatment has not been Optimized?

Till to date, the standard therapeutic regimen for severe clinical mastitis is not available even in developed countries. Therefore, veterinarian and farmers often treat with personal and herd experience. Variation in treatment outcome between natural cases and experiment model could be the possible reason for failure in optimization of therapeutic regimen. Non-inclusion of recently calved cow's, challenge the same strain with its virulent as in natural cases and often failure to have postpartum complications like compromised immune status, metabolic and environmental stresses in experimental animals collectively contribute the differential outcome between natural and experimental infections. Further, non inclusion of non-treated control in efficacy studies could also be a possible reason for differential outcome

between natural cases and experiment model. Less exploitation of recent diagnostic techniques like PCR to identify the gram (positive or negative) status of pathogens and failure to improve the *in vitro* drug sensitivity results through recent understanding of pharmacokinetics and pharmacodynamics of drugs are also believed to be important reasons for poor optimization of therapy. Severity of mastitis, parity, number of mastitis episodes in single lactation and causative pathogens are influencing the treatment outcome and these factors needs to be taken consideration while making mastitis therapy trials [13]. The following points need to be considered during evaluation of clinical mastitis treatment outcome [5].

- Utilization of naturally occurring clinical mastitis cases for clinical trial
- Inclusion of non-treated control group in experiments
- Define the severity levels studied and treatment outcome parameters
- Parameters are objective as possible, quick, easy to perform and repeatable
- Full control of researchers on cows
- Conduct of bacteriological culture prior to treatment, during treatment, immediately after milk withdrawal time and 3-4 weeks after the first treatment. For studies with chronic pathogens (e.g.,*S.aureus),* cultures should be continued for at least 2 months.
- Treatments are assigned randomly (systematically or truly random) and the method of randomization (e.g., based on parity, milk yield, etc.) should be specifically declared
- Treatment outcomes are assessed by severity level (mild, moderate, and severe).
- Treatment outcomes are assessed by etiologic agent

Assessment of Treatment Outcome

The main objective of mastitis therapy is to achieve clinical cure as early possible, reduce the disposal of milk and restore the maximum production ability of animals after treatment. Understanding the truth of small proportion of animals in herd for entire mastitis incidence has changed the concept of clinical cure into bacterial cure after mastitis therapy. Clinical cure is usually defined as appearance of normal milk after treatment of clinical mastitis. It

also includes the recovery of other clinical symptoms such as swelling of the udder, milk yield or body temperature and appetite. Bacteriologic cure is defined as the absence of isolated bacteria from milk samples obtained after 14-21 days of treatment. However, the different time periods were used by different researchers for the collection milk samples after treatment. The ability to achieve a bacteriological cure depends on the type of pathogen, severity, immunity of cows, and efficacy of the treatment protocol and the promptness of initiating treatment [14]. Collectively it suggests that better understanding of factors associated with successful therapeutic outcomes would help to make better treatment decisions. Among the various suggested parameters by different researchers, recurrence of clinical mastitis, somatic cell count, milk production, and cow survival or culling after clinical mastitis were mostly studied. Recording the complete and accurate data regarding mastitis incidence and its treatment is the foremost step in monitoring programme, but the ability to assess the results of treatment is often limited because of inadequate records [15]. Further, record systems also help to evaluate the effectiveness of treatment regimens for mastitis and culling decision. Some important questions for which the data set should provide answers are, i) the monthly incidence of clinical mastitis, ii) cause, iii) antibiotics used to treat clinical mastitis, iv) number of clinical mastitis episodes required re-treatment, v) number of cows had more than one mastitis episode in the same or different quarter in current lactation, vi) duration of cows with clinical mastitis in hospital, vii) number of quarters dried and, viii) dead or are culled due to clinical mastitis [16]. Among the various treatment outcomes, recurrence of clinical mastitis case is one of the least desirable outcomes after treatment and the possibility of recurrence is much more for animals that suffered in early lactation as compared to late lactation [17]. It suggested for more aggressive treatment protocols (e.g., longer duration therapy) for cows experiencing mastitis in early lactation as compared to treatments for cases that occur later.

Relapse and Recurrence of Mastitis

Relapse and recurrence are defined as the occurrence of clinical mastitis cases in any quarter of the same cow after treatment in particular lactation. Recurrence has been described by some researchers as *"another case of*

clinical mastitis" in the same cow, in the same quarter, or by the same pathogen [18, 19, 20, 21]. However, producers often define recurrence as another case of clinical mastitis in the same cow, independently of quarter or pathogen for practical purposes. The interval used to define a new case (rather than a recurrence) varies among studies ranging from 8 to 90 days or longer [18, 19, 20, 21]. Basically both terms are used to define the treatment efficacy, but the different duration has been suggested by different researchers. Relapse for clinical cases occur within three weeks post-treatment period [22] whereas, recurrence within clinical mastitis during 60 days follow-up period [23]. Outcome variable may be defined as "clinical cure" if no treatment for clinical mastitis in the 30 days following enrolment or as a "treatment failure" if treatment of any gland within 30 days following enrolment of the cow [24]. Recurrence can result from new infections or due to a failure to eliminate infection as a result or either insufficient treatment or treatment failure.

The probability of recurrence have been reported to be around 20% [17,18, 25]) and is known to vary with parity, days in milk, duration of treatment, bacteriological cure after treatment. For instances, the overall probability of recurrence was estimated at 13 and 23% for primiparous and multiparous cows. Further, the shorter duration of treatment caused more recurrence and cows with bacteriological cure after treatment suffered less recurrence than cows that did not experience bacteriological cure [23]. Although, detailed studies on estimation of recurrence of clinical mastitis has been just initiated across the world, no such study is available in India. Recently, the authors estimated the different episodes of clinical mastitis and its relationship with duration of treatment and seasonality and found 62% of single episodes, 15% of relapse (treated within 21 days of first treatment) and 15% recurrence (treated after 21 days of first treatment) in crossbred cows in organized dairy farm (Fig: 11.2). Further, the authors found that 53% of single episodes 29% of relapse and 6.45% recurrence were found in Murrah buffaloes in same organized dairy farm (Fig. 11.3). Interestingly, about 8% of the crossbred cows and 12% of Murrah Buffaloes were suffered by both relapse and recurrence. Collectively it suggests that more than one episodes of clinical mastitis in crossbred cows were due to both relapse and recurrence; while in buffaloes, it was mostly due to relapse than recurrence. Variations in pharmacokinetic and/or pharmacodynamics of drugs in buffaloes could be the possible reason for observed result. Thus, prediction of recurrence cases would be useful to make treatment strategy in organized farm, where treating all the animals or

episodes may not be economical. Despite the lower incidence of clinical mastitis during summer, the magnitude of relapse and recurrence was higher than single episode during summer compared to winter in our study suggest that treatment efficacy was adversely affected by heat stress. Therefore, studies on probability of recurrence of clinical mastitis by quarter, major pathogens, stage of lactation, parity, seasons, possible drug factors, cost of recurrences etc., need to be evaluated in future. For instance, among the cows with recurrent cases, 46% occurred in a different quarter and 54% in the same quarter [23], Studies on possible association between duration of treatment, severity of infections, pathogens type and recurrent episodes of mastitis in same or different quarters will improve the understanding of recurrence of clinical mastitis.

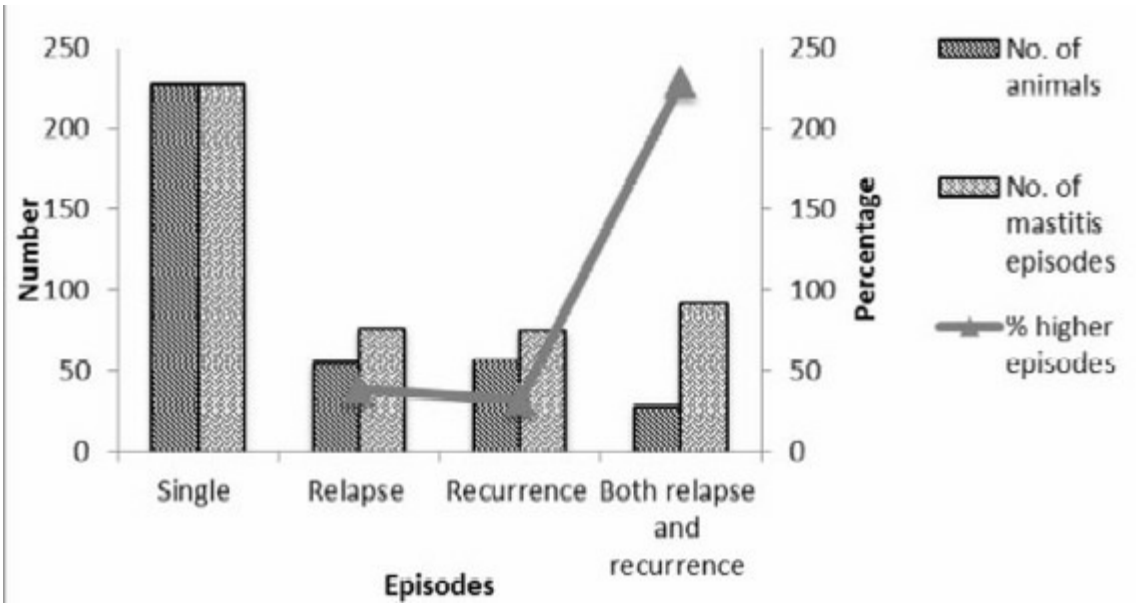

Fig. 11.2 : Different episodes of clinical mastitis in crossbred cows

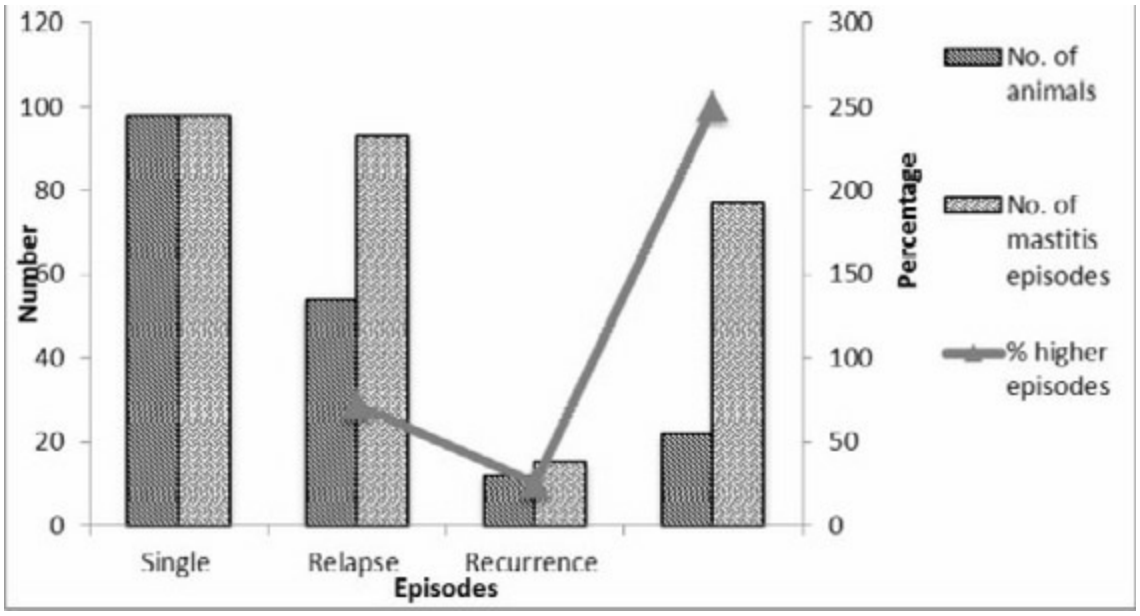

Fig. 11.3 : Different episodes of clinical mastitis in Murrah buffaloes

Evaluation of Clinical Outcome: A way to Disclose the Economics of Treatment

Although milk loss due to mastitis is believed to be major cause for an economic loss, treatment of clinical mastitis is also a cause of considerable loss to dairy farmers. Hence, the decision to treat a mastitic cow must be cost-effective and the cost of treatment must be less than the cost of untreated quarter. Apart from clinical and bacteriological cure, evaluation of treatments based on economic outcomes such as recurrence of mastitis in the same cow (should be <20% of cows), return of somatic cell counts to acceptable levels <200,000 cells/ml (within two months), retention of the cow within the herd (not culled within 60 days of mastitis event) and return to normal milk yield with no loss of quarter are important in future. Assessments of clinical outcome or efficacy studies of different antibiotics against different pathogens are the basics to understand the economic implications of treatment strategies.

Pharmacokinetics and Pharmaco-dynamic Consideration of Mastitis Treatment

Although the microbial isolation, identification and subsequent antibiogram is the basic pillar for rational use of antibiotics in mastitis treatment, failure of drugs to perform at *in vivo* conditions strongly suggesting that inadequate concentration of drugs at target site and/ or insufficient contact time of drugs with pathogens. Therefore, more understanding of pharmacokinetics (what body does do the drug?) and pharmacodynamics (what drug does to the body?) of antibiotics in milk producing healthy and mastitis animals would be very useful for mastitis therapy. Although, the understanding of basic pharmacokinetics parameters like time period of antibiotic above minimum inhibitory concentration (MIC), peak plasma concentration (C_{max}) divided by MIC and area under the concentration-time curve (AUC) divided by MIC are clear, the application of these indexes in extracellular infection and/ or in well vascularized organs are limited as these parameters are based on serum antibiotic levels [26]. On other hand, antibiotics PK/PD relationship will correlate the relationship between antibiotic concentrations in blood, target site and its clinical outcome [27]. Further, understanding of host-drug-pathogen relationship will greatly improve the clinical or treatment outcome through PK/PD-mediated optimization of dosage regimen.

Traditionally mastitis therapy has been done through systemic and intra-mammary route. Although systemic therapy has several advantages, the bioavailability of drug at milk or mammary tissue and its stasis are important criteria for clinical outcome. In both the routes, high lipid solubility, poor degree of ionization and plasma- and milk- protein binding capacity of drugs makes better bioavailability at milk compartment. Based on these physiochemical properties, it is believed that, weak organic base drugs like macrolides group attain sufficient concentration in milk after parenteral administration. On other hand, weak organic acids such as penicillin and cephalosporins group of antibiotics attains significantly lower concentration in milk than blood of normal animals. However, the changes of physicochemical properties of milk during mastitis such as composition, ions, proteins, inflammatory cells, pH, conductivity, viscosity, density, etc. were shown to be significantly affecting the kinetics of drugs. For instance, the higher pH of milk during mastitis will significantly affects the ion trapping-mediated passage of organic base drugs into milk when they administered through parenteral route, particularly if the drug has poor lipid solubility property [28]. On other hand, higher pH of milk during mastitis is believed to favor the movement of organic acid drugs into milk. However, it is possible only when milk pH is more than

7.4. Collectively, it suggests that organic base drugs have more advantages than organic acids in distribution into milk. Although drug factor favors for organic bases, the report of practical usage of antibiotics in organized farm of northern India suggested that penicillin group and their combinations were most commonly used for clinical mastitis. Similarly, penicillin has been the most commonly used drug against mastitis in conventional dairy farms at Michigan, Minnesota, New York and Wisconsin area in USA [29]. Other studies have also reported that β-lactams drugs were most commonly used for treatment of mastitis across the globe [30, 31]. Globally, weak organic base drugs were rarely used for clinical mastitis. Therefore, the existing pharmacological knowledge of drugs and its practical application needs to be revisited. Clinical evaluation of drugs in suitable clinical models and more appropriate tests for assessing the clinical or treatment outcome are important pharmacological concerns in mastitis research. Understanding of various *in vitro* and *in vivo* factors, which affects the pharmacokinetics and pharmacodynamics of drugs through new PK/PD approaches, would definitely yield better results in this concern.

Pharmacokinetic Consideration of Mastitis Therapy

The goal of antibiotic therapy in mastitis is same like all other microbial infections such as to selection of most suitable antibiotic, attaining and maintenance of effective concentrations of drugs at the target site (site of infection) for sufficient period of time, minimizing the side effects and administration of needful supportive therapy. There are three potential pharmacological compartments (therapeutic targets) has been recognized in bovine mastitis; Milk and duct system, udder tissue and cow (Table 11.1). Milk and duct system is the most common site of infection and intra-mammary administration of antibiotics is believed as most appropriate way to check the pathogens reside in this compartment. However, several limitations such as i) elimination of drugs during routine milking and through blood ii) little or no activity of available products against gram-negative pathogens iii) interference of micro abscess or fibrin casts on drug accessibility particularly in chronic intra-mammary infections, are need to be overcome for better therapeutic management through local route.

Table 11.1: Target of antibiotic therapy in clinical mastitis [32]

Organisms	Milk and duct system	udder tissue	cow
Str. agalactiae	+++	—	—
Other streptococci	+++	+	—
S. aureus	+	+++	—
CNS	+++	—	—
Coliforms	+	—	+++

Infection in udder parenchyma with *S. aureus* or *S. uberis* is another challenging pharmacological compartment and administration of drugs via systemic and intra-mammary route has been suggested for better efficacy. The ideal drug for parenteral mastitis therapy would have (1) low MIC against the majority of udder pathogens, (2) high bio-availability from intramuscular injection sites, (3) weakly basic or non-ionized form in serum, (4) lipid soluble, (5) low degree of protein binding, (6) long half-life in the body, (7) retain activity in infiammatory secretions, and (8) clearance from body normally without accumulation in specific organs [28]. Therefore, the future strategies of mastitis therapy should be, development of drugs, which can able to penetrate phagocytes and its retention for long period, no substrate or metabolism in cells, effective at low pH environment and effective through local route. Further, development of drug delivery system like micro¬particles, loposomes and nanoparticles would be very great useful to achieve the desired concentration at remote places.

Pharmaco-Dynamic Consideration of Mastitis Therapy

Pharmaco-dynamic consideration of selected drug is an important factor to gets effective clinical outcome. Among the several factors, mechanism of action and post antibiotic effects (PAE) duration are important determinants of drug effectiveness. Based on mechanism of action, antibiotics are broadly classified into time- and concentrate- dependent drugs. Maximum clinical effectiveness of time-dependent drugs like a-lactum antibiotics depends on duration of time the organisms are exposed to drug at target site. Therefore, increasing concentration of drug, higher than MIC of pathogen does not

increase the efficacy of drugs. In other words, increase the frequency of administration rather than high dose regimen will increase the efficacy of drugs. However, the degree of higher dose with maximum efficacy and no adverse effects against major mastitis pathogen for time- dependent drugs are need to be evaluated. The efficacy of concentration dependent drugs such as aminoglycosides and fluoroquinolones can be increased with higher concentration above MIC of the bacterial pathogen. In general, for concentration-dependent killing drugs, $_{Cmax}$/MIC ratios is considered as important parameter when the pathogen has a high MIC value or is rapidly proliferating [33] while AUC/MIC should be considered as a major parameter when the infection is caused by relatively slow growing bacteria, or when the MIC for the pathogen is relatively low and when there is little or no PAE. Evaluation of more sensitive drugs against major mastitis pathogens through these PK/PD parameters rather than traditional parameters may increase the clinical outcome.

PAE is defined as persistent suppression of bacterial growth after a brief exposure to antibiotics even in the absence of host defense mechanism. It is considered as very good property of drug, as ability of drugs to suppress the growth of susceptible bacterium even after the drug concentration fall below the MIC level. It is believed that the recurrent episodes of clinical mastitis are due to re-infection from the environmental pool or as a result of persistence of the organism within the mammary gland [34]. Further, the adaptive response of pathogens or inabilities of host defense to clear infections are also reasons for persistent infections [35]. Persistent of intra-mammary infection after initial treatment could be possible when some resistant "subpopulations" those are less susceptible to particular antibiotics exist and then re-populate later at infection site [36]. Therefore, maximum concentration at which prolonged or effective PAE of drug (C_{max}/MIC ratio) need to be optimized to decrease the resistant population. However, duration of the PAE depend on class of antibiotic, duration of antibiotic exposure and bacterial species. For instance, β-lactam antibiotics have prolonged PAE against gram positive pathogens, while it has brief or no PAE against gram negative organism. Further, the more variation between *in vitro* and *in vivo* conditions suggests that there should be caution while interpreting the results. At present, the drug potency are mostly assessed through MIC and MBC (minimum bactericidal concentration), which are often not same. Further, both *in vitro* parameters do not consider the effects

of serum, killing ability of drugs, PAE, etc. and drug potency are mostly evaluated against actively growing bacteria rather than G_0 phase or biofilm. Collectively, this could be the reason for variation between *in vitro* and *in vivo* conditions and better optimization of *in vitro* methods will greatly improve the treatment outcome.

Concerns of Antibiotic Usage in Food Producing Animals

Although, the use of antibiotics in food-producing animals significantly reduced the morbidity and mortality of diseases and thus more productivity of animals, there is considerable concern from public health, food safety, and regulatory perspectives about the use of antimicrobials in food-producing animals. Development of antibiotic resistance or drug residue due to usage of drugs in food producing animals is the major public health concern. However, it is not clear that how far the antibiotics usage in food animal origin contributing the resistance problems. Four important questions regarding use of antibiotics in adult dairy cows were raised (1) Are science-based data available to demonstrate antimicrobial resistance in veterinary pathogens that cause disease in dairy cows? (2) Are science-based data available to demonstrate that antimicrobial resistance in veterinary pathogens that also has public health concern? (3)Does antimicrobial resistance impact the outcome of therapy? (4)Are antibiotics used judicially in the dairy industry? They concluded that routinely used antibiotic in adult dairy cows has not increased antimicrobial resistance [2]. Further, the existing data does not support the resistance among mastitis pathogens to antibiotics have been used several decades in the dairy industry. However, it is clear that use of antibiotics in food- producing animals does contribute to increased antimicrobial resistance. They opined that those advantages of using antibiotics in adult dairy cows far outweigh the drawbacks, as clinical consequences of antimicrobial resistance of dairy pathogens affecting humans appear small as long as the milk and meat is pasteurized or cooked properly. However, concerns are with people who choose to consume raw milk and improperly cooked meat. The data related to mastitis pathogens, its antibiogram and practical usage of antibiotics during last many decades in Indian dairy farm is not available to answer those important questions in Indian context. Recently we estimated the antibiotics

usage for treatment of clinical mastitis in organized dairy farm over a period of six months and found enrofloxacin (22%), ampicilin with cloxacillin (18.57%), gentamicin (18.29) and ceftriaxone (12.57%) drugs were most commonly used against clinical mastitis cases. Further, penicillin group and their combinations (29.43%) were found to be the most commonly used antibiotic followed by fluoroquinolone (22%), aminoglycosides groups (21.43%) and cephalosporins group (16.86%) for mastitis. Tetracyclines (9.14%) and chloramphenicol (1.14%) were the least choice. Longer duration studies on this area would be useful to understand linkage between antibiotic usage and resistance development. In order to control the mastitis, problems associated with bacterial antimicrobial resistance and thus treatment failure against mastitis pathogens are to be addressed. Besides, the surveillance programs to assess the resistance phenotypes, understanding of the genetic background of resistance mechanism etc., may also aid in our efforts to control antimicrobial resistance.

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Chapter 12
Judicious Use of Antibiotics in Mastitis Therapy

Some basic knowledge of pharmacological principles is important for the success of antimicrobial therapy. The decision whether the antimicrobial therapy is required should be based on certain questions that the clinician can ask himself.

1. Is the antimicrobial therapy indicated on the basis of the clinical findings or it is prudent to wait?
2. Have the appropriate samples for the microbiological procedures been collected?
3. What is likely pathogen?
4. Is there prior evidence that the antimicrobial therapy will confer clinical benefit?

Based on these, empirical therapy is initiated then it is reassessed on the basis of the microbiological findings. If the specific pathogen is identified, can a narrow spectrum agent be substituted for the drug initially used? Similarly, it is assessed if a combination is better option.

Empirical Therapy

The antimicrobial agents are used before the pathogen is identified or susceptibility tested are performed. This use of antimicrobial agents is empirical (presumptive) therapy. This is usually based on the experience of the clinician and is given with the presumption that the early intervention will

improve the clinical outcome. The initiation of the empirical therapy should follow a specific and scientific approach. The selection of the drug for empirical therapy is based upon clinical diagnosis and spot microbiological procedures. If no information is available, then a broad spectrum agent is given which has spectrum against the likely pathogen. Among other factors which determine the selection of the drug include the disease state, pharmacokinetics of the agents, interaction with other agents, toxicity etc.

Culture and Susceptibility Test (CST)

Culture and susceptibility test not only identifies the infecting pathogen but also provides specific data regarding the drug efficacy. The distinction between gram positive and negative microorganisms can determine the choice of the agent. On an average the initial antimicrobial therapy is substituted in one third of the cases.

The susceptibility tests are performed by two methods (1) disk diffusion method (2) tube dilution method. Each method has its limitations but still provides useful information. In disk diffusion, the size of zone of inhibition is directly related to the MIC of the antimicrobial agent against particular microbe, thus this method so semi-quantitative. The drawback is that not all microbes grow rapidly and this is suitable only for rapidly growing aerobic organisms. The tube dilution method, has serial dilutions of the antimicrobial agent, determines the MIC and MBC against a particular organism. Break point MIC is the plasma concentrations that can be achieved by administering clinical acceptable doses and route of administration. More is the difference between MIC and the break point MIC, more efficient will be antimicrobial agents. Apart from the susceptibility data, the characteristics of the antimicrobial agent and host factors can also determine the choice of the antimicrobial agent.

Table 12.1: List of Bactericidal and bacteriostatic drugs used in treatment of mastitis

Bactericidal Agents	Bacteristatic Agents
Aminoglycosides	Chloramphenicol
Bacitracin	Clinamycin

Beta-lactams	Marcolides
Metronidazole	Sufonamides
Isoniazid	Tetracycline
Polymixin	Trimethoprim
Quinolones	
Rifamicin	
Vancomycin	

Bactericidal versus Bacteristatic antimicrobials: Antimicrobial agent can classified bactericidal or bacteristatic. The agents which are static have a much large difference between the concentrations which is inhibitory (MIC) and which are cidal (MBC). This is in contrast to the cidal which have less difference in these values. In general, the antimicrobial agents acting on the cell wall are cidal, whereas those acting on the protein synthesis are static.

Antimicrobial Agent Combinations: There are conditions where the combination therapy is required so as to:

1. Provide broad spectrum empirical therapy. This is usually with anti-staphylococcal agent and agent that have activity against gram negative bacteria.
2. Treat poly-microbial infections as in abscess and enhance microbe killing.
3. Reduce the emergence of resistance as in treatment.
4. Decrease the dose and related toxicity.

When two antimicrobial agents are administered together, it could lead to additive, synergistic, or antagonistic effect. The interaction between two is best explained by FIC index. FIC index value of = 0.5, 1 or = 2 indicate synergistic, additive and antagonistic effect. Synergistic effect is produced when both antimicrobials which have bactericidal effect. Additive effect is produced by antimicrobials which have bacteriostatic effects whereas antagonism is by those where one has bactericidal and the other has bacteriostatic effect. Bactericidal agents can be divided into two further

categories:

1. Exhibiting concentration dependent effect e.g. aminoglycosides, quinolones.
2. Exhibiting contact time dependent effect e.g. penicillins, vancomycin.

In concentration dependent effect, the cidal affect i.e. both extent and rate increases with the increasing concentrations. Thus maximizing the concentrations will improve the efficacy of the antimicrobial agent and decreases the selection of the resistance bacteria. Clinically these agents should be used as a single high dose rather than repeated divided doses. Drugs producing contact time dependent cidal effect, their efficacy does not increases by increasing concentrations and cidal effect is present till MBC is maintained. Thus these antimicrobial agents are given frequently in divided doses. These drugs also lack post antibiotic effect.

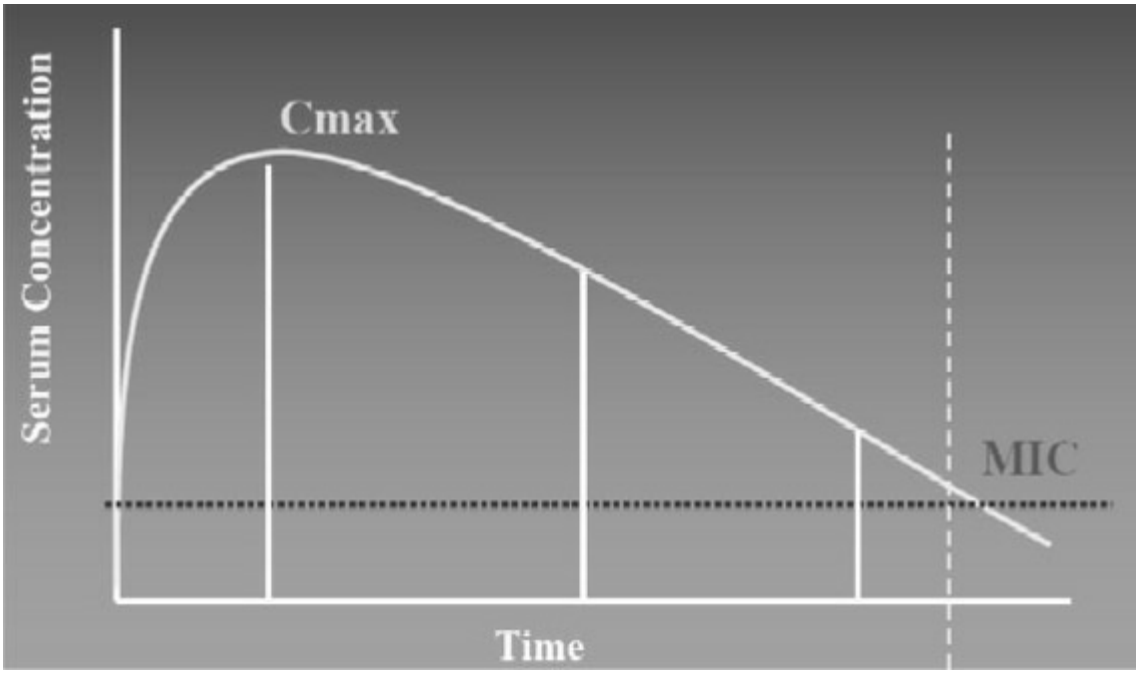

Table 12.2: Summary of the drug targets

Mastitis Pathogens	Milk and Ducts	Parenchyma	Cow
Strepts	+++	-	-
Staphs	+	+++	-

Coliforms	+	-	+++
Mycoplasma +++ Main Target	+ Some benefit	- Little benefit	+++

Antimicrobial Protocols of Clinical Mastitis Treatment

Therapy is given on the premise that treatment costs will be outweighed by production gains following elimination of infection. No significant economic losses will occur as a result of delaying therapy until bacterial culture can be completed. However, many subclinical cases selected as potential therapy candidates have chronic infections; particularly in the case of S. ***aureus,*** prediction of therapeutic outcome by ***in vitro*** testing is unreliable. Drug distribution following intra¬mammary administration may not be adequate due to extensive fibrosis and microabscess formation in the gland; it is critical to assess the cow's immune status from a perspective of duration of infection, number of quarters infected, and other variables.

