EVALUATION OF BOVINE MASTITIS CAUSING *Staphylococcus aureus* BIOFILM BASED VACCINE IN RABBITS

RATHNAMMA, D.

DEPARTMENT OF VETERINARY MICROBIOLOGY

VETERINARY COLLEGE, BANGALORE

KARNATAKA VETERINARY, ANIMAL AND FISHERIES SCIENCES UNIVERSITY, BIDAR.

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RATHNAMMA, D.

DEPARTMENT OF VETERINARY MICROBIOLOGY
VETERINARY COLLEGE, BANGALORE
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CERTIFICATE

This is to certify that the thesis entitled ***"EVALUATION OF BOVINE MASTITIS CAUSING Staphylococcus aureus BIOFILM BASED VACCINE IN RABBITS"*** submitted by **Mrs. RATHNAMMA, D.** I.D. No. **PVK 407** in partial fulfillment of the requirements for the award of **DOCTOR OF PHILOSOPHY** in **VETERINARY MICROBIOLOGY** of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar is a record of bonafide research work carried out by her during the period of her study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

Place: Bangalore
Date: 18.12.2009

(G.V.KRISHNAMURTHY)
MAJOR ADVISOR
Retd. Professor of Veterinary Microbiology

APPROVED BY:

Chairperson: _____
(G.V.KRISHNAMURTHY)

Nominated External member : _____
(V.RAMASWAMY)

Members : _____
(SHRIKRISHNA ISLOOR)

_____
(B .M .VEEREGOWDA)

_____
(U. KRISHNAMURTHY)

_____
(M. NARAYAN BHAT)

Affectionately dedicated
to my PARENTS, IN-LAWS
And
STAFF and STUDENTS of the Dept.
of Veterinary Microbiology,Bangalore

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LIST OF ABBREVATIONS / ACRONYMS

Bap/*bap*	: Biofilm associated protein
BF	: Biofilm
BTB	: Bromothymol blue
cfu	: Colony forming unit
CMI	: Cell Mediated Immune response
CMT	: California Mastitis Test
CNS	: Coagulase-negative Staphylococci
°C	: Degree Celsius
DCM	: Diffuse colony morphology
DW	: Distilled water
EDTA	: Ethylene diamine tetra acetic acid
ELISA	: Enzyme linked Immuno Sorbent Assay
EPS	: Exopolysaccharide
FC	: Free cell
FCA	: Freund's complete adjuvant
hr	: Hour
HIS	: Hyper immune serum
FIA	: Freund's incomplete adjuvant
IMI	: Intramammary infection
IM	: Intramuscular
IgA	: Immunoglobulin A
IgG	: Immunoglobulin G
IgM	: Immunoglobulin M
IROMP	: Iron regulated outer membrane protein
kDa	: Kilo Daltons
lbs	: Pounds
LPS	: Lipopolysaccharide
M	: Molar
mA	: Milli ampere
mM	: Milli molar

min	: minutes
MW	: Molecular Weight
mg	: milli gram
ml	: milli litre
µg	: micro gram
µl	: micro litre
NCM	: Nitrocellulose membrane
ng	: nano gram
OD	: Optical Density
OMP	: Outer Membrane Protein
PIA	: Polysaccharide intercellular adhesin
PBS	: Phosphate buffered saline
PMSF	: Phenyl methane sulfonyl fluoride
PP	: Per cent positivity
ppm	: Parts per million
rpm	: Revolutions per minute
RT	: Room temperature
s/c	: Subcutaneous
SCC	: Somatic cell count
Spp	: Species
Sq. cm	: Square centimeter
TSB	: Tryptic Soya Broth
V	: Volts
v/s	: versus
WST	: White side test

I. INTRODUCTION

India is the largest milk producer in the world with an annual production of 104 million tones in 2008 whereas world milk production reached at 684 million tones in 2008. The total milk production in India accounts for approximately 15 per cent of total world milk production (Indian Dairyman, 2008). With its status as the largest milk producer in the world, India has assumed an important position in the global dairy industry. Many international dairy organizations are viewing India with an eye to tap its vast growing market for dairy products as the Indian dairy industry offers opportunities to galore entrepreneurs' world wide. The 11th Five Year Plan (2007-2012) focuses on faster and more inclusive growth of the economy. The goal for agricultural sector as a whole is a growth rate of about 4 per cent and for the dairy sector, a growth rate of about 5 per cent in milk production. The dairying has been identified as an important component for diversification for the agricultural sector. Furthermore, with the current trends of increasing milk production, India will be producing more than 130 million tones of milk by 2015 (Sadana, 2006). In order to maintain its position as the largest milk producer, India should combat the costliest and complex disease like 'mastitis'. In terms of economic loss, mastitis is undoubtedly the most important disease with which Indian dairy industry has to contend. The total economic losses due to mastitis alone accounts up to Rs. 6053.21 crores per annum in India (Dua, 2001).

