II. REVIEW OF LITERATURE

*Staphylococcus aureus* is still one of the major pathogenic agents causing mastitis worldwide and therefore necessitates the use of antibiotics in dairy herds. Intramammary infections caused by *S. aureus* in bovines are very difficult to cure with antibiotics because of their association with biofilms. The increasing public concern with food safety, expressed in this context in the desire to minimize antibiotic residues in milk, on the one hand, and the need to reduce somatic cell counts on the other hand, strengthen our determination to combat *S. aureus* mastitis by means of vaccination.

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

2.1 Bovine mastitis due to *Staphylococcus aureus*

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

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

Verma (1988) examined 136 cows for subclinical mastitis and found that 42.1 per cent of animals were positive. Among isolates obtained, *S. aureus* were predominated (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.

Lakshmananchar et al. (1993) isolated 494 (67.30 per cent) Gram positive and 240 (32.70 per cent) Gram negative organisms from 975 milk samples collected from 1124 clinical mastitis cases over a period of ten years. The details of year wise distribution of the isolates revealed the predomination of *S. aureus*, thereby indicating the 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*) isolates.
Ross et al. (2001) isolated 107 bacterial isolates that included *S. aureus* (30), *S. agalactiae* (16), *S. dysgalactiae* (12), *Bacillus subtilis* (12), *P. aeruginosa* (13) and *E. coli* (22) from CMT positive animals.

Asmae et al. (2003) conducted a study to investigate the antibacterial resistance of the pathogens causing clinical mastitis in dairy cows from Estonia. The bacteria most frequently isolated were *S. aureus* (169 out of 543) and *S. agalactiae* (81 out of 543).

Wani and Bhat, (2003) examined 100 milk samples and the results revealed 95 bacterial isolates and 45 isolates of yeasts. The bacterial isolates were *S. aureus* (45 per cent), *Klebsiella* spp (8 per cent) and *Enterobacter* spp (7 per cent).

Balakrishnan et al. (2004) obtained 40 bacterial isolates from 65 milk samples. The spectrum comprised of *S. aureus* (35 per cent), *E. coli* (27.5 per cent), *S. agalactiae* (17.5 per cent), *P. 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 et al. (2008) studied the prevalence of mastitis in and around Bangalore, India. A total of 75 bacterial isolates were recovered from sixty clinical cases of mastitis affected cows. The prevalence of major bacterial pathogens isolated was 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 bacterial species accounting for 49.53 per cent of all the isolates from mastitis in buffaloes followed by *S. agalactiae* (23.83 per cent), *Staphylococcus hyicus* (*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 *S. aureus* followed by *E. coli* were the predominant pathogens causing both clinical and subclinical mastitis in bovines. 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) when compared to subclinical mastitis (14.09 per cent).

### 2.2 *Staphylococcus aureus* mastitis vaccine

Adlam et al. (1981) studied local and systemic antibody responses in cows following immunization with staphylococcal antigens in the dry period. The animals 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 cows had high colostral antibody titres which dropped to background level by two weeks. There was an indication of some local antibody being produced in those quarters of animals which had previously received two infusions of plain vaccines.

Pankey et al. (1985) evaluated protein A and a commercial staphylococcal bacterin (Somatostaph®) by experimental challenge with *S. aureus* in thirty cows in their first lactation. Studies were through three lactations and included bacteriological and
cytological analyses of quarter milk samples. Rate of intramammary infection with *S. aureus* was similar for vaccinated and unvaccinated cows. Rate of spontaneous cure within each lactation were significantly higher for vaccinated cows. For all three lactations, spontaneous cure rates were 83, 73 and 47 per cent for protein A, bacterin and control cows respectively. Somatic cell counts were significantly lower for vaccinated cows for quarters infected with *S. aureus*, but no differences were demonstrated for milk production by lactation. 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 *S. aureus* mastitis. Three cows per group were immunized in mid lactation by injections in the area of the supramammary lymph node and intramuscularly and were boosted on day 14, 42 and 70 with three variants of Smith *S. aureus* *viz.* Smith compact, nonencapsulated *S. aureus*; Smith diffuse, rigid capsulated *S. aureus*; and Smith diffuse large clearing capsulated *S. aureus* using dextran sulfate as an adjuvant. Serum agglutination and ELISA titers of cows immunized with diffuse and diffuse large clearing variants increased after immunization and after each boost and remained elevated to the end of the experiment at 112 day. Phagocytosis of diffuse and diffuse large clearing variants, read by flow cytometry, was enhanced by immunization with either organism. No antibody response to capsule or enhanced phagocytosis developed in cows immunized with compact variant. However, anti compact antibodies were opsonic for diffuse large clearing capsule.