The need for antibacterial therapy in cows should be evaluated critically. Unnecessary extension of therapy in these instances results in increased discarded milk expense for the dairy producer and risk of antibacterials in marketed milk. Based on experience of clinical veterinarians, economics and drug residues, different protocols are suggested for Gram -ve and Gram +ve bacteria. Before starting the antimicrobial therapy, the clinician should not forget to take the milk and blood samples for bacterial evaluation.

Table 12.3: Summary of the Antimicrobial Protocols for Gram -ve Bacteria [3]

Protocol No. 1

IV/IM → Sulfadiazine/Sulfadoxine + TMP - 25 mg/kg, sid for 2-4 days

I/mammary → Polymixin b SO_4/colistin SO_4 - 100 mg in 20 ml H_2O, sid for 2¬3 days

Protocol No. 2

IM ? Polymixin B SO_4 100mg or Colistin SO_4 5 mg/kg bid for 2 days

I/mammary → Cephapirin Na 200 mg after each milking for 3 i

Protocol No. 3

IM → Ampicillin trihydrate 10 mg/kg IM bid for 3 days milkings

I/mammary → Cephapirin Na 200 mg after each milking for 3 i

Protocol No. 4

IV/IM → Gentamicin SO_4 5 mg/kg sid for 3 days milkings

I/mammary → Gentamicin SO_4 150 mg sid for 3 days

Table 12.4: Summary of the Antimicrobial Protocols for Gram +ve Bacteria [3]

Protocol No. 1

IV/IM → Sulfadiazine/Sulfadoxine + TMP - 25 mg/kg, sid for 2-4 days

+

I/mammary → Hetacillin K or Amoxicillin Na 62.5 mg after each milking for 6 milkings

Protocol No. 2

IM → Ampicillin trihydrate 10 mg/kg bid for 3 days

+

I/mammary → Cloxacillin Na 200 mg after each milking for 6 milkings

Protocol No. 3

IV → Erythromycin lactobionate or Tylosin tartarate 10 mg/kg loading dose Then same drug 5 mg/kg IM bid 3 d

+

I/mammary → Erythromycin base 300 mg after each milking for 6 milkings

Protocol No. 4

IV→Sulfamethazine 100 mg/kg loading dose followed by 50 mg/kgIV,sid5d

+

I/mammary → Amoxicillin Na 62.5 mg or Cloxacillin Na 200 mg after each milking for 6 milkings.

Table 12.5: Milk Discard Times of antimicrobials commonly used in mastitis

Drug	Species	Milk Discard Time (days)
Ampicillin	Cattle	2.5
Amoxicillin	Cattle	2
Cloxacillin	Cattle	2.5
Sodium cephapirin	Cattle	4
Aminoglycosides	Cattle	3
Sulfamethazine	Cattle	4
Trimethoprim/sulfadiazine	Cattle	7
Trimethoprim/ sulfadoxine	Cattle	7
Erythromycin	Cattle	3
Tylosin	Cattle	4

Supportive Treatment of Clinical Mastitis

Many inflammatory and systemic changes seen in severe coliform mastitis, result from the effects of release of lipo-polysaccharide (LPS) endotoxin from the bacteria. By the time therapy is initiated, maximal release of LPS has likely occurred. Thus, the primary therapeutic concern is the treatment of endotoxin-induced shock with fluids, electrolytes, and anti-inflammatory drugs. IV fluids are preferred as the initial method of administration. If isotonic saline is administered, 30-40 L is necessary over a 4-hr period, which can be difficult under farm conditions. A practical alternative is 2 L of 7% NaCl (hypersaline) administered IV. This allows rapid fluid uptake from the body compartment into the circulation. Cows should then be offered free choice water to drink, and if at least 25 litres is not consumed, 12-15 liters should be pumped into the rumen. Many cows with endotoxic shock are marginally hypocalcemic, thus 500 mL calcium borogluconate should be administered SC (to avoid potential complications that could arise from IV administration). Alternatively, rapid absorption calcium gels, designed for periparturient hypocalcemia, can be given. If the cow remains in shock, continued fluid therapy should be

administered PO or IV as isotonic, not hypertonic, fluids.

Glucocorticoids may be helpful in cases of mastitis caused by endotoxin-producing coliforms if they are administered early in the course of disease. Administration of dexamethasone (30 mg, IM) to dairy cows immediately following introduction of Escherichia coli into the mammary gland has been reported to reduce mammary gland swelling and inhibition of rumen motility. Isoflupredone (10-20 mg, IM) has also been shown to reduce local mammary swelling. Cattle are sensitive to glucocorticoid-induced immune suppression; however, it is unlikely that one-time administration of a glucocorticoid will adversely affect cows with endotoxin-induced severe clinical mastitis. Care should be exercised in administering these drugs to pregnant animals; however, severe clinical mastitis in and of itself may cause pregnancy loss in cattle.

There is a little published research data on the use of glucocorticoids for mastitis caused by gram-positive bacteria. It is reasonable to expect that gram-positive infections would be less likely to benefit from the anti-inflammatory activities of glucocorticoids and as a general guide line, glucocorticoid treatment should be reserved for severe cases of gram-negative mastitis, with a single dose administered early in the disease course.

NSAID are widely used for the treatment of acute mastitis. Flunixin meglumine, flurbiprofen, carprofen, ibuprofen, and ketoprofen have been studied as treatments for experimental coliform mastitis or endotoxin-induced mastitis. Systemic use of these drugs is preferred. Phenylbutazone is prohibited for anti-inflammatory therapy for mastitis in cattle over 20 mo of age. Although ketoprofen is available as a veterinary product, has a high therapeutic index, has favorable pharmacokinetics for use in lactating dairy cattle, it is not currently labeled for food animal use. The Food Animal Residue Avoidance Databank (FARAD) recommends withdrawal intervals of 24 hr for milk, with IV or IM administration, for dosages up to 3.3 mg/kg, sid, for up to 3 days. Flunixin meglumine is the most logical choice for treating clinical mastitis. In field studies, increased survival and improved milk production have not been demonstrated following treatment of clinical acute mastitis with flunixin meglumine at a dosage of 1.1 mg/kg. However, in studies of experimental mastitis, this drug reduced the severity of clinical signs such as fever, depression, heart and respiratory rates, and udder pain. FARAD recommends withdrawal intervals of 72 hr for milk when used as specified.

Although not recommended directly for mastitis treatment, ceftiofur sodium

(2.2 mg/kg, IM, sid) decreased the mortality and cull rates of cows with severe coliform mastitis. This drug distributes poorly to the mammary gland, supporting the emphasis on treating the cow rather than the mammary gland because of the risk of septicemia.

Mastitis Caused by Unusual Pathogens

Pseudomonas aeruginosa may cause outbreaks of clinical mastitis. The organism is found in soil-water environments common to dairy farms. Other than supportive care for severe episodes, therapy is of little value. Culling is recommended for cows.

Arcanobacterium pyogenes is common in suppurative processes of cattle and produces a characteristic mastitis in heifers and dry cows. It is occasionally seen in mastitis of lactating udders after teat injury, and it may be a secondary invader. The inflammation is typified by the formation of profuse, foul-smelling, purulent exudate. Therapy is rarely successful, and the infected quarter is usually lost to production. Culling is recommended for cows.

Mycoplasma spp can cause a severe form of mastitis; *M. bovis* is the most common cause. Other significant species include *M. californicum, M. canadense, and M. bovigenitalium*. Because there is no satisfactory treatment, affected cows should be segregated at least for that lactation, or for their lifetimes.

Nocardia asteroides causes a destructive mastitis characterized by acute onset, high temperature, anorexia, rapid wasting, and marked swelling of the udder. Culling is recommended for infected cows. *Serratia* mastitis may arise from contamination of milk hoses, teat dips, water supply, or other equipment used in the milking process. The organism is resistant to antimicrobials and cows should be culled.

Complications of Antimicrobial Treatment

Mastitis due to various yeasts has appeared in dairy herds, especially after the prolonged, repetitive use of antibiotic infusions in individual cows. Yeasts grow well in the presence of some antibiotics; they may be introduced during

udder infusions of antibiotics, multiply, and cause mastitis. Yet, heifers that have never received intramammary infusions may develop yeast mastitis. If mastitis due to yeast is suspected, antimicrobial therapy should be stopped immediately.

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Chapter 13
Advances in Treatment and Control of Bovine Mastitis

Mastitis is a multifactorial disease and knowledge of the risk factors is important to understand disease complexity and deciding effective interventions for its control and treatment. In general, mastitis is an outcome of interplay between the infectious agents and management practices stressing the host defenses permitting invasion of pathogens into udder and their multiplication in milk. The invading pathogens (almost always microorganisms in mastitis) and their toxins evoke host (udder) inflammatory response to destroy or neutralize the infectious agents and to prepare the way for healing and return to normal function. The outcome of microbial invasion of the mammary gland depends on the complex interaction between pathogens that cause disease, the host responses that are needed to eliminate the infectious agent, and various risk factors that will influence pathogen virulence and mammary gland defense mechanisms. The risk factors influencing onset and severity of mastitis are categorized into three major groups- the host factors, the pathogen or microorganism factors and the environment factors including macro and micro environment and herd management practices.

Risk Factors Influencing Treatment Outcome

Host factors: Several host variables such as species, breed, age and parity,stage of lactation, milk yield, leaking milk between milking, nutrition, udder defenses and host immunity, udder structure and conformation, and peri-parturient diseases considerably influence the host susceptibility and severity of mastitis. In a study, significant risk factors identified for post-calving

subclinical mastitis included pre- calving subclinical mastitis, low minimum teat height above the ground, and unhygienic udder post-calving. Significant risk factors for post¬calving clinical mastitis included pre-calving subclinical mastitis, Friesian breed, and low minimum teat height above the ground, udder oedema, and low post-calving non-esterified fatty acid serum concentration. It is suggested that control of pre-calving subclinical mastitis and udder oedema, and enhancement of breed immunity are the critical factors in heifer mastitis management programme [1].

Breed and species: The genetic antagonism between mastitis resistance and production traits is well established. The antagonism is more pronounced between yields and clinical mastitis [2]. The high yielding breeds such as Friesian cows have higher incidence of mastitis than Jersey and Ayrshire heifers [1, 3]. A study on Swedish cows found that herds and cows of Swedish Red-breed had better udder health than those of Swedish Holstein breed, possibly due to differences in udder and teat shape and relatively more favourable metabolic and immune responses in Swedish Red Breed in the peri-parturient period [4]. Likewise, zebu cattle in India and elsewhere are less susceptible to mastitis than exotic cross-bred cows. The susceptibility to mastitis also varies between species. Most studies indicate higher resistance to intra-mammary infections in buffaloes than that in cows. Possibly the udder morphology, especially theanatomical and physical characteristics of teat canal *(ductuspapillarismammae)* of buffalo udder, provides an extra resistance to penetration of invading pathogens.

Parity and lactation stage: Relationship between mastitis caused by environmental pathogens and number of lactation is well established. Risk of udder infection increases with increasing age and parity, and the older cows show poor bacterial and clinical cure rate to mastitis therapy [5]. The cows in first lactation respond better to mastitis in terms of reduction in chronic symptoms like changes in the milk, gland or inflammatory response than those in increasing lactations [6]. This is possibly due to isolated feeding, housing and milking groups practiced for heifers and low resistance to intra¬mammary infection in older cows due to concurrent health problems such as lameness. Moreover, chances of persistence of pathogens such as *Streptococcus (Strep.) uberis* within udder increases with the increasing parity leading to higher incidence in older cows. The peri- parturient heifers are less likely to succumb to clinical mastitis and therefore are unlikely to be persistently infected and record recurrences of clinical mastitis [7]. Incidence of subclinical mastitis in

lactating buffaloes is also high in animals aged 5-9 year or in 3rd and 4th parity [8]. Some studies reported higher incidence of clinical mastitis in first- parity cows than that in the older cows in the beginning of lactation. In such situations monitoring first-parity cows especially at calving and in early lactation, reducing interactions with dry-cows with unknown udder-health status both before and at calving, provisions of a clean calving area, and quality feeding are recommended to reduce the risk of udder disorders in heifers [4].

Cows with decreasing month of lactation, dirty udders and severe hyperkeratosis of teat-ends show greater risk to clinical mastitis due to *E. coli* and *Strep. uberis*. Generally, cows during peri-parturient period are more likely to contract udder infection with increasing incidences of clinical mastitis during the first month of lactation, especially within the first week. Environmental and physiological stress (due to calving and onset of lactation) associated with negative effects on various immune responses in peri-parturient period and likely dry period infection also contribute in greater risks of intra-mammary infection during the early stage of lactation. The risk for opportunistic pathogens such as *E. coli* mastitis may be as high in the late lactation period as in the first month if presence of organisms in the environment is not kept to a minimum [7].

Nutrition, disease history and other host factors: Host nutritional status and feed and feeding practices influence the host immune response and risk for mastitis. High yielding cows in transient period are at a greater risk for negative energy balance. These can develop acetonaemia and eventually clinical ketosis when kept under poor feed and feeding management systems. In ketotic cows there is greater risk of clinical mastitis [9]. Negative energy balance is marked by elevated non-esterified fatty acids (NEFA) and β-OH-butyrate (BHB) concentrations and decreased glucose and insulin concentrations in blood. Higher blood levels of NEFA, BHB and other ketone bodies are associated with impaired immune functions and udder health. Phagocytic activity of polymorphonuclear cells (PMN) is the most important mechanism to prevent udder invasion by major mastitis pathogens including *Staph. aureus*. These cells migrate slowly in hyperketonemic cows and are outcompeted by bacteria resulting in onset of clinical mastitis. The phagocytic and bactericidal capacities of PMN are reduced in the presence of ketone bodies thus low amounts of cytokine are produced in ketotic cows [10]. All these factors enhance risk for intra-mammary infection in the cows with

negative energy balance. Other than ketosis, retained placenta, dystocia, lameness, endometritis, pyometra and udder oedema have been associated with higher incidences of mastitis. Cows with histories of previous cases of clinical mastitis respond poorly to the mastitis therapy. There are reports indicating BHV-4-serostatus as a significant risk factor for *S. aureus* mastitis [11], possibly due to compromised immune response in BHV-4 positive animals.

Vitamins E, D, C, and A, and trace minerals- selenium, zinc and copper influence the udder health and dietary supplementation with in peri-parturient period is found useful in preventing mastitis. These micronutrients are the integral part of antioxidant defense. Vitamin E is a chain breaking (free radical scavenger) lipid soluble antioxidant and protects cell membrane from attack by reactive oxygen species excessively generated during oxidative stress. Selenium is the vital component of glutathione peroxidase - an antioxidant enzyme, which is essential for protection of cells and body tissues from free radicals. These two antioxidants function in tandem. Cattle consuming storage forages are likely to be low in vitamin E unless it is supplemented in feed. The peri-parturient cows frequently show vitamin E deficiencies [9]. It is suggested that vitamin E and selenium deficiencies impair PMN activities. However, their dietary supplementation enhances PMN influx into milk, which increase intracellular killing of infecting bacteria. This helps in rapid clearance of udder from pathogenic invasion. Vitamin A and β carotene also affect immune system. Ascorbic acid is also reported to reduce oxidative stress. Zinc is an essential component and influences activities of nearly 200 enzymes in the body including those involved in oxidative burst, cell maturation, and functioning of lymphocytes and macrophages. Zinc is also required for formation of keratin, which traps and helps to kill microorganisms that get through the teat canal. Low concentration of zinc has been observed in subclinical mastitis in lactating cows [12]. Copper is another immunomodulation mineral and an important constituent of antioxidant defense. It acts synergistically with zinc in preventing mastitis.

Udder defenses and host immunity: Mammary gland defense and host immune responses are the most vital host factors affecting onset and severity of mastitis. Majority of non-antibiotic approaches to control mastitis are directed to potentiate these defenses. Anatomical location of udder in the body, its physico-morphological traits and physiological functions render udder highly vulnerable to pathogen invasions. However, pathogenic invasions are countered through a range of udder defense functioning in tandem along with

host immunological responses. For example, the incidence and severity of mastitis is greatest during peri-parturient period when mammary gland is exposed to a plethora of mastitis-causing pathogens and often host defense mechanism is compromised [13]. As such, mastitis control programmes should invariably include practices to improve both host immunity and udder defense and minimize the chances of host exposure to mastitis causing pathogens, particularly during peri- parturient period.

The udder defense systems can be broadly categorized as non- phagocytic and phagocytic defenses, which operate as inflammation independent and inflammation dependent mechanisms. The inflammation independent defenses represent intrinsic mechanism of normal gland and are largely non-phagocytic with few resident cells. The inflammatory dependent mechanism is represented mainly by phagocytic PMN. The non-phagocytic defense includes teat duct barriers, non-specific humoral defense, lymphocyte activity in the mammary gland and immunoglobulin. The phagocytic defense consists mainly of phagocytic cells in the mammary gland and those recruited in response to inflammatory reaction [14]. Non-specific defense mechanisms, comprising mainly lactoferrin (LF), lactoperoxidase system and lysozyme protect udder of lactating as well as dry cows. Cytokines such as interleukin, interferon-gamma and colony stimulating factor, antioxidants (vitamins C and E and selenium), and flushing action of milk also contribute to non-specific defense of mammary gland. These systems operate individually or interact with each other to protect udder against infections.

Anatomical and physical barriers in udder are the first line of udder defense to prevent bacterial invasion. Disruption of normal anatomy and physiology of teat orifice is associated with increased risk of bacterial colonization in streak canal and development of mastitis. A multi-spiraled, net-like integrated musculo-elastic system surrounding the teat canal facilitates automatic closing and opening of the teat canal and prevents milk from escaping from teat and eventual entry of bacteria into teat. Keratin, a fibrous protein with lipid components (long chain fatty acids) that have bacteriostatic properties is produced by cells lining the teat canal. This forms a barrier against invading bacteria. However, as approximately 12%-40% of the keratin lining is removed at each milking and since the teat canal remains dilated for 1-2 hours, the bacteria present near the opening of teat canal during milking can invade the teat canal. Loss of keratin during milking in Holstein cows is nearly 27% more than that in Jersey cows [15], which may be a contributing factor to

higher susceptibility of Holstein cows for mastitis. Trauma to teat damages keratin or mucous membranes lining the teat sinus rendering the udder more susceptible to infection.

Inflammatory response is the second line of udder defense. It is initiated when invading pathogens breach the teat defense. There are three stages of an acute response (1) immediate elimination of invading pathogens by phagocytes, (2) release of inflammatory substances, especially chemo-attractants, and (3) migration of polymorphonuclear (PMN) leukocytes into the infected udder to kill pathogens. Multiplication of bacteria, production of toxins and other chemotactic factors stimulate the production of numerous mediators of inflammation by inflammatory cells. The activated PMN migrate towards site of inflammation and phagocytize and kill the bacteria. This is the major inflammation dependent mechanism for eliminating pathogens from udder. Large number of PMN may pass between milk producing cells into the lumen of alveolus, thus increasing the somatic cell count (SCC) as well as damaging secretary cells. It is understood that pre-existing leukocytosis of milk protects the udder from mastitis and a mild elevation of PMN in milk protects against more severe infection. Although the inflammatory response is an important defense mechanism, escalation of antimicrobial defenses can have a detrimental impact on host tissue and disrupt mammary gland function. Therefore, it is important that the inflammatory response be rapid and robust during the early stages of infection to adequately eliminate the invading pathogen before significant damage to mammary tissue occurs [13]. For most types of bovine mastitis, phagocytic PMN, assisted by pathogen-reactive opsonizing antibodies are the key immune effectors in host immune response against invading bacteria [16]. When the inflammatory events fail to induce appropriate antibody and neutrophil recruitment in the infected gland, the health of mammary gland is jeopardized leading to development of clinical mastitis of acute to chronic in nature. The magnitude of inflammatory response is influenced by factors including causative pathogen, stage of lactation, age, immune status, genetic makeup, and nutritional status of the host animal [17]. For example, PMN are more effective than mononuclear cells in the protection of udder against *S. aureus* infection, whereas mononuclear cells are more important than PMN in intra-mammary infection due to *Strep. uberis*.

Environmental factors: Environment, in which cows are maintained, is often a niche for mastitis microorganisms and various in load and types of pathogens to which udder is exposed. Important environmental variables

influencing udder health include climate, season, weather, and hygienic standards of housing and feeding systems, milking practices and cleanliness of udder. Poorly designed cow sheds with hot and humid conditions enhance the risk of mastitis due to environmental pathogens. Cleanliness of rearing pen and cow is important in reducing mastitis. Farms with cleaner cows and satisfactory bed management often have low incidence of clinical mastitis. Climatic conditions of a region and its seasonal variation is an important risk factor for occurrence of mastitis. In India, the incidence of mastitis in cows and buffaloes is higher in summer and lower during spring season. Hygienic milking practices including appropriate cleaning of udder and milking machine considerably reduces the spread of infections by contagious microorganisms.

Mastitis pathogens: Onset of mastitis, and its severity, duration and therapeutic response largely depend on the type of pathogens causing the infection. More than 100 pathogens comprising bacteria, fungi, yeast, and algae are incriminated as primary or secondary causative agents of bovine mastitis. On the basis of occurrence, these are classified into three categories- major pathogens**,** minor pathogens and uncommon mastitis pathogens [18]. The major pathogens are *Streptococcus* spp., *Staphylococcus* spp., *Mycoplasma bovis, Escherichia. coli, Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp. and *Enterococcus* spp. The minor pathogens include uncommon staphylococci and *Corynebacterium bovis,* which usually do not cause clinical mastitis. Their presence is more in older than first parity cows. The uncommon pathogens including *Nocardia* spp., *Campylobacter jejuni*, some of *Enterococcus* spp., other gram negative bacteria, and fungi cause mastitis sporadically. Based on the sources and infectivity, the mastitis pathogens are further classified as- contagious pathogen *(Strep. agalactiae, Staph. aureus, Strep. dysgalactiae),* opportunistic pathogens and environmental pathogens *(Strep. uberis,Strep. bovis, E. coli, Klebsiella* spp., *Enterococcus faecium, E. faecalis).* Primary source of the contagious pathogens is udder of the infected cows and infection spreads amongst cows, primarily at milking. The environmental pathogens are persisting in the cow's surroundings in faeces, bedding material, contaminated feed and fodder and soil. Escalated load of these pathogens specifically in bedding material can often cause infection when host defense is compromised. In India, most surveys show *Staph. aureus, Strep.* spp. and *E. coli* as the major causes of bovine mastitis [8]

The success of mastitis control and treatment strategies depends on type of

mastitis pathogens involved in the infection. Mastitis control and management are more effective against mastitis due to contagious than environmental pathogens, which tend to be less adapted to survival in udder and their infection often triggers an immune response that results in mild or moderate clinical symptoms. Some environmental pathogens, such as most *E. coli* are true opportunistic bacteria and immune response successfully eliminates them after a brief period of mild clinical disease. Other environmental pathogens, such as *Strep.* spp., have become more host adapted and may cause mild clinical cases that erroneously appear to resolve; but the case has actually returned to a subclinical state. These factors create problems for success of mastitis treatments [19].Response to antibiotic treatment is poor in the environmental pathogens, especially *Strep. uberis* and the effectiveness of teat dipping against *E. coli* and *Strep. uberis* remains debatable. As such the effective mastitis control and therapeutic programmes need to be evolved in accordance to host, pathogen and environment variables and with basic understanding of pathogenesis of the disease.

Mastitis Control and Treatment Strategies

At the dairy farms, where mastitis is a persistent problem, efforts are aimed at improving udder health by using management techniques to limit the spread of major mastitis pathogens and thus reducing intra¬mammary infections. Strategies are required to control environmental pathogens as well as effective monitoring system to check new infections. Programmes for effective control of contagious mastitis are aimed at eliminating existing infection, preventing new infections and regular monitoring of udder health status with the goal to reduce *S. aureus* infection and complete elimination of *S. agalactiae.* For control of mastitis due to environmental pathogens, programmes are developed to reduce the exposure to these bacteria to which the teat end is exposed. These programmes includes practices such as improved cleanliness of cow surroundings, especially in late dry period and at calving, and to adopt procedures that ensure clean and dry teats are being milked. The goal is to reduce clinical mastitis to 3 percent in lactating cows [20].

Ten point programme for mastitis control: A five point programme based on the concept to eliminate existing infections and prevent new ones was devised by the National Institute of Research on Dairying (NIRD) and

introduced in 1970s for effective control of mastitis. The action points included:

1. *Udder hygiene and proper milking* (e.g. establishing a regular milking schedule in stress free environment, pre-milking cow preparations and teat disinfection, checking foremilk and udder for mastitis and dipping of teats in a suitable disinfectant immediately after each milking)
2. *Prompt identification and appropriate treatment* of mastitis cases during lactation (e.g. antibiotic and supportive therapy in clinical cases)
3. *Dry cow management and therapy*: cows are dried off abruptly and teats are cleaned scrupulously before dry cow antibiotics are administered, including the use of teat-end sealants if appropriate (provision of udder immune enhancers like vitamin E/ selenium in diet)
4. *Culling of recurrent cases* (e.g.chronically infected cows and buffaloes. Generally, cases of chronic mastitis with deep fibrosis and mastitis due to *Mycoplasma* and other pathogens that respond poorly to antimicrobials should be recommended for culling)
5. *Improved husbandry practices* including proper installation, function and maintenance of milking equipments (e.g.periodic evaluation of milking machine system, regular maintenance, proper transport and storage of milk).

   The above five point plan was effective in control of mastitis caused by contagious pathogens, but was not adequate for control of environmental mastitis. The following five additional practices are therefore included to make mastitis control programme more effective-'ten point programme':

6. Maintenance of appropriate environment
7. Proper record keeping
8. Monitoring udder health status
9. Revisiting udder health management programme (schedule)
10. Deciding minimum standards for udder health status

Implementing ten point mastitis control programme: The above programme can be modified as per the local needs. But its effective implementation requires willing farmers, support of a capable diagnostic laboratory, and an enthusiastic and knowledgeable veterinarian [18]. The programme is not only cost-effective, but is also beneficial to maintaining milk

quality and good animal welfare.

- *Udder hygiene and teat disinfection:* The goal of this practice is to milk clean and dry teats. The udder should be washed and dried properly before milking. On large dairy farms, hosepipes are used to wash udder. Hand washing and manual drying of teats is believed to improve stimulation and release of oxytocin and thus letdown of milk. The hand washing also limits excessive water use and reduces chances of bacterial contamination. Use of individual paper towels or latex gloves is recommended to minimize chances of contamination between cows. The practice of hand washing is much appropriate for improving udder hygiene in Indian conditions where only a small number of animals are maintained by individual farmers.
- *Pre-milking teat disinfection* using iodine sanitizers (0.5% or 1% Iodophor) is applied to improve udder hygiene. The implementation of the practice may require significant changes in management system, especially to overcome chances of iodine residue in milk. Use of paper towel to dry the teats and herbal teat dips reduce chances of iodine residues.
- *Post-milking teat dipping* is a simple and cost-effective practice that if used correctly can reduce the incidence of new udder infections by 50-90% [18]. It is the most practical management tool to kill contagious pathogens such as *S. aureus* and *Strep. agalactiae* that are transferred to teats during milking. Iodophor formulations with 0.1-1% available iodine, chlorhexidine, quarterly ammonium compounds in combination with lanolin or glycerin, sodium hypochlorite, hydrogen peroxide, gluteraldehyde based compounds are used as post-milking teat sanitizers. The iodine based teat dip in 10% glycerin is generally considered as the standard teat dip to compare efficacy of other teat dips. Cost-effective post-milking herbal teat dips consisting of components of common indigenous herbs have also been developed recently.
- *Dry cow therapy and management of non-lactating cows:* Management of non-lactating animals (dry-cows) and heifers in late-gestation is a valuable segment of the mastitis control programme. Cows are more susceptible to new intra-mammary infections in dry period than lactation, especially in the first 3 weeks of dry period and the last 7-10 days before calving. Over 60% of new infections occur in dry period [21].Yet this

period offers a valuable opportunity to improve udder health. Adoption of an effective dry cow udder health management system can eliminate existing intra-mammary infections and prevent new infection during the dry period. Dry cow therapy has been separately dealt with, in the chapter no: 18.

Dry Cow Therapy

Antimicrobial therapy at the end of lactation (dry cow therapy) is an important option for controlling mastitis. The aim of dry cow management and dry cow therapy is to minimize the number of infected quarters at calving. The dry period treatment is economically advantageous as there is no need to discard milk from treated quarters and long duration for which antibiotics remain in udder helps in better elimination of pathogens particularly *S. aureus*. It is reported that dry cow therapy can effectively eliminate 70-98% of the existing infections and reduce chances of new infections by 80%. Dry cow therapy is of limited value in controlling coliform mastitis and is not effective during the period prior to calving. But it helps controlling environmental streptococci during the early dry period [20]. Long acting antimicrobial formulations containing benzathine cephaprin, benzathine cloxacillin and sustained released preparations of erythromycin, and penicillin are recommended for dry cow therapy. However, some short acting intra-mammary antimicrobials, such as cephaprin sodium are found to be more effective against *S. aureus* infection. Since the milk withholding period of long acting antibiotics generally ranges from 30-42 days, these should not be used during the last month of gestation. The practice of dry cow therapy demands proper attention as there is a potential of introduction of intra-mammary infections during the time of infusion. Following procedure may be adopted for dry cow therapy [18]:

- Completely evacuate the udder by proper milking
- Dip all teats in an effective teat dip
- Allow dip to dry. Excess teat dip from teat ends can be removed by using a paper towel (single use)
- Scrub the teat for few second with a separate alcohol- soaked cotton swab. The disinfection should be started from the teat on far side of udder

to the near side
- A single dose antimicrobial syringe should be infused in all quarters starting from near side teat to the far side teat
- All the teats should be dipped in post-milking teat dips immediately after the treatment

Dry cow therapy may be used for treatment of all quarters of all cows (blanket dry cow therapy) or for treatment of all quarters of a cow infected in one or more quarters (selective cow therapy) or treatment of infected quarter only (selective quarter therapy). Each of the practices has its own merits and demerits. However, blanket dry cow therapy is a more practical approach to control mastitis on farms with higher prevalence of udder infections. The efficacy of dry cow antimicrobial therapy is influenced by factors such as number of quarters infected, age, nutritional status of the cows, type of pathogens and management practices used. Only approved single dose antibiotic preparations formulated specifically for dry cow therapy should be used. Use of any other preparation, which is not approved and tested for dry cow therapy, is not recommended.