Mastitis results when pathogenic bacteria are able to gain entry in to the udder, overcome the cow's immune defenses, establish an infection and produce inflammation of udder secretary tissue. The disease is often expressed as an increased somatic cell

counts (SCC) in the milk which leads to poor quality milk in the affected animals. Mastitis is a major cause of economic loss in dairy farming. This loss is primarily due to reduced milk yield, rapid spoilage of milk, discarding of milk with antibiotics, treatment and replacement costs, lower price of poor quality milk, increased culling rate or death from infection and decreased fertility (Bradley, 2002).

The vast majority of etiological agents of mastitis is of bacterial origin and just five species of bacteria *viz*. *Staphylococcus aureus* (*S.aureus*), *Streptococcus uberis* (*S.uberis*), *Streptococcus dysgalactiae* (*S.dysgalactiae*), *Streptococcus agalactiae* (*S.agalactiae) and Esherichia coli* (*E.coli*) account for almost 80 per cent of all mastitis cases (Anon., 2001, Ali *et al*., 2008). Classically, mastitis pathogens have been classified as either *contagious* or *environmental*. Contagious pathogens are considered as organisms adapted to survive within the host in particular within the mammary glands. They are capable of establishing sub clinical infection which is typically manifested by elevation in the SCC of milk from the affected quarter. In contrast, the environmental pathogens are best described as opportunistic invaders of mammary gland not adapted to survive within the host and they invade, multiply and cause clinical infections and are rapidly eliminated. They typically spread from cow to cow around or at the time of milking (Bradley, 2002).

Staphylococcus aureus is one of the most frequently (45 % - 60 %) isolated (Verma, 1988; Kaya *et al.*,1998; Wani and Bhat, 2003 and Ali *et al*. 2008) and a major contagious mastitis pathogen that cause either clinical or subclinical or chronic bovine mastitis with high economic losses to the farmers. In cows, intramammary infections

(IMI) due to *S.aureus*, which account for 25-30 per cent of total IMI, are generally subclinical. This type of mastitis impairs alveolar functions, reduces milk yield and has deleterious effect on milk composition, one of which is an increase in milk SCC (Leitner *et al*., 2000; Dego and Tareke, 2003 and Ali *et al*., 2008)). Its treatment necessitates the extensive use of antibiotics in dairy herds in contrast to increasing public concern over food safety expressed as the desire to minimize antibiotic residues in milk. Moreover, the presence of *S.aureus* in raw milk used by dairy industries is a public health problem (Leitner *et al*., 2008).

For defense purposes, bacteria have developed an interesting system. After adhering to the epithelial surface, they begin to multiply while emitting chemical signals that "intercommunicate" the bacterial cells. Once the signal intensity exceeds a certain threshold level, the genetic mechanisms underlying exopolysaccharide production are activated (Costerton *et al*., 1999). In this way, the bacteria multiply, embedded within an exopolysaccharide matrix, thus giving rise to the formation of a Biofilm / microcolony. “Biofilms (BF) are microbially derived sessile community characterised by cells that are irreversibly attached to a substratum or interface or to each other, embedded in a self produced matrix of extracellular polymeric substances / exopolysaccharide matrix and exhibiting an altered phenotype with respect to growth rate and gene transcription” (Costerton *et al*., 2003). Such BF are adherent to an inert or living surface, which constitutes a protected mode of growth that allows survival in hostile environment. Within a BF, bacteria are able to interact with each other through intercellular communication and thus rapidly adapt to changing environments. The organisms within BF are resistant to the host immune response and antibacterial agents, compared to their

free-living planktonic counterparts. The biofilm matrix plays a key role in the protection of biofilm bacteria from host defenses. However, it is important to point out that these exopolysaccharides are both chemically and physically distinct from those forming the bacterial capsule (McKenney *et al.,* 1998).

Although bacterial infections are widely reported in animals, their association with BFs is rarely discussed. *Staphylococcus aureus* is the common cause of IMI, which frequently become chronic, associated with the ability of this bacteria to produce biofilm (Cucarella *et al.,* 2001). Recently, the ability of *S.aureus* to form biofilm *in vivo* is considered to be a major virulence factor influencing its pathogenesis in mastitis. The implication of biofilm in chronic bacterial infections in many species has triggered an increasing interest in the characterization of genes involved in biofilm formation. The *bap* gene is a newly identified gene that encodes the biofilm-associated protein (BAP). The biofilm associated protein is a novel cell wall associated protein that promotes the primary attachment of bacteria to surfaces and intercellular adhesion forming biofilms which is ultimately involved in pathogenesis of mastitis, causing a persistent intramammary infection (Cucarella *et al*., 2001; Gotz, 2002; Cucarella *et al*., 2004 and Vautor *et al.,* 2008).

