II. REVIEW OF LITERATURE

*Staphylococcus aureus* is one of the major pathogenic organisms causing mastitis in dairy cattle worldwide which necessitates the use of various antibiotics in treatment. In spite of the latest generation of antibiotics available, it has become very difficult to cure mastitis caused by *S. aureus* because the organism will produce biofilms. Moreover public concern with food safety, to minimize antibiotic residues in milk and the need to reduce somatic cell counts strengthens our determination to combat *S. aureus* mastitis by means of vaccination as an effective alternative methodology.

The available literature on the relevant aspects of the present study is reviewed under the following headings.

2.1 Bovine Mastitis due to *Staphylococcus aureus* organism

Gonzalez *et al.* (1980) found that 2388 (57.3 per cent) out of 4168 quarter milk samples examined from 30 dairy farms were positive for CMT reaction. They subjected 300 samples to cultural examination, which yielded *S.aureus* (43 per cent), *S. epidermidis* (21 per cent), *S.uberis* (19 per cent), *S.agalactiae* (13 per cent) *S. dysagalactiae* (9 per cent), 7 per cent of *Corynebacterium bovis* (*C.bovis*), 1.3 per cent of *Corynebacterium pyogenes* (*C.pyogenes*), Coliforms (1.7 per cent) and mixed Streptococcal and Staphylococcal cultures.

Ferriro *et al.* (1985) screened 4268 quarters from 1067 lactating cows and found that 1046 (24.50 per cent) quarters produced suspect milk. Among them 896 bacterial cultures were obtained mainly from quarters with subclinical mastitis. The predominant
isolates were *S.aureus* in 152 quarters (16.96 per cent) and *S. epidermidis* from 119 quarters (13.25 per cent). The Streptococcus species distribution in different quarters was 108 (12.05 per cent) *Streptococcus agalactiae*, 79 (8.82 per cent) *Streptococcus uberis* and 46 (5.14 per cent) *Streptococcus dysgalactiae*. Further, they also isolated 12 yeasts (majority *Candida albicans*), 12 Nocardia and three mould fungi.

Verma (1988) examined 136 cows for udder infection and found that 42.1 per cent of animals were positive for subclinical mastitis. Among the different organisms isolated, *S. aureus* predominated in 34 of 61 samples. Other organisms isolated were Streptococcus spp (4), *E. coli* (7), Klebsiella spp (2), Corynebacterium spp (6), *Proteus mirabilis* (2) and the fungi *Aspergillus fumigatus*, Geotrichum and Saccharomyces species.

Schukken *et al.* (1989) examined a total of 1140 clinical cases of mastitis. The microorganisms most frequently isolated were *E. coli* (16.2 per cent), coagulase negative Staphylococci (13.0 per cent), *Staphylococcus aureus* (9.6 per cent) and *Streptococcus uberis* (8.0 per cent). They found that as the incidence of clinical mastitis increased, the proportion of *S. aureus* also increased, while the proportions of *E.coli, Streptococcus uberis* and *Streptococcus dysgalactiae* remained uniform.

Lakshmana *et al.* (1993) isolated 494 cases of (67.30 per cent) Gram positive and 240 cases of (32.70 per cent) Gram negative organisms from 975 milk samples collected from 1124 clinical mastitis cases over a period of ten years. The distribution of different isolates revealed that *S. aureus* was predominant indicating high prevalence of
Staphylococcal mastitis in cows in and around Hyderabad, India followed by *E. coli*, Streptococci and others.

Kaya *et al.* (1998) examined 141 milk samples collected from cows with clinical mastitis for pathogenic bacteria. They isolated *S. aureus* (57 per cent), Streptococcus spp (8 per cent), *E. coli* (5 per cent), Lactobacillus species (5 per cent), *Klebsiella pneumoniae* (5 per cent), *C. pyogenes* (4 per cent) and three per cent of *Pseudomonas aeruginosa* (*P.aeruginosa)*.

Gracious *et al.* (2001) isolated 105 bacterial isolates which included *S. aureus* (30), *S. agalactiae* (16), *S. dysgalactiae* (12), *Bacillus subtilis* (12), *P. aeruginosa* (13) and *E. coli* (22) from CMT positive animals.

Wani and Bhat (2003) examined 100 milk samples for the presence of bacteria and they recorded 95 bacterial isolates and 45 isolates of yeasts. Among the bacterial isolates, *S. aureus* (45 per cent), Klebsiella spp (8 per cent) and Enterobacter spp (7 per cent) were present in majority of the samples.

Balakrishnan *et al.* (2004) obtained 40 bacterial isolates from 65 milk samples. The spectrum of different bacteria comprised *S.aureus* (35 per cent), *E. coli* (27.5 per cent), *S. agalactiae* (17.5 per cent), *Pseudomonas aeruginosa* (12.5 per cent), *S. dysgalactiae* (2.5 per cent), *Pasteurella haemolytica* (2.5 per cent) and *Actinobacillus capsulatus* (2.5 per cent).
Palinivel et al. (2005) obtained 12 bacterial isolates from 80 mastitis positive milk samples and the bacterial agents isolated were *S. aureus, S. uberis, S. dysgalactiae, E. coli*, Corynebacterium and Pseudomonas species.

Sumathi (2005) studied the prevalence of mastitis in and around Bangalore, India. A total of seventy five bacterial isolates were recovered from sixty clinical cases of mastitis affected cows. The prevalence of major bacterial pathogens isolated were 24 per cent for *S. aureus*, 20 per cent for *E. coli* followed by 16 per cent for *S. epidermidis* and Streptococcus spp and 10 per cent for Klebsiella spp.

Ali et al. (2008) reported that *S. aureus* was the most frequently recovered organism from mastitis in buffaloes accounting for 49.53 per cent of all the isolates, followed by *S. agalactiae* (23.83 per cent), *S. hyicus* (8.88 per cent), *S. epidermidis*, Bacillus spp. (3.74 per cent), *Staphylococcus hominis* (1.40 per cent), *E. coli* (1.40 per cent), *Staphylococcus xylosus, S. dysgalactiae* and Corynebacterium spp. (0.93 per cent each).

Rajeev et al. (2009) reported that the *S. aureus* followed by *E. coli* were the most predominant pathogens causing both clinical and subclinical mastitis. The prevalence of *S. aureus* was high in subclinical mastitis (24.36 per cent) when compared to clinical mastitis (13.95 per cent), whereas prevalence of *E. coli* was marginally high in clinical mastitis (15.7 per cent) as compared to subclinical mastitis (14.09 per cent).
2.2 Somatic Cell Count (SCC)

Ruffo (1968) recorded predominantly more differential cell count in *Staphylococcus aureus* mastitis.

In a normal non infected and non inflamed quarter, the somatic cell population consisted of polymorphs, macrophages, lymphocytes and epithelial cells (Concha, 1986). The majority of cells consists of macrophages approximately 60% cells, lymphocytes approximately 30% and polymorphonuclear cells approximately 10% and the epithelial cells 2% of the population (Paape *et al.* 1991, Burvenich *et al.* 1994).