Nickerson *et al.* (1993) studied the influence of a *S. aureus* mastitis vaccine on immunologic status and rate of new IMI in dairy cows. At drying off, cows were
vaccinated either intramuscularly or subcutaneously in the area of the supramammary lymph node, boosted at six weeks later. Serum antibody concentrations, bacteriologic status and SCC of quarter milk samples were determined. Four weeks after vaccination, cows were challenged by intramammary infusion of *S. aureus*. Mean serum anti staphylococcal antibody titer of vaccinated cows during the trial was 4.7-fold to that of controls. Challenge studies resulted in IMI rates of 92, 36 and 60 per cent for controls, cows vaccinated intramuscularly and cows vaccinated in the area of the supramammary lymph node respectively. Leukocyte infiltration was greater in quarters from cows vaccinated in the area of the supramammary lymph node than in quarters from unvaccinated controls. Plasma cell populations producing IgG₁, IgG₂, IgA and IgM were greatest in quarters of cows vaccinated in the area of the supramammary lymph node followed by those in quarters of cows vaccinated intramuscularly and control cows.

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, α and β toxoids were used. Mineral oil was used as an adjuvant. The heifers were injected in the area of the supramammary lymph nodes twice before calving and were observed and sampled throughout the first lactation. None of the vaccinated cows suffered from clinical *S. aureus* mastitis and only 8.6 per cent suffered from subclinical *S. aureus* mastitis, but a total of 16 per cent of the control cows suffered from clinical or subclinical *S. aureus* mastitis. Mean SCC in vaccinated and control cows were the same throughout the lactation. Local swellings at the injection site were palpable in a substantial proportion of the vaccinated cows. In the statistical analyses, when cow was used as the unit of concern, no significant differences 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 and 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 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; no differences in antibody concentrations in milk were significant between groups. The antibody response to the pseudocapsule consisted of the IgG1 and IgG2 isotypes, but in milk, only the concentration of Ig G1 was significantly increased in the vaccinated cows during the lactation compared with the control cows.

Guidry et al. (1994) tested two modes of immunization for the ability to induce anticapsular opsonins. Cows were immunized at drying off and boosted on day 14 and 28 by injection of Smith diffuse *S. aureus* plus dextran sulfate in the area of the supramammary lymph node or intramammarily. In cows immunized at supramammary lymph node, IgG1 and IgG2 sera antibody titers to capsule increased and remained elevated to the end of the study i.e 120 days post calving. Response of serum IgG1 and
IgM to intramammary immunization was similar to that with supramammary lymph node immunization, but more delayed and lower in magnitude. Antibodies of all isotopes, IgG1, IgG2, IgA and IgM increased in dry secretions following immunization via lymph node, IgG1 antibodies remained elevated throughout the study, but IgG1 antibodies dropped to baseline 15 days post calving. In cows immunized with intramammary, only IgA antibodies increased significantly in lacteal secretions and remained elevated throughout the study.

Watson et al. (1996) assessed the efficacy of a new staphylococcal mastitis vaccine under commercial 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 prevalence of subclinical mastitis compared to controls. An unexpected feature of the trial as a whole was the low incidence of clinical mastitis from which S. aureus was 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 reduction of clinical mastitis and prevalence of subclinical mastitis in herds 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 seven 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 s/c at one and five weeks after calving. The control group
received two injections of a placebo s/c 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. 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 a vaccine 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 four month period. Two doses of the vaccine were administered s/c to 82 cows in the brachiocephalicus muscle of the neck within a four 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. 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 than in control heifers. Regarding 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.* (2003a) developed a vaccine composed of three field isolates of bovine mastitic *S. aureus* and administered to nine uninfected cows while 10 other cows were used as controls. All cows were challenged with a highly virulent *S. aureus* strain administered into two quarters of each cow. Quarters were tested for clinical signs, secretion of *S. aureus* and SCC. No systemic effects were observed in any of the cows, vaccinated or control. Vaccinated cows had 70 per cent protection from infection compared with fewer than 10 per cent in the controls. Moreover, all quarters challenged in the vaccinated cows, regardless of whether they were successfully infected or not with *S. aureus* exhibited very mild inflammatory reactions, identified by their low SCC (<100,000).