A multitude of strategies have been applied to compare gene and protein expression patterns in biofilms with those in planktonic cultures. When assessed by DNA microarrays, gene expression in biofilms differed from that of planktonic cultures in *Bacillus subtilis* and *Pseudomonas aeruginosa* (Whiteley, 2001 and Stanley, 2003). Biofilm formation in *S.aureus* is considered to be a two step process in which the bacteria

first adhered to a surface, followed by multiplication and cell to cell adhesion forming multilayered biofilm /microcolonies. This is mediated by polysaccharide intercellular adhesin (PIA) and biofilm associated protein (Bap), a surface associated protein. The intercellular locus consisting of the genes *icaA,D,B* and *C* encode the proteins mediating the synthesis of PIA in *Staphylococcus* species (Cramton *et al*., 1999). Among the *ica* genes, *icaA* and *icaD* have been reported to play an important role in biofilm formation in *S. aureus* and *Staphylococcus epidermidis* (*S.epidermidis*) (Yazdani *et al*., 2006). On the other hand, the biofilm associated protein locus encodes a novel cell wall associated protein that promotes the primary attachment of bacteria to surfaces and intercellular adhesion forming biofilms which ultimately involve in pathogenesis of mastitis, causing a persistent intramammary infection (Cucarella *et al*., 2001 and Cucarella *et al*., 2004).

Biofilms adopt their own strategy of survival by way of altering their cell wall proteins and other components. Microcolony or sessile bacterial cells under BF mode of growth, may release low level of antigen, stimulating immune response and induce antibody production. However, these low levels of antibodies may not be effective in killing bacteria inside the BF. Therefore, by stimulating the immune response effectively by exogenous administration of BF antigens, early humoral responses can be induced against the exopolysaccharides responsible for biofilm formation, thereby avoiding the appearance of these microcolonies and controling the infections associated with BF or preventing establishment of BF on the mucosal or epithelial surfaces. This could be achieved by *in vitro* growth of the bacteria in BF mode, which simulate natural *in vivo* conditions to express novel immunogenic proteins. Hence, such BF grown bacteria can be exploited as potential vaccine candidate against mastitis causing organisms in bovines.

Experimental and field trials conducted in the Department of Microbiology, Veterinary College, Bangalore using these BF based vaccines against important avian bacterial pathogens have shown promising results (Shivaraj and Krishnappa, 2002; Veeregowda, 2003; Prakash, 2004; Prakash, 2006 and Ramesh, 2006).

Intramammary infections caused by *S.aureus* in bovines are very difficult to cure. In the context of the high prevalence and economic consequences of *S.aureus* IMI and the relative inefficiency of control measures, the development of a vaccine against *S.aureus* IMI is of great interest. Vaccination has been employed as an adjunct to therapy as well as a preventive measure for *S. aureus* mastitis. Several vaccines have been formulated based on bacterial cell wall components (protein A), adhesion factors (bacterial factors that allow *S. aureus* to attach to mammary epithelial cells) and *S.aureus* pseudocapsules which have been evaluated for protection against *S. aureus* mastitis (Ruegg, 2001). The outcome of these studies has been inconsistent and confusing. Although, *S. aureus* bacterins like Somatostaph® / Lysigin® and 'Mastivac I' (Leitner *et al.,* 2008) are commercially available in the United States of America (USA) and Israel respectively, these vaccines have limited ability to prevent new IMI infections. A three - lactation trial failed to demonstrate a reduction in the number of new *S. aureus* infections in cows vaccinated with a commercial vaccine (Pankey *et al.,* 1985). Experimental vaccines for *S. aureus* composed of pseudocapsule-enriched bacterins supplemented with α – and /or β - toxoids appear promising, but none of these have been commercialized (Yancey *et al.,* 1999). Many other conventional vaccines are also commercially available against *S.aureus* mastitis. The efficacy of such vaccines in reducing the severity of clinical disease has been demonstrated (Nordhaug *et al.,* 1994a; Giraudo *et al.,* 1997;

Leitner *et al*., 2003b and Lee *et al*., 2005) but the vaccines seem unable to prevent new intramammary infections. As yet, no commercial vaccines are currently available in India and other developing countries and it is unlikely that vaccines themselves will give the whole answer to bovine mastitis for sometime to come. Recently, bovine mastitis causing *E.coli* BF and free cell (FC) based vaccines were compared by vaccination trials in pregnant rabbits (Kavitha, 2008 and Jyothi, 2009) and in lactating cows (Chandrashekhara, 2009). These studies have indicated the superiority of biofilm vaccine as serum and milk IgG and IgA levels detected by ELISA were significantly higher in BF vaccinated than FC vaccinated and control animals.

Rabbits have been considered to be good animal models for mastitis studies as the lactating mammary gland of the rabbit is susceptible to natural infection by staphylococci and the disease could be reproduced in the laboratory by injecting low numbers of organisms into the mammary tissue. The type of disease produced varied with the strain used to infect the lactating mammary gland in rabbits (Adlam *et al.*,1977 and Adlam *et al.*, 1980). Further, they are economical and have more number of teats than ruminants. Hence, they may help to reduce the cost and the number of animals involved. They are also larger than mice and may thus be easier to handle them for intramammary injections or infusions (Amorena *et al.*, 1991 and Reinoso *et al*., 2002) and collection of milk.