Batra and McAllister (1984) made a comparison of various methods for detection of mastitis in dairy cattle by screening 2131 milk samples. The authors reported that the error rate in identifying the infected quarters was lowest for CMT score (9.1 per cent), followed by SCC (13.9 per cent) and conductivity (28.4 per cent).

Vianni and Filho (1989) screened 159 quarter milk samples and found 14 were positive and 145 negative by CMT. Of the CMT positive samples, six were found positive by total leucocyte count (5, 00,000 cells /ml).

Somatic cell counts are used as quality parameter for raw milk. The assumption is that high cell counts are associated with poor quality raw milk, and lower cell counts indicate better quality. The somatic cell count penalty limits for saleable milk differs between countries, with the European Union, Australia and New Zealand at 400,000, Canada at 500,000 and the United States at 750,000 per ml of milk (Paape *et al.* 1997).
Giraudo et al. (1997) developed a vaccine against bovine mastitis based on inactivated, highly encapsulated \textit{S.aureus} and tested on 30 heifers during a 7-months period. This vaccine did not produce observable effect on somatic cell counts.

Schukken et al. (1989) opined that the somatic cells in the milk played an important role in the innate immunity of uninfected mammary gland. A complete absence of cells would put cows at risk for disease and a very low concentration of somatic cells increases the risk of clinical mastitis.

Leitner et al. (2003a) developed a vaccine composed of three field isolates of bovine mastitic \textit{S. aureus} and administered to nine uninfected cows while 10 other cows were used as controls. All cows were challenged with a highly virulent \textit{S. aureus} strain administered into two quarters of each cow. There were no systemic effects observed in the nine cows. All quarters challenged in vaccinated cows exhibited mild inflammatory reactions and low SCC counts.

Leitner et al. (2003b) tested the efficacy of MASTIVAC I in 452 Israeli Holstein heifers in a study conducted over two consecutive years. When SCC and milk yields were measured, a significant difference was found between the cows vaccinated during first and second lactation with respect to SCC (42 and 54 per cent respectively) whereas, the milk yield was 0.5 kg per day higher than the control cows. They also showed that SCC in the vaccinated heifers was significantly lower than those in control animals. They proved that contaminants of one quarter had no influence regarding SCC on the other quarters of the same cow.
Ebling et al. (2001) demonstrated elevation of milk SCC in HF cows with *Staphylococcus aureus* strain. They attributed this to glandular changes in blood and milk barrier of glands.

2.3 **Bacterial biofilm (BF)**

Bacterial biofilm (BF) is a structural community of bacterial cells enclosed in a self-produced exopolysaccharide (EPS) matrix and adherent to an inert or living surface, which constitutes a protected mode of growth that allows survival of organism in hostile environment (Costerton et al., 1999).

Baselga et al. (1993) studied 144 *S. aureus* strains from ovine mastitis for slime production. *In vitro* slime production was detected in 21 strains by tube BF formation and colonial morphology on Congo red agar. The majority of the cells (85 per cent) from slime producing strains and a small number of cells (5 per cent) from non-slime producing strains showed a condensed EPS matrix (slime) surrounding the bacterial cell wall detected by electron microscopy and immunofluorescence. The BF production was also demonstrated immuno-histochemically *in-vivo* after experimental infection of the mammary gland. They concluded that the slime producing variants showed a significant higher colonization capacity than the non-slime producing variants of the same strain.

Azad et al. (1996) studied the development of BF by *Aeromonas hydrophila*, using Tryptose soya broth (TSB). The optimum concentration of TSB was found to be 0.225 per cent with 0.3 per cent chitin flakes. Biofilm cell population reached its peak on day four with a cell density of $10^{10}$ cfu/g of chitin. On the other hand, the planktonic cell density decreased from day one and it was only $1.48 \times 10^6$ cfu/ml on fourth day,
indicating that the BF cell population was inversely related to planktonic cell population in the presence of inert material.

Naveen kumar (2005) reported that optimum concentration of nutrient media required for maximum BF formation of *S. aureus* was 0.32 per cent TSB with 0.3 per cent bentonite clay. The BF cell population peaked on day three with a cell density of $8.13 \times 10^{10}$ cfu/g of bentonite clay and persisted even after 50 days with a count of $5.75 \times 10^{7}$ cfu/g. On the other hand, free cells attained a peak on the first day after inoculation with an average viable count of $9.74 \times 10^{9}$ cfu/ml which declined rapidly thereafter.

### 2.4 Optimization of infective dose for the induction of mastitis in lactating rabbits

Amorena *et al.* (1991) used $5 \times 10^2$, $5 \times 10^4$, $5 \times 10^6$ viable *S. aureus* bacteria in PBS for inducing mastitis through teat canal (48 hrs post-parturition) in rabbits. They maintained rabbits only for two days with first evaluation at 24 hr and second evaluation at 48 hr. Mastitis development was observed in both the cases.

Adlam *et al.* (1977) used minimum $10^3$ (low dose) and maximum $10^4$ (high dose) *S. aureus* in eight to ten days post-parturient rabbits by injecting bacterial suspension at the base of teat. Development of infection was noticed more in $10^4$ dose and they are monitored daily for eight days by measuring the areas of blue discoloration and thickening in the mammary tissue.
Ward et al. (1979) used $10^4$ viable *S. aureus* as challenge organisms in 0.2 ml nutrient broth for challenge studies injecting at base of the teat. Lesions were noticed and measured at 4, 24 and 48 hrs post challenge.

2.5 Gross and Histopathology

Watson et al. (1985) found that the virulence of *S. aureus* depends on alpha toxin, beta toxin, leucocidin and they suggested several important factors of ruminants immune system responsible for eliminating *Staphylococcus aureus*. The authors also suggested that elevated opsonic concentrations were necessary for enhanced PMN cells activity and protective immunization also helped in enhanced phagocytic capacity of PMN cells.

When *Staphylococcus aureus* was inoculated into the mammary glands of mice, the organisms multiplied rapidly and produced an acute inflammatory reaction which was characterized by epithelial cell necrosis. However, neutrophil infiltration in the mammary glands was noticed after intramammary inoculation of endotoxins 6 hrs before the staphylococcal challenge (Anderson, 1977).

Adlam et al. (1976) described both naturally occurring and artificially induced *Staphylococcus* mastitis in Rabbits. In naturally occurring mastitis the mammary gland was thickened and indurated. The animals were lethargic, dull and anorexic. Histopathologically degenerated epithelium and cellular debris along with large PMN cells in alveolar spaces with organisms were noticed. In experimentally induced mastitis, the glands of rabbits were edematous, hemorrhagic and bluish indurated with abscess. Histopathologically at 48 hr PI, the organisms in alveoli, desquamation of epithelium,
PMN cells infiltration and necrosis were seen. Finally, it resulted in gangrenous mastitis and persisted up to two weeks.

The bovine mastitis strains of *E.coli* produced a typical acute mastitis when inoculated to the mouse mammary glands through the teat canal. Clinical mastitis was evident in all the cases by 48 hr. More severe mastitis was observed at 48 hr p.i., associated with necrosis of the epithelial cells of the ducts and alveoli. The general architecture of the tissue was distorted, hemorrhagic and edematous exudation was noticed. (Chandler and Anger, 1977).