Teat sealers: During dry period, teat canal is naturally sealed by a keratin plug to prevent new intra-mammary infections. The formation of complete keratin plug in the streak canalis, however, delayed if significant proportion of the quarters and teat ends remain open for a considerable time in the beginning of the dry period. The quarters that remain open and have cracked teat-ends are more likely to develop new intra-mammary infection during the dry period [22]. Teat sealers are introduced as a promising management practice to prevent entry of microorganisms into udder of dry cows. Both external and internal sealants have been tested under field conditions with proven efficacy and are available commercially. Internal teat sealers are available for use by itself or in combination with dry cow antibiotics. The use of internal teat sealers without antibiotics is recommended only in uninfected quarters. Infusion of a bismuth sub nitrate based internal teat sealer in uninfected quarters was as effective in reducing new dry period infections and was used as in long-acting dry cow antibiotic formulation [23]. The combined use of teat sealers and antimicrobials can improve the effectiveness of dry cow antibiotic therapy. Teat sealants and cephalonium containing dry cow therapy increased the cure rate of existing intra-mammary infections associated with major pathogens along with increased likelihood of pathogen free post-calving and

reduced likelihood of developing clinical mastitis in the first 100 day of the subsequent lactation [24]. Risk of clinical and subclinical mastitis in the subsequent lactation was significantly reduced by the application of a dry cow therapy comprising intra-mammary infusion of a combination of 600 mg cloxacillin with an internal teat sealant containing 2.6 g bismuth subnitrate immediately after the final milking [25]. Infusion of long acting antibiotics with a commercially available internal sealant reduced new intra-mammary infection between dry off and day - 3 in lactation by 31% and the risk of clinical mastitis by 33% between dry off and day 60 in lactation [26]. Several other trials have also reported effectiveness of internal sealants in preventing udder infection in dry cows.

External teat sealants are adjuncts to antimicrobial infusion and applied to teats by dipping. These products do not persist for long duration inside animal body and infection is prevented till the teat end remains covered with sealant. Therefore, external sealants require reapplication every 5-7 days throughout the dry period for continuous protection, which increases extra labour cost and limit routine use of external sealant. Infusion of quarters with an internal teat sealer requires careful attention to avoid introducing pathogens into untreated quarters [26]. Although application of teat sealants is promising but their use is an additional practice and may not necessarily replace currently available successful mastitis prevention practices.

Other management practices suggested in dry period to reduce the risk of clinical mastitis during the next lactation include vaccination with a leptospirosis vaccine, dry-cow treatment specific for individual cows within a herd rather than its application at herd level, routine body condition scoring of cows at drying off, and a pasture rotation policy of grazing dry cows [27]. Periodic monitoring of dry cows for swollen quarter, reducing nutrient intake one or two week prior to drying off, and provision of clean, dry, cool, comfortable and insect free environment reduces chances of udder infections. The dry cows need adequate quantity of balanced and optimum nutrition, as risk of clinical mastitis is associated with the negative energy balance, and deficiencies of vitamins E, A and D, and certain micro-minerals (selenium, copper and zinc) during the transition period. Recommended nutrients intake guidelines can be used to calculate optimum nutritional requirement for an individual cow and a herd. Provision of adequate levels of vitamin E and selenium in the ration of dry cows improves udder immunity and reduces chances of new infections. Vitamin E @ 500 IU per day for dry cows and 1000

IU per day for lactating cows along with 3 mg and 6 mg selenium per day respectively is recommended to reduce the cases of clinical mastitis. The dry cow therapy will be detailed in the chapter no: 16.

Lactation therapy

A nearly detection and effective treatment of clinical cases of mastitis is an important component of mastitis control programme. Information on mastitis epidemiology is beneficial in selecting proper antibiotic. The current approaches include administration of antimicrobials by intra-mammary or intramuscular or by both the routes depending upon the type and severity of infection. But use of antibiotic for treatment of mastitis should be critically assessed and the treatment strategies should focus on efficacy, economics, animal welfare issues and drug withholding time. The deciding factors for opting antimicrobial treatment include:

- Type of pathogen
- Type and severity of inflammatory response
- Duration of infection
- Stage of lactation
- Age and pregnancy status of animal

- *Therapeutic consideration for antibiotic use:* The clinical efficacy of almost all antibiotics is debatable to treat the chronic cases of *S.aureus*. The antibiotic treatment is mostly ineffective in mastitis caused by *Mycoplasma bovis, Arcanobacterium pyogenes, Nocardia* spp., *Pseudomonas aeruginosa, Serratia* spp, *Prototheca*zopfii, *P. wickerhamii* and various yeast. Treatment of cows exhibiting abnormal milk may not be economical except in the case of *Strep. agalactiae*. Periodic episodes of mild to moderate cases of mastitis caused by contagious pathogens usually resolve without treatment. Antibiotic therapy is considered economically viable option only when the probability of cure is high and the rate of recurrence is expected to decrease after the treatment [19]. Treatment during the late stage of lactation is mostly considered uneconomical. Older cows and those with the previous history of clinical mastitis or recurrent cases are less likely to respond to the antibiotic therapy. Further, certain intra-mammary antibiotics such as penicillin, oxytetracycline, lincomycin and neomycin are reported to influence the phagocytic properties of PMN by inhibiting

oxidative burst. Heat, pain and swelling of udder quarter indicate need for antimicrobial therapy. Animal welfare issues also emphasize that a cow with abnormal gland or systemic reaction must be treated with appropriate antimicrobial and supportive therapy. The most effective treatment strategies include early detection of mastitis, identification of pathogens and selection of appropriate antibiotic and course of the treatment.

- *Selection of antibiotic:* Success of the antimicrobial treatment of mastitis depends on judicious use of appropriate antibiotic, which is influenced by type of pathogen involved and its virulence and severity of mastitis. Mostly an antibiotic is selected based on isolation, identification and drug sensitivity profile of the causative pathogens. However, laboratory report may not be available readily, and *in vitro* sensitivity profile may be of little relevance on field trials. Initiating mastitis treatment could be started using antibiotic based on available retrospective epidemiological and therapeutic data. The knowledge of target site and pharmacokinetic and pharmacodynamics of antimicrobials is helpful in making these decisions. Penetration and distribution of parenterally administered or intra-mammary infused antibiotic into udder gland depends on their lipid solubility, degree of ionization, extent of binding to serum and udder proteins, and the type of vehicle for antibiotic administration. Activity of some of antibiotics is generally affected in milk. Broad-spectrum antimicrobials such as third or fourth generation cephalosporins should not be used as first alternatives for mastitis, as they may increase emergence of broad spectrum a-lactam resistance [28]. Narrow-spectrum bactericidal antibiotics with better diffusion and binding capacity to mammary tissue and low minimum inhibitory concentration (MIC) value are usually preferred for mastitis therapy. A written on-farm treatment protocol should be developed for judicious use of antimicrobials.
- *Route and duration of treatment:* Preference for route of administration of antibiotics and its duration depends on the anatomical location of mastitis pathogens and the severity of infections. Mastitis pathogens may present in different locations. Some of them are predominantly present in udder parenchyma (*S. aureus*, *Arcanobacterium pyogenes)* and some in milk and teat canal (*Strep. agalactiae* and other streptococci, Coagulase negative staphylococci and *Corynebacterium bovis). Arcanobacterium pyogenes, Mycoplasma bovis* and coliform infections are often

associated with systemic reaction. These anatomical locations are the target sites for antibiotic action and the route is selected to ensure the maximum diffusion of antibiotic at the target site. Theoretically, parenterally administered antibiotics will have better (moderate to excellent) penetration of the udder parenchyma than those infused in udder (moderate). As such, the systemic route is considered more effective for treatment of clinical mastitis caused by *Staphylococcus* [29]. But it is difficult to produce and maintain therapeutic concentrations of drugs in milk following systemic administration of commonly used broad spectrum antibiotic such asoxytetracycline, trimethoprim- sulphonamide and ceftiofur. This is a major drawback of parenteral antibiotic therapy in mastitis treatment. Udder infusions are recommended for treatment of pathogens located in teat canal and milk as higher concentrations of antimicrobials are achieved in milk *via* this route, even with lower consumption of the drug. The limitations of intra-mammary infusion are uneven distribution of drug throughout the udder, risk of introducing infection during drug infusion and variation in the therapeutic response [28]. In general, infections confined to milk and teat canal can be treated easily by intra-mammary infusions. Both intra-mammary and parenteral route are recommended to achieve better efficacies against pathogens such as *S. aureus* present in udder parenchyma. Infections such as coliform mastitis, associated with systemic reaction require parenteral antibiotic as well as intra-mammary infusion along with a supportive therapy comprising non-steroidal anti-inflammatory drugs (e.g., meloxicam, flunixinmeglumine, ketoprofen) and intravenous fluid infusion to ameliorate toxaemia (7.5% saline 4-5 ml per kg). Higher than normal dose of parenteral antibiotics with an extended schedule for therapy (5-8 days) than routine intra-mammary therapy (2-3 days) are suggested for the treatment of mastitis caused by *S. aureus* and *Strep. uberis*. However, the benefits of extended therapy are debatable

Alternative Approaches to Mastitis Control

Limitations associated with antibiotics restrict their use in treatment and prophylaxis of mastitis. This has necessitated need to find effective natural alternatives for prevention and control of intra¬mammary infections. A wide

range of practices consisting of vaccination, use of immune-biologicals, medicinal herbs, homeopathic drugs, vitamins, micronutrients and hormones have been tried and tested for mastitis management with some encouraging results. Some of these practices are routinely used by farmers or veterinary practitioners for treatment and control of mastitis. However, these alternatives are often valued for their supportive or adjunct role in combination with conventional mastitis treatment and control practices.

Treatment of subclinical mastitis: There are controversial opinions on the treatment of sub-clinical mastitis. Poor bacterial cure rate and high cost of antimicrobial therapy do not favour the treatment of subclinical mastitis. Untreated mild udder infections can recover spontaneously. If effective preventive and control measures are adopted at farms, chances of new infections are considerably reduced. In this case treatment of subclinical mastitis will not affect the over-all incidences of clinical mastitis. The treatment of subclinical mastitis is advised in herds encountering incidences of highly contagious mastitis pathogens such as *S. aureus* or *Strep. agalactiae.* Decision for undertaking treatment of subclinical mastitis also depends on the age of cow, stage of lactation, days in milk, early detection, identification of bacteria and SCC level. Treatment is avoided in older cows, which usually respond to treatment poorly. Treatment of cows in the late lactation may not be economically beneficial. Such cows should be dried off unless bacteriological examination justifies the antibiotic therapy. A new technique based on infusion of casein hydrolyzate (CNH) into treated glands is suggested for drying-off milk secretion from the treated gland. The technique is advantageous as individual glands can be treated during the lactation leading to minimal milk loss during the treatment (no withdrawal time), improve milk quality and reduce SCC level. In many cases the CNH treated gland returns to full functionality in the following lactation [30]. The practical goals of mastitis treatment are to- get a rapid reduction in clinical symptoms, achieve an eventual reduction in SCC, prevent recurrence of additional clinical cases, and maintain expected milk yield [19]. Therefore, treatment should be performed on only 'true' udder infections, characterized by bacteriologically positive quarters along with increase in SCC and/or change on distribution of leukocytes in milk. Some recent publications [31, 30] may be useful for developing guidelines to decide on treatment of mild or moderate and subclinical mastitis.

Vaccination for mastitis: Development of effective vaccine to raise

immunity against mastitis pathogens has been the most desired goal of researchers for the past several years. But attempts to develop efficacious mastitis vaccines have met with variable results. The complexity and heterogeneity of pathogen virulence factors and limited knowledge of adaptive immune responses of the mammary gland is a significant hurdle in successful development of mastitis vaccine. A single vaccine may not be effective in preventing udder infections. Development of J-5 *E. coli* vaccines from rough (Rc) mutant strain (J- 5) of *E. coli* and its field application is one of the major successes. This is characterized by a T helper 1 (Th1) response and is mediated by memory cells inside the mammary gland resulting in enhanced PMN migration subsequent to an intra-mammary infection [32]. J-5 vaccination schedule include first inoculation at 6-8 weeks pre-calving followed by booster at 3 weeks before calving. Cows should be revaccinated at 3 weeks prior to the breeding.

Limited successes have been achieved in the development of effective vaccines against other major mastitis pathogens such as *S. aureus, Strep. uberis, Strept. agalactiae and Strept. dysgalactiae. S. aureus* vaccine (somatic antigen containing phage types I, II, III, IV and miscellaneous groups of *S. aureus*) has been commercially available for some years. The vaccine improves spontaneous cure and is effective in reducing the chronic infections rather than preventing new infections, which is the major limitation of this vaccine and also probably a reason for the limited use of vaccine in mastitis control programs. Presently several studies are directed to the development of vaccines against *S. aureus* based on different types of capsular polysaccharide (CP) like CP5, CP8 and CP336 associated to protein carriers, combination of mucus (slime) in liposomes, and *S. aureus* pseudo capsules (a biofilm matrix that surrounds the bacteria and reduces the ability of leukocytes to destroy it). But outcomes of these studies have been inconsistent and confusing to interpret [33]. Experimental vaccines composed of *S. aureus* pseudo capsule-enriched bacterins supplemented with a- and/or β-toxoids, and *Strep. uberis* bacterin appears promising, but none of these has been commercialized [34]. Combined vaccine has also been introduced in the market to immunize healthy cows and heifers against *S. aureus,* coliforms and coagulase-negative staphylococci (CNS) mastitis. The vaccination schedule consisted first vaccination (intramuscular inoculation 2 ml) on day 45 before expected calving, thereafter 2nd injection 10 days before calving and 3rd inoculation 52 days after parturition. The vaccination schedule is repeated with each gestation. Full

immunization scheme is claimed to induce protection approximately for 130 days post-parturition. A bacterin based *Strep. uberis* vaccine is also now available commercially for vaccinating herds with known or suspected *S. uberis* infections.

Bacteriocins: Bacteriocins (lysostaphin) are protein compounds produced by lactic acid bacteria and are capable of killing closely related organisms [35]. Certain bacteriocins also elicit bactericidal activities against several bacterial species not closely related to the bacteriocin producing strains. Nisin, a natural antimicrobial peptide of 34 amino acids is produced by *Lactococcuslactis* ssp. *Lactis*. This bacteriocin show antibacterial activity against a wide range of pathogenic bacteria including *S. aureus, S. epidermidis, Strept. agalactiae, Strept. uberis, Bacillus cereus, Listeria monocytogenes, Clostridium botulinum, Clostridium butyricum,* and *Clostridium tyrobutyricum*. Considering its activity against major mastitis pathogens, nisin has been incorporated into some of the commercially available teat dipping products with considerable therapeutic success. Intra-mammary infusion of a nisin based formulation (2,50,000 IU nisin dissolved in 20 ml saline) once daily for 3 days resulted in bacteriological cure for subclinical mastitis in lactating cows caused by *Strep. agalactiae, S. aureus,* coagulase negative staphylococci and other pathogens [36]. Nisin has been granted 'Generally Recognized as Safe (GRAS)' status by United State Food and Drug Administration (USFDA) in 1988 and is widely used as biopreservative in a number of food products. Poor solubility at physiological pH is one of the major drawbacks of nisin. *Lacticin 3147,* another bacteriocin produced by *Lactococcus lactiss* sp. *lactis* DPC 3147, is also reported effective against mastitis pathogens such as *S. aureus, Strept. agalactiae, Strept. uberis* and *Strept. dysgalactiae* in vitro assessments. Incorporation of Lacticin 3147 into teat sealant formulations resulted in an excellent antimicrobial efficacy and has been suggested for effective prevention of mastitis during drying off [37]. Being the natural biodegradable products, bacteriocins are safe for animals as well as human beings. The milk withdrawal time following the treatment with bacteriocins is also less. In conclusion, these are proposed as a valuable alternative to antibiotics.

Bacteriophage and endolysins: It is now widely accepted that the future approaches to prevent and control mastitis in dairy animals will be based on to assist and boost host immune response to making it robust enough to counter the invasion by pathogenic microorganisms. PMN cells, assisted by opsonizing

antibodies are the most effective defense weapons for killing majority of mastitis pathogens. The animals capable of effectively clearing new intra-mammary infections not only have rapid and massive neutrophil recruitment capabilities, but also high blood levels of pathogen-reactive IgG2 and an effective early inflammatory response that enables significant leakage of these blood antibodies into milk before neutrophils arrive [16]. In recent years, many synthetic immune molecules have been evaluated for their potential to improve these components of udder defense to eliminate mastitis pathogens.

Cytokines: More than 50 cytokines have been purified and characterized for their therapeutic value. Of these, colony-stimulating factors (CSF), interferon - *g* (IFNg) and interleukins (particularly IL-2) have shown pronounced influence on mammary immune cells and were found useful for effective management of mastitis [38]. Enhancement of the bactericidal effect of certain antibiotics by IL-2, IFN-c or GM-CSF, can be attributed to the substantial mobilization of innate and adaptive immunity. Although cytokine immunotherapy, particularly with IL-2 and IFN-c, showed promise of prophylactic activity in normal mammary gland, the resistance to *S. aureus* infection is not enhanced [39]. High cost may limit the wider application of cytokines under field conditions.

Lactoferrin: It is a non-specific glycoprotein, normally present in milk, bile, saliva, tear and PMN granules. It is released in high concentrations by the secondary granules of neutrophils and epithelial cells in response to inflammatory reactions. Lactoferrin appear to have many biological functions, including bacteriostatic, bactericidal, anti¬inflammatory, and immune-modulatory activities. The best-known effect of lactoferrin is to bind iron that is essential for bacterial growth. Lactoferrin has demonstrated broad-spectrum antimicrobial activities against *E. coli, S. aureus, Pseudomonas aeruginosa* and *Klebsiella pneumoniaea* indicating its potential use in the treatment of mastitis. Lactoferrin combined with penicillin provided an effective combination for the treatment of stable *S. aureus* infections resistant to a-lactam antibiotics [40]. Intra-mammary infusion may cause some local irritation to mammary tissue but no systemic reaction and signs like fever and anorexia are reported following lactoferrin infusion. High cost and low production may limit the commercial use of lactoferrin based immunotherapy.

Complement: It is a collection of proteins that is present in serum and milk and functions together with a specific antibody to cause lysis of invading

bacteria. Concentrations of complement are highest in colostrum, inflamed mammary glands, and during involution. In contrast, concentrations of complement are lowest during lactation. Because of its intermittent presence in milk, complement is thought to play only a minor bactericidal role in the mammary gland. However, complement-sensitive organisms including some strains of *E. coli* are killed by the alternative complement pathway. CD14 is a complement protein, naturally found in bovine milk and expressed on bovine macrophages. It helps the immune system to fight infection. CD14 binds to lipopolysaccharides (LPS) located on *E. coli's* outer membrane. Enhancement of the level of CD14 in milk would boost the immunity of mammary gland. When CD14 is inserted into mammary gland through teat opening, it binds to *E. coli* and triggers the cow's immune response, which reduces inflammation in udder. This process helps to neutralize and clear bacterial toxins thereby reducing the chances of an excessive immune response. Clinical trial against bovine *E. coli* mastitis using sCD14 was found to be very effective [41].Scientists have been able to synthesize recombinant bovine sCD14 (rbosCD14) by transfected insect sf/9 cells which was 100% effective in preventing *E. coli* mastitis [42]. Similarly, plant derived recombinant bovines sCD14 (Prbos CD14) has been also synthesized and reported effective in reducing the severity of intra-mammary *E. coli* infection in lactating cows [43].

Plant-based products: They constitute a major source of alternative therapies for mastitis. Several herbs have been evaluated for their potential use in mastitis treatment [44, 45, 46]. Some of the plants and their parts assessed *in vitro* with promising results against mastitis pathogens have been used as intra-mammary infusions in sub-clinical or clinical mastitis. These include *Tinospora cordifolia*, *Curcuma longa, Phyllanthusemblica, Allium stivum, Azadirachtaindica, Ocimumsanctum, Terminaliachebula, Morindacitrifolia, Taraxa- cummongolicum, Eucalyptusglobulus, Panax ginseng* and *Ocimum sanctum.* Herbal preparations are commercially available and some products are highly popular in the Indian market for management of mastitis with varied clinical efficacy. Most of the herbal preparations developed so far are usually for external application and their use are of limited value to cure the infection in mammary gland completely. Further research on clinical efficacy of promising herbs, especially those with anti-inflammatory, antibacterial, immune modulatory and antioxidant properties can provide an effective alternative for the treatment of mastitis.

Homeopathy: The homeopathic drugs modulate the system in such a way

that it can either kill or repel the infectious agents from the body. The homeopathic drugs do not directly kill the infectious agents like antibiotics and other drugs. Various homeopathic drugs individually or in combinations have been reported effective for the treatment and prevention of bovine mastitis. The homeopathic remedies are also used by farmers of developed country for organic herd farming [47]. Homeopathic remedies either in combination or individually has been already described in the chapter on ethno-veterinary medicines for the treatment of mastitis.

Poly-homeopathic remedies are becoming more popular as researches support higher cure with homeopathic combinations than a single drug. Combination homeopathic drugs are also available commercially and tested for veterinary use. An intramammary homeopathic ointment comprising *Belladonna 1 dH, Calendula MT, Echinacea 1 dH, Dulcamara 1 cH* showed beneficial therapeutic effect in the early stages of udder inflammation and for restoring udder health and function [48]. In India, oral use of commercial pills composed of *Phytolacca, Calcareafluorica, Silica, Belladonna, Bryonia, Arnica, Conium* and *Ipecacuanha* showed 86.6% efficacy with a mean recovery period of 7.7 days in the treatment of acute non-fibrosed mastitis [49]. Homeopathic preparations of use in mastitis treatment is detailed in the chapter no: 13.

Preventive Homeopathy: Nosodes are the homeopathic remedies that are prepared from the specific products of a particular disease or pathogen. These are like homeopathic vaccines developed using products of species of bacteria causing infection in the herd. Nosodes can be used as preventive remedies. Claims have been made that Nosodes can reduce and maintain low SCC in addition to providing a treatment for both subclinical and clinical mastitis [50] but certain controlled trials failed to support these claims [51]. Trials with combined homeopathic and tuberculinum nosode did not yield beneficial effect on subclinical mastitis [52].

Other homeopathic remedies that have been tried and claimed to be effective in treatment and prevention of mastitis include homeopathic phototherapy products- kelp and aloe. *Kelp* is a variety of seaweed known to be effective against different types of bacteria. It is reported effective in prevention of mastitis. *Aloe* is reported useful in the treatment of udder injuries that often predispose to staphylococcal mastitis. Application of aloes quickly heals the tissue damage. It works both as an anti-inflammatory and

anticoagulant.

Minerals and Vitamins: Role of micronutrients and vitamins in improving the host immunity has provided an important basis to facilitate mastitis control and treatment. Most of the available information links zinc, selenium, vitamin E, vitamin A, β carotene, vitamin C and copper to status of udder health. These micronutrients are antioxidant and immuno-modulators. Oxidative stress has been associated in several inflammatory conditions and incriminated in the pathogenesis of many diseases including inflammatory udder conditions. Increase in erythrocyte lipid peroxidation and decrease in blood concentrations of antioxidant micronutrients have been reported in clinical and subclinical bovine mastitis. Incorporation of mineral supplementation in mastitis management plan is recommended for effective outcome [53]. Cows with mastitis show low concentration of zinc, and intra-mammary infections can be reduced by supplementation of zinc and methionine complexes. Copper acts synergistically with zinc in preventing mastitis.

Selenium together with vitamin E is one of the most widely used micronutrient combinations to prevent mastitis. These micronutrients increase bactericidal capabilities of neutrophils. It is observed that low levels of selenium and vitamin E enhance risk of clinical mastitis and higher SCC. Dietary supplementation with these micronutrients (500-750 IU per day of vitamin E and 0.2-0.3 ppm selenium) during dry period and early lactation considerably reduces frequency and the duration of clinical mastitis after calving.

Vitamin A and β- carotene also influence host immunity. Cows fed on low dietary levels of vitamin A show higher incidence of mastitis. Supplementation of vitamin A @ 170, 000 IU per cow per day reduced intra-mammary infections. β-carotene supplementation @ 300 mg per cow per day reduces clinical mastitis. The effect of beta-carotene on mastitis is independent to vitamin A. Vitamin C is a potent antioxidant, immune-modulator and most essential component for the synthesis of collagen required for tissue healing. Leukocytes carry the highest concentration of vitamin C than any of other vitamins. The subcutaneous administration of vitamin C @ 25 mg per kg body weight for 5 days was found beneficial as an adjunct to antibiotics therapy in mastitis [54].

Deficiency of serum levels of 25-hydroxyvitamin D_3 has been correlated with increased risk of infectious diseases such as tuberculosis and influenza.

Recently, USDA scientists have reported therapeutic potential of intra-mammary infusion of 25-hydroxyvitamin D_3 (100 pg in 10 ml fetal bovine serum) in experimental *Strep. uberis* infection of udder. It is suggested that expression of genes encoding important antimicrobial proteins depends on concentrations of 1,25- dihydroxyvitamin D_3 produced by activated immune cells at the sites of infection and synthesis of 1,25-dihydroxyvitamin D_3 is dependent on the availability of 25-hydroxyvitamin D_3 [55]. In general it should be noted that the nutritional approaches are more preventive than curative and can support the mastitis control programme.

Other products and practices: In recent years several alternate and supportive approaches have been tested for their potential use in prevention and treatment of mastitis. Some of these include oxytocin, ozone therapies, acupuncture, clay therapies, Sugar Poly-X (natural polysaccharide occurring in cell wall of certain yeasts), caprylic acid, monocaprylic acid, and water soluble fraction of *Mycobacterium phlei*. Most of these approaches are either in preliminary stage of evaluation or have been evaluated on limited field cases.

Milk let down properties of oxytocin was first reported in 1910. This property has been used to treat intra-mammary infections. It is claimed that administration of oxytocin flushes residual bacteria-laden milk, which aid in elimination of infection. In addition, evacuation of milk stimulates production of more milk, enhances movement of leucocytes into the quarters and activates other anti-microbial components. However, the use of oxytocin alone or in combination with antibiotics for treatment of mastitis is debatable as some controlled trials failed to support the efficacy of oxytocin treatment. Ozone is a polymerized oxygen (O_3) and a potent germicidal against a range of bacteria, virus, fungi and yeast. It is used for sterilization of hospitals, food storage facilities and decontamination of water and wastewater. The ozone treatment for mastitis involves immersion of affected quarters in the gas. Ozone trial on Holstein cows suffering from acute mastitis resulted in 60% cure rate without any additional antimicrobial treatment. Affected quarters were infused 1-5 liter of ozone per quarter. No adverse reaction in udder tissue and residual ozone were recorded in the treated milk [56]. Ozone treatment does not usually have any adverse interaction with other drugs. Milk of animal recovered from clinical mastitis after ozone treatment can be used for industrial or nutritional purpose. Recurrence of mastitis is reported to be delayed following ozone

therapy.

Several organic milk producing farmers use a variety of treatment and practices for cows at dry-off and for clinical mastitis in their dairy herds where antimicrobials are not approved for organic production. These products include ultrafiltered bovine whey products, isoflupredone, garlic tincture, *Aloe vera,* vitamin C and B, vegetable or olive oil, homeopathy drugs, corticosteroids, microbial supplements and electrolytes. However, many of these products are neither clinically evaluated nor approved for veterinary or human medical use. Approximately one-half of the conventional and one-third of organic farmers successfully treat about one-half of the clinical mastitis cases using non-antimicrobial approaches [47]. These ethno-veterinary practices used by farmers for treatment of mastitis need to be scientifically validated for providing cost-effective alternate way for mastitis management.

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Chapter 14
Ethno Veterinary Approaches for Treatment of Bovine Mastitis

The common mode of therapy for mastitis involves the use of antibacterial agents that are also used for other infections, either parenterally or through the unique route of intra-mammary administration. In the recent days there is a growing resentment to the use of synthetic drugs/antibiotics for the treatment of mastitis owing to the increasing levels of awareness about the ill effects of residues of drugs in food produce such as milk. Governments are placing huge restrictions by virtue of fixing MRLs and withdrawal times and banning drugs for food animal use. The above measures force the veterinarian or the farmer to look at alternate avenues for the treatment of mastitis.

Problems in Conventional Anti biotic Therapy

The so called conventional therapy for mastitis refers to the treatment of mastitis with antibacterial agents, which are natural or synthetic. As on date they remain the mainstay in the therapy of mastitis. However, their regular use poses many problems.

1.*Widespread Resistance*

The foremost problem with respect to antibiotic use is the widespread reports of resistance of mastitis causing bacteria against drugs. Reports of resistance by bacteria against some of the common drugs such as ampicillin, cloxacillin, penicillin etc., have been reported. While even some other sensitive drugs are also only efficient to the tune of 60-70%. This leaves a gap in efficacy and leaves the veterinarian in a great dilemma as to the appropriate

choice of drug. In a study conducted by the authors on the status of resistance, out of about 2500 isolates of mastitis causing bacteria tested, the sensitivity pattern revealed the following. Enrofloxacin (73.9%), Chloramphenicol (68.3%), Neomycin (62.07%). Maximum resistance were observed against the following antibiotics: Nalidixic acid (19.43 %,), Penicillin (20.74), Ampicillin (24.10 %) and Cloxacillin (28.90%) (unpublished data). It need to be observed with great concern that, the last three drugs are commonly used against mastitis in the field. In another study, the antimicrobial susceptibility test revealed that most of the bacterial strains (gram positive, gram negative, and mixed) isolated from subclinical mastitis milk samples, were highly sensitive to enrofloxacin 53.91%, least sensitive to oxytetracycline 17.39% and ampicillin 7.83%, and resistant to streptomycin.

2. *Cross Resistance*

Cross resistance is a phenomenon in which once a bacterial strain is resistant to a drug, resistance is also seen for the congeners of the drug which act through the same target site. Some of the groups of antibacterial drugs are also currently the drugs of choice in treating human infections. These drugs themselves or their congeners are used in treating human infections, sometimes life threatening infections. Since many of the pathogenic bacteria are common there is a high possibility, that once resistance against these drugs sets in, it will become very difficult to treat these infections in human beings.

3. *No New Drugs in Pipeline*

With reference to the therapy of mastitis with antibiotics, it is to be noted that the last two decades have not seen any new drug molecule with an entirely new bacterial target. Drugs that have been introduced are only congeners of existing molecules with an established mechanism of antibacterial action. Therefore these drugs have every chance of having been made ineffective. For instance, authors in one of their *in vitro* studies have observed, rampant resistance in *E. coli* isolated from animals against drugs such as sparfloxacin a newer fluoroquinolone even though this drug has never been used in animal therapy. Even if new drugs were to be introduced the preference for use will be for treating life threatening infections of man, thereby precluding their use in animals especially in food animals.

4. *Chemical Pollution of Milk*

The residues of drugs, if present in milk, are likely to cause ill effects in the consuming public apart from causing the survival of resistant bacteria. There has been a growing awareness about the need to minimize or totally avoid any residues of drugs in milk. Globally, governments have issued guidelines regarding the use of synthetic drugs and given the Maximum Residues Limits (MRL) and Withdrawal periods for drug use in milk and other produce. In case of withdrawal period, the milk has to be discarded without any productive use for it, which may cause a huge loss for the farmer. Moreover, even when discarded, there is a concern for the impact of such chemicals when they get into the environment and may ultimately reach the public at large.