Keeping this information in background, the present study was undertaken to evaluate the bovine mastitis causing *S.aureus* biofilm vaccine in rabbits.

The objectives of the study were:

- Molecular Characterization of *S.aureus* isolates derived from subclinical and clinical bovine mastitis cases with reference to Biofilm associated protein (*Bap*) gene.
- Analysis of Proteins of *S.aureus* grown under biofilm and planktonic mode with reference to their immunogenicity and cross reactivity.
- Production and experimental evaluation of *S.aureus* biofilm based vaccine in rabbits.

The following parameters were used in the present study;

- *Bap* specific Polymerase Chain Reaction of *S.aureus* isolates.
- Sub clinical mastitis test - California Mastitis Test (CMT) and Somatic cell count.
- Western blot analysis of proteins of *S.aureus* grown under biofilm and planktonic mode
- Immunoglobulin G (IgG) based serum Enzyme Linked Immunosorbent Assay (ELISA).

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.

Lakshmanachar *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. dysgalactie* (12), *Bacillus subtulis* (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. agalactaie* (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. S*mith 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 IgG_1, IgG_2, 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 IgGl and IgG2 isotypes, but in milk, only the concentration of Ig Gl 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 1.48×10^6 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 9.8×10^9 cfu/g of chitin. On the other hand, the free cell (FC) density decreased from day one and was only 5.2×10^8 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 1.33×10^9 cfu/g of bentonite clay. On the other hand, the FC density decreased from day one and was 5.2×10^8 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 4.2×10^{11} cfu/g of bentonite clay, whereas the FC density peaked on the first day after inoculation with an average viable count of 2.0×10^{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 5.70×10^9

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 9.60×10^9 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 8.13×10^{10} cfu/g of bentonite clay and persisted even after 50 days with a count of 5.75×10^7 cfu/g. Free cells on the other hand, attained a peak on the first day after inoculation with an average viable count of 9.74×10^9 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 *ica*A and *ica*D. 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 *ica*A and *ica*D 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 (*Enteroccocus 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 biofim 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^1 - 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 ($P \leq 0.05$) 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 5×10^2, 5×10^4, 5×10^6 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.

Chakraborthy 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 8^{th} 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.

III. MATERIALS AND METHODS

3.1 General considerations

The glasswares used in the present study were of either Corning or Borosil. The culture media, buffers and other biochemical reagents were prepared in double glass distilled water. The chemicals of Analar, Excellar or Molecular biology grade were used for the preparation of various solutions and reagents. These chemicals were obtained from M/s. Hi-media, Mumbai, Sisco Research Laboratories Pvt, Ltd, Mumbai, Bangalore Genie, Bangalore and Sigma Aldrich, USA.

Plasticwares including centrifuge tubes, microcentrifuge tubes, cryovials, Petri plates and microtips were procured from M/s.Tarson products Pvt. Ltd., Bangalore.

3.1.1 Preparation of glasswares

The glasswares used in the study were prepared by soaking them in detergent solution over night. Next day, they were washed thoroughly in running tap water, followed by rinse in deionised / DW. The air-dried glasswares were packed and sterilized in hot air over for one and half hour at 160°C.

3.1.2 Preparation of media

All the media employed were prepared as per the guidelines of Cruickshank *et al*. (1975). The media used were ;

1. Tryptose soya broth
2. Mannitol salt agar
3. Nutrient agar

4. Bentonite clay (M/s Loba Chemie, Mumbai)

3.2 *Staphylococcus aureus* cultures

In the present study, 25 isolates of *S.aureus* (SA1 to SA25) which were isolated from the cases of clinical (SA1 to SA6) and sub clinical bovine mastitis (SA7 to SA25) and maintained at the Department of Microbiology, Veterinary College, Bangalore were used. These cultures were routinely sub cultured and maintained on nutrient agar as per the standard procedures.

3.3 Molecular characterization of *S.aureus* isolates with reference to '*bap*' gene.

3.3.1Isolation and assessing the purity of genomic DNA

Equipments

a.Refrigerated centrifuge

b.Spectrophotometer

c.Ependroff tubes

d.Micropipettes

Reagents

a. Genomic DNA extraction buffer (100ml)

Final concentration:

Triton X-100 (2per cent)	2 ml
SDS (1per cent)	1 g
NaCl (100mM)	0.5844 g
Tris base, pH 8 (10mM)	0.12114 g

EDTA (1mM)	0.029225 g
DW up to	100 ml

The ingredients were weighed out separately and solution was heated to 68ºC to dissolve. The pH of the Tris base was adjusted to 8 by adding a few drops of 1N NaOH and volume was adjusted to 100 ml with DW. The solution was sterilized by autoclaving.

b. TE buffer

Tris base (10mM) pH 8	0.12114 g
EDTA (1mM)	0.029225 g
DW up to	100 ml

The solution was sterilized by autoclaving.

c. Saturated Ultra Pure Phenol (USB): Chloroform: Isoamylalcohol (25:24:1)

d. Acid washed glass beads (Sigma): Size: 212- 300µ

e. Ribonulcease (RNase) (10µg)

Working solution (3µg /ml) was prepared by diluting 1.5µg of Ribonuclease in 2.5 ml of DW and stored in aliquots at - 20ºC.

f. Sodium acetate (3M) pH 5.2

Sodium acetate	10.206 g
DW up to	25 ml

Following dissolution of Sodium acetate, pH was adjusted to 5.2 with glacial acetic acid and the volume was made up to 25 ml.

g. Absolute ethanol (E-Merck, Germany)

h. Ethanol (70 per cent)

Absolute alcohol 70 ml

DW 30 ml

The mixture was chilled at 4ºC.