Leitner *et al.* (2003b) tested the efficacy of MASTIVAC I in 452 Holstein heifers in a study conducted over two consecutive years. Antibody response was detected in all vaccinated animals four to five weeks post-primary immunization and it was sustained throughout the experimental period (300 to 330 days). About 1.3 per cent *S. aureus* infection could be detected in the vaccinated group and 2.7 per cent in the control group. However, when SCC and milk yields were considered, 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. These results suggested that the new vaccine elicited a non-specific health improvement of the udder in addition to specific protection against *S. aureus*.

Lee *et al.* (2005) evaluated a novel bovine mastitis trivalent vaccine, containing *S.aureus* capsular polysaccharide type 5, 8 and 336 on antibody production and neutrophil phagocytosis in pregnant heifers. Animals were immunized with either the trivalent alone, trivalent emulsified in Freund's incomplete adjuvant (FIA), trivalent in aluminum hydroxide or adjuvant only, 30 days before the expected calving date followed by two boosters in a two week interval. Serum antigen-specific IgG1 and IgG2 were significantly increased in all the vaccinated groups before parturition and sustained until three weeks postpartum. In comparison with the trivalent alone, formulation with either adjuvant enhanced production of IgG2, but not IgG1. Immune sera which contained the highest titer of antibodies slightly increased neutrophil phagocytosis to the three serotypes of killed *S.aureus*.

Shakoor *et al.* (2006) evaluated four *S.aureus* mastitis vaccines for milk yield, fat, protein and SCC in five different groups of non-mastitic healthy pregnant buffaloes. These vaccines (live attenuated, simple bacterin, dextran sulphate adjuvanted and oil adjuvanted) were administered to 20 healthy pregnant buffaloes. Each vaccine was administered twice at 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. Difference of these parameters among the vaccinated groups of buffaloes was non significant. All the vaccines reduced the SCC significantly as
compared to control group and concluded that *S.aureus* mastitis vaccines were helpful in improving the quality and quantity of milk in buffaloes.

Pellegrino *et al.* (2008) evaluated the response of heifers vaccinated with a *S. aureus* avirulent mutant to the intramammary challenge with a *S. aureus* virulent strain for clinical signs, production of milk, shedding of *S. aureus*, SCC and antigen-specific IgG in blood and milk. Two subcutaneous doses of a culture of the mutant used as vaccine, was administered to pregnant heifers 30 and 10 days before calving. The vaccinated and non-vaccinated heifers were challenged 10 days after calving with the homologous virulent *S. aureus* strain, which was inoculated by intramammary route into two quarters of each animal. No local tissue damage was observed due to the administration of the vaccine. A significant increase of specific IgG to *S. aureus* RC122 was detected in blood and milk of vaccinated heifers as well as a slight increase in daily milk yield during the trial. No significant difference on shedding of bacteria in milk and SCC were found among groups.

Middleton *et al.* (2009) evaluated a commercially available *S. aureus* bacterin in protecting against staphylococcal IMI (*S. aureus* and coagulase-negative staphylococci, CNS) for milk SCC and milk antibody isotype response to vaccination in Holstein-Friesian lactating dairy cows. Vaccinates received two doses of the bacterin at 14 days interval. No animals in either group developed a new *S. aureus* IMI after vaccination. 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 *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 milk to facilitate phagocytosis and clearance of staphylococci from the mammary gland.

### 2.3 Bacterial biofilms

Bacterial biofilm is a structural community of bacterial cells enclosed in a self-produced exopolysachharide (EPS) matrix and adherent to an inert or living surface, which constitutes a protected mode of growth that allows survival in hostile environment (Costerton et al., 1981).

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 very small number of cells (5 per cent) from non-slime producing strains showed a condensed EPS matrix (slime) surrounding the bacterial cell wall, as revealed by electron microscopy and immunofluorescence. The BF production was also detected 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 BF cell population was inversely related to planktonic cell population.