5.*Organic farming*

The concept of organic farming is becoming increasingly popular. A lot of interest is shown by farmers to produce obtained by organic farming methods, which totally precludes the use of any synthetic chemicals. The farmers are increasingly taking to organic farming, since there is a better price for the produce and consumer awareness about the need to minimize chemical residues. Extended to dairy farming, the concept of organic farming requires complete abstinence from the use of synthetic drugs. In such a scenario, management of mastitis with antibiotics is not possible. This challenge demands the development of effective and appropriate natural products for treatment of mastitis.

Alternatives to Antibiotic Therapy

Herbs have always been a great source of interest for the common man to combat many illnesses affecting either himself or his animals. One of the hallmarks of the civilizations has been the use of these traditional plants for medicinal purpose. Herbs have also found their way into modern medicine and are still one of the potential sources of new drugs. Of late, a renewed interest in the fields of Indian traditional medicine systems such as Ayurveda, Siddha, Unani etc. is being observed. Even modern scientists are involved in the search for newer drugs using the wisdom of traditional medicinal practices.

Advantages of Herbal Therapy

- Accessibility

  Usually in traditional practices herbs that are available in the vicinity are used and useful concoctions are derived out of them. This makes the preparation to be made easily available and can be practiced by local healers or even by the patients / farmers themselves.

- Cost effective therapy

  Since the plants are made use of with little transportation and processing requirements, herbal drugs represent one of the most cost effective methods of therapy.

- Rich heritage of traditional medicine

  Often, the herbs are chosen based on a pre-existing knowledge of their pharmacological activities as mentioned in the scriptures of Ayurveda/ Siddha *etc.,* the chances of their failure is minimal.

Ideal Herbal Therapy for Mastitis

An appropriate drug for the therapy of mastitis would mean that the drug is primarily an antibacterial agent. It would be desirable that herbal drug or combination of plant based drugs with ideal inhibitory activity. The drug should be able to inhibit bacteria at low concentrations so that Since a wide variety of bacteria including Gram positive and Gram negative bacteria are involved in the etiology of mastitis, it would also be desirable to be produce antibacterial activity against a wide variety of bacteria associated with etiology of mastitis.

Since mastitis is complicated by the presence of inflammatory process, it would be appropriate that the drug also possesses anti¬inflammatory activity. Since research on herbal drugs has shown a number of plant based drugs with immunomodulatory activity, the third dimension to an ideal herbal preparation for mastitis will be an immunomodulator to minimize the damage. Table 14.1 indicates the name of the plants used in mastitis treatment.

Some of the Herbs Popularly Tested in Bovine Mastitis

Ocimum sanctum

Commonly known as Tulsi, the extracts of different parts of the plant have found wide use in herbal medicine. Seeds contain a pale yellow colored fixed oil. The fixed oil of the herb was found to possess significant analgesic, anti-inflammatory, immunomodulatory and antimicrobial activity. The oil contains a-linolenic acid, an omega-3 fatty acid, which on metabolism produces eicosapentaenoic acid and the same appears to be responsible for the antibacterial activity against Staphylococcus aureus. The oil alone or in combination with cloxacillin, a beta-lactamase resistant penicillin, has been found to be beneficial in bovine mastitis, an inflammatory disorder resulting from staphy¬lococcal infection. When extract of Tulsi was used for the treatment of subclinical mastitis, the results revealed that the aqueous extract of O. sanctum treatment reduced the total bacterial count (TBC) and increased neutrophil and lymphocyte counts with enhanced phagocytic activity and phagocytic index. The bioactive principle of leaf extract of *O. sanctum,* like ursolic acid, oleanolic acid and salrigenin possess immunomodulatory potential which is indicated by the increase in neutrophil and lymphocyte and enhanced activity of the phagocytosis of the PMN cells in the bovine mammary gland and resulted in the reduction of TBC. The results suggest that the crude aqueous extract of *O. sanctum* (leaf) possesses some biologically active principles that are antibacterial and immune-modulatory in nature [13].

Plant Names	**Herbal Formulation**	**Activity Spectrum**	**Reference**
Brachiaria sp. Cenchrus ciliaris, Abutilon indicnm and *Coccinia grandis*	Aqueous and methanol extracts	S. *aureus, E. coli, S. agalactiae* and *K. pneumonia.*	[1]
Devdaru oil, Nilgiri oil, *Haridra ghansatua, Gandhaprasaarinee ghansaWa, Madhnyashti ghansatva,* and *Shudh*	Emulsifier gel base	coagulase-negative staphylococci (CNS), coagulase-positive staphylococci (CPS), *Micrococcus* spp.,	[2]

gandhaka		*Streptococcus agalactiae,* and *Escherichia coli.*	
Gloriosa superb	Crushed roots are boiled in whey (per os)	Not available (Ethnoveterinary practice)	[3]
Acacia nilotica Curcuma longa Musa paradisica Salvadora persica	Gum Paste of rhizome powder Fruits mixed with milk and fed Leaf juice is applied locally	Not available (Ethnoveterinary practice)	[4]
Aloe vera	Leaf pulp	Not available (Ethnoveterinary practice)	[5]
Glycyrrhiza glabra, Curcuma longa, Cedrus deodar a, Paederia foetida	Gel (Mastiliep ® Gel) supportive therapy to intra¬mammary antibiotic treatment	*Staphylococcus sp., Sterptococcus sp.*	[6]
Capsicum annuum ,Lepidium sativum, Allium sativum,Sesamiun indicum Citrus limon, Zingiber officinale Roscoe, Citndlus colocynthis Curcuma longa, Cuminum cyminum, Rosa indica,Centratherum	For details refer the article for the compounding of formulations of various herbs listed	Not available (Ethnoveterinary practice)	[7]

anthelmisticum, Triticum aestivum, Nigella sativa, Peganum harmala			
Cinnamomum Zeylanicum	Essential Oil of leaves	*Staphylococcus sp.*	[8]
Azadirachta indica	Hydromethanolic extract	—	[9]
Persicaria enegalense	crude extracts	Staphylococcus aureus, Candida albicans and Corynebacterium bovis, Pseudomonas aeruginosa	[10]
Tinospora cordifolia	Hydromethanolic extract		[11]
Tinospora cordifolia, Terminalia chebula and Vitis vinifera		*In vitro* testing against *Candida albicans* from mastitis	[12]

Azadirachta indica.

The immunotherapeutic potential of hydro-methanolic extract intramammary treatment significantly decreased the SCC, TBC, milk neutrophil percent and significantly enhanced milk lymphocyte percent, hydrogen peroxide and superoxide production by milk cells. The IL-2 and IFN-gamma were expressed in normal healthy cows and diseased cows after *A. indica* treatment, whereas both the cytokines could not be expressed in cows treated with antibiotic and in untreated diseased cows. The results of the study indicated anti inflammatory, antibacterial and immunomodulatory potential of the herb, these activities could be due to the presence of bioactive principle in the extract [9].

Panax ginseng

Cows with subclinical mastitis caused by *S. aureus* were subjected to subcutaneous injection with an extract from the root of Panax ginseng at a dose of 8mg/kg body weight per day for 6 days. After the end of treatment, the numbers of *S.* aureus-infected quarters and milk and somatic cell count tended to decrease in ginseng-treated cows, but not in the control group [14]. The findings indicated that ginseng treatment may activate the innate immunity of cows [15] and may contribute to the cow's recovery from mastitis [16].

Persicaria senegalense

The possible remedial effect of *Persicaria senegalense* in bovine subclinical mastitis was studied by in vitro and in vivo antimicrobial tests, using crude extracts and the leaf in different forms. The *in vitro* test showed that isolates of *S. aureus, Candida albicans* and *Corynebacterium bovis* from subclinical cases and an isolate of *Pseudomonas aeruginosa* from a clinical case of mastitis were all inhibited by the three crude extracts. The 0.77 kg of leaf powder, equivalent to 3 kg of wet leaf, was fed per day for 5 days resulted in an apparent cure rate of 92.8% (52.8% actual as there was a 40% spontaneous cure rate in the negative control group, in contrast to 80% (40% actual) in the positive control group treated with an intra-mammary antibiotic preparation [10].

Houttuynia cordata

An aqueous intra-mammary solution obtained from Houttuynia was made for the treatment of bovine clinical mastitis. A total of 104 acute and subacute mastitis cases were randomly assigned into two groups (with 52 cases in each group) — a treatment group and a control group (in which intra-mammary administration of 800,000IU penicillin G in combination with 1g streptomycin was conducted). No statistically significant difference was found between the treatment group and control groups in the treatment of acute and subacute mastitis. In addition, an inhibitory effect was seen on the growth of lactic streptococcus in the milk collected within 48 hours of intramammary treatment with penicillin G in combination with streptomycin. However, for the herbal preparation, a mild inhibitory effect on lactic streptococci was detected in the milk within 12 hours of treatment [17]. Other herbs suggested in treatment of mastitis are given in the table 14.2.

Table 14.2: Other herbs suggested in the treatment of mastitis

Achillea millefolium	**Achyianthes aspera,**	**Aictium lappa,**
Ascophyllum nodosum	*Calendula officinalis*	*Calpurinia aurea*
Croton macrostachys,	*Ficus caria,*	*Galium aparine*
Houttuynia cordata	*Malvi parviflora*	*Nicotiana tabacum,*
Salix alba,	*Solanum hastifolium*	*Symphytum officinale*
Teucrium scorodonia	*Vernonia species*	*Ziziphus spina- christi*

Clues from Siddha Medicine

Some of the common Siddha preparations as follows (Table 14.3) have also shown to be effective against bacteria and could be of value in the treatment of mastitis.

Table 14.3: Siddha preparation for treatment of mastitis

Name of the preparation	Organism inhibited
Triphala choornam	Klebsiella, Staphylococcus aureus, Vibrio sp.
Sangu parpam	Beta haemolytic streptococci
Nandu theneer	E. coli, Neisseria sp., Strptococci
Kukil parpam	E. coli, Proteus, Klebsiella, Pseudomonas
Idi vellathi	E. coli, Proteus, Klebsiella, Pseudomonas
Nandi mezhugu	E. coli, Proteus, Klebsiella, Pseudomonas
Vediuppu churnam	E. coli, Proteus, Klebsiella, Pseudomonas

Besides, a large number of plants commonly found in India and reported in Siddha literature have been found to be associated with antibacterial property against pathogens causing mastitis [18].

Staphyloccoccus aureus 90 plants

Streptococcus pyogenes	30 plants
E coli	101 plants
Proteus	16 plants
Klebsiella	13 plants
Vibrio sp.	34 plants
Pseudomonas sp.	23 plants

In the above plants, extracts of various plants have been suggested to possess the activity. Some of the plants such as *Acacia spAzadirachta indica, Lawsonia inermis, Eclipta alba, Ocimum sanctum etc,.* seem to possess broad spectrum of activity showing activity against many species of bacteria.

Ethnoveterinary Practices for Mastitis

The use of ethnoveterinary medicine (EVM) may present a cheaper and sustainable alternative to synthetic medicines. These herbal preparations, drawing upon centuries of traditional belief and use, are in practice over time by pastoralists and farmers for the treatment of different diseases of livestock. However the treatment procedure and type of plant varied largely from area to area and person to person. Some of the reported ethnoveterinary practices seemingly in vogue for the treatment of mastitis are as follows [19, 20].

- Apple cider vinegar (1/2 cup) added to the grain and fed twice a day
- Woodsage *(Teucrium scorodonia)* tincture is infused in the udder. An infusion of cleavers *(Galium aparine)* is made by steeping 1 table spoon of cleavers in 1 cup of boiling water for 15 minutes. This is then drenched to help boost circulation in the udder and for lymph support.
- A tea of yarrow *(Achillea millefolium),* honey, sea salt, burdock root *(Arctium* sp.) and white willow bark *(Salix* sp.) is given. It is made with 1/3 cup of yarrow (whole chopped plant with flowers), 1/3 cup chopped burdock root and 1/3 cup chopped white willow bark. Three cups of boiling water are poured over the herbs and steeped for 15 - 20 minutes. Sea salt and honey is added. When cool, the herbs are applied as a

poultice, or a cotton cloth is dipped in the warm infusion and put around the udder until the poultice cools.

- Topical applications of Comfrey (*Symphytum offinale*) extract (in propylene glycol base with allantoin, ascorbic acid, and chlorophyll or carotene) to the teats of dairy cows and infusion of the extract into the udder cured acute mastitis after two applications.
- Injection of 20 to 60 mL of aloes (in gel or juice form) into the infected quarter at least once a day.
- Paste made of Aloe vera, turmeric and lime water to be applied on the udder
- Chamomile *(Matricaria recutita)* or cabbage made into a poultice
- The fresh root of *Achyranthes aspera* is chopped and bounded together with a leaf of *Commicarpus podunculosus.* This will be mixed with water and given orally for treatment of bovine mastitis.
- The fresh leaves of *Calpurinia aurea, Ficus caria, Nicotiana tabacum or Ziziphus spina-christi* is grounded with small amount of water and then applied topically to the udder.

Homeopathic Treatment for Mastitis

Homoeopthic therapy for mastitis is one of the commonest causes of homoeopathy use in animals among a variety of disease conditions. The Pan European Livestock Standards stipulate that phyto- therapeutic and homoeopathic products shall be used in preference to chemically synthesised allopathic veterinary medicinal products and antibiotics, provided that they are therapeutically effective. Some of the therapies for mastitis in homoeopathy are given in the table 14.4., and severity based treatment options are detailed in the table 14.5.

Table 14.4: General homeopathy therapy associated with bovine mastitis [21]

S.No.	Details of therapy	Remarks
1.	Belladonna 30 or 200	When the udder is hot, painful, and edematous. Dose: Belladonna 30 - one dose every two hours 4 to 5 times till relief & Belladonna 200 : B.I.D for 2 days

2.	Bryonia 30 or 200	When udder is hard, painful and hot, animal is disinclined to move. Dose : One dose every 3 hrs , till relief
3.	Urticaria urens 30	When the udder is hard, painful, edematous with allergic reactions and let down problems, dysagalactia, Dose : One dose every 1 hr till relief
4.	Combination: Belladonna, Bryonia, Urtica aa 30	Dose : 1 dose once in 2 hrs till temperature comes to normal
5.	Phytolocca 200	When the udder is hot, with flakes and clots in milk and refuse to allow the calf for suckling or milking Dose : 1 dose 2 hourly 4-5 doses, for 2-3 days
6.	Conium 200	When the udder is very hard, with yellowish and cheesy milk and painful udder. Dose : B.I.D. for 2 - 7 days
7.	Merc sol 200	When the udder is hard and when the milk is watery or serosanginous in appearance (Foot and Mouth affections) Dose: B.I.D. for 2 days
8.	Silicea 6x	Udder indurated, milk cheesy in consistence, with yellow clots. Dose : T.I.D for 1 week to 10 days
9.	Biochemical Preparation 1: Kali mur 6x (when clots in milk) & Calc Flur 6x	Dose : B.I.D. for 1 week to 10 days
10.	Biochemical Preparation 2: Silicea 6x (When the udder is hard) & Calc Sulph 6x (clots in milk)	Dose : Q.I.D for 1 week
11.	Homeopathic Combination 1:	Indications : Inflammation of the udder

	For intra mammary use. Calundula Q, Bella donna 30, Dulcamara Q, Echinaea 30 aa 1ml, made up to 20 ml with distilled water	with loss of appetite, fever, congestion and trauma. Dose : 10 ml Morning and 10ml Night, intra mammary injection for 2 to 3 days. Massage the udder to disperse the medicine uniformly
12.	Homeopathic Combination 2 : For External use only. Phytolocca decandra 30, Calendula officinalis Q, Apis mel 30, Belladonna 30 aa 1 ml made up to 20 ml with glycerine	Indicated in fissures, wounds, ulcers, congestions, hematomas, inflammations, contusions etc. Dose: Apply on the affected teats and udder, B.I.D. for 2 - 4 days
13.	Homeopathic Combination 3: For internal use, Phytolocca 200, Calc. Fluor 200, Silicea 30, Belladonna 30, Arnica 30, Conium 30, Ipeca 30 aa 0.5 ml, Made up to 30 ml vimeral	In acute, subacute and chronic mastitis. Dose: 2 -5 ml, B.I.D. orally for One week

In a study in Somerset, in two farms the effects of homoeopathic treatments were analysed and it was found that though the therapies suggested had variable results against individual bacteria causing mastitis, an overall cure rate of 65 and 53 per cent respectively were observed. However, the study included some of the good management practices, which could also account for the success rate [22]. In a comparative efficacy study of allopathic and homoeopathic therapies for mastitis, the overall effectiveness of homoeopathy was found to be higher and also highly cost effective than antibiotic therapy [21]. However,[23] suggests although clear evidence of any therapeutic advantage is lacking with respect to homoeopathic medicines in mastitis, producers still value their use based on personal experience.

Table 14.5: Severity based treatment approach for mastitis

Homeopathic remedy	**Symptoms**	**Dose**
Belladona 1 m	For acute and per-acute	1 dose every 4 hour till

	postpartum mastitis.	cure
Aconitum 6 x	Routine treatment for all	1 dose every half hour. 6
	acute cases.	doses or till cure
Apis Mellifica 6c	Udder edema and acute	1 dose every 3 hours. 4
	mastitis in first calvers	doses or till recovery
Bryonia Alba 30 c	Chronic mastitis (Fibrosed Udder) and other type of mastitis	Acute cases: 1 dose every 4 hours till cure
		Chronic cases: 1 dose 2
Arnica Montana 30 c	For mastitis occurring due to udder injuries and also for	times daily for 1 month 3 doses per day for 3 days or till cure
Phytolacca 30 c	blood in milk Acute and chronic mastitis	***Acute cases:*** 3 times per day for 3 days, followed by 1 dose per day for 4 doses or till recovery. ***Chronic cases:*** 1 dose every 3 hours, 4 doses for 10-30 days.
S.S.C. 30 (Mixture of Sulfur, Silica and Carbovegetabilis)	Clinical and subclinical mastitis	3 dose for 3 days or till recovery

Hepar Sulfuris 6 x	Summer mastitis (***C.****pyrogenes)*	1 dose every 3 hours till cure
Silica 200 c	All type of mastitis	2-4 doses daily till cure
Ipecac 30 c	Blood in milk	3 doses a day for 3 days

Commercial Herb Based Products Available for the Therapy of Mastitis

A product by name 'mastilep®' is already available commercially in India (Rahman and Sharma, 2000[6]). As per the literature it contains, (per 100 g) *Glycyrrhiza glabra* (5 g), *Curcuma longa* (2 g), *Cedrus deodara* (10 g), *Paederia foetida* (5 g) and sulfur (10 g) in gel base - and is to be applied topically on the udder and teats. The product has been shown to produce 80% recovery when combined with parenteral antibiotics and 30% after topical application without antibiotics. It has been shown to be a good supplement for clinical mastitis and however can be used alone in subclinical mastitis with a recovery of 88%. Another product by name *Phytomast*® is also available abroad which is also shown to possess protective effect in mastitis [24]. However the ability of these products to completely replace antibiotics is still questionable.

Issues in Herbal Therapy of Mastitis

1. Low efficacy

Most of the antibacterial studies indicate that the MIC levels achieved for phyto-chiemicals or plant extracts is far higher than those of conventional antibiotics. This is suggestive of the low efficacy of the plant extracts, which would weigh against their utility. Thus the herbs a theoretical basis of efficacy but it is difficult to be transform into clinical success. A great amount of processing and purification would be required to improve their efficacy.

2. Validation issues

As far as the study on newer research drugs is considered facilities for clinical trials are virtually non-existent in our country. There are not many specialized institutes carrying out clinical trials in animal drugs. A complete systematic study to prove the efficacy by laboratory and clinical research is lacking. Any approval for use by government agencies would require a lot of validation process thus delaying or denying effective use of these compounds. Their use will continue to be unconventional and marginal and never seeing the light of legality.

3. Formulation issues

When it comes to herbal drugs, there is a perennial dilemma of having to use a raw plant part, crude or semi-purified extract or a

pure compound isolated from the plant. While a raw plant part may suffer from poor efficacy, a pure extract would necessitate too much processing which deifies the purpose of simple cost effective nature of herbal medicines.

4. Identifying the 'pharmacophore'

A conventional pharmacologist tries to establish a clear link between the compound, its mechanism of action and clinical efficacy and adverse reactions. With respect to herbal drugs, it is very difficult to isolate the active compound. Sometimes, the activity is attributed to a combination of plants where the single pharmacophore cannot be identified and the logical drug discovery process cannot be followed.

5. Residues of herbal drugs

In addition to the above issues pertaining to any herbal drugs, drugs for bovine mastitis have to be viewed from an additional perspective of drug residues. Though it is believed that herbal drugs are safe, may not require any withdrawal times, the legal position is still not clear. In the Uk, there is a mandatory two day withdrawal period for all phototherapeutic and homoeopathic medicines except for dilutions 6C and above. It would be advantageous for the livestock industry if some of these potentially useful herbal products were properly assessed and licensed to ensure quality of production, concentration and use, including the stipulation of withdrawal periods.

6. Sourcing of plant products

In view of the conservation of biodiversity issues, use of plants from natural wild sources is often discouraged. Thus the availability of adequate plant will be a challenge. Plantation / culture of such rare plants will be the only option which is a daunting task considering the lesser availability of land and other natural resources and competition among plantations for human food, fodder, cash crops *etc*.

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Chapter15
Current Clinical Practice and Strategies in Bovine Mastitis Management

Mastitis remains every day challenge for the practicing veterinarians, besides dairy animal keepers, farmers and the milk industry in India and around the world. India, being the largest milk producer in the world means it also has the largest challenge in terms of mastitis. Unfortunately, for such a vast country with small dairy animal holdings spreading across every part of the villages of the country, we do not have any National Agencies dedicated to mastitis, like the National Mastitis Council in UK and elsewhere, or National Guidelines on Mastitis Therapy or a National Vision and Mission to contain mastitis and thereby improve the economic returns and their sustainability for not only the millions of poor animal keepers but also for the Indian milk industry, through supply of infection free and drug residue free milk and ultimately better human health and safety.

The goals of mastitis therapy and the treatment plan include

- To ensure complete recovery of the animal from the ill health caused by mastitis and to enable a mastitis free and drug residue free milk to their calves.
- To ensure supply of infection free and drug residue free and good quality, nutritious milk to human food chain and milk product industry
- To constrain development of drug resistant pathogens/ mastitogens and their impact on animal health and public health

Understanding the Complexity Before Treating

About 137 infectious causes of bovine mastitis are known so far, but the majority of cases are caused by only a few common bacterial pathogens, namely *Staphylococci, Streptococci, Coliforms* and *Arcanobacterium pyogenes* [1]. To simplify understanding of mastitis, one need to consider that three major factors are involved in this disease:

- The microorganisms that cause mastitis,
- The cow as host and
- The environment, which can influence the cow and the microorganisms.

While more than 100 different microorganisms can cause mastitis, they vary greatly in the route by which they reach the cow and the nature of the disease they cause in the cows. Cows contract udder infection at different ages and 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 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. Hence mastitis management strategies are three pronged such as, therapeutic management of the affected cows, udder heath and mastitis control practices and environmental care and management practices.

Different Conditions of Clinical Mastitis

It is categorized based on severity of immune response

Peracute Mastitis

- Sudden onset
- Severe inflammation of the udder
- Serous milk
- Systemic illness often precedes the symptoms manifested in the milk and mammary gland.

Acute Mastitis

- Sudden onset,
- Moderate to severe inflammation of the udder,
- Decreased production,
- Occurrence of serous milk/ fibrin clots,
- Systemic signs are similar but less severe than for the peracute form.

Subacute Mastitis

- Mild inflammation,
- No visible changes in udder,
- But there generally are small flakes or clots in the milk,
- The milk may have an off-color.
- There are no systemic signs of illness.

Chronic Mastitis

- Chronic mastitis may persist in a subclinical form for months or years with occasional clinical flare-ups.
- Treatment usually involves treating the clinical flare-ups, or culling the cow from the herd.

Clinical Mastitis and Sub-Clinical Mastitis

For every clinical case of mastitis in a herd or dairy holding 20-40 cases of subclinical cases of mastitis is present (Fig. 15.1). Detection of these remains a major challenge.

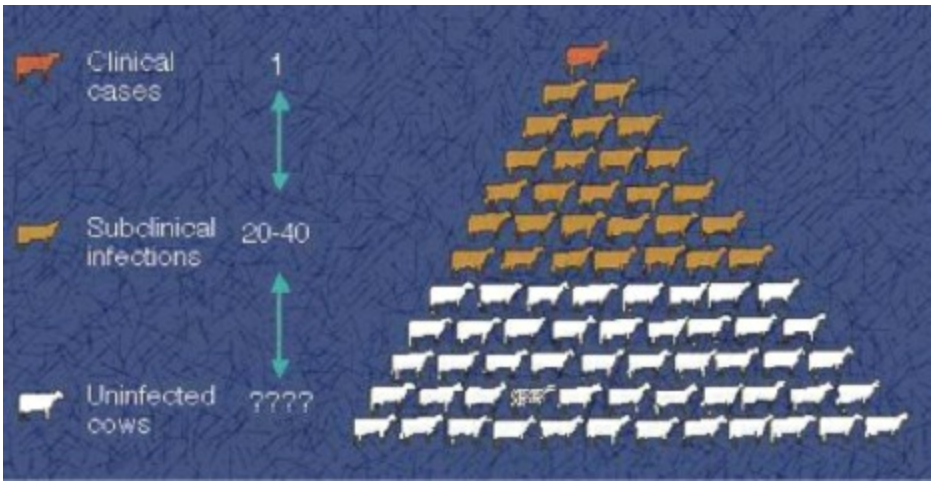

Fig. 15.1: Pictorial representation of incidence rate of clinical and subclinical mastitis in a herd

Table 15.1: Differentiation of Clinical versus Subclinical Mastitis

Subclinical Mastitis	**Clinical Mastitis**
Almost 90 -95% of all mastitis cases	Almost 5 - 10% of all mastitis cases
Udder appears normal	Inflamed udder
Milk appears normal	Clumps and clots in milk
Elevated SCC (score 3-5)	Acute type

- Major type of clinical mastitis
- Bad milk
- Loss of appetite
- Depression

- Prompt attention needed

Lowered milk output (~ 10%) Chronic type

- Bad milk

- Cow appears healthy

Longer duration

Table 15.2: Clinical signs as indicators of common pathogens causing mastitis

Sl. No.	Clinical sign	Organism
1	Swelling of udder milk is thick clots & pus, gangrene of the gland, Secretion; blood stained serous fluid without odour	*Staphylococcus*
2	Discoloration of milk due to blood staining	*Streptococcus*
	or watery Clots & flakes -discoloration	*agalactia*
3	Watery milk to thin Yellowish milk-flakes & clots	*E.coli*
4	Pink or red tinged milk from all quarter Udder soft & pliable	*Leptospira*

Table 15.3: Clinical signs as indicators of lesser known pathogens causing mastitis

Sl. No.	Clinical sign	Organism
1	Secretion purulent resembling cooked cereal or discolored small curds of cottage cheese Gray or brownish or blood tinged milk-all quarter.	*Mycoplasma*

2	Abscess with a discharge of thick greenish yellow pus with foul odour later purulent greenish sanguineous or chocolate brown secretion	*Corynebacterium*
3	Gangrene of the udder. Secretion- blood tinged milk containing gas bubbles. Teat blue,cold & crepitating	*Clostridium perfringens*
4	Secretion bluish tinged with smell &flakes or clots. Blood tinged milk	*Pseudomonas*
5	Serous flocculent & purulent secretion become blood tinged Yellowish viscid in fore milk	*Pasteurella*
6	Milk grossly normal color changes through an white to amber color with fine floccule. Soapy frothy appearance-shaking	*Mycobacterium*
7	Fore milk- gray white or mucoid secretion. Udder- extreme distention & edema	*Yeast*

Table 15.4: Differential diagnosis of contagious versus environmental mastitis

	Contagious Mastitis	**Environmental Mastitis**
Etiology	• *Streptococcus agalactiae (S. agalactiae)* • *Staphylococcus aureus (S. aureus)* • *Streptococcus dysgalactiae (S. dysgalactiae)*	**Coliforms** *Escherichia coli* *Klebsiella pneumonia* *Klebsiellaoxytoca* *Enterobacteraerogenes* **Environmental streptococci** • *S. uberis* • *S. bovis* • *S. disgalactiae* • *Enterococcus faecium*

		• *Enterococcus faecalis*
Primary source	Udders of infected cows	The environment of the cow
Method of spread	From infected quarters to other quarters and cows primarily at milking time	Through cow's environment
Indicators	Bulk tank somatic cell count (SCC) above 300,000 cells/ml SCC score above 3 More than 15 percent of cows with a SCC score of 5 or greater Frequent flare-ups of clinical mastitis, often in the same cows Bacterial culturing of cows shows *S. agalactiae* and/or *S. aureus* infections	High rate of clinical mastiti, usually in early lactation or during hot weather; somatic cell count may be low (less than 300,000)
Control	Develop program to prevent the spread of bacteria at milking time Eliminate existing infections by treating all cows at drying off and culling chronic cows bacteria	Reduce the number of to which the teat end is exposed Improve cleanliness of cow surroundings, especially in late dry period and at calving Improve prepping procedures to ensure clean, dry teats are being milked
Goals	Eradicate *S. agalactiae* from the herd Reduce *S. aureus* infections to less than 5 percent of the cows in the herd	Goal: Reduce clinical mastitis to less than 3 percent of the milking cows/ month

Therapeutic Planning in the Field Practice

The immediate goal is to return the quarter and milk to clinically normal, while paying attention to

- Cost effectiveness
- Without causing drug residue
- Using rational treatment protocol.

The Secondary goal is

- To eliminate mastitis-causing organism
- Prevent further damage
- Sustain future milk production & low Somatic Cell Count.

Selection of appropriate treatment

The decision is based on the following;

- The likely bacteria causing mastitis. Ideally the cause should be identified by milk culture.
- Time of the season e.g. certain bacteria (especially *Strep. uberis)* are likely to be more prevalent around calving and milk discard may be less of an issue early in the season when feeding calves.
- Age of the animals e.g. treatment for heifers may differ to that for an old cow.
- Clinical presentation e.g. single quarter therapy may differ to multiple quarter infections or very swollen udders.

Ensure the gland is milked out as completely as possible.

- This removes millions of bacteria, milk (which is the primary growth medium for the bacteria) and clots and debris that can hinder the spread of antibiotic treatment.
- Some bacteria produce toxins that can make the cow very sick.
- In these cases of toxic mastitis, milking the cow out also removes these toxins.
- Note in severe cases it is recommended to milk the cow more frequently than twice daily.
- Oxytocin stimulates milk letdown and contraction of muscle cells that

'squeeze' milk from the glands and into the udder. This drug can help the cow milk out completely especially if the cow is being stripped out in between regular milking.