3.3.1.1 Method of Isolation of genomic DNA

Genomic DNA was isolated from all the 25 *S. aureus* isolates according to the method described by Hoffman and Winston (1987).

- 4 ml of overnight broth culture was harvested by centrifugation for 30 sec at 12,000 rpm.
- The culture pellet was resuspended in one ml of water and harvested again as before.
- The supernatant was decanted and the cells were resuspended in residual volume of water by vortexing briefly.
- 200 µl of genomic DNA extraction buffer was added.
- 200 µl of Phenol: Choloroform: Isoamylalcohol mixture was added.
- 0.3g of acid-washed glass beads was added.
- The solution was vortexed at maximum setting for 4 min.
- 200 µl of TE buffer was added, mixed and centrifuged at 14,000 rpm for 5 min.
- The top aqueous layer was transferred to a new tube and 1ml of ice-cold ethanol was added and then mixed by inversion and centrifuged at 14,000 rpm for 2 min.

- The pellet was resuspended in 200 µl of TE and Ribonuclease was added to final concentration of 3 µg/ml and incubated for 5 min. at 37°C.
- 1/10 volume of 3M Sodium acetate of pH 5.2 and 2 volume of ice-cold ethanol was added and mixed by inversion and centrifuged at 14,000 rpm for 2 min.
- The supernatant was decanted, centrifuged again at 14,000 rpm for 1 min. and the residual ethanol was removed using pipette.
- 500 µl of 70 per cent ethanol was added and centrifuged at 14,000 rpm for 2 min.
- The pellet was resuspended in 50 µl of water, tapped gently and the DNA was stored at -20ºC until use.

3.3.1.2 Determination of purity and yield of the DNA samples

The purity and concentration of the extracted genomic DNA were estimated by spectrophotometry. Twenty microlitre of DNA sample was dissolved in 0.98 ml of sterile DW. The diluted DNA was transferred into 1ml microcuvette and checked for the optical density (OD) at 260 nm and 280 nm in a UV spectrophotometer. Sterile DW was used as blank.

The ratio of 260/280 OD was calculated and a ratio between 1.7 to1.9 was considered as pure. The concentration of the DNA was estimated by the equation:

1 OD at 260 nm = 50 µg/ml of DNA.

3.3.1.3 DNA confirmation by Agarose Gel Electrophoresis

Equipments

a.Horizontal electrophoresis apparatus with power pack (Bangalore Genei, India)

b.Microwave oven MX100 (M/s. BPL Pvt.Ltd)

c.U V transilluminator (M/s. Pharmacia, Sweden)

d.Gel documentation unit (Biorad, USA)

Reagents

a.Agarose

b.TBE buffer (Tris-Borate EDTA buffer) (0.5X , pH 8.2)

A stock solution of 10X TBE was prepared as given below.

Tris base		10.8 g
Boric acid		5.5 g
EDTA (0.5M, pH 8)		4 ml
DW	up to	100 ml

The solution was sterilized by autoclaving and diluted 20 times to get working strength of 0.5X TBE.

c.Gel loading dye (6X)

Bromophenol blue	50 mg
Xylene cyanol	50 mg
Sucrose	8 g
DW	20 ml

The above mixture was stirred well and stored at 4ºC.

d.Ethidium bromide (10mg /ml)

Ethidium bromide	100 mg
DW	10 ml

The suspension was stirred to ensure that the dye had dissolved. The container then wrapped in aluminum foil and stored at room temperature.

The purity of the DNA sample was further checked by electrophoresis on 0.8 per cent of the agarose gel as described below.

a. Required quantity of agarose was weighed and dissolved in proportionate volume of 0.5 per cent of TBE and melted in a microwave oven for 1min.

b. The gel tray was sealed at both ends using adhesive tape. The comb was placed in proper position and the melted agarose was poured into gel tray carefully avoiding air bubbles and allowed to solidify.

c. On solidification, comb and the seals on either side were removed carefully.

d. The gel tray was held in electrophoresis tank and 0.5X TBE buffer was poured to submerge the gel in the tank.

e. The DNA samples were mixed with $1/6^{th}$ volume of 6X loading dye and carefully loaded into the wells using micropipette.

f. The electrophoresis was carried out at 5 v/cm at room temperature till the Bromophenol blue dye had just reached the end of the gel.

g. Following the electrophoresis, the gels were stained for 30 min in 0.5 µg/ml ethidium bromide and the DNA bands were visualized at 300 nm with a UV transilluminator.