Shivaraj and Krishnappa, (2001) reported that the optimum concentration of nutrient media required for maximum BF formation of *E.coli* was 0.08 per cent TSB with 0.3 per cent chitin flakes. Biofilm cell population peaked on day two with a cell density of $9.8 \times 10^9$ cfu/g of chitin. On the other hand, the free cell (FC) density decreased from day one and was only $5.2 \times 10^8$ cfu/ml on the fourth day.

Prakash and Krishnappa, (2002) found that optimum concentration of TSB for maximum BF formation of *Salmonella gallinarum* (*S.gallinarum*) was 0.16 per cent with 0.3 per cent bentonite clay as inert surface. Biofilm cell population peaked on day four with a cell density of $1.33 \times 10^9$ cfu/g of bentonite clay. On the other hand, the FC density decreased from day one and was $5.2 \times 10^8$ cfu/ml on the fourth day.

Vadakel and Krishnappa, (2002) reported that the optimum concentration of TSB for maximum BF formation of *Pasteurella multocida* was 0.32 per cent with 0.3 per cent bentonite clay as inert surface. Biofilm cell population peaked on day three with a cell density of $4.2 \times 10^{11}$ cfu/g of bentonite clay, whereas the FC density peaked on the first day after inoculation with an average viable count of $2.0 \times 10^{10}$ cfu/ml, which declined rapidly thereafter.

Veeregowda, (2003) reported that optimum concentration of nutrient media required for maximum BF formation of *E.coli* was 0.16 per cent TSB with 0.3 per cent bentonite clay. The BF cell population peaked on day seven with a count of $5.70 \times 10^9$
34
cfu/g of bentonite clay. Free cells on the other hand, attained a peak on the day seven after inoculation with an average viable count of \(9.60 \times 10^9\) cfu/ml of 3 per cent TSB which declined rapidly thereafter.

Naveenkumar, (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. Free cells on the other hand, 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 Molecular characterization of \(S.aureus\) genes for biofilm formation

Cramton et al. (1999) investigated a variety of \(S.\ aureus\) strains and found that all strains tested contained the ica locus and of that, several can form biofilms in vitro. Sequence comparison with the \(S.\ epidermidis\) ica genes revealed 59 to 78 per cent amino acid identity. Deletion of the ica locus resulted in a loss of the ability to form biofilms, produce polysaccharide intercellular adhesion or mediate \(N\)-acetylglucosaminyl transferase activity in vitro.

Arciola et al. (2001) described a simple, rapid and reliable PCR method to detect icaA and icaD. The method was applied for the detection of ica genes in two reference strains, 15 strains each of \(S.\ epidermidis\) and \(S.aureus\) from prosthesis infections and 10 strains from the skin and mucosa of healthy volunteers. The icaA and icaD were detectable only in slime-producing strains (tested for slime production on Congo Red agar) and never in non slime-producing ones. This method was a
straightforward way of detecting the slime-producing ability by *S. epidermidis* and *S.aureus*. In clinical specimens, PCR method enabled rapid diagnosis of virulent slime-producing strains with respect to the traditional culture method on Congo red agar, which was time consuming.

Cucarella *et al.* (2001) used a biofilm-producing *S.aureus* isolate to generate biofilm-negative transposon (Tn917) insertion mutants. Two mutants were found with a significant decrease in attachment to inert surfaces (early adherence), intercellular adhesion and biofilm formation. The transposon was inserted at the same locus in both mutants. The locus *bap* [ biofilm associated protein] with a size of ‘971’ bp encodes a novel cell wall associated protein which showed global organizational similarities to surface proteins of gram-negative (*Pseudomonas aeruginosa* and *Salmonella enterica* serovar Typhi) and gram-positive (*Enterococcus faecalis*) microorganisms. The biofilm associated protein’s core region represented 52 per cent of the protein and consisted of 13 successive, nearly identical repeats, each containing 86 amino acids. The *bap* was present in a small fraction of bovine mastitis isolates (5 per cent of the 350 *S. aureus* isolates tested), but it was absent in the 75 clinical human *S. aureus* isolates analyzed. All staphylococcal isolates harboring *bap* were highly adherent and strong biofilm producers. In a mouse infection model *bap* was involved in pathogenesis, causing a persistent infection.