If cows are having difficulty releasing or ejecting milk because of clots and flakes that plug the teat canal, these should be stripped out. 20 IU of Oxytocin before each milking for 6 milking were able to clear about 25% of experimental *Strepuberis* infections. However, after the oxytocin dosing, sustained use of intra-mammary antibiotics is effective to eliminate all infections. Oxytocin use alone is not beneficial as compared to antibiotic use.

Antibiotic Treatment

Many bacteria can cause the same signs and severity, but not all bacteria respond the same to any on treatment. It is good to identify the types of infections to make the best choices.

Good Records of Antibiotic Therapy

Records of Therapy are absolutely necessary for an effective clinical mastitis therapy program

- Types of infections causing clinical mastitis
- Past mastitis history
- Success with available treatment
- Stage of lactation
- Stage of pregnancy
- Value of cow as cull or price of replacement.

Table 15.5: Preferred antimicrobials for various infectious agents

Organism	Route of administration	Antimicrobial drugs
E.Coli,	Parentral	Cefrioxone/Amoxycillin/Gentamycin/
Klebsiella		Ciprofloxacin
	Intramammary	Cefrioxone/ Amoxycillin/Ampicillin

S.aureus	Parentral	Caoxacillin / T etracycline /Penicillin-G/Cefrioxone
	Intramammary	Cefrioxone/Cloxacillin/Erythromycin
Streptococci	Parentral	Amoxycillin/Ampicillin / T etracycline / Penicillin-G
	Intramammary	Ceftrioxone/Cloxacillin/Ampicillin/ Penicillin-G
P.aeruginosa	Intrammary	Gentamycin/Colistin/Ciprofloxacin/ Enrofloxacin
F. necrophorum	Intramammary	Amoxacillin /Ampicillin /Penicillin-G/ Erythromycin/ Chloromphenicol
C.perfringens, B.cerus	Intramammary	Ceftroxone /Amoxycillin/Penicillin-G
Nocardiaspp	Intramammary	T rimethoprim-Sulfamethoxone /Amikacin
Mycoplasma	Parentral	Ciprofloxacin/Enrofloxacin

Administration of Intra-Mammary Therapy

Many a time, inappropriate handling of intra-mammary tubes, either by farmers or animal health care providers itself can cause much harm. The appropriate process is as given below

- Milk samples should be taken before the treatment begins. These can be frozen and cultured later if needed.
- Strip out the quarter fully before infusing antibiotic into the quarter. This may be assisted by the injection of 2 - 3 mL of oxytocin into the muscle prior to milking.
- Clean all teat ends thoroughly (wipe all teat ends with teat wipes followed by spraying with 70% alcohol).
- Hold the barrel of the syringe in one hand and remove cap by gently twisting. Do not bend the nozzle. Take care not to contaminate the nozzle.

- Partially insert (3mm) the nozzle of the intra-mammary into the teat canal and apply steady pressure on the syringe until the full dose has been delivered into the quarter.
- Massage the infusion up into the udder.
- Record treatment date, product used, and withholding periods.

Table 15.6: Treatment plan for various mastitis conditions

Severity	Symptoms	Treatment Plan
Mild	Abnormal milk	• Oxytocin & milk out Antibiotic : IMM (depending on the need)
Moderate	Abnormal milk swollen quarter pain, no fever	• Oxytocin & milk out • Antibiotic: IMM and / or systemic
Severe - Acute	Temp >102°C pain, swelling	• Oxytocin & milk out • Antibiotic: IMM and / or systemic • Anti-inflammatory drugs • Systemic antibiotics
Toxic	Severely ill depressed, off-feed dehydration	• Oxytocin & milk out • Anti-flammatory drugs • Fluids (intravenous or oral) • Systemic antibiotics

Acute Coliform Mastitis Therapy

Shock is the primary concern. Correction of tissue perfusion deficits is the major goal.

- 40 to 60 L of isotonic saline or 2 L of 7.5% saline (hypertonic) is recommended
- Hypocalcemia is most consistent serum chemistry change in such cases.

AABP (American Association of Bovine Practitioners) guidelines for therapy of clinical mastitis in lactating dairy cows

The role of the veterinarian is to design rational treatment protocols that help cows recover, improve the owner's net profit and protect the consumer from violative drug residues.

- Mastitis management must focus on prevention
- Therapy of clinical mastitis should be part of an
    - Udder Health Program that includes
        1. Milking hygiene,
        2. Management of the cows' environment,
        3. Milking equipment evaluation and maintenance,
        4. Evaluation of milking technique,
        5. Appropriate immunizations and
        6. A culling protocol.
- The veterinarian's recommendations for therapy must be based on knowledge of the likely etiology for each herd, based on recent culture results.
- Severity of clinical signs and the appearance of the milk are not reliable evidence of etiology.
- Coliform mastitis, for example, can be mild and chronic or peracute and severe. Therapy of a given cow often begins before the cow's culture results can be known. However, a treatment protocol can be designed based on the known pattern of pathogens involved in the etiology of clinical mastitis on the farm. This may be done by culturing pre-treatment milk samples from cows with clinical mastitis or a high SCC.
- Good records are a prerequisite for an effective therapy program and are

needed to document residue prevention efforts. It is especially useful to know the cow's past history of mastitis problems.

- Mastitis caused by *Mycoplasma sp., Serratia sp., Pseudomonas sp., Arcanobacterium sp.* (formerly *Actinomyces* sp.), *Nocardia sp., Prototheca sp., Mycobacterium sp.,* yeasts, fungi and most other unusual pathogens is refractory to all known therapy.
- Mastitis caused by *Staphylococcus aureus* is refractory to treatment in most cows.
- Untested combinations of extra-label products should not be formulated. There is no scientific evidence for their efficacy and safety, withdrawal times generally are unknown and their formulation for sale is illegal.
- Multi dose containers should never be used because of the risk of contamination with resistant organisms such as Mycoplasma and yeast.
- Antibiotics are unlikely to be of benefit in clinical mastitis caused by gram-negative organisms. Thorough milk out with supportive therapy, possibly including anti-inflammatory drugs, should be the basis of treatment. Severely ill cows may benefit from systemic antibiotics.

AABP Guidelines on Clinical Mastitis Therapy

- Clinical mastitis should be classified according to severity.
- Severe mastitis, where the cow is depressed and off feed, should be treated with supportive therapy aimed at counteracting the effects of endotoxin through the use of treatments such as
    - Fluids,
    - Calcium,
    - Hypertonic saline,
    - Anti-inflammatory drugs and
    - Complete and frequent milk out of the affected quarter(s).
- Studies have shown that antibiotics make little difference in the outcome of severe coliform mastitis.
- Intra-mammary antibiotics are poorly distributed in a severely swollen gland.
- Successful treatment of these cows may require frequent veterinary interventions

Clinical mastitis caused by *Streptococcus agalactiae* should be treated

with approved intra-mammary antibiotics.

Clinical mastitis caused by *Staphylococcus aureus* can be treated with intra-mammary antibiotics to reduce clinical signs, but few cows will be cured during lactation.

Mild Clinical Mastitis in herds with no history of mastitis caused by *S. agalactiae* may be allowed to recover with no antibiotic therapy, relying only on complete milkout, perhaps with the aid of oxytocin injections.

Intermediate cases caused by gram-positive organisms may benefit from intra-mammary antibiotics or a combination of intra-mammary and systemic antibiotics. Antibiotics approved for systemic use in lactating cows may not cross the blood-milk barrier in therapeutic concentrations. Anti-inflammatory therapy may be used to reduce udder swelling and to help cows feel better.

Staphylococcus aureus mastitis: The Never Ending War

Staphylococcus aureus is often considered the most common cause of contagious mastitis in dairy herds. There are estimates that 80-100% of all herds have at least some *S. aureus* mastitis, with from 5 to 10% of cows infected.

- *Staphylococcus aureus* is also an important cause of mastitis in heifers, with wide variability in levels of infection among herds.
- *Staphylococcus aureus* is often considered the most common cause of contagious mastitis. There are estimates that 80-100% of all herds have at least some *S. aureus mastitis*, with from 5 to 10% of cows infected.
- Herds with excellent milking hygiene practices and management have lower levels of *S. aureus* intra-mammary infections (IMIs), as compared to those herds with poor hygiene or management.

The numbers of *S. aureus* bacteria found in the milk of an infected cow often show cyclic variation. Bacterial numbers in milk may be high for a while, followed by an intervening period with much lower to non-detectable numbers of bacteria. A negative result or very low numbers of *S. aureus* might be found in an infected cow when milk samples are collected during the period when the numbers of bacteria are at their lowest (or non-detectable) levels.

- A single negative result is not proof that a cow is uninfected with *S.*

aureus.

- A more accurate determination of a cow's infection status could be obtained by testing 2 or 3 milk samples collected on different days.

Therapy and Management of S. *aureus* Mastitis

Mastitis due to *S. aureus* is a major challenge in clinical practice. Many times it remains on responsive to all the available treatment protocols. Bacteriological cure for *S. aureus* remains unattainable goal.

- Bacteriological cure rate during lactation is low (about 30-60%) because
  - *S. aureus* causes micro-abscesses in the udder,
  - Survives inside cells, and
  - Some forms are resistant to commonly used antibiotics(e.g. strains with the enzyme beta-lactamase are resistant to penicillin).
- The best hope for successful treatment is in young cows with recent infections (of less than two weeks duration).
- Treatment of clinical mastitis may reduce Staph shedding, and result in milk returning to clinical normality.

Staphylococcus aureus intramammary infections (IMIs) are difficult to eliminate. Average cure rates have been reported to be only

- About 50% (17-95% range) for subclinical cases,
- Approximately 55% (26-92% range) for clinical cases, and
- Around 60% (14-100% range) for dry-cow therapy.

Several different strategies have been introduced to improve the therapy success rate.

They include

1. Using extended antibiotic therapy protocols,
2. Vaccinating animals, and
3. Combining extended antibiotic therapy with vaccination.

Appropriate withholding times for meat and milk must be observed in such antibiotic usage times.

- Treatment of cows infected with *S. aureus* may be successful when infections are of short duration (< 2- weeks), in young cows and in early lactation.
- Usage of extended duration of intra-mammary therapy (8 days) may further improve cure rates [2, 3].
- The bacteriologic cure rates for newly acquired (< 2-weeks duration) *S. aureus* infections were reported to be 70% after treatment with intra-mammary penicillin product [4].
- Cure rates for chronic (> 4-weeks duration) *S. aureus* infections were only 35%.
- Cure rates for mastitis caused by *S. aureus* have been shown to decrease with age (from 81 % for cows <48 months of age to 55% for cows >96 months), the number of infected quarters (from 73% for 1infected quarter to 56% for 4 infected quarters) and SCC [5].
- Cows infected in more than 1 quarter were less than half as likely to be cured as compared to cows with only 1-quarter infected [5].

Extended Antibiotic Therapy Protocols

Extended therapy protocols involve multiple infusions of Pirlimycin per quarter. The extended therapy approach appeared to work because of the extended period of effective antibiotic levels in the mammary gland. In one study, the product was used according to label (2 treatments at 24 hour intervals, followed by a 36-hour milk withholding period) for 3 consecutive series of treatments.

Cure rates from extended therapy with Pirlimycin reportedly exceeded 60%, compared to 49% with the label use of 2 infusions at 24 hour intervals. In studies in the European Union, extended therapy trials have been conducted with as many as 8 infusions of Pirlimycin on consecutive days. In those studies, treatment success improved with increasing number of infusions.

Another strategy observed to increase treatment success in *S. aureus* mastitis is to combine systemic with intra-mammary therapy. In one study, the cure rate was only 25% when udder infusions of Amoxicillin were administered for six milkings. Cows in another group that received both the udder infusions plus systemic procaine penicillin G for 3 days had a cure rate of 51%. This cure rate, however, is still lower than desired.

Vaccination for *S. aureus*

The current strategy in the management of *S. aureus* mastitis is use of vaccination practices. It has been employed as an adjunct to therapy as well as a preventative measure for *S. aureus* mastitis. Several vaccine products have been evaluated for protection against *S. aureus* mastitis and their results are variable. Some studies have demonstrated decreased new infections or increased cure rates in both cows and heifers.

One study with heifers vaccinated at 6 months of age, 14 days later and then at6 month intervals showed a

- 43% decrease in quarters exhibiting chronic IMI during pregnancy,
- A 45% decrease in new IMIs during pregnancy, and
- A 45% decrease in new IMIs at freshening.

In other studies with cows, extended therapy with Pirlimycin has been compared with vaccination alone or in combination with extended therapy. The studies have shown that extended therapy plus vaccination generally produced results that are better than either extended therapy or vaccination alone.

Guidelines in Control of S. *aureus* Mastitis

Control of *S. aureus* should be focused on three areas:

1. Preventing new infections;
2. Elimination of existing infections; and
3. Monitoring progress after implementation.

- The single most important step in preventing new infections is to dip every quarter of every cow after every milking with an effective teat dip.
- Dry cow treatment is an essential step in eliminating existing infections, and is also a step which can reduce new infections by 50-75%.
- Monitoring for quality of milk and for presence of contagious and subclinical mastitis has to be undertaken.
- Our veterinarians should also specialize to be milk quality specialists, so as to enable an orchestrated mastitis control strategy.
- The level of mastitis caused by *S. aureus* can be minimized when milk producers are educated on mastitis control strategies and if they started

following proven practices, which unfortunately is missing in Indian dairy production system.
- Day to day interactions and consultations among veterinarians, extension specialists, fieldman, milk handler and dairy plant personnel will help in establishing a less mastitis status and more profit from dairy business.

Therapy for mastitis due to *Streptococcus agalactiae:*

- *Strep. agalactiae* lives only in the udder of cows and is not a frequent cause of clinical mastitis in most herds.
- *Strep. agalactiae* is highly sensitive to most of the commonly used antibiotics, and a high cure rate (>90%) can be expected using the correct antibiotic.
- Treatment stops shedding of *Strep. agalactiae* by cows with clinical mastitis.
- Intra-mammary treatment with penicillin type drugs continues to be highly effective resulting in 80-90% cure rates.
- To eradicate *Strep. agalactiae,* all four quarters of all culture positive cows in the herd should be treated with an appropriate commercially marketed intra-mammary antibiotic [6].
- A small percentage of animals will not be cured, therefore cows that continue to have high SCC values should be resampled and cultured at 30-day intervals.
- Cows that remain infected can be retreated but should be segregated from the herd to prevent reinfection.
- Treatment of the herd should be accompanied by an effective teat dipping program and comprehensive dry cow therapy.
- Treatment of cows subclinically infected with *Strep agalactiae* usually results in increased production.

Therapy for mastitis due to environmental Streptococci

- Spontaneous cure rate for clinical mastitis caused by environmental causes, usually *Strep. uberis and Strep. dysgalactiae,* may exceed 50%, but frequent relapses occur if the cows do not receive appropriate antibiotic therapy [7].
- Clinical cases of mastitis caused by environmental streptococci should be

treated with approved intra-mammary antibiotics for an appropriate number of treatments.
- The use of aggressive treatment of induced *Strep. uberis* infections has been shown to result incur rates that exceed 90% [8].
- Experience shows some cases readily respond to treatment and others are quite refractory to treatment.
- Field strains of *Strep. uberis* are able to invade and live in epithelial cells, which may partially explain why infections are refractory to treatment

Therapy for mastitis due to Coagulase-negative Staphylococci (CNS):

- CNS usually lives on teat skin and can colonize the teat canal.
- Dry cow therapy is found to be effective in controlling these organisms.
- The rate of spontaneous cure is high but intra-mammary treatment of cows infected with CNS is often highly successful [9].
- While CNS are not a frequent cause of clinical mastitis, surveys in herds that have controlled major pathogens generally attribute 3-10% of clinical cases to CNS.

Therapy for Mastitis Due to Gram-negative Bacteria

Toxins produced by *Escherichia coli* cause the clinical signs of mastitis. In many cases, bacterial numbers are falling when clinical signs appear. Treatment aims to remove toxin by frequent stripping out and use of 30-60 IU oxytocin, and to minimise the effects of toxin by using anti-inflammatory agents and possibly intravenous fluids. Systemic antibiotics are given when the cow is extremely ill or when intra-mammary infusions are unlikely to diffuse through tissue because the udder is greatly swollen.

- The hydration status of the cows should be evaluated and cows should be given hypertonic or isotonic fluid therapy and appropriate anti-inflammatories.
- In more than 40% of severely ill animals, bacteria may escape the udder and circulate throughout the blood stream [10].
- A study demonstrated more favourable clinical outcomes for cows with

severe clinical coliform mastitis that received intramuscular Ceftiofur once daily as compared to cows that received only supportive therapy [11].

- Mastitis vaccines had been developed and successfully deployed in clinical practice for mastitis due to Gram-negative bacterial organisms
- Many reports had documented the use of J-5 vaccines in reducing the amount of severe mastitis caused by Gram-negative bacteria.
- Most mastitis caused by Gram-negative bacteria is mild or moderate because the immune response is highly successful in destroying these bacteria.
- As the bacteria are destroyed, they release endotoxin from their cell walls.
- In 5-15% of these cases, enough endotoxin is released to result in seriously ill cows.
- These cows require rapid diagnosis and immediate supportive therapy.

Principles of Mastitis Therapy

To make the mastitis therapy successful, selection of appropriate antibacterial mastitis therapy is the single most important factor [12].

To be successful,

- The selected drug must attain and maintain concentrations exceeding the minimum inhibitory concentration (MIC) at the focus of infection for sufficiently long enough to break the production and toxin-producing cycle of the pathogens.
- Therapy may also have poor results owing to tissue damage and introduction of new infections during treatment, and/or failure to eliminate the management factors that predispose to mastitis.
- While the primary goal of antibacterial therapy is to kill bacteria and that the normal udder is sterile, usually the best that can be achieved is temporary reduction or suppression of the bacterial population to allow the host to overcome the infection.
- Udder infections tend to be dynamic, and stress may contribute to udder infections becoming clinically apparent. Hence all these factors must be borne in mind before instituting appropriate therapeutic protocols.

Correct diagnosis, appropriateness of the route of administration and the drug selected, stage at which treatment is initiated, severity of udder pathology, supportive treatment, and elimination of predisposing factors are the other factors that determine the success of therapy.

Intramammary infusion Therapy: Newer Concepts

- Intramammary treatment is practical and effective for cases where the inflammatory response does not occlude the teat canal or cistern.
- The inflammatory process in affected glands may impede distribution of antibiotics.
- Dry Cow Treatment preparations should never be used in lactating cows.
- Inadvertent use of Dry Cow Treatment would require milk to be discarded for extended periods of time.
- Intramammary infusion of drugs is the route of choice in subclinical, mild or moderately severe mastitis, and is used as an adjunct to parenteral administration in severe mastitis.
- For effective intramammary treatment, drugs should get distributed throughout the udder and be rapidly absorbed into the general blood circulation.
- Significantly better results were reported to be obtained when the drug is administered intra-cisternally in 1 L of 0.5 % glucose solution, rather than in 50ml of saline.
- Local application of antimicrobials has the disadvantage of slow and uneven distribution of certain drugs in the infected udder.
- In acute, severe disease, distribution through the udder is impaired by inflammation or blockage of milk ducts by debris.
- Parenteral administration generally overcomes these problems; although it is a common clinical practice to administer agents concurrently by the intramammary route.
- Severely inflamed udders should be milked out frequently, with the aid of oxytocin if necessary.
- In peracute or acute clinical mastitis cases with systemic signs, combined systemic and intramammary treatment with compatible antibiotics, supplemented with supportive therapy, is recommended.

Systemic Antibiotics

Acute mastitis cases may benefit from both intra-mammary and systemic antibiotics. Peracute cases often require systemic antibiotics and anti-inflammatory preparations, and possibly intravenous fluids. The prognosis for peracute cases in cows with severe clinical signs (as indicated by body temperature, dehydration, etc.) is poor regardless of treatment. Systemic antibiotics have the advantage that drug distribution is not impeded by local inflammatory reactions in the udder. However, to be effective, systemic antibiotic treatments must be absorbed from the injection site and pass from the blood into the udder. Their major difficulty is penetration of the "blood-milk barrier".

Drugs move across the blood-milk barrier by passive diffusion of the non-ionised parts of the molecule according to the principle of osmosis. This barrier is penetrated by the non-ionized, lipid soluble, non-protein-bound drug fractions.

- Weak acids (e.g. Penicillin G) are almost completely ionised in blood and have poor tissue penetration.
- On the other hand, Penethamatehydroiodide achieves concentrations in the milk that are 5-10 times higher than other penicillin salts due to its basic and lipophilic properties. This treatment results in high levels of penicillin in the udder because it is hydrolysed as it crosses into milk liberating active benzyl penicillin.

Allowing for antibiotic sensitivity patterns, antibiotics with high milk-to-plasma ratios are most suitable for systemic administration. Many clinical reports and studies suggest that the combined systemic and intramammary antibiotic treatment results in a slightly but significantly higher rate of bacteriological cure in the treatment of acute staphylococcal and Streptococcal mastitis.

Strategies missing with Field Practitioners in India

- In Indian scenario, it is common to treat first and obtain an accurate diagnosis later, if such one is needed or obtained at all. Many times accurate diagnosis with regard to specific pathogen involved in mastitis

is not at all arrived.
- If a precise diagnosis is not immediately available, it is advised that the Practitioners or Farmers themselves can submit milk samples to labs for rapid provisional diagnosis and then readjust therapy when the pathogen is diagnosed 24-48 hours after beginning treatment.

Therapeutic Response varies with the age of the Cow

- Older cattle were observed to be a greater risk for both subclinical and clinical mastitis, and have poor response to treatment, when compared to younger cattle.
- Bacteriological cure rates were shown to decline with parity and this necessitated longer duration of therapy for cows experiencing second clinical episodes
- Longer duration of therapy was also required for older cows with a history of two to three months of high SCC.
- SCC plays major role in treatment decisions of cows with chronic mastitis. If the SCC of a treated cow remains increased for months and the cow has recurrent mild cases of mastitis, it is very essential to determine the pathogen and then go for therapeutic planning and appropriate decisions, with regard to cost and the outcome.
- The complexities of the cow's immune status as well as their age were attributed to have such an effect.

Duration of intra-mammary therapy

- To document the appropriate duration of intra-mammary therapy for mastitis, we need more clinical trials, as such studies are lacking.
- Based on the limited evidence, five days of therapy may be appropriate for mastitis caused by environmental streptococci.
- If the cow had developed mastitis caused by *S. aureus,* it is recommended that eight days of intra-mammary therapy be used.

Extended duration of intra-mammary therapy

- Extended duration therapies are always targeted at cows that are likely to benefit, for example in cases like, a new *S. aureus* mastitis in a young cow or in case of a third lactation cow with an environmental strep

infection in the first week of calving (during which period there will be peri-partum immune depression).

- When extended duration therapy is routinely used for all cases without regard to causative pathogen, we need to keep in mind the residues in milk and that considerable milk will have to be discarded unnecessarily.
- While deciding for extended therapy, we also need to ensure that drugs are used properly. Giving just antibiotics to cows that won't respond is both a waste of money and an unnecessary potential introduction of the compounds into the dairy food production system.

Supportive Treatment

Anti-inflammatory Drugs

Flunixin meglumine inhibits prostaglandin production and limits exudate at the site of inflammation. In contrast with corticosteroids, Flunixin does not inhibit white blood cell mobilisation at the infection site. Passage of Flunixin from blood to milk is poor, with levels in milk about 1% of those in blood. Nevertheless, it has a useful systemic effect and helps reverse the clinical signs of shock in toxic forms of coliform or staphylococcal mastitis.

Other drugs such as Salicylates (Aspirin), may help reduce fever and inflammation but have allow potency and relatively short half¬life. In contrast, Phenylbutazone has a long half-life (36-72 hours in cattle depending on the dose) but its action may be cumulative and toxic.

Fluid Therapy

Large volumes of isotonic intravenous fluid (25-40 L) can markedly improve the chances of survival of cows suffering from acute toxic mastitis. In the early stages of shock (for example, in cows that had a normal fluid status two hours earlier) small volumes of hypertonic saline have been used as an initial treatment to help restore the circulatory blood volume.

Current Practices in Mastitis Vaccination Programme

Vaccinating cows as a preventative mastitis treatment has made all the difference to the control of mastitis on a wide range of herds in many countries. Some herds have been able to cut mastitis rates by up to 50%, with a big reduction in the retreatment rate also seen. Currently a commercially available Startvac mastitis vaccine, is been used to help tackle the two primary causes of mastitis - *E. coli* and *S. aureus*.

Currently there are two mastitis vaccine programmes that can be used. The first involves vaccinating cows at 45 days pre-calving, 10 days pre-calving, and then after 52 days in-milk. This programme can be adapted to make it more convenient for farmers by injecting at drying off, as cows move into the transition group, and then at the fertility check at 45-55 days in-milk. As an alternative, which many farmers in western economies prefer, is a rolling quarterly programme. This involves vaccinating the cattle twice - 30 days apart - and then every three months thereafter. As a result of using the vaccine, it was also observed that the lower-grade antibiotics are working to a greater effect to treat mastitis cases in herds on the vaccination programme. And that has been observed to be a really big benefit, because that they can move away from having to use the more modern drugs and reduce the risks of resistance, which the medical profession around the world is concerned about.

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Chapter 16
Transition Cow Management for Boosting Udder Immunity

The transition or periparturient period is defined as the three weeks before and after calving. During this period, stressors associated with calving and lactation are at their maximum. There is a change in state of the animal from non-lactating to lactating. To adapt the animal to this altered state a number of orchestrated series of changes occur in its metabolism. There is a dramatic increase in the requirements for energy, glucose, amino acids and other nutrients in dairy cattle. Simultaneously, feed intake is often depressed which results in negative energy balance. This suppresses the immune function and promotes metabolic disorders, thus potentially explaining relationships between infectious and noninfectious transition disorders.

During transition, there is an acute adipose tissue mobilization, breakdown of liver glycogen and liver triglyceride accumulation. Cytokines promote the breakdown of fat storage through decreased feed intake, impaired insulin sensitivity and direct stimulation of lipolysis, which may lead to ketosis and fatty liver in dairy cattle [1]. Clinical ketosis has also been associated with a two-fold increase in the risk of clinical mastitis and the concentration of beta-hydroxy butyric acid (BHBA) shows a strong positive correlation to the severity of induced *E. coli* mastitis during this period.

Tumor necrosis factor alpha (TNF a) produced around transition decreases liver glucose production and promotes triglyceride accumulation. A sudden change in the dietary shifts during the transition period results in ruminal production of endotoxin and subsequent transfer of these endotoxins into the bloodstream [2]. Cows during this period start producing acute phase proteins (APP) which subsequently lead to low milk production and reduced

reproductive efficiency. Reactive oxygen species such as hydrogen peroxide are produced in the liver and activate inflammatory cascades, which in turn alter nutrient metabolism, harm immune cells and can decrease the ability of the immune system to respond to infections. During suppressed immunity, chances of invasion by the pathogen increases. To remove these pathogens, leucocytes particularly neutrophils have to migrate to kill the pathogen. It has been observed that their migration is faster for cells from cows with low serum BHBA levels than for cells from cows with high BHBA levels in their blood. If cells move slowly, they are out competed by bacteria, resulting in clinical mastitis. Glucocorticoids (immune-suppressants) are elevated around the time of calving and have been postulated to be at least partly responsible for peri-parturient immunosuppression [3]. Furthermore, changes in estradiol and progesterone just prior to calving may directly or indirectly affect immune-competence. As a combined result of these dysfunctions the transition cows may be hyposensitive and hypo- responsive to antigens, and therefore more susceptible to infectious diseases such as mastitis.

Onset of Mammary Infections During Involution

Mammary gland has sufficient fighting arsenals against mastitis. But, the infection occurs after bacteria gain entrance to the mammary gland via the teat canal and overcome the anatomical defense. This happens during drying off, at calving and during milking with fluctuations of milking vacuums. The graph below (Fig. 16.1) shows the rate of intra-mammary infections during different phases of the lactation cycle of a cow. The incidences of infections are maximum during peri-partum period and minimum during mid-lactation. During the beginning of the dry period (involution) the cow is not milked. But the mammary gland continues to secrete milk with maximum accumulation occurring 2 to 3 days after milk removal is stopped. Pressure in the gland can cause the streak canal to widen and the teat sphincter to dilate, allowing pathogens to enter inside the gland. Also during colostrogenesis there is an increase in the intra mammary pressure which may sometimes cause leakage allowing an easy path for the pathogens.

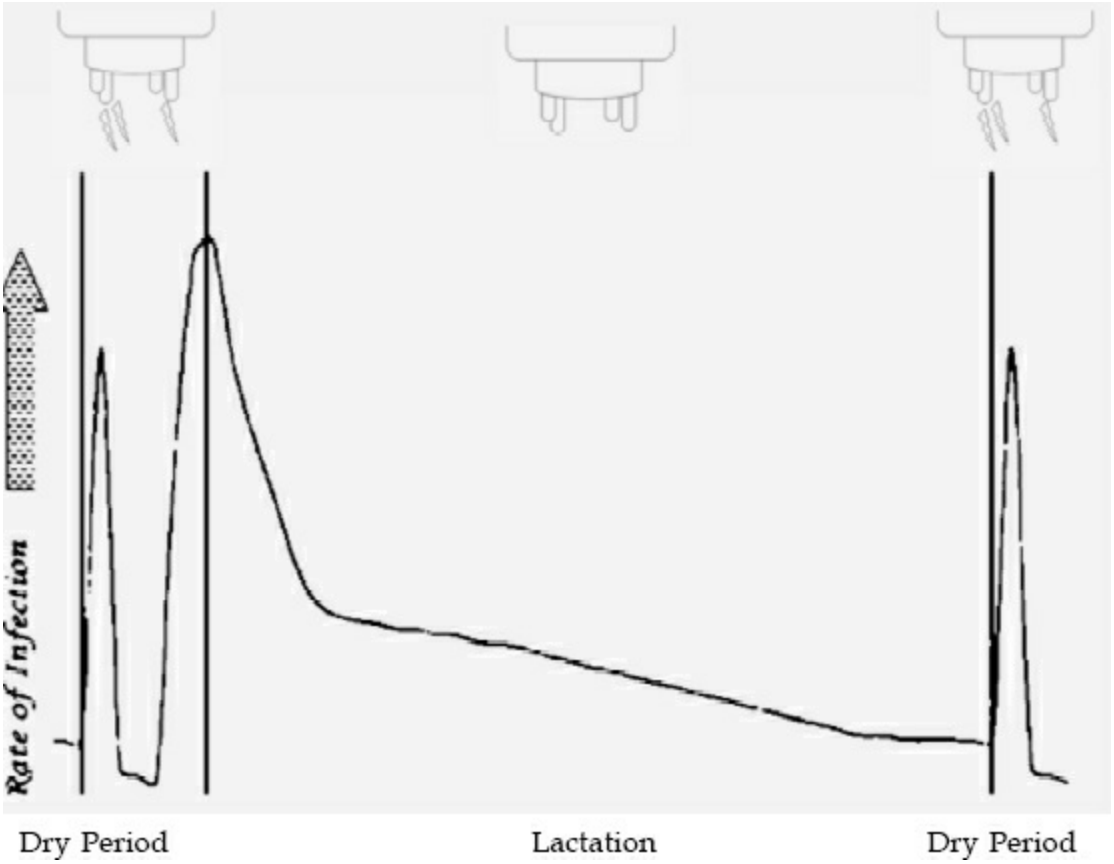

Fig. 16.1: Rate of intra-mammary infections during different phases of the lactation cycle

Once they enter, bacteria pass the keratin lining and travel to the milk producing epithelial cells. A host of signaling molecules like nitric oxide, prostaglandins, and cytokines are released by activated immune cells of the mammary gland. Chemical signals are sent and many more cytokines like TNF a, IL-8, IL-1, PGF2a and APP are released, which attract the leucocytes to come and kill bacteria. If the neutrophils fail then macrophages follow and try to clear the bacteria. The number of somatic cells (leucocytes and epithelial cells) in milk continues to increase and concomitantly, tissue damage is worsened. The alveoli in the gland start to lose structural integrity and the blood-milk barrier is breached. This allows extracellular fluid to enter the gland and mix with the milk. Visible changes in the milk and the udder start to occur. If the leucocytes are not able to kill the invading pathogens, it leads to mastitis.