3.4 *bap* specific-Polymerase Chain Reaction (*bap* -PCR)

Reagents

a.*Taq* DNA polymerase (Bangalore Genei, Bangalore)

b.10X PCR buffer (Bangalore Genei, Bangalore)

c.*bap* primers (Bio serve Biotechnologies Pvt.Ltd. Hyderabad)

d.Deoxy nucleotides mix (dNTPs) (Bangalore Genei, Bangalore)

e.500 bp DNA ladder (Bangalore Genei, Bangalore)

3.4.1*bap* specific Primers

For amplification of *bap* gene, specific primers published by Cucarella *et al.* (2001) were procured commercially. They are enlisted with their base sequences in Table1. These primers were reconstituted in TE to a concentration of 20 pmol /µl and stored at -20°C.

Table 1 : Nucleotide sequence of *bap* specific primers (Cucarella *et al.*, 2001)

Sl. No.	Primer Code	Nucleotide sequence
1	*sasp-6m(forward)*	5'-CCCTATATCGAAGGTGTAGAATTGCAC-3'
2	*sasp-7c(reverse)*	5'-GCTGTTGAAGTTAATACTGTACCTGC-3'

3.4.2 *bap* specific PCR

The amplification reactions were carried out in 0.2ml microcentrifuge tubes using a programmable thermal cycler (Palm Cycler, Corbett Research, Australia) following the method described by Cucarella *et al.* (2001)

a.A 25µl of the PCR mixture comprised of 250 ng (3 µl) of *S.aureus* DNA, 2 µl (40 pmol) of each forward and reverse primers and 1 µl (100 µM) of each dNTPs, 2. 5µl

of 10X PCR assay buffer and 0.66µl of *Taq.*DNA polymerase. Water was added to make a final volume of 25µl.

b.The contents were mixed gently using a micropipette and centrifuged for 5 sec. at 5000 rpm.

c.A drop of mineral oil was overlaid in all the tubes to avoid evaporation at high temperature during PCR.

d.The PCR amplification was carried out by initial denaturation of DNA at 94°C for 2 min. followed by 40 cycles of denaturation at 94°C for 20 sec, annealing at 42°C for 20 sec and extension at 72°C for 50 sec. Final extension step of 72°C for 5 min was included to complete the synthesis of unfinished products.

e.After completion of PCR reaction, amplified products were subjected to electrophoresis in 0.8 per cent agarose gel with 6X gel loading dye. As molecular weight marker, 500 bp DNA ladder was used.

3.5 Analysis of proteins of BF and FC of selected *S.aureus* isolates

3.5.1 Selection of *S.aureus* isolates

S.aureus SA16, which was isolated from sub clinical mastitis was used as Biofilm vaccine candidate and also for homologous challenge studies and *S.aureus* SA2, which was isolated from clinical mastitis was used for heterologous challenge studies. The selection of these strains was based on possessing *bap* gene which was characterized in the present study and *ica*A gene which was based on earlier studies carried out by Rajeev (2006) in the Dept. of Veterinary Microbiology, Veterinary College, Bangalore.

3.5.2 Extraction of Proteins

*S.aureu*s SA16 and SA2 strains were grown in BF mode according to the method standardized by Naveenkumar (2005) using 0.32 per cent TSB with 0.3 per cent bentonite clay as an inert surface for three days, whereas in FC mode, bacteria were grown in 3 per cent TSB for 16 hrs. *S. aureus* cultures grown under the above conditions were pelleted at 4000 rpm for 10 min at 4°C. The pellet was washed three times in 10mM HEPES buffer and finally re-suspended in 10 mM HEPES buffer and stored at –20°C until further processing.

Equipments and reagents

Ultra centrifuge (Sorvall-OTD 65B, U.S.A)

Sonicator (SONICS®, Vibra Cell, Sonicator U.S.A)

Spectrophotometer (Spectronic 21, Bausch and Lomb)

Sodium-n-lauryl sarcosinate (Sigma Aldrich, U.S.A)

Protein estimation Kit (M/s Bangalore Genei, Bangalore)

3.5.2.1 Protein extraction

The proteins were extracted from BF and FC of *S.aureus* SA16 and SA2 as per the methods of Bolin and Jensen (1987).

1. The culture pellet suspended in 10 mM HEPES buffer was added with a final concentration of 1 mM phenyl methane sulfonyl fluoride (PMSF) and 1 mM EDTA; subjected to sonication at the rate of 70 per cent amplitude corresponding to 16.1 μ in an ice bath for ten cycles of thirty seconds each with thirty seconds rest between each

cycle. Following this, whole cells and debris were pelleted out by centrifugation at 4000 rpm for 20 min. at 5°C and the supernatant containing the cell membranes was collected.