Götz, (2002) reported the ability of staphylococci to form a biofilm affords at least two properties: the adherence of cells to a surface and accumulation to form multilayered cell clusters. A trademark is the production of the slime substance PIA, a
polysaccharide composed of beta-1, 6-linked N-acetylglucosamines with partly deacetylated residues, in which the cells are embedded and protected against the host's immune defence and antibiotic treatment. Mutations in the corresponding biosynthetic genes (*ica*operon) lead to a pleiotropic phenotype; the cells are biofilm and haemagglutination negative, less virulent and less adhesive on hydrophilic surfaces. *ica* expression is modulated by various environmental conditions, appears to be controlled by SigB and can be turned on and off by insertion sequence (IS) elements. A number of biofilm-negative mutants have been isolated in which PIA production appears to be unaffected. Two of the characterized mutants are affected in the major autolysin (*atlE*) and in D-alanine esterification of teichoic acids (*dltA*). Proteins have been identified that are also involved in biofilm formation, such as the accumulation-associated protein (AAP), the clumping factor A (ClfA), the staphylococcal surface protein (SSP1) and the biofilm-associated protein.

Cucarella *et al.* (2004) analyzed 195 bovine mastitis *S. aureus* isolates by PCR using *bap* specific primers and *ica*ADBC specific primers. Results revealed that 94.36 per cent were *ica*ADBC positive and 25.6 per cent were *bap* positive isolates. They also reported that all the *bap* positive isolates were also *ica* positive. They reported the relationship between the ability to produce chronic bovine mastitis and biofilm formation. The presence of anti-Bap antibodies in serum samples taken from animals with confirmed *S. aureus* infections indicated the production of Bap during infection. Furthermore, disruption of the *ica* operon in a *bap*-positive strain had no effect on *in vitro* biofilm formation, a finding which strongly suggested that Bap could compensate for the deficiency of the PIA. Altogether, these results demonstrated that, in the bovine
mammary gland, the presence of Bap may facilitate a biofilm formation connected with the persistence of *S. aureus*.

Vautor *et al.* (2008) reported that the implication of biofilm in chronic bacterial infection in many species has triggered an increasing interest in the characterization of genes involved in biofilm formation. The biofilm associated protein (*bap*) gene is a newly identified gene that encodes the biofilm-associated protein, which is involved in biofilm formation in *S. aureus*. So far, the *bap* gene has only been found in a small proportion of *S. aureus* strains from bovine mastitis in Spain. They tested 262 *S. aureus* isolates obtained from animals for the presence of *bap* gene by PCR, using published primers and dot-blot. The results indicated that none of the isolates carried the *bap* gene suggesting that the prevalence of this gene among *S. aureus* isolates could be very low in Spain.

2.5 Western blotting

Bolin and Jensen (1987) studied the role of iron regulated outer membrane proteins (IROMPs) of *E. coli* in conferring protective immunity. They detected IROMPs in OMP- enriched fractions of *E. coli* grown in media containing α, α'-dipyridyl as iron chelator. They raised antibodies in rabbits against these IROMPs and passively immunized 18-day-old turkeys. On challenge with the same culture by air sac route, they observed that fatalities occurred only in the control groups and not in the passively immunized groups and thus confirmed the role of IROMPs in protective immunity.

Prakash and Krishnappa, (2002) carried out a comparative antigenic analysis of BF cells and FCs of *S. gallinarum* and reported repression 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) studied the OMPs of BF and FC of fowl cholera causing Pasteurella multocida A:1, a reference strain and a field isolate of Pasteurella multocida 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.

Veeregowda, (2003) studied the variations in the OMP profiles of E. coli BF cells, which revealed repression of the 58 and 42 kDa and over expression of the 61, 35.5, 34 and 28.8 kDa OMPs in BF cells and nutrient restricted cells, when compared to the FC; three unique OMPs of 93.2, 81 and 28.8 kDa were detected only in BF cells. Whereas, 61, 35.5, 34 and 28.8 kDa proteins were involved in cross protection while the 95.2, 93.2, 81 and 28.8 kDa proteins were identified as IROMPs.