Anderson (1977) showed two strains of *S.aureus* isolated from chronic bovine mastitis produced an acute reaction when inoculated into the mammary glands of mice. Histopathologically at 18 hr post infection there was infiltration of neutrophils. Detached epithelial cells in the alveoli and many neutrophils with engulfed *S.aureus* were also seen. At 48 hr, interalveolar cell infiltration, collapsed alveoli, hyperplastic epithelium, cellular debris and fibrosis were recorded. He stated that intracellular location of *Staphylococcus aureus*, abscess formation, deep tissue penetration of bacteria, fibrosis and presence of milk in the glands were responsible for the failure of antibiotic therapy.

The mice which were challenged with *E.coli* 24 hrs after colonization by *Staphylococcus epidermidis* were clinically normal after challenge. Histological examination of mammary glands 24 hrs after challenge with *E.coli* showed that there were neutrophils and neutrophil debris in the alveoli and ducts. The epithelium was vacuolated and liquefactive necrosis was observed. Coliforms were not detected but Staphylococci were seen in the neutrophils, alveoli and ducts. At 48 hr after inoculation
of *E. coli*, the alveoli were contracted, epithelium was hyperplastic and the interalveolar septae were infiltrated by neutrophils, mononuclear cells and mast cells (Anderson, 1978).

Anderson (1978) isolated *Staphylococcus aureus* from bovine mastitis and injected to mouse mammary gland to study the lesions. At 24 hr, histopathologically there were degenerative changes and necrotic changes in the epithelium of the mammary gland. Bacteria were present in the lumen with conspicuous liquefactive necrosis of the gland. At 48 hr there was a hyper plastic appearance of the gland with accumulation of interalveolar fat, infiltration of neutrophils, mononuclear cells and mast cells. *Staphylococcus aureus* was present in the neutrophils. Atrophy of alveoli with decreased secretions was also noticed in many glands.

Jain (1979) reviewed *Staphylococcus aureus* mastitis. Toxin and toxin products were thought to be involved in mastitis and gangrene. Alpha toxin was most potent factor because it caused vasoconstriction leading to ischaemic necrosis and gangrene. Coagulase and other bacterial products were involved in enhancing infection and phagocytosis. Gamma toxin was also most irritating bacterial toxin of staphylococcus. Peptidoglycan fraction of cell wall was involved in hyper sensitivity reaction of the gland. Leucocytosis in milk was not successful in clearing *Staphylococcus aureus* bacteria because, a) Bacterial products, alpha toxin and leucocidin are involved in damaging the neutrophils b) Protein A is antiphagocytic. c) Certain bacterial products protect them from intracellular killing. Adherence of bacteria to parts of epithelial cells was considered to be more pathogenic.
Ward et al. (1979) studied the role of alpha and beta toxins of *Staphylococcus aureus* in the pathogenesis of mastitis in rabbits and compared with the natural infection. In the natural infection, grossly, glands were hemorrhagic, dark colored and edematous. Adjacent skin of the gland showed PMN cell infiltration. Microscopically detached secretory epithelium, undifferentiated small cells infiltration, intense PMN cells infiltration and accumulation of fat droplets in alveoli were recorded at 24 hrs post infection. The experimental infection was produced by injecting rabbit lactating mammary gland with alpha and beta toxins at the rate of $4 \times 10^{11}$ HU/ mg. Two types of pathological syndromes such as Acute: blue breast appearance and Chronic: abscess form was observed. In the acute form, secretory epithelium was detached within the alveoli or floating freely in darkly staining material in the alveoli and PMN cell were less in alveoli but more in surrounding areas. The alveoli were necrosed and distorted. Staphylococci were seen in the alveoli. In the chronic form intense PMN cell infiltration was usually caused by beta toxin, thickening of the septa and atrophy of alveoli with occasional bacteria were seen. Comparison of these findings in the experimental infection, they opined that hemorrhagic necrotic syndrome known as blue breast was directly attributable to alpha toxin. The less PMN cells in the alveoli in acute form was due to direct lysis of PMN cells by alpha toxin. More PMN cells in beta toxin were due to chemotactic effect of inflammatory response by the infection.

Craven and Anderson (1982) established an acute mastitis by inoculating $10^6$ cfu of *Staphylococcus aureus* to the normal lactating mouse mammary glands. At 18-24 hrs after intramammary inoculation, liquefactive necrosis of epithelium, lysis of neutrophils and large number of staphylococci were seen in the mammary glands. Alpha toxin was
detected in 4-5 glands of mice. Vacuolation of epithelium and debris in the lumen was a consistent feature recorded.

Bramley et al. (1989) studied the pathogenesis of Staphylococcus mastitis in mouse model. They studied sequential pathology at 2, 6, 8, and 24 hours post infection using $10^8$ CFU. Histopathologically from 20 mins to 2 hours there were bacteria, cell debris and milk components present in the alveoli. At 6-8 hr there was accumulation of PMN cells in the alveoli, distention and vacuolation of secretory epithelium. At 8 hr large areas of glands were affected, PMN cell infiltration in the alveoli and interalveolar spaces was noticed at 24 hours along with pockets of abscesses. There was severe infiltration of neutrophils and macrophages with epithelial hyperplasia.

Amorena et al. (1991) developed an experimental model in rabbits to study ovine mastitis. A total of 19 ovine mastitis bacterial strains (seven Staphylococcus aureus, four S.chromogenes, four S.hyicus and four Escherichia coli) were used to infect mammary glands of rabbits by inoculating the bacterial suspension through the alternate teat ducts. The histopathological evaluation showed that the ovine mastitis corresponded with experimental infections produced in the rabbits. They also established that the most pathogenic species was Staphylococcus aureus followed by E. coli. The lesions consisted of fibrosis and infiltration of PMN cells in the alveolar septa.

Lam et al. (1996) opined that E coli was an environmental pathogen which did not generally spread from one quarter to another quarter, but S.aureus mastitis was a contagious disease that spread from infected to uninfected quarters.
Jones (1998) described the pathogenesis of *Staphylococcus aureus* mastitis. *S. aureus* produced toxins that destroy cell membranes and could directly damage milk producing tissue. Initially, the bacterium damages the tissue lining the teat and gland cisterns within the quarter. Then they move up into the duct system and establish deep seated pockets of infection in the alveoli. This is followed by walling of bacteria by scar tissue and the formation of abscesses which might cause poor response to antibiotic treatment. Alveolar and duct cells will be destroyed and there is a reduction in milk yield. These degenerated cells may combine with leucocytes and clog the milk ducts that drain the alveolar areas contributing for scar tissue formation. The abscess becomes quit large and detected as lumps in the udder – swelling and chunks of milk clinically. The reasons attributable for difficulty in treating infection were because of bacteria living inside the WBCs and produces enzymes that inactivate most penicillin based treatments. The bacteria may remain inactive inside the neutrophils for 5-7 days. When the cell dies the bacteria are released to resume cell division and the infection process continues. The development of antibiotic resistance and formation of L-forms during treatment are also additional reasons for therapy failure.