2. The cell membranes in the supernatant were pelleted out by ultracentrifugation at 1,05,000g for 60 min at 5°C.

3. The pellet (membranes) was suspended in 10 mM HEPES buffer (pH 7.4) containing two per cent sodium-n-lauryl sarcosinate and incubated for one hour at 22°C.

4. The detergent-insoluble protein-enriched fraction was pelleted out by ultra centrifugation at 1,05,000 g for 60 min at 5°C; the pellet was again resuspended in 10 mM HEPES buffer (pH 7.4) and stored at -70°C until use.

3.5.2.2 Quantification of proteins

The protein content in the extract was estimated using a protein-dye-binding method according to Bradford (1976), using a ready made kit procured from M/s Bangalore Genei, Bangalore.

Bradford reagent

Coomassie brilliant blue (R-250)	25.0 mg
Methanol (95 %)	12.5 ml
Ortho phosphoric acid	25.0 ml
DW up to	250.0 ml

The reagent was prepared freshly each time.

Procedure

Two mililitre of Bradford reagent was added to an aliquot of protein sample to be assayed, after ten minutes of adding the reagents; the absorbance was measured at 595 nm. Finally, the protein content was calculated by comparing the absorbance value with standard BSA ranging from 10 to 80 μg.

3.5.3 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis of proteins was carried out according to the method described by Laemmli (1970).

Equipment

a. Slab gel apparatus (vertical gel system, M/s Bangalore Genei, Bangalore)

b. Glass plates of 10 × 10 cm one of them having a 1.5 × 7 cm notch

c. Plexiglass comb, spacers and gel casting unit

d. Power pack, vacuum pump (M/s Bangalore Genei, Bangalore)

e. Gel rocker (M/s Bangalore Genei, Bangalore)

Reagents

1. **Solution A (Acrylamide and bisacrylamide stock)**

Acrylamide	29.2 g
Bisacrylamide	0. 8 g
DW up to	100.0 ml

2. **Solution B (1.5 M Tris Cl, pH 8.8)**

Tris base	18.2 g

Sodium dodecyl sulphate (Sigma)		0.4 g
Hydrochloric acid	up to	2.0 ml
DW	up to	100.0 ml

3. Solution C (0.5 M Tris Cl, pH 6.8)

Tris base	6.1 g
SDS	0.4 g
Hydrochloric acid	4.2 ml
DW up to	100.0 ml

4. Solution D (10 % Ammonium per sulphate)

Ammonium per sulphate	0.1 g
DW	1.0 ml

5. TEMED (N, N, N, N - Tetra methylene diamine)

6. Protein molecular weight marker (M/s Bangalore Genei, Bangalore)

Myosin, rabbit muscle	205,000 Da
Phosphorylase b	97,400 Da
Bovine serum albumin	66,000 Da
Ovalbumin	43,000 Da
Carbonic anhydrase	29,000 Da
Soyabean trypsin inhibitor	20,100 Da
Lysozyme	14,300 Da
Aprotinin	6,500 Da

Insulin (α and β chains)	3,000 Da

7. Buffers

a. Separating gel (15 %)

Solution A	9.0 ml
Solution B	4.5 ml
Solution D	80.00 µl
TEMED	10.00 µl
DW	4.5 ml

b. Stacking gel (4.5 %)

Solution A	0.9 ml
Solution C	1.6 ml
Solution D	20.0 µl
TEMED	10.0 µl
DW	3.6 ml

c. Running buffer

Tris base	1.50 g
Glycine	7.20 g
SDS	0.50 g
DW up to	500.00 ml

d. Sample buffer

10 per cent SDS	1.60 ml

2-mercaptoethanol	0.40 ml
Solution C	1.00 ml
Glycerol	0.80 ml
Bromophenol blue (1%)	0.40 ml
DW	3.80 ml

8. Staining solution

Coomassie Brilliant Blue R 150	0.25 g
Methanol	45.00 ml
Glacial acetic acid	10.00 ml
DW	45.00 ml

The stain was filtered through Whatman No.1 filter paper to remove any particulate matter.

9. Destaining solution

Methanol	45.00 ml
Glacial acetic acid	10.00 ml
DW	45.00ml

3.5.3.1 Method

Clean, grease-free and protein-free glassware and hand gloves were used while handling the gel.

a. The glass plates, spacers and comb were cleaned thoroughly and dried.

b. The glass plates with spacers were assembled in to the gel-casting unit.

c. Separating gel was prepared and poured in between the plates with a gentle continuous flow avoiding air bubbles, up to about three fourth of the height of the plate (about two inches below the level of the notch).

d. A thick layer (approx. 0.5 cm) of water-saturated butanol was immediately overlaid carefully avoiding mixing of the two, using a syringe fitted with a thin needle. The gel was allowed to set.

e. Water saturated butanol layer was blotted off using filter paper strips.

f. The gel was washed by overlaying DW and blotted off using filter paper strips.

g. Stacking gel was prepared and poured up to the required height, the comb was inserted into the stacking gel, to make the wells of required height and the gel was allowed to set.

h. The comb was removed and the slab was fixed to the electrophoresis apparatus and the running buffer was poured into both upper and lower tanks.

i. Sample preparation and loading:

An aliquot of the sample corresponding to the required amount of protein (25 μg) was mixed with $1/4^{th}$ volume of sample buffer and heated to 100°C in a dry bath for five minutes. Each sample was then carefully charged into each of the wells of the stacking gel using a micropipette. Similarly one of the wells was loaded with pre-stained standard protein molecular weight marker (MW), after keeping in the water bath for 100°C for five minutes. Electrophoresis was carried out at a constant current of 10 mA, 50 V for the

stacking gel and 15 mA, 100 V for the resolving gel, until the dye front reached the lower end of the gel.