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 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.
Naveenkumar, (2005) studied the proteins of bovine mastitis isolates of S. aureus BF and FC by western blotting. The biofilm and free cell proteins when probed with BF hyper immune serum showed that the over expressed 79, 65, 60, 48 and 40kDa proteins were found to be immunogenic. He also found that the unique proteins of BF cell 67, 37, 26 and 20.8 kDa were immunogenic.

Sumathi, (2005) studied 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.

Prakash, (2006) evaluated E.coli BF based vaccine in broiler breeders. The molecular basis of cross protection as revealed by western blot analysis identified OMPs of 61 and 34 kDa proteins as cross reactive.

Kavita, (2008) studied the OMPs of mastitis isolates of E.coli BF and FC by western blotting and found out that the 24.4 and 28.5 kDa polypeptides in case of OMPs of both E.coli O9 and O147 grown under BF mode and 34.5 kDa polypeptide in case of E.coli O147 grown under BF mode 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 expressed when E.coli was grown under BF mode.

2.6 Biofilm vaccine

Azad et al. (1999) studied humoral and protective responses to different doses and duration of oral administration of an Aeromonas hydrophila biofilm vaccine in three
species of carp viz., catla, rohu and common carp. Among three doses ($10^7, 10^{10}$ and $10^{13}$ cfu/g fish/day) administered for 15 days, $10^{13}$ cfu/g fish/day, elicited the highest serum antibody titre and protective response in all the three carp species. Of the three vaccination schedules studied (10, 15 and 20 days at $10^{10}$ cfu/g fish/day), days 15 and 20 induced higher responses than day 10. Among the three carp species, catla produced the highest antibody and protective response followed by rohu and common carp. Independent of dose and duration, the antibody titer and protective response increased with time following vaccination up to day 60.

Shivaraj and Krishnappa, (2002) carried out a preliminary vaccination trial to evaluate and compare the protection pattern conferred by two killed vaccines i.e., *E.coli* BF grown on chitin flakes and conventional *E.coli* vaccine. Chicks were fed daily with killed vaccines ($10^9$ 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 IM challenge infection.

Veeregowda, (2003) standardized *E.coli* O78 BF using bentonite clay as inert material and conducted vaccination trial to evaluate and compare the protection pattern conferred by BF vaccine and conventional FC vaccine by different routes of vaccinations. Subcutaneous route of vaccination with BF vaccine offered 100 per cent cross protection compared to 58 per cent with FC vaccine by IM live challenge infection. Oral BF vaccination was effective in significantly reducing the colonization of *E.coli* compared to FC vaccine, upon intra nasal challenge. Field trials with BF vaccine conferred cross
protection to the extent of 100 per cent and 80 per cent in s/c and oral vaccinated birds respectively. The BF vaccine given through s/c route resulted in reduced feed conversion ratio when compared to unvaccinated controls to the extent of 1.68 and 2.06 respectively.

Prakash, (2006) evaluated *E.coli* BF based vaccine in broiler breeders. The efficacy of maternal antibodies to offer protection against possible *E.coli* infection was studied. He found that the progeny of BF vaccinates had only 1.55 per cent mortality due to colibacillosis, compared to 4.65 and 5.44 per cent mortality in two different commercial farms where the progeny of unvaccinated were reared. Also, the viable counts of *E.coli* in the heart, liver and intestine and body weight gain were significantly (P ≤ 0.05) less in the progeny of vaccinated, compared to the progeny of un vaccinates. After challenge/infection, at three different intervals on days 15, 29 and 39 revealed a mortality of 100 per cent in all the three challenges upon O2 challenge and 66.64, 50 and 66.64 per cents upon O78 challenge in progeny from unvaccinates, whereas progeny from vaccinates had only 16.66 per cent mortality on homologous (O78) and 16.66, 33.32 and 16.66 per cent mortality on heterologous (O2) in first, second and third challenge respectively.