Shibahara and Nakamura (1999) studied the pathology of *Staphylococcus aureus* in the cow. They noticed gross pathological changes like firmness, enlargement, and edema in the udder of the cow. Histopathologically, there were debris, bacterial clumps and necrotic foci in the alveoli. Fibrinous necrosis was a feature. Liver showed centrolobular fatty change.
Paape et al. (2003) opined that the macrophages were the predominant cells in the normal mammary gland, which acted as sentinels to invading mastitis causing pathogens. Once the invaders were detected, macrophages release chemical messengers called chemoattractants that caused the migration of PMN cells into the infected area. Migration of neutrophils into mammary tissue provided the first line of defense against bacteria that penetrated the physical barrier of the teat canal. The authors explained that the PMN cell were phagocytosing and destroying the invading pathogens, they released chemicals which induced swelling of secretory epithelium, sloughing of secretory cells, and decreased secretory activity. Resident and newly migrated macrophages helped to reduce the damage to the epithelium by phagocytosing PMN cells that underwent programmed cell death through a process called apoptosis. In response to infection, freshly migrated leukocytes expressed greater numbers of cell surface receptors for immunoglobulins and complement and were more phagocytic than their counterparts in blood.

Cucarella et al. (2001) stated that the pathogenesis of Staphylococcus aureus was attributed to the combined effects of extracellular factors and toxins, together with the invasive properties of the strain such as adherence, biofilm formation and resistant to phagocytosis. Two steps involved in the formation of biofilms were a) Attachment of bacterial cells to the surface (early adherence) and the growth dependent accumulation of bacteria in multilayered cell clusters (intercellular adhesion). All Staphylococcus isolates harboring bap gene were highly adherent and strong biofilm producers. Bap is an important factor determining persistence of infection. During development of infections such as subclinical mastitis, biofilm formation could be an efficient way of persisting in the micro environment of the udder, where shear forces arose during milking. The
presence of bap in *Staphylococcus aureus* strains was responsible for mastitis that might enhance mammary adherence and biofilm formation leading to inefficacy of antibiotic treatment.

Benites *et al.* (2002) examined the mammary parenchyma from 184 slaughtered dairy cows for existence of microorganisms and histopathological changes. Of all the samples from which microorganisms were isolated, only 3.1% did not show histological changes. The remaining 96.9% of samples showed inflammatory response characterized by edema, mammary epithelial cell damage, and PMN cell infiltration and/ or tissue repairative process. In contrast, 33.9% of samples from glands without evidence of microorganisms showed no histological changes. The authors opined that the presence of microorganisms was associated with the tissue damage during mastitis.

Beytut *et al.* (2002) investigated *Staphylococcus aureus* mastitis in bovines from Kars areas. They described small nodular regions and abscess formation in the udders of the cow. Histopathologically fibrosis, hemorrhage, edema and necrosis were recorded in natural infection of cows with *Staphylococcus aureus*.

Reinoso *et al.* (2002) studied pathology of *Staphylococcus aureus* strain RC 122 using bovine and rabbit models. The results clearly showed that RC122 was less virulent than its RC108 parental strain in the rabbit skin model. Macroscopic lesions were characterized by swelling, hyperemia and bluing. Histopathologically, necrosis, inflammation and fibrosis were noticed. Hyperemia and congestion were seen in epidermis, dermis, hypodermis and muscle layers.
The enzymes involved in bovine mammary tissue destruction were investigated by Mehrzad et al. (2005), using an endotoxin-induced mastitis model. They reported that mastitic milk proteases hydrolyzed casein, gelatin, collagen, hemoglobin, mammary gland membrane proteins and lactoferrin, and mastitic milk proteases had a broad spectrum of activities. Further, the direct involvement of proteases in epithelial cell damage was demonstrated by the fact that co-incubation of normal mammary tissue with mastitic milk, produced tissue degradation, but not normal milk.

Zhao and Lacasse (2007) opined that the mammary tissue damage could be induced either by apoptosis or necrosis. The authors postulated that it could be due to the release of wide range of cellular and extracellular products from the bacterial pathogens or lysosomal enzymes and oxidative products released from phagocytes during phagocytosis of invading organism. They opined the proteases from blood and cytokines released during the immune response could also play an important role in tissue destruction. Mastitis was characterized by an influx of somatic cells, primarily polymorphonuclear neutrophils, which could harm the mammary tissue by releasing reactive oxygen intermediates and proteolytic enzymes.

Strandberg et al. (2008) stated that transcriptional response within bovine mammary epithelial cells subjected to challenge with Staphylococcus aureus determined susceptibility of the tissue and the ability to resolve the infection. The secretion of proinflammatory cytokine and chemokines from mammary epithelial cells stimulated by the bacteria triggered recruitment and activation of neutrophils in the mammary tissue.
Viana et al. (2008) recorded spectrum of lesions associated with natural and chronic staphylococcal mastitis in 130 rabbits. Mastitis cases were classified as, a) abscess type; presence of one or several well differentiated abscesses which consisted of purulent material, heterophils, debris and bacteria b) Rosette type; presence of large non encapsulated inflammatory areas which consisted of abundant necrosis and bacteria c) Sandwich type; characterized by broad band of inflammatory tissue in the periphery of mammary gland which extended into subcutaneous tissue and abdominal muscles d) Mixed type; which had two histological characteristic of either abscess type or rosette type.

2.6 Ultrastructural Pathology

Chandler and Anger (1977) showed that there was no selective adhesion of staphylococcus to the secretory epithelium at ultrastructural level. They also studied the ultrastructural changes in mouse mammary glands after intramammary inoculation with various E.coli strains isolated from bovine mastitis. The ultrastructural pathology of E.coli infections demonstrated a broad spectrum of changes. Bacteria were usually seen in the lumen and appeared normal at 48 hr post inoculation. The epithelium showed involution with sparcity of microvilli and loss of apical cytoplasm. The milk protein particles in the lumen were coagulated into round masses and the bacteria were located in the remaining amorphous matrix. When endotoxin producing strains were inoculated, clear zones were evident surrounding the bacteria and there was some correlation with the general pathogenicity of the strains studied. The J2 strain of E.coli, a K88-enterotoxin producing strain caused necrosis of secretory epithelium characterized by the loss of cell membrane, dissolution of endoplasmic reticulum and vacuolation as early as 5
h p.i. At 48 h p.i, the basic architecture of the acini was difficult to visualize, the epithelial cells being necrosed and displaced by milk proteins, lipid globules, cell debris and bacteria.

An investigation was made using light and electron microscopy of the progressive pathological changes in nine experimental and two natural cases of severe *Escherichia coli* mastitis in dairy cows. The duration of infection varied from 18 hrs to 13 days. Epithelial lesions were not found in glands which had been infected for more than 24 hrs. However, the epithelia of the sinuses and large ducts became hyperplastic after 60 hr of infection and by six days hyperplasia was extensive on the crests of folds. The leucocyte response in the lumina of the glands and subepithelial tissue showed a progressive change from an acute neutrophil reaction to a chronic mononuclear cell infiltration within the first 36 hr of infection. (Hill *et al.*, 1991)

Transmission electron microscopy of bovine mammary cells invaded by *S. aureus* showed intracellular replication of the bacterium within membrane-bound vacuoles (Almeida *et al.*, 1996). Invasion was reduced significantly when bovine mammary epithelial cells were treated with inhibitors of F-actin microfilament polymerization but it was not seen when these cells were treated with inhibitors of microtubule formation. Results indicated that *S. aureus* was capable of invading and replicating inside bovine mammary epithelial cells. Data also suggested that *S. aureus* invasion of bovine mammary epithelial cells required active participation of specific components of the cytoskeleton of the epithelial cell.
Shibahara and Nakamura (1999) studied the pathology of *Staphylococcus aureus* in the cow. Ultra structurally they recorded bacteria as round with thick cellular wall.