Involution of a dairy animal can be divided into three stages i.e. active, steady and redevelopment withcolostrogenesis. The changes occurring in the

mammary gland during involution of pregnant cows have been presented in table 16.1

Table 16.1: Changes during mammary gland involution

I	**ACTIVE INVOLUTION**		**Udder immunity low**
a.	Accumulation of milk	⇑	Causes leakage of mammary gland
b.	Lactose synthesis	⇓	Little present (are good growth medium for bacteria)
c.	Milk fat	⇓	Still present (good growth medium for bacteria)
d.	Caseins, a-lactalbumin, β-lactoglobulin	⇓	Present (growth medium for bacteria)
e.	Immunoglobulins, serum albumin	⇑	Provide some immunity
f.	Tight junctions between epithelial cells	⇑	Loss in the integrity of alveolar shape
g·	N-acety l-l^-D-gl ucosam in idase	⇑	(lysosomal enzyme), provide immunity
h.	Lactoferrein	⇑	Protective effect is not absolute and new gram negative bacterial infections do occur. Also citrate:lactoferrin ratio is very high
i.	Neutrophils	⇑	More involved in clearing milk debris
j·	Macrophages	⇔	Not changed much
k.	Lymphocytes	⇔	Not changed much

Intra-mammary dry-cow antibiotic therapy if given after last milking i.e. at the beginning of dry period helps in preventing intra-mammary infections

II	**STEADY STATE INVOLUTION**		**Udder immunity higher than active state**
a.	Teats sealed	⇔	No leakage, entry of pathogens closed
b.	Small fluid volume in the gland	⇔	Composition of the fluid less conducive to bacterial growth
c.	High concentrations of leukocytes	⇑	Provide maximum immunity
	Neutrophils	⇔	
	Macrophages	⇑	More phagocytosis
	Lymphocytes	⇑	More cytokine production
d.	Little milk fat, casein or debris left	⇔	Leukocytes are more effective
e.	Lactoferrin concentration higher than active involution stage	⇑	Provide immunity citrate:lactoferrin ratio is lowered
f.	Immunoglobulin concentrations higher than active involution stage	⇑	Provide immunity

Period of greater resistant to intra-mammary infections

III	**REDEVELOPMENT AND COLOSTROGENESIS**		**Udder immunity low**
a.	Accumulation of fluid in the udder as calving approaches	⇑	leakage from the teats begins
b.	Synthesis of milk components	⇑	Good medium for bacterial growth Hinder effective phagocytosis by leucocytes

c. Leucocyte concentration ⇓ Immunity decreases
decreases

Intra-mammary dry-cow antibiotic therapy if given earlier is significantly reduced in this stage, therefore, intra-mammary infection chances are more

During transition, in some animals, accumulations of excessive interstitial fluid in extravascular spaces of the udder and adjacent tissues have also been observed. Greater incidence and persistence of udder edema has been observed in first-calf heifers than in multiparous cows due to less developed vascular circulation in heifers. This occurs as a result of a threefold increase in mammary blood flow during three weeks before calving. Decreased plasma colloidal pressure, increased capillary blood pressure, obstruction of lymphatic drainage and retention of sodium and water are the basic causes of udder edema. Although the swelling usually diminishes after parturition, it may damage the mammary gland and increase the stress level in newly calved animal. During this stage many oxidative species are produced which may lead to lipid peroxidation, damage to critical molecules, and ultimately disease conditions may result. Possible changes relative to udder edema include injury to membrane integrity or damage to specific steroidogenic enzymes, thereby altering synthesis of steroid hormones.

Onset of mammary infections during early lactation

There is always a risk of mastitis in high producing cows during early lactation as they are more likely to leak milk, and they are slower to form a keratin plug in the teat. Therefore they cannot act effectively to prevent pathogenic organisms from entering the mammary gland. When milk neutrophils were isolated from lactating buffaloes of different parity and studied for their *in vitro* phagocytic activity (PA), it was found that *in vitro* PA was lowest during early lactation. So, early lactation is the most critical period followed by late and mid lactation [4], The figure 16.2 shows the changes in phagocytic activity of milk neutrophils in buffaloes of different parity (1, 2,3 and 4) during different days of lactation.

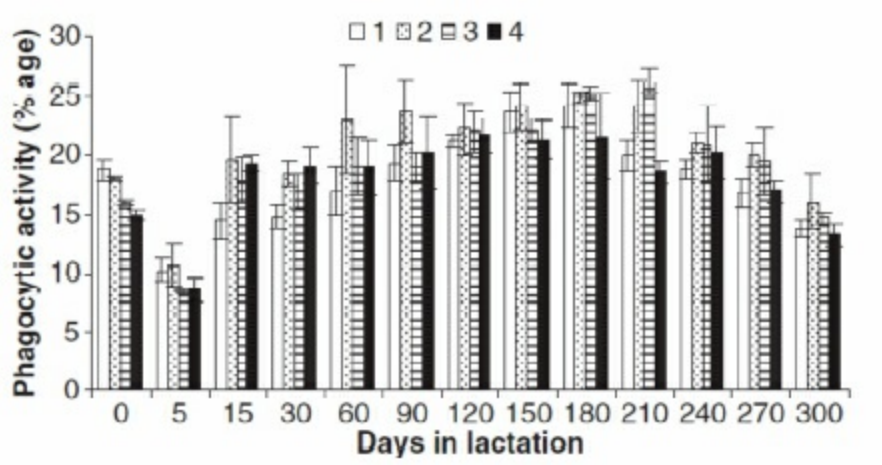

Fig. 16.2: The changes in phagocytic activity of milk neutrophils

Role of Neutrophils Around Calving

The peripartum immunosupression is characterized by the impairment of neutrophil trafficking, phagocytosis and killing capacity of neutrophils. Cytokine and hormonal changes around parturition are closely related to impaired neutrophil development and immunity- related activities. The molecular mechanism behind these processes is poorly understood. However, the negative energy balance associated with the transition period is a critical determinant of immune dysfunction of the neutrophils and lymphocyte proliferation. The trafficking of the polymorphonuclear leukocytes (PMN) drastically gets reduced and the endothelial migration, rolling of the neutrophils gets compromised during peripartum period.

The neutrophilic response to the cytokines is also drastically reduced and this decreases the phagocytic ability of the neutrophils. Ability to produce the free radicals in the intracellular environment is decreased, causing a very poor phagocytosis of the foreign microbes. Neutrophil extracellular trap (NET) is considered as another mechanism by which neutrophils confer immune response to the pathogens. During peripartum, the ability of the neutrophils to produce the NETS is reduced significantly and this causes poor antipathogen function of neutrophils [5].

At calving and few days early to calving, the pregnant animal exhibits high circulating concentration of cortisol as a mode for normal parturition. This rise

in the circulating concentration of the cortisol causes downregulation of chemokine receptors and an upregulation of death receptors. The down regulation of chemokine receptors causes a reduced response of neutrophils to the pathogens leading to poor phagocytosis and impaired immune response. The up regulation in the death receptors promotes the activity of Fas ligands that are considered as the promoters of neutrophil apoptosis. Rise in the apoptotic neutrophils predispose the animals to a number of infections around peripartum.

Molecular biological studies have demonstrated a down regulation of TLR2 and TLR4 receptors on neutrophils during peripartum period. This down regulation imposes a very poor recognisation of the pathogens by the neutrophils. The neutrophil surface receptors like CD11b and CD62L are down regulated causing a reduced and compromised endothelial rolling and migration of the neutrophils to the site of infection. Altogether, these lead to a state at which neutrophils become very poor in regulating the functions of immune system [6].

Large quantities of calcium are required for milk synthesis and an inadequate adaptation to this calcium sink at the onset of lactation results in hypocalcemia (milk fever). Calcium is also important for intracellular metabolism and signaling in most cell types, including the leukocytes of the immune system. The peripartum reduction in the circulating levels of the Ca++ is an important factor associated with immune dysfunctions. Milk fever (parturient paresis) occurs when the level of calcium in the blood gets too low (<5 mg/ dl), nerve and muscle functions are lost. Milk fever also predisposes cows to other health problems including ketosis, retained placenta, displaced abomasum and mastitis. Milk fever reduces feed intake resulting in ketosis. Cows experiencing milk fever release more cortisol which inhibits the immune system promoting retained placenta, metritis and mastitis. The teat end is often slow to close following milking due to Ca deficiency around this period, thus predisposing mastitis.

The Ca++ serves as a major intracellular molecule for downstream signal transduction mechanism in leukocytes. Decreased Ca++ causes a compromised signaling in leukocytes causing a reduced intracellular killing of pathogens by the neutrophils. Reduction in Ca++ is also associated with a decreased expression of adhesion molecules on the surface of neutrophils causing a poor transmigration of neutrophils to the site of infection [7].

Mammary immunity is compromised to a larger extent as compared to all other structures of the cow's body around parturition. The increased load of mammary synthesis partitions the nutrients available for the leukocytes. The transmigrated and resident leokocytes are deprived of the nutrition and hence exhibit a poor immune mechanism in the udder. The deficiency of micronutrients also reduces the antioxidant enzymes in the neutrophils. The cell protecting thiols get reduced and promote dysfunctions of mammary leukocytes. Peripartum period associated reduced expression of neutrophil receptors is predominantly followed by poor migration of the neutrophils to the mammary lumen causing poor immune activity of the neutrophils.

A striking change in morphology occurs when PMN leave blood and enter milk during transition period in dairy cows. In milk, PMN ingest milk fat globules and casein that result in large intracellular membrane bound vacuoles. Internalization of cell membrane leads to loss of pseudopodia and cell rounding. Cytoplasmic granules migrate and fuse to vacuoles forming phagolysosomes. This loss of membrane and cytoplasmic granules results in diminishment of PMN phagocytic and bactericidal activities and compromises intra mammary defence. During transition, the phenomemnon of neutrophil rounding is more profound in dairy cows indicating a reduced phagocytic ability of the neutrophils.

The phagocytic ability of the milk neutrophils dramatically starts falling in and around of the parturition and lasts up to early lactation [8]. The surface architecture gets altered and is an indication of the morpho-physiological variation of the neutrophil during transepithelial migration and the loss of surface architecture is predominant during transition period. The ruffledness of the neutrophil surface gets reduced during the period of transition causing a reduction in the surface area of the neutrophils. Milk neutrophils exhibit a rapid reduction in the surface area as compared to the blood neutrophils and hence exhibit poor immune response during the time of transition [9].

Intra mammary defence against invading microorganisms is dependent on an increase in number of PMN recruited to the mammary gland, whereas, resolution is dependent on diminishment in elevated numbers of PMN. Both of these processes are mediated, in part, by changes in PMN life span and inhibition and/or acceleration of PMN apoptosis. Apoptosis is a guided and dynamic process that regulates the fate of infection. Timely removal of the apoptotic neutrophils are required to inhibit the initiation of secondary

inflammations where as excessive apoptosis leads to immune compromised state of the gland. During peripartum period the degree of apoptosis of the neutrophils dramatically increases causing a reduced immune response of the neutrophils. This also causes poor udder immunity during the transition period [10].

The ability of neutrophils to migrate into infected tissues is dependent upon recognition of inflammatory mediators by cytokine, chemokine and complement receptors. Two chemokine receptors present on neutrophil surfaces, CXCR1 and CXCR2, are required for maximum neutrophil function during infection. At the time calving, cortisol downregulates these receptors and as a consequence the net activity of neutrophils gets reduced providing a diminished udder immunity.

Effective neutrophil recruitment to the site of infection requires adhesion molecules from the selectin and β2 integrin families. Neutrophil selectin CD62L slows down cells upon contact with its specific ligands and allows neutrophils to roll along vascular endothelial cells. Neutrophils activated by cytokines or chemoattractants subsequently shed CD62L as a prerequisite for β2 integrin-mediated tight adhesion. The most important β2 integrin involved in neutrophil recruitment in to inflamed tissue is CD11b/ CD18, which is predominantly stored in cytoplasmic granules. These receptors are down regulated during peripartum in dairy cows and hence the migratory activity of the neutrophils gets compromised causing a poor immune response in the udder to the pathogens [3, 5].

Role of Lymphocytes Around Calving

Lymphocytes are the mediators of humoral and cell mediated immunity. The role of lymphocytes becomes highly pertinent during the time of parturition to prevent aberrant immune response. Shifting of the lymphocytes occurs from Th1 to Th2 causing a persistent immune suppression during peripartum. The blastogenic potential of the B-lymphocytes gets reduced causing a reduction in the circulating number of B- lymphocytes. Lymphocyte proliferation response gets diminished around the peripartum. This causes a reduced cellular and humoral immunity in the udder udder [11]. The reduction in the ability of B-lymphocytes to produce antibodies mediates ease in colonization of bacteria in the udder during postpartum period. The reduced lymphocyte activity leads to

poor activation of complement proteins and hence the immunity of the udder decreases as a whole during transition period. As a result of this, the udder immunity gets lowered and mediates the colonization of the pathogens entering the mammary gland [12]. A significant increase in lymphocyte proliferation response was observed in B-lymphocytes in Sahiwal cows supplemented with Vitamin E and zinc supplemented group as compared to un¬supplemented animals during the pre-partum period [13]

Role of Macrophages Around Calving

Macrophages are considered as the resident cells of udder and are involved in normal physiological protection of mammary gland from pathogen challenge. Their number remains constant during lactation but during transition period, the number of milk macrophages gradually reduces [14]. The reduction in the macrophage number allows the dead and apoptotic neutrophils to accumulate in the mammary gland. This accumulation of neutrophils initiates the process of secondary inflammation and causes tissue damage. During transition period, the macrophage exhibits a reduced size as well as a reduction in the vacuolar contents. The phagocytic response of the macrophages along with antigen presentation and processing dramatically decreases. This facilitates the entry and invasion of pathogens. During transition, there is reduction in the uptake and removal of neutrophils by the macrophages and hence it predisposes autoinflammation in the mammary tissues [15, 16].

Potential Interventions for Boosting Udder Immunity

Nutritional Supplements

Nutrition serves as the best measure of all types of illness. So nutrition should be given the highest priority out of all interventions during the transition period. Animal should be fed with extra allowance other than the normal requirement to maintain a positive energy balance in the body. Dry matter feed intake should be increased. Concentrate feeding should be increased as a mode to fulfill the requirement of pregnancy, parturition and early lactation. Nutrition influence the cow's resistance to mastitis but cannot influence the exposure of teat ends to pathogens. Ensuring that the cow has adequate energy, minerals and

vitamins for optimal milk production are essential for the maintenance of udder health and immune status. Microminerals like Zn, Se, Cu and Co should be fed to boost the immune system. Antioxidant vitamins like Vitamin A, Vitamin E and Vitamin C should be given to reduce the oxidative stress to the animals. Maintaining the calcium balance avoids hypocalcemia and milk fever.

One of the older and promising technologies to boost the udder immunity of dairy animals during peri-partum periods of immunosuppressionis by supplementation of various micronutrients. There is a depression in the blood levels of micro minerals and vitamins around the peri-parturient period [17]. Increased incidences of mastitis have been reported at calving when the concentrations of VA and VE are decreased [18]. A decrease in reproductive efficiency of cattle has also been observed due to Cu and phosphorus deficiencies accompanied by a low level of feeding management. Copper and Zn deficiency has been found to affect various physiological functions that may be important in immunological defense to pathogenic challenge [19].

Dietary supplementation of cows with Se and Vitamin E results in a more rapid PMN influx into milk following intra-mammary bacterial challenge and increased intracellular killing of ingested bacteria by PMN as well as lowering the frequency and shortening the duration of clinical mastitis. Vitamin E and selenium supplemented during transition may also prevent retained placenta. Low plasma vitamin E concentrations have also been seen to be associated with increased incidence of fatty liver and displaced abomasum. Zinc is required for keratin formation and cows receiving supplemental zinc methionine have more teat canal keratin and numerically reduced SCC. Micronutrient supplementation has also been found to increase the total immunoglobulin secreted in colostrum [20]. Cortisol levels are also maintained towards the basal values during peri-parturient period when micronutrients are fed to the peri-partum buffaloes and thus enable the mother to bypass the state of immune suppression and enhance the resistance of mother to combat infections during the subsequent periods [21]. Vitamin A requirement for cows is 50 IU/lb of body weight or about 70,000 to 77,000 IU/day of adult cows, whereas, Vitamin E requirement recommended by National Research Council, (2001) is 80 IU/Kg feed during transition period which comes to nearly 1000 IU/cow/day [22]. But as buffaloes are heavier than cows, they have to be supplemented with 1,000,00 IU of Vitamin A and 2000 IU of Vitamin E. The Cu and Zn requirement are also doubled to that of [22] as these trace mineral requirement are considered to be higher for immune

function than the normal recommendations.

Agonists for peroxisome proliferator-activated receptors (PPAR) have been found to promote metabolic health of transition cows [23]. They can improve liver metabolism and decrease plasma NEFA concentration, whereas, those targeting PPARa (the primary isoform in liver) promote fatty acid oxidation in liver, limiting triglyceride accumulation and production of lipid peroxides. Choline limits oxidative stress by limiting lipid peroxide formation by decreasing plasma NEFA concentration and promoting clearance of triglycerides from the liver. Supplementation of rumen-protected choline has been shown to increase plasma a-tocopherol concentration during the transition period presumably contributing to immune function and modulation of inflammation. Feeding of choline and L-carnitine combination has also been found to have beneficial effect on improved reproduction indices and reduction of milk SCC in cattle.

Short-term non-steroidal anti-inflammatory drugs (NSAIDs) treatment has been used as a preventative measure against transition disorders like mastitis and has been found to reduce body temperatures of diseased cows. Carprofen, another NSAID was shown to partially alleviate the decrease in ruminal contractions during mastitis and could help prevent a subsequent displaced abomasum. Cows treated with acetyl-salicylate (aspirin) for the first five days of lactation had significantly lower plasma concentrations of acute phase proteins and tended to have greater peak milk production [23]. Also yeast-derived glucans and mannans are being used to enhance immune function in several species, including cattle. They have been found to reduce milk SCC.

Vaccinations

Timely vaccination of dairy cows against mastitis is essential so as to keep the immune system active against the pathogen challenge. Vaccination with *E. coli* J5 bacterin at drying off, 30 days after drying off and within 24 hours of calving reduces the incidence by 70-80%. Vaccinations like hipramastivac have also been reported to reduce the incidences of mastitis significantly. Farmers expect mastitis vaccines to reduce the severity and frequency of mastitis, prevent new infections and eliminate existing infections. However, till today, not a single vaccine has been developed which can achieve all of the above three expected outcomes.

Housing of Transition Cows

Calving areas should be cleaned and sanitized after 1-2 calvings. The bedding provided to the pregnant cows is the primary source of environmental pathogens. Therefore pregnant animals should be provided with dry beddings and changed at frequent intervals. Inorganic bedding like sand supports less bacterial growth than sawdust, straw, recycled paper, or manure. The pens for pregnant animals should be stress free with *adlib* provision of water and feed and free from any flies, insects. Proper ventilation in the lying area of lactating animals helps to keep bedding dry which favors good mammary health. Good ventilation during hot weather may also lead to greater milk productivity as maintaining air movement in the feeding area makes animals to consume more dry matter. A recently calved cow should be placed in a special area for at least one week for frequent observation before rejoining the milking herd.

Milking of Newly Calved Cows

The teat cistern, teat canal and teat apex can be colonized by a variety of microorganisms. Therefore, teats should be cleaned before and after every milking and udder should be kept dry between different milkings. Teat dips solution when applied after milking reduces the somatic cells coming in milk. Milking equipmentsshould be properly cleaned and maintained in good operating conditions. Milk should be regularly drained /evacuated and residual milk should be stripped out. Clean milk production practices should be maintained to avoid the occurrence of infections [24].

Monitoring of Milk Total and Differential Somatic Cell Counts

Milk somatic cells include both the leukocytes and the epithelial cells which are sloughed off during the normal process of milking throughout the lactation cycle. The epithelial cells are capable of synthesizing milk, whereas, the white blood cells serve as a defense mechanism to fight infection and assist in repairing damaged tissue. Counting of total milk somatic cells is widely accepted criteria for measuring udder health and milk quality in all major milk

producing countries throughout the world. It is very cost effective in determining the udder health of dairy animals [25]. Milk SCC increase whenever the mammary gland is under stress, not milked properly or kept under unhygienic conditions. Maintaining a low somatic cell count in the dairy herd is a constant ongoing battle facing the dairy industry worldwide. On differentiating the milk cells, it has been seen that most cells in normal bovine milk are macrophages. At the onset of any infection, macrophages present in the udder signal the cow's immune system to send neutrophils to engulf and destroy the bacteria. In normal milk, neutrophils range from 10-20%, but, 80-90% of SCC in infected (mastitic) glands are composed of neutrophils [13]. Estimation of total and differential somatic cells can provide a better picture of the physiological status of the mammary gland. If counting of milk SCC is done simultaneously with California Mastitis test, accuracy is above 90% in detecting udder health. Further on culturing of milk samples of cows with high SCC can provide an insight into pathogens that need to be targeted with dry cow therapy with the aim of maximizing cure rates.

Conclusion

Transition period in the life cycle of the cow brings many dramatic physiological and metabolic changes. These changes not only help in preparation for the upcoming lactation but also determine the productive integrity of the cow in the succeeding lactation. By providing appropriate nutrition and management we can keep our cows stress free and minimize postcalving disorders. Over the years, the majority of researches on health issues in transition dairy cows have focused on nutrition, physiology and metabolism. Despite great advances made in understanding these areas, the incidence of disease after calving remains high. Therefore by understanding the action of immune cells and modulating their functions by nutritional supplements we can formulate new treatment strategies and keep our animal healthy.

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Chapter 17
Dry Cow Therapy for Mastitis Control

Dry period is one of the crucial stages in production cycle of dairy animal and proper maneuvering of this stage has effective impact on both form of mastitis. This chapter discuss the procedures and impacts of dry period management in dairy animals as a measure of mastitis control.

Meaning and Objectives of Dry CowTherapy (DCT)

Treating or infusing teat of lactating dairy animals with long acting antibiotics or teat sealants or both at drying-off following last milking for management of subclinical mastitis is called dry cow therapy. The dry cow therapy not only helps in preventing the risk of udder infection during dry period but also the milk yield in subsequent lactations is increased. This therapy is also being adopted either to cure existing infections or to prevent new infections in the early dry period. Many cows infected during lactation do not show signs of infection or mastitis immediately following infection and in some instances clinical symptoms are not observable even for whole lactation. This type of infection remains sub-clinical [1] and may become clinical during dry period or just after parturition. Therefore, the benefits of DCT can be drawn as under:

- Reduced incidence of new intra-mammary infections during the first 3-4 weeks of the dry period.
- Efficacy of treatment is being higher during the dry period than during lactation i.e. 80 to 90 % v/s 30 to 40 % [2]. This may be due to infusion of higher doses of antibiotics without being removed through milk.
- Retention time of antibiotic in the udder is longer.

- The risk of contaminating milk drug residue is reduced.
- No milk is being discarded due to mastitis incidences.
- A concomitant 'self cure' to subclinical mastitis takes place during dry period. This may be the probable reason for normal milk production by many quarters dried earlier during lactation.
- When long-acting antibiotic infusion product for DCT is prepared after the use of waxes, benzathine, aluminium or smaller particle size, the efficacy of action get improved due to slower absorption from the site of application.

These advantages should be considered carefully, especially if the cow is due for early drying-off or the bacterial culture tests indicate the infecting organism is not *Streptococcus agalactiae.* This is because *Streptococcus agalactiae* is the only common mastitis causing organism that can be treated readily during lactation.

Need of Dry Cow Therapy

During the early dry period tremendous stress is exerted on the udder because the gland get break down and absorb retained milk as well as millions of dead milk secreting cells. It is during this time and 2-3 weeks prior to calving that approximately 40 to 50% of new udder infections occur. Research has shown that dry cow therapy can reduce the number of new infections during this period by up to 30%. In order to create awareness among the dairy farmers in particular and the related professionals in general through this chapter, attention will be focused only on the factors which may result in increased SCC leading to improper quality milk production and causing economic loss. In Israel and other developed countries, milk is sold at a premium when the SCC is within permissible limits. In India too, the milk plants and multinational companies, dealing with milk products, check the bulk tank milk for SCC.

Specific Requirements of Dry Cow Therapy

Specific changes in udder during drying off period justify the need of DCT.

However, few more bodily changes also justify need of DCT during dry period which is as under:

Depressed host immunity*:* It is well known that dairy animals undergo a suppression of immunity at 2 week before and 2 week after the calving. The foetus, which is in the uterus, is being treated as foreign body by dam. This leads to continuous hypersensitivity reaction in dam's body which depresses the immunity of dams. This makes these cows more susceptible to disease especially towards the end of gestation period or nearing drying off period. Therefore, pregnant females must be treated during dry period for protection of these animals against any possible microorganism invasion through teat canal due to poor immunity.

Reduced dry matter intake*:* The dry matter intake starts to fall approximately 2 weeks pre-calving *i.e.,* from 2.5% of body weight to less than 2%. There is further reduced DMI on the day of calving probably due to slower or no rumination. This may further disturb the energy balance probably more towards negative side and make animal more susceptible for infection. Hence, DCT will probably protect the dairy animals from mastitis during this stressful phase.

Udder Physiology and Its Significance at Drying-off

With an objective to achieve proper foetal growth and recouping of body tissue for next lactation, dairy animals are being dried for approximately 60 days after a lactation length of about 305 days. However, notorious opportunistic microorganisms like *Escherichia coli* and *Streptococcus uberis* get access to udder during dry period, remain dormant i.e. subclinical, throughout the dry period, but are then an important cause of clinical mastitis in the first few months of the next lactation. Physiological changes in udder during dry period up to next lactation have events as per following pattern:

1. During first 2 weeks after the drying, slow closure of the teat canal starts. A plug of keratin and lipid is excreted into the lumen of the canal to form a teat seal. Simultaneously, slow regression of mammary alveoli also ensues.
2. During mid-dry period, also known by 'rest phase', the alveoli or secretary tissue become dormant and there is a piling-up of natural

inhibitory substances, such as lactoferrin, neutrophils, N-acetyl glucosaminidase (NAGase) and immunoglobulins, especially towards the end of this phase.
3. During the last 2 weeks before calving, formation of new mammary alveoli take place and the keratin plug slowly dissolves for the start of the next lactation.

During first and last 2 weeks of dry period, when the teat canal keratin plug formed and dissolved, the dairy animals are more susceptible to new infections. The environmental pathogens especially *S.uberis* has higher prevalence during the first weeks of lactation, while during last week pathogens like coliform bacteria are 4 to 5 folds greater during the dry period than during lactation [3]. New infection rates with environmental organisms are being up to 10 times higher than during lactation [3].

It has also been observed that the period just before and just after calving is the time of major risk for new dry period infections, and this is when management of cow and buffalo should be at its highest [4]. However, these infections do not develop into clinical cases during dry period; the majority remain dormant in the udder until next lactation after which they show mastitis incidences. It has been observed that most new cases of mastitis occur in the first 4 week of lactation, and, of these clinical cases, some 60% originate from infections that have become established during dry period [5]. It has also been noted that dry period infection continued to cause clinical mastitis up until the fifth month after calving [4].

The keratin plug formation in the teat canal during first 2 week as described above, sometime fails, and unfortunately, many cows do not form an effective teat seal, and in such cows the risk of infection remains very high. Effectiveness of the teat seal formation depends on various factors, some of which is as under:

1. Overall production: Cows having higher lactation yields generally develops less effective teat seal.
2. Milk flow rate: Fast milkers are more prone to develop mastitis due to higher incidences of leaky teat and less effective seal formation. Leaky teats are four times more likely to develop mastitis than normal teat during dry period [6].
3. Milk yield at drying off: Higher milk yield at drying off usually

responsible for the higher incidences of ineffective keratin plug formation in teat canal, access to microorganisms and finally mastitis incidences after calving. A study observed that 26% cows having 21 kg or more milk yield, developed mastitis as compared to 16% of cows having less than 13 kg yield at drying off [7]. It has also been observed that every 1 litre increase in yield at drying off produces a 6% increase in the risk of a new dry period infection [8].Hence, suitable management strategy may be adopted to decrease the milk yield before drying off. However, it should not be achieved by milking once a day or alternate-day milking but can be achieved either by reduced nutrient feeding or other suitable management strategies.

4. Teat or udder damage: Damage to any part of teat or udder increases the risk of mastitis both during dry and lactation period. Cows with a significant level of teat-end damage were 1.7 times more likely to develop a new dry period infection [7].
5. Dry cow therapy (DCT): Cows receiving DCT usually develops good seal due to absence of any microorganisms in teat canal [9].It has been observed that teat canal organisms degrade keratin and DCT leads to their removal and more effective seal development.

Methods of Dry Cow Therapy

There are 3 methods of dry cow therapy *viz.,* long-acting antibiotic therapy, teat sealants and combination of antibiotics and teat sealants.

Long- acting antibiotic therapy: This includes administration of a long-acting antibiotic into each quarter at drying off to remove existing infections and to prevent some new infections. This is most effective and widely used method for controlling mastitis in dry cows. It has been estimated that 70-98% of infections present at drying-off can be eliminated with this therapy [10]. It has been reported that use of intra-mammary antibiotic dry cow therapy increased the rate of development of functional keratin plug [11]. Quarters receiving dry cow therapy at drying-off had a significantly higher closure rate of teat canal in the first four weeks of the dry period than untreated quarters [12]. This implies that DCT facilitates physical sealing of the canal. DCT by this method probably kills resident bacteria that colonise the teat canal and also minimises debris built-up associated with the action of bacterial enzyme.