Ramesh, (2006) evaluated *Pasteurella multocida* biofilm vaccine in comparision with conventional vaccines against fowl cholera in poultry layer birds and reported that BF vaccinated birds had higher antibody titers compared to broth and commercial vaccine. He also observed that there was 100 per cent protection in BF vaccinated birds upon challenge compared to 80 per cent in broth vaccinated and 70 per cent protection in birds vaccinated with commercial vaccine.
Kavita, (2008) evaluated bovine mastitis causing *E. coli* O9 BF and FC vaccines in pregnant rabbits for gross lesions of mammary glands, SCC, CMT and serum IgG level by ELISA after homologous and heterologous challenge. Serum IgG level detected by ELISA was significantly higher in BF vaccinated rabbits than FC vaccinated and control ones. Higher cross protection conferred by BF vaccine was noticed 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 using *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 of percentage of CD4 and CD8 T cells as analysed by flow cytometry and increased serum IgG levels significantly (P<0.001) as detected by ELISA in the *E. coli* BF vaccinated groups than FC vaccinated and control groups.

### 2.7 Rabbit as an animal model to study mastitis

Adlam *et al.* (1977) conducted experiments to determine whether immunization of female rabbits with highly purified staphylococcal alpha or beta toxins would protect them against intramammary challenge with staphylococci. High circulating anti-alpha-toxin titers reduced the lethal hemorrhagic edematous form of the disease ("blue-breast") produced by strains BB and Compton 201 to a localized chronic abscess form. No such protection was afforded by high anti-beta-toxin titers. Immunization with alpha - or beta-toxins produced no change in the clinical picture of the disease produced by CN 6708, a
strain of *S. aureus* responsible for a natural outbreak of abscess-type rabbit mastitis. From these experiments it would appear that alpha-toxin is a key antigen in the blue-breast form of rabbit mastitis. Since the abscess form of the disease was not prevented by immunization with either alpha- or beta-toxin, other virulence factors must be acting to produce more localized disease.

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 a strain of *S. aureus* (CN 6708) responsible for a natural outbreak of abscess type rabbit mastitis. The protection was not obtained against the abscess disease produced by this organism, as several animals contracted the lethal spreading type of disease (“blue breast”), even though circulating antibodies to all three toxins were present. They concluded that the “blue breast” produced in a proportion of animals challenged by strain CN 6708 may be caused by a different and unidentified toxin.

Amorena *et al.* (1991) developed an experimental model in rabbits to study ovine mastitis. A total of 19 ovine mastitis bacterial strains (seven *S. aureus*, four *S. chromogenes*, four *S. hyicus* and four *E. coli*) were used for mammary gland infections. The histopathological results showed that the ovine mastitis types corresponded to experimental infections produced in the rabbit with the ovine strains. These results helped the grading of the bacterial species tested according to the severity of their effects on the mammary gland. The most pathogenic species was *S. aureus*, followed by *E. coli*, *S. hyicus* and *S. chromogenes* in that order. The procedure was simple and consisted of
introducing bacterial suspensions through alternate teat ducts of animals with the help of a cannula. It helped to minimize the number of animals required in the experiments.

Reinoso et al. (2002) studied the avirulent RC122 S. aureus mutant strain in rabbit and bovine infection models. The results clearly showed that RC122 was less virulent than its RC108 parental strain in a rabbit skin model. The ability to produce skin lesions in rabbit and the reduced virulence as an udder pathogen made the RC122 mutant strain interesting as a potential strain for an experimental vaccination trial in dairy herds.

2.8 Optimization of infective dose for the induction of mastitis in lactating rabbits

Adlam et al. (1977) used minimum $10^3$ (low dose) to maximum $10^4$ (high dose) S. aureus in eight to ten days post-parturient rabbits by injecting bacterial suspension at the base of teat. Infection was 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 injection at the base of teat. Lesions were measured at 4, 24 and 48 hrs.

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 hrs and second evaluation at 48 hrs.

2.9 Tests to detect Mastitis

2.9.1 Direct microscopic Somatic Cell Count (SCC)
Batra and McAllister, (1984) made a comparison of various methods for detection of mastitis in dairy cattle by screening 2131 composite milk samples. These 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 of which 14 were found positive and 145 negative by CMT. Of the CMT positive samples six were found positive by total leucocyte count (5,00,000 cells /ml).

### 2.9.2 California mastitis test

Schalm and Noorlander, (1957) developed CMT by modifying the WST after analyzing its difficulties and disadvantages, by the addition of alkyl aryl sulfonate. The authors tested 573 fore milk, 136 stripping milk, 234 bucket milk and 154 bulk milk samples by CMT and by leucocyte count. California mastitis test reaction was pronounced with the increase in the total cell counts, which was indicative of udder inflammation. They finally concluded that CMT could be used as a rapid screening test for mastitis.