### 2.7 *Staphylococcus aureus* vaccine against mastitis:

Schalm *et al.* (1964) stated that cows parentrally immunized against *Staphylococcus aureus* showed marked leucocytosis in response to second intramammary challenge with killed whole *Staphylococcus aureus* bacterin. The leucocytosis might indicate CMI as a result of vaccination. Leucocytes might increase *Staphylococcus aureus* multiplication due to mammary tissue damage from the immune inflammatory response. Large repeated injections of vaccine were required to produce high serum antibody titres and these titters were only maintained for a few months. Vaccine strains were specific and do not protect adequately against heterologous strains.

Adlam *et al.* (1977) studied the effect of highly purified alpha and beta toxins from *S.aureus* on mastitis in rabbits and found that high circulating anti alpha toxin titres reduced the lethal, hemorrhagic form of the disease to a localized chronic abscess form but anti-beta-toxin titres failed to afford protection. Alpha toxins produced blue breast and beta toxin produced abscess formation and chronic mastitis in rabbits. Immunization of animals with alpha and beta toxoid vaccines had less effect on clinical course of infection produced by *Staphylococcus aureus* strains. In his experiment all the five animals developed abscesses upon challenge by strain CN 6708.

Mc Dowell and Lascelles (1971) studied local immunization of ewes with *Staphylococcus* cell and cell toxoid vaccines. Grossly inflammatory changes following challenge at 24 hours revealed gangrenous mastitis, swelling and edema. TLC and
bacterial counts were increased in non immunized ewes but decreased TLC was observed in immunized goats after challenge with virulent Staphylococci. Antibody titres were increased in serum following immunization with polyvalent cell vaccine but these titers were decreased substantially by 48 days after immunization to low levels in serum.

The mouse mammary glands were colonized by *Staphylococcus epidermidis* before challenging with *S.aureus* or *E.coli*. Chronic mastitis was induced and *Staphylococcus epidermidis* was found to be more effective against *E.coli* than *S.aureus* (Anderson, 1978).

Adlam *et al.* (1980) used purified Panton-Valentine leucocidin or delta-toxin either alone or in combination with alpha-toxoid to immunize female rabbits. Challenge was carried out by injecting lactating mammary tissue with low numbers of staphylococcus (CN 6708) strain responsible for a natural outbreak of rabbit mastitis with abscesses. The immunization did not protect from the abscess disease produced by this organism, but several animals contracted with the lethal spreading type of disease (“blue breast”), though the circulating antibodies to all three toxins were present. They concluded that the “blue breast” produced by strain CN 6708 might be caused by a different and unidentified toxin.

Adlam *et al.* (1981) studied local and systemic antibody response in cows following immunization with staphylococcal antigens during the dry period. The animals that received local infusions of plain vaccine into two quarters of the udder two weeks before calving and agglutinating antibodies in serum, colostrum and milk were measured. All the cows had high colostral antibody titres which were dropped to background level.
by two weeks. They opined that some local antibody being produced in those quarters of animals, which were previously received two doses of plain vaccines.

Pankey et al. (1985) evaluated protein A and a commercial staphylococcal bacterin (Somatostaph®) by experimentally challenging with \textit{S. aureus} in thirty cows during their first lactation. The studies were carried out through three lactations and included bacteriological and cytological analyses of quarter milk samples. The rate of intramammary reaction with \textit{S.aureus} was similar for both vaccinated and unvaccinated cows. Somatic cell counts were significantly lower for vaccinated cows for quarters infected with \textit{S.aureus}, but there was no difference demonstrated for milk production by any of the lactation. They concluded that the incidence of clinical mastitis was higher in unvaccinated cows.

Guidry et al. (1991) studied the effect of anticapsular antibodies on neutrophil phagocytosis in cows immunized against \textit{S.aureus} mastitis. Serum agglutination and ELISA titers of cows immunized with diffuse and diffuse large clearing variants increased after immunization and after each booster and remained elevated till the end of the experiment. They stated that major obstacle to produce a protective immune response to \textit{Staphylococcus aureus} was development of extracellular polysaccharide. The exopolysaccharide allowed antibiotics to cell wall and complement to penetrate but masks recognition of antibiotics by PMN cells and could prevent activation of complements (C3).

Nickerson et al. (1993) studied the influence of a \textit{S.aureus} mastitis vaccine on immunologic status and recorded series of events following \textit{S.aureus} bacterin vaccine
(Somatostaph®) in cows. Four weeks after revaccinations cows were challenged by intramammary infusions of *S.aureus* and then killed at 24 and 72 hr to record series of events. Leucocyte infiltration was greater in quarters from cows vaccinated in the area of supramammary lymph node. The SCC in cows vaccinated in the area of supramammary lymph node was lowest. Mean serum antibody titre was 4.7 fold higher than that of control. Increased plasma cells were located in the tissues. Increased leucocytosis was seen. Leukocyte infiltration was significantly higher from cows vaccinated in the area of supramammary lymph node. This has been done to see whether vaccination influenced the recruitment of phagocytes and seeding of mammary tissue with lymphoid cells which played a role in immunity. This parameter was significantly higher at 48 hr and 72 hr. They opined that vaccination provided partial protection against *S. aureus* challenge.

Yancey (1993) showed that *Staphylococcus uberis* vaccine could not protect against heterologous challenge strains in bovine mastitis.

Nordhaug *et al.* (1994a) conducted field trial with an experimental vaccine against *S. aureus* mastitis in cattle. *Staphylococcus aureus* mastitis vaccine contained whole, inactivated bacteria with pseudocapsule. Mean SCC in vaccinated and control cows were same throughout the lactation. Local swellings at the injection site were palpable in a substantial proportion of the vaccinated cows. In the statistical analysis, no significant differences were seen between groups. However, when all parameters on udder health were considered together, the results indicated a potential protective effect of this vaccine during the entire lactation.
Nordhaug et al. (1994b) studied antibody response in heifers vaccinated with a *S. aureus* vaccine containing whole, inactivated bacteria with pseudocapsule and alpha, beta toxoids with a mineral oil as an adjuvant. Heifers were injected in the area of the supramammary lymph nodes with vaccine or placebo twice before calving and observed and sampled throughout their first lactation. Antibody response towards the pseudocapsule and the α toxin was significant in serum from the vaccinated cows. These antibody concentrations were significantly higher in the serum and milk during the entire lactation compared with that of the controls. The antibody response to the β toxin was moderate in serum from vaccinated cows. The antibody response to the pseudocapsule consisted of the IgG1 and IgG2 isotypes, but in milk, only the concentration of IgG1 was significantly increased in the vaccinated cows during the lactation compared with the control cows.