Most new infections occur during the first and last 2 weeks of the dry period [5]. This method of DCT will help to reduce the number of new infections at drying off. One main disadvantage of this method is that by the time of calving, antibiotic levels reduced significantly and probably seems infective during calving and sometimes resulted in mastitis. However, framycetin containing antibiotics persists at bacteria-killing level throughout drying-off period. Cows given DCT produced 179 kg more milk during the first 120 days of the next lactation, had a 10-fold reduction in clinical mastitis in the dry period, showed a 3-fold reduction in infection at calving and had a 3-fold reduction in clinical cases in the first 21 days after calving [13].

The preparations of DCT should be effective against *S. aureus* (carried in the udder from one lactation to other), *S. uberis* and *E. coli* (both contracted as new infection during drying off period). Therefore, the drugs most commonly used in DCT should be effective against these microbes; which may be Cloxacillin, Cephalosporins, Nafcillin, combination of Penicillin and Streptomycin etc. Some workers suggest that DCT preparation should be changed occasionally, to avoid the development of antibiotic resistance. As no strains of *S. aureus* have ever been found resistance to cloxacillin or cephalosporins, no benefit is likely to be obtained from changing the antibiotic, although additional antibiotic cover to prevent new coliform infections during the dry period may be more effective. Furthermore, failure of DCT may not be a fault of the drug but may be due to wrong method of administration, probably in udder at the time of the drug administration.

Antibiotic therapy may be used either by blanket or selective dry cow therapy as mentioned below:

- ***Blanket dry cow therapy:*** Treatment of all quarters of all animals at the time of drying-off is called "Blanket dry cow therapy". Due to this method, infections arising just after drying-off are prevented and laboratory or screening procedures to decide which quarter to treat are eliminated. Blanket dry cow therapy has added advantages like treatment of all infected quarters at drying-off, prevention of new infection during the first week or two of the dry period and economical as no testing or screening cost is involved.
- ***Selective dry cow therapy:*** Treatment of infected quarters at the time of drying-off is called "Selective dry cow therapy". Selective quarter treatment requires initial screening to identify an infection and general

preventive benefits of total dry cow therapy are lost. The advantages of this technique are saving in cost, especially when only 8-10% of quarters are infected, and the avoidance of complete sterilization of all quarters, with a resulting possible increase in susceptibility to infection. However, selective dry cow therapy is hard to justify on the basis of cost. In a study comprising selective and blanket dry cow therapy on this merit alone, the benefits associated with blanket use of dry cow therapy included a low rate of clinical mastitis, a marked reduction in somatic cell count (SCC) and an increased milk production in the next lactation [14] was observed.

- ***Teat sealants :*** The main objection to dry cow antibiotic therapy comes from continued use of antibiotics in the dry period for either prophylactic or therapeutic purposes. The blanket and overuse of antibiotics might be associated with antibiotic residue in milk or emergence of antibiotic resistant in human and animal pathogens. An alternative strategy in form of teat sealant may be adopted to resolve above concerns. It is believed that a teat canal keratin plug should form early during the dry period to establish a natural protective mechanism to prevent penetration of bacteria and reduce the incidence of new intra-mammary infection [12]. This natural teat seal i.e. keratin may take a minimum of 2 weeks to form and up to 23% of teat may never be closed naturally [12]. Hence, synthetic teat sealants may be used.

The infusion of an internal wax sealant in to the teat canal and base of teat prevents new infections. This had dramatically reduced the incidence of mastitis in early lactation. Two types of teat seal are available:

1. External film sealant and
2. Internal teat canal wax plug
    - ***External film sealant :*** It provides a flexible barrier film over the teat end for up to 7 days. This seal is no longer commonly used, although it can be used in the late dry period if no internal seal has been administered.
    - ***Internal teat canal wax plug:*** This is by far the most effective and most commonly used sealant. It contains bismuth salt in wax base that is infused into the teat canal at drying off. Due to absence of antimicrobial activity in this sealant, strict hygiene during administration is essential.

- ***Combination of antibiotics and teat sealant :*** In this teat sealants are commonly used in addition to dry cow antibiotic tubes. Here the antibiotics should be administered first. The effect of both on incidence of mastitis in the next lactation are additive. Ina study cows were divided into four groups *viz*. control (no treatment at drying off), antibiotic (cephalonium), teat seal and combined antibiotic (cloxacillin) and teat seal. All treatments reduced clinical mastitis in the next lactation by 50% compared with the controls [9]. In the same study, incidences of new intra-mammary infections decreased by 10-fold and the combination of antibiotic and teat seal gave the best protection. A similar trial in the UK, comprising dry cow therapy plus teat seal with teat seal alone, showed a 30% reduction in the incidence of new infection in the first week of the subsequent lactation [4].

Preventing Drug Residues

Attention must be given to preventing drug residues in milk and meat. Label directions must be followed exactly to avoid residues after freshening, especially for cows with shorter than normal dry periods. Tests are available to determine antibiotic residues in milk. Most dairy cooperatives, cheese plants, and some veterinary clinics will run these tests or producers can buy their own test kit. If a question arises concerning whether or not the milk may contain antibiotics, a test should be conducted. Caution: all antibiotic residue test kits do not detect all antibiotics. Care must be taken to ensure the test used can detect the antibiotic residue in question.

Infusion Technique During Dry Cow Therapy

Following proper infusion procedures is a key component of the dry cow therapy program. Teats must be cleaned and sanitized before infusing antibiotics into a quarter. Without proper preparation, organisms present on the teat end may be forced into the udder and result in an infection more severe than the one for which treatment was intended. Administration of DCT tubes requires gentle and hygienic. Specific points for the administration of antibiotics or internal teat sealants are as under:

- Identify treated cows and remove them from the milking herd to prevent antibiotics from entering the milk supply.
- Infusion in teat canal should be done after lactation phase has been completed by animal in question.
- Administrator's hand must be properly cleaned and if possible gloved hand should be used for infusion.
- Clean and dry teats with a single service paper towel or cloth.
- Dip teats in an effective germicidal product. Allow 30 seconds of contact time before wiping teats with single service paper towel or cloth.
- Thoroughly clean and disinfect each teat end by scrubbing with cotton soaked in 70 percent alcohol or surgical spirit or commercial wipes (e.g. Mediwipes). Use a separate piece of cotton for each teat. Prepare teats on the far side of the udder first, followed by the teats on the near side.
- Treat quarters in reverse order; near side first, far side last.
- During administration of teat sealants alone, strict hygienic procedure should be adopted as sealants have no antibacterial property.
- Diameter and length of nozzle or tube to be inserted in teat canal should be small and short, respectively. Wider diameter of tube or nozzle results in excess dilatation of the teat canal which produces cracks in its lipid and keratin layers by which defence mechanism of teat may become poor. Similarly, nozzle should be inserted for shorter distance in teat canal rather than full insertion up to teat sinus. This is because short distance insertion of teat canal and squeezing of antibiotic in to it usually kills the colonizing bacteria of teat canal, which may not be possible during nozzle insertion up to teat sinus.
- After infusing the antibiotic in teat canal, this should be back stripped up to teat sinus and udder. After infusing the teat sealant, base of teat should be held between finger and thumb to retain the sealant in teat.
- Teat dip should be applied immediately after tubing to remove any bacterial colonization at teat end and to avoid any new infection.
- Proper record of drying off and details of DCT medication should be properly maintained.
- Careful checking of cows for mastitis within 5 days after drying off should be ascertained.

Dry Cow Infusion Products

Only scientifically approved single dose intra-mammary infusion antibiotic products formulated specially for dry cow therapy should be used. These products contain high levels of antibiotics in a slow release base that will maintain therapeutic levels in the dry udder for longer periods of time than infusion products intended for use in lactating cows or in dry cows within a month of freshening. Cows treated at drying off will have a high antibiotic residue in their bodies. Refer to the label of the drug used for specific recommendations.

Most of the dry cow therapy products are designed to eliminate existing *Staphylococcus aureus* and *Streptococcus agalactiae* infections in the early dry period. In some herds, especially where confinement has become more intense, environmental bacteria cause a higher percentage of new infections during the dry period. Most of the dry cow therapy products are reasonably effective against environmental streptococci but are ineffective against coliform bacteria. Consult the expert to determine which dry cow product to use in a particular herd.

Products used for dry cow therapy should be stored in accordance with the Pasteurized Milk Ordinance and discarded when the expiration date is reached. Outdated intra-mammary antibiotics may have little antibacterial activity. At the end of lactation period and at the start of dry period, after completely evacuating the udder, teats are washed and dipped in 3% solution of iodine and intra-mammary inclusion of suitable antibiotic is given.

Preventive measures to minimize the requirement of dry cow therapy

The requirement of dry cow therapy can be minimized by

- Management factors
- Milking techniques, procedure and equipments
- Stress related factors
- Heredity, physiological status and milk yield.

Management Factors

Hygiene & Sanitation: The risk of new intra-mammary infection is greatest during the early and latter part of the dry period. Because udders are

not milked during these times, pathogens are not flushed from the lower portion of the teat canal and may lead to new intra¬mammary infections. The number of new infections is related to the bacterial population on the teat end. Therefore, exercise lots, loafing areas, stalls, and maternity pens should be clean and dry. Animals on pasture should not be allowed in ponds or muddy areas.

Animal Housing: The animal house should be such that it is comfortable for the animal, easy to clean and flexible in nature for the variations in different climates so that it can be covered with curtains in winter and have sufficient area for ventilation in the hot humid period. The udder should be well protected from cold and damp floor. If required, bedding material free from mouldy and fungal growth can be provided. Moreover, it should be regularly changed. Scientific reports indicated that use of saw dust can decrease the somatic cell count (SCC). A dirty animal house leads to increased SCC. Scientifically one sq. ft. of light area (may be open or glass) is sufficient for every 15-20 sq. ft. of floor area in the conventional animal shed.

It is a well established fact that cows produce more milk in a properly ventilated shed; however, it should be well protected from draughts or western hot winds in summer months. The ventilation system should be capable of moving upto100 cu. ft. of air per minute for each 450 kg animal. Indirect ventilation area in winter, hot-dry summer and hot-humid summer is approximately 10, 25 and 60 % of the floor area, respectively. Crevices, holes in the animal sheds and the marshy areas where the animals may go for grazing are the breeding grounds for disease causing organisms. Sometimes mud or dung cakes are formed on udder and teats which may be a source of infection. Brick laid open yards and fencing of marshy area is desirable because cleanliness in animal area is directly associated with decreased infected udders. When loose/semi-loose animal housing system is compared with conventional (tie system) then the later is observed to have more infected animals. However, it will depend upon the cleanliness and other preventive measures taken.

Milking Parlours: Milking parlours require much more cleanliness and protection from flies and insects as compared to general livestock houses to maintain lower infection rate in the animals. They should be regularly disinfected.

Milker's Hygiene: Unhealthy and dirty milkers may contaminate the milk

and also affects the health of the animal. Persons suffering from typhoid, paratyphoid, bacillary and amoebic dysentery, salmonellosis, diphtheria, streptococcal and staphylococcal infection and tuberculosis infection should not be allowed to milk the animal. Tuberculosis infection contracted from the milker may result in tubercular mastitis in the animals. These disease causing organisms may also find entry into milk and can infect the consumer. Therefore, milkers should trim their nails regularly, put on clean clothes, and wash their hands before milking. They should disinfect their hands after milking the infected animals. The milker's experience plays a vital role in completing the milking efficiently and method is the best to reduce infection rate and SCC.

Udder Washing and wiping: Before the start of milking procedure, the teats and udder should be washed so as to remove the dirt, dust and faecal matter adhered to it. This may be done with lukewarm water in winter. If required, a sanitizer can also be used. It has been reported that when the udder is washed with chlorine solution before milking and followed by wiping with a dry towel and then using iodophor teat dip, it results in reducing bacterial as well as SCC count in raw milk. Instead of using the same cloth or towel for wiping or drying of udder after washing, if a paper for each cow is used that will help to stop the spread of microorganisms from one cow to another.

Teat Dipping: Starting with a clean udder, pre-dipping is designed to replace water preparations and reduce the number of bacteria on the outer surface of teat. It works especially against *Streptococcus uberis* and coliform. The relative importance of a teat dip depends on its ability to kill the bacteria, remain active on the teat and promote healing. The effect of use of various pre-dip compounds such as iodine, chlorhexidine peroxide and sodium chlorite lactic acid was significantly associated with reduced somatic cell count and mastitis rate in the herd. Scientific studies indicate that with the use of an iodophor teat disinfectant, the incidence of clinical mastitis was reduced by 57% and total bacterial count by 70%.

Post Milking Preventive Measures: Management practices followed immediately after milking plays a very vital role in improving udder health status. After milking, teat sphincter remains open for at least 30 minutes. During this period the microorganisms may find access to enter mammary gland if the surrounding environment of the animal is not hygienic. To overcome this problem either some feed should be offered or some other measures should be adopted to keep the animal in standing position.

Alternatively apply post milking teat dip and keep the floor clean and free from any type of pathogenic microorganisms.

Milking techniques, procedure and equipments

Milking Techniques: Proper milking procedures are important in prevention of udder problems of dairy animal and ensuring that one gets complete milk removal from udder. The main methods employed for milking of animals are:

- **Stripping:** Normal microflora of teat canal and opportunistic organisms are present in the milk that is in the teat canal. These opportunistic organisms may find access to the mammary tissue, thereby resulting in infection and lowering down of milk production. So it is better to drain the milk present in teat canal to get rid of these bacteria. Scientific reports indicate that the failure of milkers on some dairy farms to strip cows before milking have significant effect on bacterial count and SCC.
- **Hand Milking**: It is an age old and traditional method followed in most of the parts of the country. It is of three types:
    - Full palm method or fisting: It is most suitable.
    - Stripping: Employed if teats are small. Only thumb along with index and middle finger are used.
    - Knuckling: It is a wrong but commonly used method. It can lead to injury and fibrosis and results in narrowing of teat canal.
- **Machine Milking**: This method is followed by progressive dairy farmers, at government farms as well as private or commercial farms. Generally on such farms the number of dairy animals is quite large. Scientific studies indicate that microbial count is generally higher in hand milking milk than in the machine milked milk. Similarly total milk yield and keeping quality is higher in machine milked milk than hand milked milk. Machine milking with stripping by hand is an efficient method for milking high yielding cows. When machine milking is followed then we must be familiar with the proper operation of the machine. It is considered that faulty operation of milking machine can influence the amount of SCC in the herd as under:
    - It may cause injury to the teat sphincter particularly when milking is prolonged or operating vacuum is very high.
    - In case of over milking, the mucous membrane of teat canal may also

be damaged particularly when hard liner is used and vacuum is very high.

- When there is fluctuating vacuum at the teat cup then there is possibility that infected milk will reflux over teats and may even enter the udder through relaxed teat sphincter.
- When pulsation ratio is too wide and pulsation rate is too high then that may cause injury to teats and teat sphincter may get damaged.
- The teat clusters may transfer infection from animal to animal during milking if not properly cleaned, washed and disinfected.
- Teat cup liners should be changed after 1200-1500 hrs. or use in order to minimize bacterial and somatic cell count.

Milking Frequency: Scientific studies indicate that there is an increase of 15-20% milk and total milk fat when an animal is milked thrice a day as compared to 2 times a day. It also leads to improve feed efficiency. Secondly, it may also lead to decreased SCC due to lowered stress on the udder.

Sequence of animals for milking: In order to keep the infection rate and SCC at lower level, the sequence should be as follows: first milk the healthy animals (first time calvers in the beginning followed by multiparous animals) and then milk the suspected animals and then the infected animals at the end.

Preparation of udder: If the udder is not prepared properly for the milk letdown and the cups in case of machine milking are applied then may lead to injury to the udder which may cause discoloration of the milk and increased SCC. Moreover, milking should be completed within 6-7 minutes.

Drying off and the Early Dry Period: Reducing the grain ration and sudden cessation of milking is the recommended practice for drying off cows. High producing cows should be taken off concentrates two weeks prior to dry off to help reduce production. Cows should be observed closely for two weeks after drying off to ensure udders are involuting (not swollen or inflamed) properly. Udders with swollen quarters should be examined for mastitis. Cows showing visible signs of illness should be provided supportive therapy; however, re-infusion of antibiotics into the mammary gland is not recommended. Supportive therapy may include intramuscular or intravenous administration of antibiotic and/or anti-inflammatory compounds. In severe cases electrolyte therapy may be warranted. Be careful to follow drug withdrawal recommendations closely to avoid possible residue violations. In

instances where a severe problem with mastitis threatens a producer's milk market, consideration should be given to using DHI somatic cell counts to aid in selecting cows for early dry off and dry cow therapy. This is particularly true if bacterial cultural tests indicate the infecting organism is not *Streptococcus agalactiae.* However, care should be taken so as not to provide cows with a dry period in excess of 100 days.

Number of infusions: To date, research indicates there is little, if any, value in treating cows at drying off and again two or three weeks later. Subsequent treatments may pose the additional risk of forcing bacteria into the gland and increasing the risk of antibiotic residues in milk after freshening.

Total vs. selective dry cow therapy: When subclinical mastitis in a herd has been reduced to a very low level (e.g. every cow in the herd has less than 1,00,000 somatic cells/milliliter of milk), some dairy producers and veterinarians have considered selective dry cow treatment. However, selective treatment may fail to reach 20 to 40% of sub clinically infected quarters in the herd. Also, quarters not treated at drying off are more likely to be prone than treated quarters to become infected during the early dry period. Treating every quarter of every cow at drying off will reach all infected quarters, is more effective than selective treatment in preventing new infections during the early dry period, and does not require screening of cows to determine those to treat. Additionally, studies indicated that if the decision is based on economics (i.e. the cost of dry cow therapy compared to the return to the producer), treating every quarter on every cow at drying-off is preferable. Selective, rather than "blanket" dry cow therapy with long acting intra mammaries is recommended in Nordic countries. In Norway selective dry cow therapy is recommended but practiced on only 1-2 % of dairy cows, in Finland the rate is about 20%, in Sweden 25% and in Denmark 6-7%. To evaluate the effect of dry cow therapy at national level it is of interest to compare these figures with the level of SCC and incidence rate of clinical mastitis.

The new infection rate during the dry period is very much in focus in the arguments for dry cow therapy. The question of total or selective dry cow therapy is the question of the necessity of treatment of the 61.1% that are healthy at the next lactation or an attempt to select some of those 38.9% that are not "healthy" at the next lactation. Some of the "unhealthy" cows have chronic subclinical mastitis and will not cure anyway and thus do not need therapy. The question of "blanket" or selective dry cow therapy is also the question of it you

view distribution of antibiotics as an insurance against infection or whether you emphasize other means of preventing cows from being infected during the dry period. The Nordic philosophy has always been to stress the importance of truly preventive measures instead of trying to control the new infections with antibiotics [15, 16]. Therapy at drying off does not have to be at random, and prognosis for treatment can be predicted [17]. Selection or blanket dry cow therapy thus is a question of being able to and having the benefit of this selection process. This will depend on information available, herd situation and health situation.

Teat dips: Dipping teats with a disinfectant is considered one of the most important steps in the prevention of new mastitis infections. When the practice of teat dipping is employed, the rate of new infections during lactation can be reduced approximately 50 percent within one year. After a two-year period, up to 75 percent of the infections can be prevented. If teat dipping is discontinued, the infection rate increases rapidly. A wide variety of teat dips under various trade names are on the market. Use only authenticated products. Contact the appropriate authorities for a current list of tested teat dips.

Dipping vs. spraying: Early reports indicated that, the practice of spraying teats could be up to 50 percent effective as dipping. However, more recent data show spraying to be just as effective as dipping provided at least the lower two-thirds of the teat is covered.

Milking Equipment: If hand milking is done then the milking pail should be thoroughly cleaned and sterilized. Moreover it should be preferably with a dome shaped top that will prevent anything falling in the milk.

Stress Related Factors

Overcrowding: In conventional dairy sheds a cow requires at least 42 sq. ft. of covered area whereas this requirement in buffaloes is 48 sq. ft. If the space provided is less the animal will remain under stress. In case of loose and semi-loose housing systems an additional space (2 times of covered space) can be provided for comfort as well as for higher productivity. In case of large breeds like Holstein Friesian if the standing platform is not of desired length (5 % ' -6') then stress is there on the udder as it hangs over gutter edge. To overcome this problem the sheds should be planned according to the breed

and species.

Inadequate and unbalanced feeding: The type of feed fed to the animal also plays an important role in increased number of unhealthy udders, because of variation in pH which in normal milk is between 6.5 - 6.8. At this pH bacteria cannot grow properly. Any change which turns the pH of milk to alkaline, favours bacterial growth. In such cases scientific reports indicate that oral administration of 12 gm of Trisodium citrate per day for 3-4 days helps in restoring normal udder pH. Another factor which makes milk unacceptable is the pesticide residue in the milk. This condition arises due to feeding of fodder crops which are sprayed with higher amount of pesticides and insecticides. These pesticides like Aldrin, Chlor Aldrin, Dieldrin, DDT etc. when present in milk may results in allergic reactions in the body.

Exogenous Oxytocin Injections: Oxytocin, a hormone secreted by the posterior pituitary gland, is responsible for letdown of milk. Before milking when the animal is shifted from shed to milking parlour, washed, offered feed, udder and teat are massaged, sound of milking equipment or suckling by calf leads to passage of nerve impulses from udder to brain, which in turn releases oxytocin and it is responsible for letdown of milk. Some people want to cut short the whole process and inject the animal with exogenous oxytocin. Therefore, when the udder is not properly prepared it may lead to increased somatic cell count.

Heredity, Physiological Status and Milk Yield

Heredity: Udder conformation and resistance to diseases in dairy animals is directly related with heredity. Daughters of certain bulls are more resistant to infection. Pendulous udder and long bulbous teats are liable to injury. Teats that are wrongly placed and pointing outwards are liable to injury by being bruised by cow's hind legs while walking. Studies indicate that dairy animals having udders with better depth and forward extension, high rear attachment and collapsibility with long and cylindrical teats of medium thickness were superior milkers, which indirectly shows better udder health status. While selecting the dairy animals one must take care that soft milkers are more prone to infections as compared to hard milkers because of loose sphincter muscle in the former case.

Parity, age, stage of lactation and milk yield: Milk production by a cow is maximum in third to fifth parity or lactation. Similarly, within lactation it is at its peak after about 60 days of calving. This higher production lays down an additional stress on udder resulting in lowering down of defence mechanism of udder. This condition makes the udder more susceptible to infection. Various reports on udder health status in different parities and stages of lactation have indicated that the percentage of affected quarters was higher in the 3rd and 4th parities and the early stage of lactation due to higher milk production in these periods. Therefore, dairy farmers should provide additional care to the high yielders.

Length of dry period: While dry cow treatment is beneficial in preventing new infections during the early dry period, the udder is vulnerable to new infections during the last two to three weeks of the dry period when dry cow therapy is no longer effective. Special attention must be given to springing cows and heifers. These animals must be kept clean and dry if mastitis is to be avoided during early lactation. Weather permitting, a clean dry bedding, preferably straw or inorganic bedding, are recommended during inclement weather.

Recent studies on the use of persistent barrier teat dips starting 10 to 14 days pre-partum may prove a viable management option for reducing new intra-mammary infections at calving. Studies indicate barrier dips persisting greater than three days may result in up to a 50 percent reduction in total, major pathogen and environmental streptococcal infections at calving in cows and heifers. This practice may be particularly beneficial in herds experiencing high rates of mastitis in early lactation or when environmental conditions are less than ideal. Inadequate duration of dry period affects udder health and consequently milk yield. The animal needs rest from the work of synthesizing milk towards last stage of lactation i.e. at least 2 months prior to the next calving because:

- The pregnant animal has to direct the nutrients for the development of foetus.
- To rebuild body reserves by the animal.
- To regenerate the mammary system for synthesizing milk during next lactation.

Generally with increased somatic cell count milk yield falls and length of

calving interval increases.

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Chapter 18
Risk Management Approach for Udder Health in Dairy Herds

Mastitis is a serious herd health issue in small and large dairy operation, alike. Over and above the serious economic implications, it also raises issues like impaired animal welfare, decreased work satisfaction by the farmer and risk of increased antibiotic residues in milk [1]. The complexity of mastitis control lies in its multifactorial etiology and numerous risk factors associated (Fig: 18. 1). Mastitis management requires adequate attention to the contributing causalfactors, especially as the pathogenic factor is ubiquitously present in the environment. So, every mastitis control programme needs sufficient information regarding cow history, environmental conditions, production data, management data, etc. Hygiene is the most important issue and it goes far beyond the hygiene during milking, to all levels from housing, feeding, cows in the barn and milkers even [2].

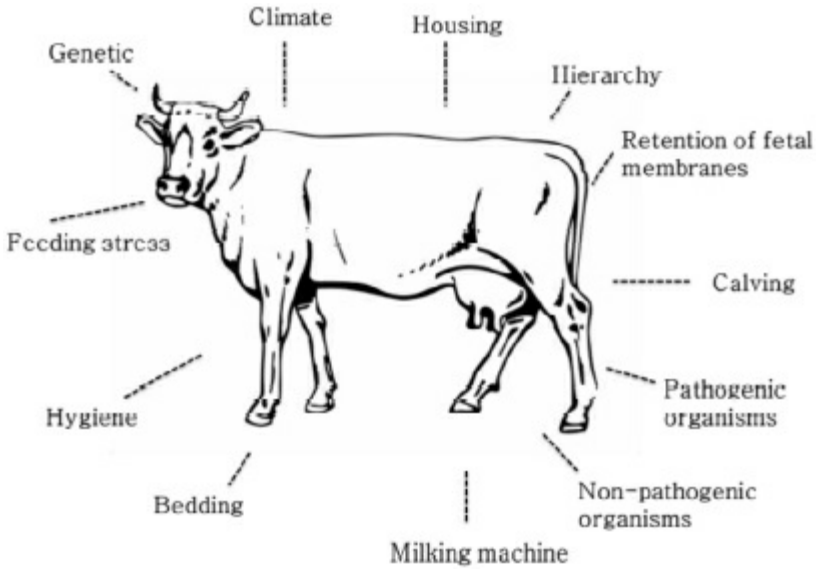

Fig. 18.1: Risk factors associated with incidence of mastitis

This chapter will brief the risk factors associated with mastitis, mechanism of incidence with points of possible interventions to evolve a suitable mastitis management strategy focused at reducing and eliminating the risk factors at the herd level. But the most important of all is that, once an udder health control programme (UHC) is designed and implemented, it warrants a persistent and protocol-based approach by boththe farmer (and his co-workers) and a coaching veterinarianin all areas of udder health [3, 4].

Risk Factors Contributing to Mastitis

Multifactor etiology makes mastitis a difficult problem to comprehend. Although the microorganisms are responsible for the infection, the mode of entry to the mammary glands to establish themselves is more significant from the management point of view. Estimate shows that 25% of the susceptibility to infection is attributable to environmental factors, 20% to genetic factors and 50% to herd management [5].

Table 18.1: Risk factors that increase the susceptibility to mastitis

S. No.	Risk Factors	Description	Reference
1	*Environmental factors*		
	□ Climate	Exposure to intense cold, heat and their rapid changes	[6, 7]
	□ Housing	The risk of udder injury is reduced in tied stalls and by partitioning between the stalls	[8, 9]
	□ Quality of in door air	Relative humidity influences insect and flies and even spread of bacterial infections	[10]
	□ Bedding	Sawdust and shavings encourage the rapid develop¬ment of coliform,	[11, 12]

		chopped straw favours Klebsiella	
	□ Stress	Excessive density of animals and stress adversely affect immune status	[10]
2	*Animal factors*		
	□ Species/ Breed	Cattle more susceptible than buffaloes, exotic cattle breeds or their crosses are more susceptible than zebu	[13, 14]
	□ Age and parity	Sub clinical mastitis is higher in older animals compared to younger animals. Buffaloes between 3rd to 4th parity (5-9 yrs) had higher prevalence of sub clinical mastitis	[15, 16]
	□ Stage of lactation	Animals in first 2-3 weeks postpartum has negative energy balance, high oxidative stress, hence, vulnerable to mastitis	[17]
	□ Milking interval	With increase in the milking interval, the microbial load increases	[18]
	□ Udder and teat condition	The teat having injury is highly vulnerable for the infection	[19]
	□ Dry period management	Lactation phase during dry period has 2-12 times higherchances of intra¬mammary infection is	[20]
3	***Managemental factors***		
	□ Nutrition		
	• Nitrogen source	Non-protein source of nitrogen (e.g. urea and ammonia) has harmful effect	[21, 22]
	• Concentrates	A high energy content in rations	[23]

	increased the incidence of mastitis in first lactation cows whereas it had the opposite effect on the other cows	
• Calcium-phosphorus ratio	An inadequate calcium to phosphorus ratio in rations has been correlated withincidence coliform mastitis	[24]
• Silage and hay	Poor quality silage has a very negative effect on the immune system	[25]
• Alfalfa and other legumes	Legumes (alfalfa) contain estrogenic substances which encourages premature udder growth, increasing incidence of environmental mastitis	[5]
• Selenium and vitamin E	Adequate level of Se and Vit. E prevent mastitis, reduce the severity of infection	[26]
• Silica	Reduce the formation of free radicals, lipid peroxidation and modulates macrophage activity	[27]
□ Physical & Ethological		
• Post-milking lying	Higher chances of mastitis in the cows that lay down within 40min post milking.Manipulating the feed, feeding time around milking is beneficial.	[28, 29]
• Needs of the calf	Frequency of calf suckling is greater than cow milking, giving very little time formultiplication of microorganisms	[30]
• Herd hierarchy	Least dominant members of the herd are often harassed, have a greater tendency to develop diseases	[10]
• Uterine health	Retention of fetal membranes after calving is associated with mastitis	[31]

• Ruminal status	Rumen acidosis fosters *Streptococcus, Candida* etc.The toxins they produce favor gram-positive bacteria in udder	[32]

Mode of Transmission

The approach to udderhealth problems in which all fragments of different mastitis control programmes are integrated, structured and formalized could only solve the riddle, called mastitis, in dairy farms. To evolve a technically viable approach, that may yield better results than present methods, a thorough knowledge on the various risk factors involved (table 18.1) and the mode of infection and its transmission is needed (Fig.18.2).Ultimately, this holistic understanding on etiological factors (infectious agents, sources, propagation factors etc.), symptoms and preventive measures associated with mastitis would, fruitfully and case specifically, minimize the incidence of mastitis at individual dairy farms.