Miljkovic and Milojevic, (1962) studied the application of Schalm test for detecting mastitis under Yugoslav conditions and found that 81.3 per cent of 1022 individual milk samples examined were CMT positive, out of which 39.3 per cent were culturally positive and remaining 42 per cent were culturally negative. Only 7.6 per cent of CMT negative samples were bacteriologically positive. The authors concluded Schalm test as a useful screening test for the detection of cows with disturbed milk secretion prior to bacteriological examinations.
Sharma and Rajani, (1965) modified CMT reagent by substituting alkyl aryl sulfonate with teepol. The authors tested 2624 milk samples by CMT and cultural methods. Out of 495 CMT positive samples, 177 were found positive and out of 2129 CMT negative samples only one per cent samples were positive by cultural examination. So, the modified reagent was advocated for routine use in the diagnosis of mastitis.

Chakraborty and Hazarika, (1977) compared various indirect tests with cultural examination by screening 850 quarter milk samples from 200 cows and 20 buffaloes for subclinical mastitis and found that CMT was very close to total leucocyte count in identifying the true positive and CMT percentage was less (1.5 per cent) compared to total leucocyte count (3.05 per cent) in false negative cases.

Okello, (1992) screened 335 cows quarter fore milk samples in 18 dairy herds in Switzerland by CMT and modified WST and concluded that both the tests were of value in identifying the infected quarter milk samples.

Mohinikumari and Janakiramguptha, (2002) subjected a total of 81 milk samples to CMT along with WST, Strip cup test and BTB indicator card. Of these, only 19 (23.45 per cent) were positive by strip cup test, while 71 (87.65 per cent) were found positive by CMT, BTB indicator card test and WST. The cultural examination revealed Staphylococcus spp. (35.21 per cent), E.coli (28.39 per cent), followed by Streptococcus spp. (18.51 per cent), Pseudomonas spp. (4.9 per cent), Corynebacterium spp. (2.46 per cent) and Klebsiella spp. (2.46 per cent).
Mdegala et al. (2004) carried out a study to establish the prevalence of mastitis in small holder dairy farms in Kibaha and Morogoro districts (Tanzania). A total of 57 herds comprising 114 milking cows in Kibaha and 48 herds consisting of 96 milking animals in Morogoro were included in the study. California mastitis test and microbiological assessment of milk was carried out to establish the status of mastitis and responsible etiological agents. Based on CMT, the cow based prevalence of subclinical mastitis was 82.4 per cent in Kibaha and 62.4 per cent in Morogoro.

2.10 Enzyme Linked Immunosorbent Assay (ELISA) for detection of *S.aureus* antibodies

Loeffler and Norcross, (1987) used an 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 did the non-infected cows (P<0.002). Milk antibodies to 2 to 8 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 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 determine anti staphylococcal serum IgG titers in cows vaccinated with a cell-toxoid adjuvanted preparation of S. aureus strain. Mean anti 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 pre treatment titers throughout the trial. At week 8 and 10 (2 and 4 wks after booster injections), titers in vaccinates tended to be higher than at other times during the trial and were elevated (P <0.05) over those at fourth week.

Gilbert et al. (1994) assessed 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 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 one, 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 responses 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 entails both antibody responses against CP5 and carrier - specific recruitment of cells in milk of immunized animals.

Nordhaug et al. (1994 b) studied antibody response in heifers vaccinated with a S. aureus vaccine containing whole, inactivated bacteria with pseudocapsule and alpha and beta toxoids with a mineral oil as adjuvant using ELISA. 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 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.

Prakash, (2004) assayed the antibody titers of both serum and egg yolk of *S.gallinarum* BF vaccinated birds at a single serum dilution of 1:320 by ELISA. The antibody titer was high in the birds at the peak egg production, which were vaccinated at 8th week followed by a booster vaccination when compared to the birds that were given a single dose before laying. The antibody titers in the egg yolk and the serum of vaccinated chicks also followed the same pattern.

Kavita, (2008) used an indirect ELISA to assess the serum IgG levels in rabbits immunized with *E.coli* O9 BF and FC 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 inability of FC vaccine to confer cross protection against infection with heterologous serotype. When BF vaccinated 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.