Watson et al. (1996) assessed the efficacy of a new staphylococcal mastitis vaccine under drying conditions by vaccinating pregnant cows twice during the last 10 weeks of pregnancy. Vaccinated animals had significantly lower incidence of clinical staphylococcal mastitis and subclinical mastitis compared to controls. The incidence of clinical mastitis was very low from which *S. aureus* were isolated (26.3 per cent) and the high incidence of clinical *S. uberis* mastitis (22.7 per cent). The trial showed that the vaccine was effective in reducing the clinical mastitis and subclinical mastitis in the herd that had a serious Staphylococcal mastitis problem.

Giraudo et al. (1997) developed a vaccine against bovine mastitis based on inactivated, highly encapsulated *S. aureus*; a crude extract of *S. aureus*
exopolysaccharides; and inactivated, unencapsulated *S. aureus* and *Streptococcus* spp. cells and tested on 30 heifers during a 7-month period. The prepartum group received two injections of the vaccine at eight and four weeks before calving, and the postpartum group received two injections subcutaneous at one and five weeks after calving. The control group received two injections of a placebo subcutaneous at eight and four weeks before calving. The frequencies of intramammary infections caused by *S. aureus* were reduced from 18.8 per cent for heifers in the control group to 6.7 and 6.0 per cent for heifers in the prepartum and postpartum groups respectively. This vaccine had no observable effect on somatic cell counts. Clinically, no reaction and fever was observed in heifers after vaccination. Transitory swellings around the teats were observed after the vaccination in heifers. The results of the trial indicated the effectiveness of the vaccine in decreasing the incidence of intramammary infections caused by *S. aureus*.

Calzolari *et al.* (1997) evaluated the vaccine prepared based on inactivated, highly encapsulated *S. aureus* cells; a crude extract of *S. aureus* exopolysaccharides; and inactivated unencapsulated *S. aureus* and *Streptococcus* spp. in 164 cows from two commercial dairies during a 4-months period. Two doses of the vaccine were administered subcutaneously to 82 cows in the brachiocephalicus muscle of the neck within a 4-week interval. The results revealed significantly fewer intramammary infections caused by *S. aureus* at various levels of severity (clinical, subclinical, and latent) in cows that were vaccinated. The colony counts for *S. aureus* in milk from infected quarters of vaccinated cows were significantly lower than those in milk from infected quarters of control cows. Also, the somatic cell counts in milk from vaccinated
cows were significantly decreased when the initial somatic cell count was <500,000 cells/ml at the start of the trial.

Tenhagen et al. (2002) evaluated a herd-specific vaccine against *S.aureus* on IMI, SCC and clinical mastitis in heifers. Heifers in the vaccination group were vaccinated twice, *i.e.* five and two weeks before their expected calving date. The prevalence of *S. aureus* in quarter milk samples taken at calving and three to four weeks post-partum did not differ significantly between the vaccine and control group. The incidence of clinical mastitis during the first three months after calving and the prevalence of *S. aureus* in quarter milk samples taken before the onset of treatment did not differ significantly between the groups. The SCC was lower in vaccinated heifers than in control heifers. The prevalence of IMI with *S. aureus* and incidence of clinical mastitis, the use of a herd-specific vaccine against *S.aureus* did not prove to be efficient.

Leitner et al. (2003) reported that a new *Staphylococcus aureus* vaccine “Mastivac-I” elicited a non-specific health improvement of the udder in addition to specific protection against *S.aureus*. The vaccine consisting of three field isolates of *S.aureus* from bovine mastitis was tried on lactating cows and challenged with a highly virulent strain of *S.aureus*. Vaccinated cows had 70 per cent protection from infection compared to less than 10 per cent in the controls.

Leitner et al. (2003a) developed a vaccine consisting of three field isolates of *S. aureus* bovine mastitis and administered to nine uninfected cows, while ten cows were used as controls. All the cows were challenged with a highly virulent *S. aureus* strain infusing into two quarters of each cow. Quarters were tested for development of clinical
signs, secretion of *S. aureus* and SCC. No systemic alterations were observed in any of the cows, vaccinated or controls. There were no systemic effects observed in the nine cows. All quarters challenged in vaccinated cows exhibited mild inflammatory reactions and low SCC counts. Neutrophils were increased in proportion soon after vaccination and were the main cell types present in all quarters. These findings were consistent with typical mastitis. Moreover, all the quarters challenged in the vaccinated cows, whether they were successfully infected with *S. aureus* or not exhibited very mild inflammatory reactions, identified by their low SCC (<100,000).

Leitner *et al.* (2003b) in a study conducted over two consecutive years tested the efficacy of MASTIVAC I in 452 Israeli Holstein heifers. The antibody response was detected in all the vaccinated animals for four to five weeks post-primary immunization and they observed that it was sustained throughout the experimental period (300 to 330 days). They also showed that SCC in the vaccinated heifers was significantly lower than those in control animals. They proved that contaminants of one quarter had no influence regarding SCC on the other quarters of the same cow. Further they recorded local swelling in the udder of cows after vaccination which persisted for 10 days. These results suggested that the new vaccine elicited a non-specific health improvement of the udder in addition to specific protection against *S. aureus*.

Shakoor *et al.* (2006) evaluated four *S. aureus* mastitis vaccines with respect to milk yield, fat, protein and SCC in five different groups of non-mastitic healthy pregnant buffaloes. These vaccines (live attenuated, simple bacterin, dextran sulphate and oil adjuvanted) were administered to 20 healthy pregnant buffaloes. Each vaccine was
administered twice at rate of 5 ml IM at 60 and 30 days pre-partum. There was a significant difference in the milk yield, fat and protein percentage between the vaccinated and non-vaccinated groups. While difference in these parameters among the vaccinated groups of buffaloes were non significant. All the vaccines reduced the SCC significantly as compared to control group and concluded that \textit{S.aureus} mastitis vaccines were helpful in improving the quality and quantity of milk in buffaloes.

Pellegrino \textit{et al.} (2008) evaluated the response of heifers vaccinated with a \textit{S. aureus} avirulent mutant and intramammary challenge with a \textit{S. aureus} virulent strain for clinical signs, production of milk, shedding of \textit{S. aureus}, SCC and antigen-specific IgG in blood and milk. No local tissue damage was observed due to the administration of the vaccine. A significant increase in specific IgG to \textit{S. aureus} RC122 was detected in the blood and milk of vaccinated heifers. Also a slight increase in daily milk yield during the trial was noticed. No significant difference on shedding of bacteria in milk and SCC were found among groups.

Middleton \textit{et al.} (2009) evaluated a commercially available \textit{S. aureus} bacterin against staphylococcal IMI (\textit{S. aureus} and Coagulase-negative staphylococci-CNS) for milk SCC and milk antibody isotype response to vaccination in Holstein-Friesian lactating dairy cows. The number of mammary quarters that developed a new CNS IMI, time to new CNS IMI, milk SCC and milk antibody isotype sample-to-positive ratio did not significantly differ between groups (P>0.05). In a herd with a three per cent prevalence of \textit{S. aureus} IMI and a 30 per cent prevalence of CNS IMI, the vaccine did not reduce the new staphylococcal IMI rate. There might be insufficient vaccine-induced
opsonizing antibody in the milk to facilitate phagocytosis and clearance of staphylococci from the mammary gland.