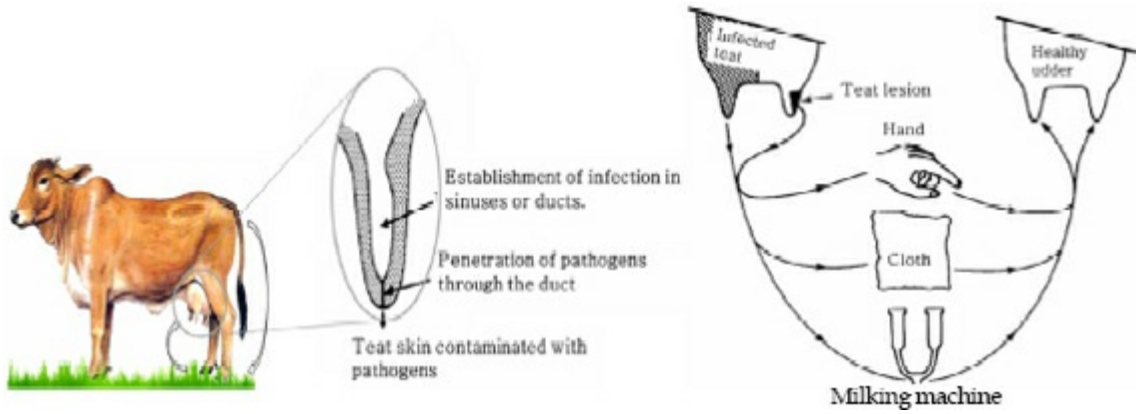

Fig. 18.2: A comprehensive representation of mode of infection and transmission

Udder Health Control (UHC)

The complexity of mastitis is evident from figures18.1&18.2. Regulating the herd dynamics including various interactions between infected and non-infected cows, variations in housing, climate, feed quality, milking machine

conditions, milking method and hygiene practice are crucial in maintaining good udder health. Moreover, the issues of animal welfare, farm economics are also important. Among udder health control programmes, the most commonly adopted five point plan predominantly focused on contagious mastitis, e.g., *Streptococcus agalactiae* [2]. The five points involve (1) controlling clinical and subclinical mastitis,(2) drying off therapy, (3) culling of chronically infected cows, (4)the proper milking machine function and (5) appropriate milking methods including teat dipping. The main goals of this five point plan were to prevent new infections from occurring and reducing the number of existing infections.

Implementing this programme resulted in a change in the pathogen profile of dairy herds. Coliform bacteria have emerged while streptococci got reduced, indicating a rise in udder infections due to environmental bacteria [33], This paved way for the emerging concept of reducing diverse risk factors at certain critical point levels, to sustain optimal udder health. In this concern an on farm quality risk management (QRM) approach similar to HACCP programme to control hazards and risks in the areas of food safety - public health, and animal health and welfare, recommended by European Union hygiene directive [34], appears to be well suited in the control and management of mastitis.

HACCP: concept and application

Hazard analysis critical control points (HACCP)is basically a method to assure product quality through the control of the production process.With a focus on UHC problems, the HACCP-like approach will focus the farmer's attention on relevant risk factors. In this QRM approach, all areas with significant impacton incidence of mastitis are addressed at the same time and that too, in an extensive manner [2]. The eight key components in the HACCP concept are: analysis of main hazards and associated risks on a farm (e.g., mastitis), production process diagrams, definition of critical control points (CCPs) with their standards and tolerance limits and points of particular attention (POPA) with their targets, the monitoring of theseCCP's and POPA's, the designing corrective measures, working instructions and guidelines, maintenance of records and finally verification of risk control [2, 35, 36].

Steps Programme

1. Assemble an on-farm QRM-team; describe the general farm geography
2. Identify the most significant hazards
3. Determine risk factors associated with the hazards,which are applicable on the dairy farm
4. Draw farm process flow diagrams (detailed for the hazard) and check them on site
5. Define critical control points (CCP) and points of particular attention (POPA)
6. Determine the respective standards and tolerance limits (CCP) and target values (POPA)
7. Weigh the various risk factors for their probability of occurrence and their impact
8. Design a formal monitoring scheme (method of monitoring, frequency, person, measures)
9. Determine sets of corrective measures for deviations occurring at CCP's and/or POPA's
10. Develop good dairy farming guidelines and technical working instructions
11. Introduce the necessary documents and install training programmes for farm workers
12. Install internal validation procedures and external auditing procedures

Hazard identification: An individual hazard causing serious challenge to the udder health in each dairy farm is identified in this step. To facilitate a better understanding on this concept, let’s consider the hazard S. aureus, as an example, to continue the discussion. Based on a herd inventory and history, a herd treatment advisory plan is designed for several categories of disease severity groups (mild mastitis, severe mastitis andvery severe mastitis), with further actions or interventions. Next step is the identification of risk factors

for S. aureus, applicable to the particular farm. These risk factors were weighted and ranked in order of estimated relevance, see table 18.3. Weighting can be done qualitatively by making a best possible estimate [34].

Table 18.3: Risk factors contributing to S. aureus mastitis with relative ranking [2]

	Risk Factors	Relative Weightage
(a)	Deficiencies in the milking machine function	1
(b)	High teat end callosity scores in the herd	2
(c)	Contaminated hands of milkers, bedding material, flies	3
(d)	Previous udder infections with *Staphylococcus aureus*	4
(e)	Poor milking method and hygiene	5
(f)	Poor culling policy regarding problem cows	6
(g)	Age of cows	7

Then, each of them was screened for CCP or POPA, and a tolerance limits or a target is set respectively. Then a monitoring scheme is set. The scheme includes: setting CCP or POPA, method of monitoring, frequency of monitoring, person responsible of monitoring, result of monitoring (Example with S. aureus is given in table18.4).For example, the targets envisaged for yearly incidence rate of clinical mastitis cases should be less than 25%, the somatic cell counts at cow level should be less than 150,000/ml, the level of new udder infections per time unit should be less than 10% etc.

Table 18.4: Hazards & risks management with targets and frequency of monitoring Γ21

Risk area	Target	Monitoring
Milking machine function	Optimal*	Daily + Once weekly +
(e.g. vacuum level)		Twice / yr

Teat liner condition	Optimal	Once weekly
Overall hygiene on the farm (e.g. barn)	Optimal	Once weekly
Udder health state (pathogen profile)	Optimal	Daily + Once weekly
Milking method and hygiene (milker)	Optimal	Daily
Culling rate of problem cows	Optimal	Daily + Once weekly

*'Optimal' means according to prescriptions of the manufacturer or at best possible practice

Items listed under "risk area[7] in table 18.4 should be further detailed as specific as possible. But decisions on implementation of the HACCP- based control measures should be taken only in compliance with the existing socio-economic aspects of the dairy farm. For that, a fruitful communication between the farmer, technical expert (veterinarian), and the feed-back from already established farm protocols is required (Fig. 18. 3).

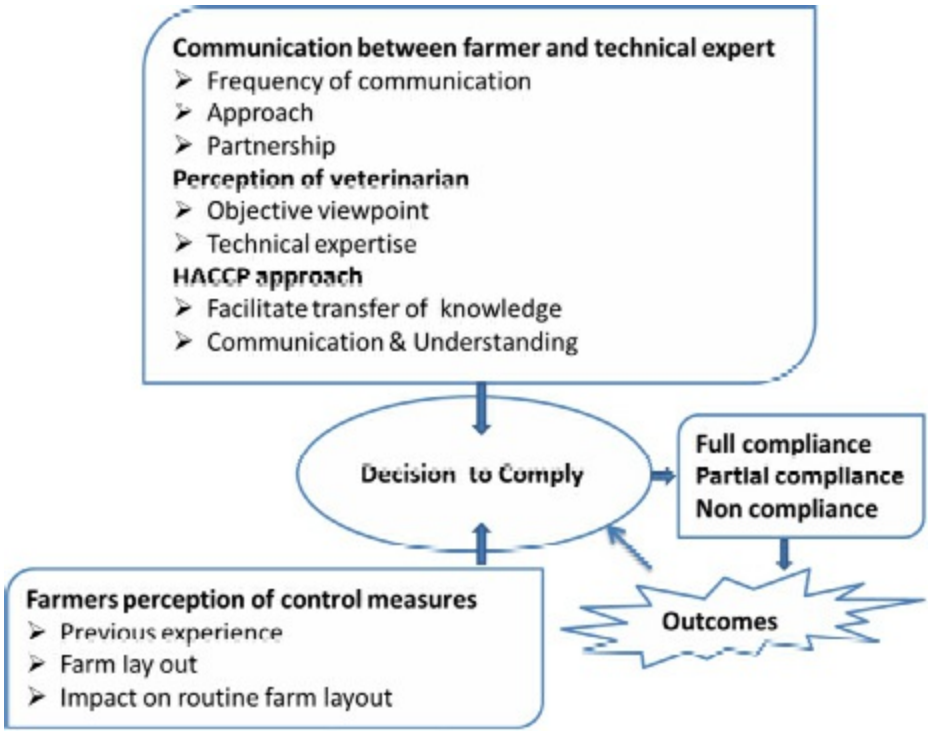

Fig.18.3: Sociological factors considered during implementation of the HACCP- based control measures [37]

Results of each monitoring action are recorded on amonitoring log with date and findings; when deviations havebeen noticed, the intervention conducted is recorded too.The proposed interventions (corrective measures) areshould be updated when needed,e.g., after a follow-up evaluation. Such revisits on management strategy are essential in avoiding the inherent flaws and lack of compliance. A model mini HACCP -like protocol for mastitis management with a summary of critical control points, their control measures, monitoring, verification and corrective actions is described in the table 18.5.

Table 18.5: Summary of critical control points (CCP) for mastitis control [37]

CCP	Control measures	Monitoring (Records and Visual inspection)	Verification	Corrective actions
Udder Preparation	Washing; Drying; Fore-	Preparation; Milk socks.	Recent infection rate;	Udder preparation and cleanliness

	milking; Pre-dipping.		Total bacterial count.	records
Cluster attachment	Cluster disinfection; Milking hygiene; Liner quality.	Frequency of detergent change and its quality and quantity; Machine washing protocol; Number of milkings/liner; Milk recording.	Recent infection rate; Chronic infection rate; Clinical mastitis rate; Thermoduric count.	Management of chronic infection; Segregation strategy; Examining records of milking and washing protocols Rubberware care.
Post milking teat disinfection	Teat disinfection.	Application; Detergent quality and quantity	Recent infection rate.	Quality and quantity of teat disinfection.
Milking machine	Optimum milking machine functioning.	Teat end scoring; Assessing liner slippage; Manual vacuum test; Liner change date.	Milking machine report.	Milking machine performance; Teat end scoring.
Drying off process	Teat preparation; Treatment protocol.	Drying off procedure.	Cure rate; New infection rate; Mastitis cases in dry animals	Teat preparation; Protocol verification.

Calving	Hygiene; Shed layout; Stocking density.	Visual inspection.	Clinical mastitis cases first 60 days	Time spent in area; Pen hygiene; Stocking density.

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Chapter 19
Evaluation of Udder and Teat Conditions for Udder Health Management

Teat health is important for udder health management and quality milk production. The physical condition of bovine teat is an indicator of the milking management and possible intra-mammary infections in a dairy herd. Therefore daily monitoring of teat and udder conditions are important for udder health management. Since, most of the mastitis associated pathogens spread through teat canal; mastitis risk is greater when, the number of bacteria near the teat end lesions is more. Besides, teat lesions results in poor letdown due to pain, and thus provide better environment for bacterial multiplication. Although, mastitis has been considered as a management problem, the positive genetic correlation between milk yield and mastitis suggests that control of mastitis may be more complex. Therefore, an understanding on various animal factors that keeps some animals more resistant to mastitis such as udder and teat morphology, may aid us in our efforts to control mastitis.

Teat Canal-an Important Defense Barrier

Teat canal protect against bacterial invasion through tight closure or sealing between milking, adherence of bacteria to the keratin layer, which eventually is sheared off during milk flow and re-sealing of the canal during the early post-milking period. Besides, formation of a lipid film allows, easy opening of the teat canal during milking along with effective cleaning of any adherent bacteria and effective re-sealing after milking. Therefore, disruptions to teat canal barriers will increase the susceptibility of the udder to infection. Further, the following alterations in teat canal structure will increase the infection rate

- Wider diameter of the teat canal
- Shorter teat canal
- Incomplete sealing of the keratin plug after milking

Healthy teat ends are the key determinants of any mastitis control strategy. Any lesions in teat end will increase the new intra mammary infections. Teat end lesions can be due to faulty milking machine, environment and/or infectious causes. Milking machine-induced changes in teats can be observed after removal of the cluster, usually within 30-60 seconds. However, environment-induced changes can be observed even prior to milking. Teat color varies within and among cows in a herd according to breed characteristics. Black teats hide machine milking induced changes or environment mediated hypersensitivity reactions.

Table 19.1: Milkina and environment induced teat lesions in dairy animals

Milking-induced	**Environmental effects**
Discoloration	Skin dryness or roughness
Firmness or swelling	Hyperkeratosis
Wedging of the teat end	Chapping
Openness of the teat orifice	Abrasions and cuts
Petechial haemorrhages	Photosensitization
Hyperkeratosis (thickening of the skin)	Chemical damage
	Allergic reactions
	Fly bites

The milking and environment induced teat lesions can be classified into short, medium and longer-term changes in dairy animals (Table 19.2). Short-term changes are mostly due to faulty milking machines or milking management. Medium-term vascular damage or changes are due to tissue responses against faulty milking machine or poor milking management, which usually takes few days or weeks for its clinical manifestations. Vascular changes are usually due to pulsation failure along with high vacuum pressure resulting in over milking. Medium-term effect causes low flow rate (<1

litre/minute) for longer periods and/ or over milking. Long-term effect develops typically over a period of 2-8 weeks and very long-term changes occur over a period of few months even.

Table 19.2: The milking and environment induced changes in teat conditions

Short term changes	**Medium term changes**	**Long term changes**
Discoloration	Haemorrhages of the teat skin	Teat end hyperkeratosis Changes in size
Swelling at or near to teat base		
Firmness at or near to teat end		Tissue fibrosis and thickness of teat
Openness of the teat orifice		

Infectious lesions are mostly due to viruses, bacteria and fungi in teat skin, teat end or udder. Infectious lesions of teat skin are indicators of farm hygienic practices, mastitis control and milk quality management programmes and any deterioration of teat skin condition may adversely affect the milk quality and udder health. Pseudo cow pox, bovine herpes mamillitis, teat warts papilloma, foot and mouth disease and vesicular stomatitis are some important viral diseases in which teat lesion occurs. *Staphylococcus aureus, Streptococcus dysgalactiae* and *Arcanobacterium pyogenes* are the primary bacterial organism present in the pustules or normal skin of dairy cows and these pathogens are a major source of new intramammary infections and clinical mastitis during lactating and dry period. Black spot is an important bacterial lesion that occurs due to poor machine milking- mediated colonization of the *Fusiformis necrophorum.* Black spot is a major risk factor for intramammary infection and often leads to incomplete and very slow milking. The most common fungus associated with infection of skin keratin is *Trichophyton* spp.

How to Assess/ Score the Teat end Lesions?

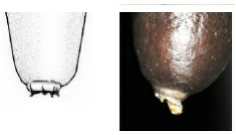
The teat end conditions should be systematically evaluated in sufficient number of animals. In a herd with 500 cows, at least 10% of the animals should be evaluated, while in herds of more than 500 cows, at least 50 randomly selected cows should be checked for better interpretation of farm status [1].The teat condition should be evaluated immediately after the cluster removal and before application of a teat disinfectant. For greater understanding, teat skin condition should be evaluated before milking also. The following instructions should be maintained

- Proper restraining of animals
- Evaluation in a regular pattern
- Visual observation before handling and dry the teat end with a paper towel for better visualization if required
- Examination should be done with good lighting
- Teat end should be evaluated from all age groups or management groups
- Interesting teat conditions should be photographed for subsequent discussions with the udder health experts

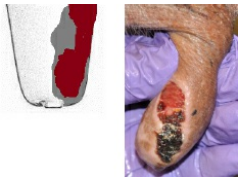
Table 19.3: Teat end condition score card [2]

Score	Description	Photos
1	**No ring:** The teat end is smooth with a small orifice.	
2	**Smooth or Slightly rough ring:** Smooth or slightly rough raised rings around the orifice but no fronds of old keratin are evident.	
3	**Rough ring:** Raised, roughened ring with fronds of old keratin for about 1-3 mm from the teat orifice.	
4	**Very rough ring:** Very rough and cracked ring with rough fronds of	

old keratin for more than 4 mm from the teat orifice.



5 **Open Lesions or Scabs:**
Severely damaged teat end with ulcerative scabs or open lesions.



Teat end Lesions and Mastitis

Milking machine mediated higher probability of intra mammary infection is a known concept. Severe teat-end lesions are positively associated with the prevalence of subclinical or clinical mastitis. Data from Netherlands indicated a lower risk of clinical mastitis in teats with mild or moderate smooth rings compared to no ring or rough rings [3]. In contrast, another research conducted in UK showed a higher risk of sub-clinical mastitis (based on CMT positive) with poorer teat-end scores [4].

Stage of lactation could be an important factor as cows with clinical mastitis had severe teat end lesions than healthy animals, particularly when clinical mastitis occurred between the second and fifth month of lactation [5].Further, detailed studies on influence of parity, stage of lactation, milk yield and breed are important to understand the risk factors associated with teat end lesions and their impact on the incidence of mastitis.

Threshold for Interpretation of Teat end Lesions

Interpretation of teat end lesions requires a better understanding on threshold level for various conditions. An Australian data based threshold levels (Table 19.4) can be considered for investigations of the influence of milking machine, environmental and infectious factors of teat end condition.

Table 19.4: Threshold levels for different changes

Conditions	Threshold levels
Changes in colour of teats like reddening or cyanotic	>20%
Teats with marked swelling or palpable rings at near or the top of the teat	>20%
Teats ends with firm, hard or swollen, or noticeably wedged lesions	>20%
Teat orifices with openness	>20%
Teats with vascular damage such as haemorrhage, petechiae	>10%
Teats with open lesions such as cracks	>5%
Teat end with hyperkeratosis	>20%

Teat Morphology and Mastitis

The relationship between teat size and shape **(Plate 1)** with udder infection has been studied as they are clinically relevant to infection rate. For instance, higher incidence of mastitis was found in cylindrical shaped teats than in funnel shaped teats [6, 7]. The prolapses of teat orifices were also found higher in cylindrical shaped teats. Mastitis was found to be associated with bottle shaped teat and round end teats in Sahiwal and cross bred Sindhi cows. Subclinical mastitis was related with bottle shaped teats [8]. In other studies, mastitis was reported to be associated with funnel shaped teat, conical shaped teat, cylindrical teats and inverted teat end [9, 10, 11]. In contrast, some studies have reported that there exists no relation between incidence of mastitis and teat shape [12]. Breed and other differences might be responsible for the variation in these studies.

Along with udder health, teat morphology was also associated with milk production. Higher milk yield was observed in cows with funnel shape, pear

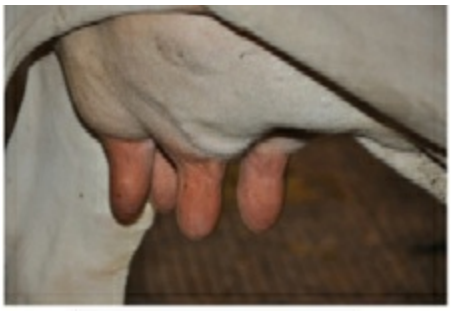
shape, bottle shape and cylindrical teats [9, 13, 14]. In contrast, no relation between milk yield and teat shape was observed by several other researchers [8, 15]. Since the teat characteristics are heritable in nature, understanding the association between teat morphology with production and infection rate and its genomic background will be useful to select the high yielders with mastitis resistance.

Relationship Between Udder Morphology and its Health

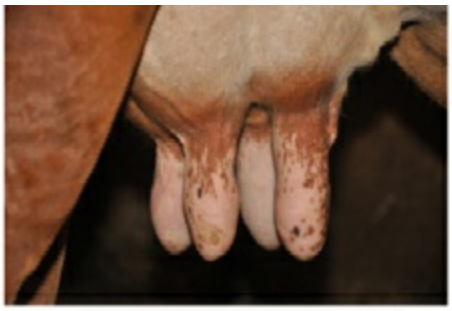
Udder morphological characters such as fore and rear udder heights (distance from floor to udder), udder levelness (front udder height minus rear udder height) and distances between teats were related to milking management and udder health. For instance, teat cup liner slips during milking have been related to udder health as it cause an abrupt change in vacuum leading to the movement of mastitis causing pathogens from teat opening or within the streak canal into the teat cistern. Further, liner slip and manual adjustments increase the labor involvement. Udders that were lower (closer to the floor) had more liner slips, produced more milk yield and required more milking time [15]. In addition, cows with front udder higher than rear udder and wider teats had more liner slips. Several researchers concluded that deeper udders have higher somatic cell counts and more incidences of mastitis, while animals with non-pendulous udders were having higher resistance to mastitis [16]. Since zebu cows or buffalo are different from exotic animals, understanding the teat and udder morphology in relation to production and intra mammary infection are important for genetic selection and improvement. Studies at NDRI on Karan Fries and Sahiwal cows revealed that round and trough shapes udder were more frequent in Karan Fries and Sahiwal cows, respectively. Trough shaped udder had more milk yield. Distributions of cylindrical and conical teats were more frequent in Karan Fries and Sahiwal cows, respectively and shape of teat had no effects on milk yield. The of front teats were longer than rear teats and distance between front to front teats were longer in these animals. Both length of teats and distance between teats were significantly correlated with milk yield in these animals [17, 18]. Collectively, it suggests that selection of cows having proper shape and size of udder or teat will improve the yield and health conditions.

Conclusion

Morphological characteristics of udder and teats are moderately to highly heritable. Pendulous udders are more susceptible to mastitis than firmly attached higher udders. Although, teat-end shape or lesions seems to be correlated with incidence of mastitis, there is lack of agreement on relationships between other teat anatomical traits and mastitis incidence. Lack of universal standard scoring system, breed variations and different method of interpretation of data could be the reason for difference in opinion.

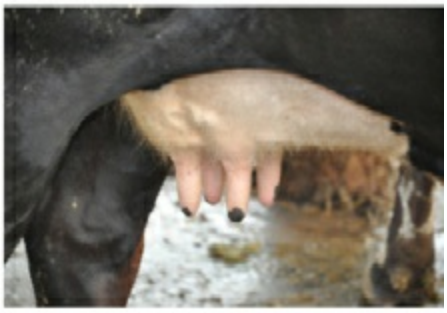


Wide

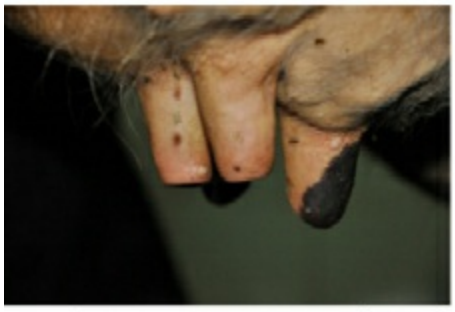


Cylinder

Pointed

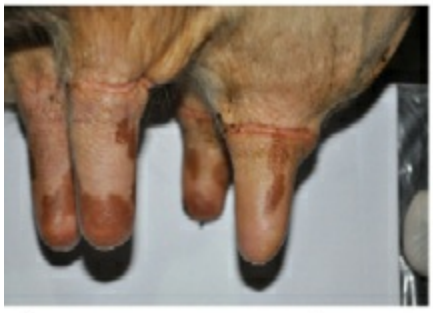


Round

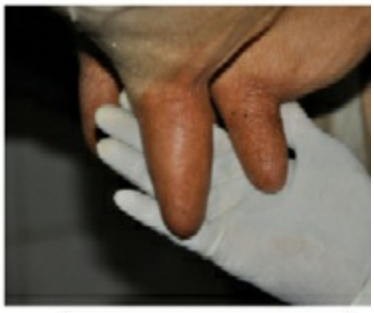


Flat

Funnel



Dryness of teat



Pustules

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Chapter 20
Epilogue

The dairy industry worldwide has achieved many significant laurels during the last fifty years. However, still various hurdles including mastitis need critical attention to achieve the desired targets. Mastitis is accounted worldwide a very serious problem due to its massive economic losses in dairy industry. Huge efforts are going on for the reduction of such diseases since many decades; nevertheless complete success has yet to be achieved. The following points are the excerpts of the foregoing chapters, given in a concise way, for quick understanding of the facts and figures about bovine mastitis.

- The data calculated as mean of more than 100 studies carried out in 21 states of India indicates that the overall prevalence of bovine mastitis in India was 44.67% (ranged from 25.63 to 97.61%). This significant increase in the occurrence of bovine mastitis is an alarming phase for the dairy sector. In spite of decades of research, mastitis still remains as a complex disease condition difficult to resolve due to the involvement of a wide range of etiological agents. Considering that different pathogens are the predominant cause of mastitis in different countries, mastitis control programmes need to be developed to meet the specific requirements of an individual country or segment of the dairy industry. A more in depth knowledge of the etiological agents is crucial to gain better understanding of pathogenesis and epidemiology of bovine mastitis. Identifying the microorganisms responsible for culture negative, clinical mastitis and assessing changes in bacterial populations throughout infection will improve our understanding of the disease process allowing us to identify more effective intervention strategies. Thus, an accurate profile of microbial diversity of the mastitis milk is highly essential to design appropriate preventive and treatment strategies to curtail mastitis. The

constantly changing predominance of etiological agents in different geographical locations must be considered while adopting and developing mastitis control strategies. The application of culture independent bacterial community profiling represents a powerful approach to understand long-standing questions in animal health and disease including mastitis.

- Enhancing the natural ability of the host to resist intra mammary infections without introducing undesirable residues into the food chain is highly desirable in view of public concerns and demands. A thorough knowledge of the immune defense mechanism of the udder is essential to evaluate immunomodulatory strategies for the control of intra mammary infections.
- Candidate genes and genetic markers database are helpful to develop molecular approach to select the animal at early stage with improved udder health. Mastitis is polygenic in nature and several candidate genes (BolA-DRB3, β-defensins, TLRs, IL-6, etc.) have been reported in the recent years, which showed potential role in detection of disease resistance or susceptibility. One database is available, which is consisted of 943 genes and genetic markers involved in mammary gland health and function. Currently, many methods and techniques viz. PCR-RFLP, SSCP, RAPD, AFLP, RT-PCR, micro-arrays etc. are used in detection of mastitis in animals.
- Mastitis also affect reproductive performance of dairy cows in many ways resulting in longer interval from calving to first service, days open, and number of services per conception. Therefore, the appropriate management of lactating dairy cows to minimize the incidence of mastitis should increase the profitability of dairy herds not only by improving milk quality but also by reducing involuntary culling and improving reproductive performance. It is also important to give focus on peripartum health status of animals in order to improve reproductive efficiency in dairy animals. As genetic correlations between mastitis and lactation average somatic cell scores are strong (from 0.7 to 0.8), somatic cell count may be considered as an indicator trait for mastitis, which in many countries is used as a criterion in indirect selection, for improving mastitis resistance. Strategies to prevent metabolic disorders, incidences of mastitis and maintaining proper nutritional and health status and minimizing overcrowding of cows especially in the fresh cow pen by following appropriate management practices are essential during the peri-

partum period.

- Among the several aids to diagnose mastitis, alterations in milk quality in terms of Somatic Cell Count (SCC), Electrical Conductivity (EC) and pH have been shown to be strongly associated with mastitis thus assessment of these parameters have been employed to diagnose mastitis in smaller dairy units. However, the standards of these parameters to detect subclinical mastitis have been developed elsewhere in highly organized farms but the suitability, practicability, sensitivity and specificity of these standards in unorganized sectors have not been studied in detail. In spite of having significant contribution from unorganized farms, no consistent efforts have been taken up to apply these tests and standards to check the milk quality in small holder production system.
- A number of c onventional tests have been developed for detection of subclinical and clinical mastitis. Recent advances in nucleic acid and proteomic based techniques have resulted in identification of novel biomarkers of mastitis. No test in isolation confirms the occurrence of subclinical mastitis. Therefore, more than one test is required to confirm the occurrence of mastitis. However, more research is required for development of a quick, simple, easy, cheap, specific and sensitive "cow side test" for detection of mastitis in farm animals.
- Therapeutic management of mastitis is traditional and new strategies are rarely followed in treatment despite of improvement in understanding of the pathophysiology. Weakness in clinical studies is believed to be major reason for poor adaptation in field conditions. Therefore, to produce reliable results, clinical trials should be randomized and balanced and confounding factors taken into account in statistical models. Further, antibiotic residue and possibility of antimicrobial resistance should always be taken into consideration as milk is used for human consumption. Since the mastitis pathogens are varies between different geographical locations,formulation of treatment strategies based on causative agent along with route, duration, supportive therapy, etc. and factors affecting the treatment response are need to be considered during clinical trials. Farm level trials in different field conditions are necessary to explore economic benefits of treating different types of mastitis. Diagnosis of mastitis as per severity (mild, moderate and severe) to decide the required cost-effective treatment protocol and to establish the clinical as well as bacteriological cure is the important pharmacological

concern of mastitis treatment. Better optimization of the existing *in vitro* methods and evaluation of more sensitive drugs against major mastitis pathogens through more reliable PK/PD parameters rather than traditional parameters will greatly improve the treatment outcome. Further, the maintenance of proper record system will be useful to identify the relapse and recurrence of clinical mastitis and analysis of treatment records will be useful to improve the existing protocol in farms.

- In the present trend, though herbal medicines seem to be useful and possessing many advantages, their efficacy as sole agents in the therapy of mastitis is not yet proven. They may be used preventively and in controlling subclinical mastitis with some effect. Herbal drugs seem to be the best option available to replace antibiotics. However, a lot of research in veterinary institutes is to be done before venturing to suggest a suitable drug. The research facilities has to be strengthened in the areas of photochemistry, purification, large scale production of standardized extracts and clinical trials to carry out efficacy studies to realize a suitable alternative therapy. As a science it offers great scope for research, given the challenges in sourcing, purification, formulation and validation.
- Introduction of machine milking made the milking quick, complete and contamination free. Moreover, the quality and shelf life of the end product has increased many folds. However, there is need of proper precaution in using machine milking to reduce machine generated incidence of mastitis. The device should work properly with all the parts functioning well, personnel related should be well trained to operate the device properly and finally cows should be properly managed during milking. For preventing mastitis, the machine milking should be operated by following '3 P' functions properly -

- Pressure - Maintain pressure so that there is no much fluctuation.
- Pulsator - Pulsator should maintain proper milking and massage phase.
- Personnel - Well trained personnel to operate the milking machine properly.

In developed countries, using either a five or ten point plan, the prevalence of mastitis has been brought down significantly, however the situation in India is not encouraging. With the increase in milk production, the cases of mastitis

have also increased. There are no much studies on the epidemiology of the mastitis at various time intervals, geographic locations and host-environment-pathogen interaction. Such studies are essentially required to develop strategic plans to control mastitis since the factors like herd size, agro-climatic conditions of the region, variations in socio-cultural practices, milk marketing, literacy level of the animal owner, system of feeding, and management affects the incidence of mastitis.

Golden Rules for Keeping Udder Healthy

- Keep your Cow Healthy
- Keep her Udder Clean
- Keep her environment clean and dry
- Follow proper milking procedures
- Regularly screen your cows for higher milk SCC
- Teat dip after every milking
- Keep her udders dry between milking
- Properly dry your milch animals
- Feed Vitamins, minerals, supplements during stressful periods
- Keep yourself aware of Early Mastitis symptoms
- Cull chronic mastitis cases
- If possible genetically select your cow against mastitis.

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