2.8 Biofilm vaccine

Cucarella et al. (2002) examined the influence of biofilm associated protein (Bap) expression on *Staphylococcus aureus* adherence to host proteins, epithelial cell cultures and mammary gland sections and showed that Bap positive strain V 329 lower adherence to immobilized fibrinogen than isogenic Bap deficient strain m556. Bacterial internalization into the cells of mammary glands was lower in Bap positive strains than the Bap negative strains.

Shivaraj and Krishnappa (2002) carried out a preliminary vaccination trial to evaluate and compare the protection pattern conferred by two killed vaccines *i.e.* BF form of *E.coli* grown on chitin flakes and conventional *E.coli* vaccine. Chicks were fed daily with killed vaccines (10⁹ cfu/ bird) after mixing with feed from day three to eight and boosted on days 21 and 23. They reported a maximum of 88.33 per cent protection in BF vaccinated group; followed by 33.33 and 16.6 per cent protection, in FC vaccinated and unvaccinated control group respectively after homologous intramuscular (i/m) challenge infection.

Kavitha (2008) evaluated *E.coli* O9 BF and FC vaccines in pregnant rabbits which were isolated from bovine mastitis cases. The gross lesions of mammary glands, SCC, CMT and serum IgG level by ELISA after homologous and heterologous challenge were recoded Serum IgG levels were significantly higher in BF vaccinated rabbits than FC vaccinated and control rabbits. The author noticed higher cross protection by BF
vaccine based on challenge studies using homologous (E.coli O9) and heterologous (E.coli O147) serotypes. E. coli BF and FC based vaccination trials in rabbits indicated the superiority of E.coli BF vaccine over the FC vaccine against experimentally induced mastitis in rabbits with E. coli isolates from bovine mastitis.

Chandrashekhara (2009) evaluated E. coli BF and FC vaccines in lactating cows for cell mediated as well as humoral immune responses and reported a significant (P<0.001) increase in CD4 and CD8 T cells percentage as analyzed by flow cytometry and significantly increased serum IgG levels were detected by ELISA in the E.coli BF vaccinated groups than FC vaccinated and control groups.

2.9 Immunohistochemistry

Mammary glands of pregnant, lactating and resting goats were studied by immunohistochemistry for lymphocyte subpopulations using a panel of monoclonal antibodies. All T lymphocyte subpopulations - CD2+, CD4+, CD8+, T cells and subsets, were present in the mammary gland and were noted to increase in number progressively during pregnancy, decrease significantly during lactation and moderate increase during the resting period. CD4+ cells, the predominant cell type in the mammary gland, were located mainly in the connective tissue, whereas CD2+ and CD8+ cells were predominant in the intraepithelial areas (Ismail et al., 2002).

Sordillo et al. (1997) studied the udder immunology by Flow cytometry and reported that CD8+ lymphocytes had higher activation and during the postpartum period than during later lactation. They also reported that CD8+ lymphocytes immediately
following parturition were of the suppressor type and from mid to late lactation were more of cytotoxic nature.

The existence and distribution of T lymphocyte subpopulations in the mammary tissue of cows were immunohistochemically detected by Yamaguchi et al. (2000). CD2+, CD4+, and CD8+ T lymphocytes were localized primarily in the mammary parenchymal tissue. CD8+ T lymphocytes were predominant over CD4+ T lymphocytes and occurred in close contact with the alveolar epithelium and between epithelial cells in the central area of the upper mammary gland. CD4+ T lymphocytes were present in equal numbers in the epithelial and connective tissue area. Occasionally, both CD4+ and CD8+ T lymphocytes formed cell clusters in the interalveolar connective tissue. The ratio of CD4+ T lymphocytes to CD8+ T lymphocytes were less than 1.0 and was lower in the epithelial area than in the connective tissue.

Soltys and Quinn (1999) recorded that the CD4+ T lymphocytes were increased in the mammary glands during an infection of staphylococcal mastitis whereas both CD4+ and CD8+ T cells increased during streptococcal mastitis.

Yamaguchi et al. (2000) demonstrated immunohistochemically that the bovine mammary glands had a higher percentage of CD8+ mammary intraepithelial lymphocytes which expressed γ/δ T cell receptors as well as ACT 2 receptors. The ratio of CD4+ to CD8+ cells was less than 0.3 in the mammary glands.

Riollet et al. (2001) examined the cell subpopulations and cytokine expression in cow milk in response to chronic *Staphylococcus aureus* infection using flow cytometry to
assess the expression of specific antigens on the surface of lymphocytes and neutrophils. They reported the CD8+ T-lymphocytes were mainly recruited in milk compared with the CD4+ T-lymphocytes, suggesting that the CD8+ T-lymphocytes played an important role in chronic *Staphylococcus aureus* infection.

Gomez *et al.* (2002) observed an increase in percentage of IFN-Y-producing CD-4+, CD8+ T-cells after *S. aureus* intramammary challenge in the immunized mice compared to control. They suggested that IFN-Y production induced by intramammary immunization played a vital role in the eradication of intracellular *S. aureus*. By 6\textsuperscript{th} day after *S. aureus* intramammary challenge, a significant increase in the percentage of CD-4+ and CD-8+ T-cells were observed from immunized mice compared to control mice.

Yong *et al.* (2004) analyzed the lymphocyte subpopulations of mastitis-resistant and susceptible cows using monoclonal antibodies specific for bovine leukocyte differentiation antigens and flow cytometry. They reported that susceptible cows had CD4:CD8 ratios of less than one in both their mammary gland secretions and peripheral blood and asserted that the CD4: CD8 ratio could be used as an indicator of susceptibility to bovine mastitis.

### 2.10 Western blotting

Prakash and Krishnappa (2002) carried out a comparative antigenic analysis of BF cells and FCs of *S. gallinarum* and reported expression of 89, 86, and 34 kDa OMPs and an increased expression of 66, 43 and 38 kDa proteins in BF cells when compared to OMPs of the FCs. Unique OMPs of 59, 57, 54 and 31 kDa were observed in the BF cells and subsequent immunoblotting proved that the BF OMPs were immunogenic.
Arun (2002) analysed the OMPs of BF and FC of *Pasteurella multocida* A: 1 causing fowl cholera. A reference strain and a field isolate of *Pasteurella multocida* were analysed by western blotting and reported that a maximum of eight and five BF OMPs of homologous and heterologous strains respectively were detected by *Pasteurella multocida* A: 1 BF hyper immune serum indicating immunogenicity and cross reactivity of BF OMPs.

Prakash (2004) detected the passive transfer of IgY antibodies to 66, 53, 48, 30 and 20 kDa OMPs of *S. gallinarum* from BF vaccinated birds to the progeny via egg which were characterized by western blotting. The western blot analysis using antisera raised against BF and FC heat inactivated bacterins in chicks indicated that 66 kDa protein was immunogenic in both FC and BF cells. Two immunogenic proteins of 57 and 31 kDa were present only in BF cells but not in FCs. The 38 kDa protein was present in both but, showed immunogenicity only in BF cells.

Naveen kumar (2005) analyzed proteins of bovine mastitis isolates of *S. aureus* BF and FC by western blotting. The biofilm and free cell proteins when analysed with BF hyperimmune serum showed the over expression of 79, 65, 60, 48 and 40 kDa proteins and were found immunogenic. He also found that the unique proteins of BF cell 67, 37, 26 and 20.8 kDa were immunogenic.

Sumathi (2005) analyzed the OMPs of mastitis isolates of *E. coli* BF and FC by western blotting. The unique protein of BF cell 59.5 kDa and the over expressed 53 kDa protein were found to be immunogenic.
Kavita (2008) analysed OMPs of mastitis isolates of *E. coli* BF and FC by western blotting and found that the 24.4 and 28.5 kDa polypeptides in OMPs of both *E. coli* O9 and O147 serotypes grown under BF mode and 34.5 kDa polypeptide in *E. coli* O147 were detected when probed by hyper immune serum against OMP of *E. coli* O9 grown under BF mode indicating the immunogenicity and cross reactivity of novel proteins.

### 2.11 Enzyme Linked Immunosorbent Assay for detection of *S. aureus* antibodies

Loeffler and Norcross (1987) used an enzyme-linked immunosorbent assay (ELISA) to quantitate milk and serum antibodies (IgG) to *S. aureus* alpha and beta toxins and *S. aureus* 2-8 and Smith diffuse strain capsular antigens. Serum samples taken from 13 infected and 4 non-infected cows also indicated that significant elevations in anti-alpha toxin and anti-beta toxin IgG were present in *S. aureus*-infected cows, compared to non-infected cows. All groups of infected cows, regardless of SCC, had significantly higher milk antibody levels to alpha and beta toxins than non-infected cows. Milk antibodies to two to eight capsules were significantly elevated only in infected cows with SCC greater than $10^6$/ml compared to non-infected cows. Significant increase in milk and serum antibodies to alpha and beta toxins in cows with chronic staphylococcal mastitis apparently resulted from a systemic immune response to these toxins.

Grove and Jones (1992) evaluated the ability of an ELISA to identify *S. aureus* IMI and reported that the test was 96 per cent accurate; sensitivity was 90 per cent, and specificity was 97 per cent. The test was used to screen preserved milk samples rapidly in 10 herds. Prevalence of IMI was more than one per cent in six herds at the first test.
Average prevalence of cows scoring +2 (suspect) and +3 (positive) was 12.6 per cent. Prevalence declined during the 12-month study. Incidence of new IMI decreased from 7.9 per cent at six month to 3.6 per cent at 12 month. Milk antibody concentrations changed quadratically with increasing SCC. The SCC increased as milk antibody concentration increased.

Nickerson et al. (1993) used an indirect ELISA to detect anti staphylococcal serum IgG titers in cows vaccinated with a cell-toxoid adjuvanted preparation of \textit{S. aureus} strain. Mean anti \textit{S. aureus} IgG titers in serum across the trial for vaccinates remained elevated approximately 4.7-fold (P < 0.05) over those of controls and pretreatment titers throughout the trial.

Gilbert et al. (1994) assessed \textit{S. aureus} type 5 capsular polysaccharide antibodies in sera by ELISA. Six dairy cows were immunized subcutaneously with purified type 5 capsular polysaccharide (CP5) of \textit{S. aureus} or CP5-ovalbumin conjugate in Freund’s incomplete adjuvant. At the doses tested, the purified CP5-ovalbumin conjugate did not induce a humoral response in the cows. Immunization of two cows with CP5-ovalbumin conjugate elicited a CP5 antibody response mainly of the IgG2 isotype, which culminated four week later. A second injection of conjugate, three months after the first injection resulted in a rapid and durable anti-CP5 responses without exceeding the antibody peak value. Intramammary infusion of purified CP5 failed to provoke an inflammatory response in the milk of the immunized cows. In contrast, a marked recruitment of cells was recorded in the milk of the sensitized cows after intramammary infusion of ovalbumin. These results demonstrated that injection of CP5 - protein carrier conjugate in
cows entailed both antibody responses against CP5 and carrier-specific recruitment of cells in milk of immunized animals.

Nordhaug et al. (1994 b) studied antibody response by ELISA in heifers vaccinated with a *S. aureus* vaccine containing whole, inactivated bacteria with pseudocapsule and alpha and beta toxoids with a mineral oil as an adjuvant. The antigens were used in dilutions (vol/vol) of 1:2000 for pseudocapsule, 1:3200 for α-toxin and 1:100 for β-toxin and incubated overnight at 4°C in microtiter plates. The serum samples were diluted 1:800 in the β-toxin ELISA and 1:2000 in the two other ELISA. Heifers injected with a *S. aureus* vaccine before calving showed a marked and long-lasting serum IgG response against cellular (pseudocapsule) and soluble (α toxin) antigens. These antibody concentrations were significantly higher in serum and milk during the entire lactation period compared with that of the controls. The antibody response to the β toxin was moderate in serum from vaccinated cows; no differences in antibody concentrations in milk were significant between groups.

Herbeline et al. (1997) used an indirect ELISA to measure the antibodies in sera and milk samples of the dairy cows immunized with *S. aureus* α-toxin. Sera samples were diluted at 1:2000 and 1:4000 whereas milk samples were diluted at 1:100. The antibody titres in sera and milk samples were increased after immunization. Ten lactating Holstein cows that were free of intramammary infection received systemic immunization by subcutaneous injection of FIA with α-toxin, α-toxin mixed with type 5 capsular polysaccharide. The magnitude of antibody response was similar for all cows that had been immunized either with α-toxin alone or with α-toxin that was conjugated with CP5.
Leitner et al. (2000) used ELISA to study systemic and local antibody response in cows infected chronically with *S. aureus* in serum and milk samples. Specific antibodies of IgG class were detected in sera of 82.6 per cent of the cows chronically infected by *S. aureus*, while in 17.4 per cent no such antibodies could be detected. No specific IgG antibodies were detected in sera of cows free of mammary infection or in the cows that were infected with different coagulase negative staphylococci (CNS).

Kavita (2008) used an indirect ELISA to assess the serum IgG levels in rabbits immunized with *E.coli* O9 Biofilm and Free cell vaccine and challenged with homologous and heterologous serotypes. The difference in the level of antibodies in the sera collected from BF vaccinated group challenged with homologous serotype v/s heterologous serotype, on days 0, 15, 22 , 29, 30, 31, 35, 43 and 50 was ‘non-significant’ (P>0.05) indicating the ability of BF vaccine to confer cross protection against infection with heterologous serotype. When comparison was made between FC vaccinated group challenged with homologous serotype and FC vaccinated group challenged with heterologous on days 43 and 50, the difference was significant, indicating the inability of FC vaccine to confer cross protection against infection with heterologous serotype. When BF and FC vaccinated groups were compared based on PP values of serum samples collected at day 0, 15, 22 , 29, 30, 31, 35, 43 and 50, the serum IgG levels detected by ELISA were significantly higher in BF vaccinated than FC vaccinated and control rabbits.