3.5.4 Western blotting

Electroblotting

Electroblotting of proteins from SDS-PAGE gel to nitrocellulose (NC) membrane and Ponceau S staining.

Equipments

a. Semi dry electro blot apparatus (Locally designed)
b. Power pack (M/s Bangalore Genei, Bangalore)
c. Nitrocellulose membrane (Pall corporation, USA)
d. Whatman 1 MM filter paper

Reagents

a. Transfer buffer (pH 8.3)

Tris base	5.81 g
Glycine	2.93 g
SDS	0.37 g
Methanol	200.00 ml
DW up to	1000.00 ml

b. Ponceau S stain

Ponceau S (M/s Loba chemie)	2.0 g
Trichloroacetic acid	30.0 g
Sulfosalicylic acid	30.0 g
DW up to	1000.0 ml

3.5.4.1 Method

a. Graphite plates of Western blot apparatus were rinsed with transfer buffer.

b. Wearing gloves, one piece of NC membrane (Pall corporation,USA) and twelve pieces of Whatman1MM filter paper were cut to the exact size of the gel and soaked in transfer buffer.

c. After completing the SDS-PAGE, the gel was separated from the glass plates and transferred to the transfer buffer.

d. The bottom electrode (anode) was laid flat on the bench, with graphite side up.

e. Six pieces of 1 MM filter paper, soaked in transfer buffer were placed on the anode. These pieces were stacked exactly one over the other. Using a glass pipette as a roller, air bubbles trapped if any between the pieces were squeezed out.

f. Now, the soaked NC membrane was placed on the stack of filter papers, care was taken to remove trapped air bubbles between NC membrane and filter paper.

g. The gel (from step c) was placed exactly on the top of the NC membrane. The gel was oriented such that, the pencil mark on the NC membrane corresponded to the bottom left hand corner of the gel and trapped air bubbles were squeezed out with gloved fingers.

h. Finally six pieces of 1MM filter paper were placed with exact alignment on the gel.

i. Upper electrode (cathode) was placed on the top of the stack, with graphite side down. Electrical leads were connected and a constant 50V was applied for 90 min.

j. After the run, electrical power was turned off and the leads were disconnected. The transfer apparatus was disassembled from the top downward, peeling each layer in turn.

k. Ponceau staining of proteins in NC membrane and Coomassie blue staining for the gel after electroblotting was done to confirm transfer of proteins from gel on to NC membrane.

3.5.4.2 Ponceau S staining

Ponceau staining of proteins electroblotted onto NC membrane was carried out as described by Sambrook *et al*. (1989).

a. After disengaging the sandwich of 1 MM filter papers, gel and NC membrane, the membrane was transferred to a tray containing working solution of Ponceau stain.

b. The membrane was incubated for 10 min. at RT, immersed in stain with gentle agitation.

c. When the bands of protein were visible, the membrane was washed with several changes of DW at RT and allowed to dry.

3.5.5 Immunoblotting

Reagents and materials

a. Hyper immune sera

Hyper immune sera were raised against *S.aureus* SA16 BF and FC proteins which were extracted as described in Section 3.5.2.

b. Blocking buffer

Skimmed milk powder	5 g
Tween-20	100 µl
PBS	100 ml

c. Wash buffer

Tween-20	100 µl
PBS	100 ml

d. Goat anti-rabbit IgG - HRP conjugate

Working solution	1:10000

e. ODD – Substrate solution

Ortho dianisidin dihydrochloride (ODD)	5-10 mg
Acetate buffer (4X)	6 ml
DW	18 ml
H_2O_2 (30 %)	6 µl

i. Acetate buffer (0.2 M)

Solution A

Acetic acid	6 ml
DW up to	500 ml

Solution B

Sodium acetate	16.4 g
DW up to	1000 ml

Mix 148 ml of Solution A and 352 ml of Solution B to get 500 ml of 0.2M Acetate buffer

ii. Acetate buffer (4X)

Acetate buffer (0.2 M)	50 ml
EDTA	74.4 mg
Triton X 100	0.4 ml

3.5.5.1 Raising of hyper immune serum

Four adult rabbits were procured from a commercial rabbitry and randomly divided into two groups of two rabbits each and were used for raising hyper immune serum (HIS) against *S.aureus* SA16 BF and FC proteins.

One group of rabbits received *S.aureus* SA16 BF protein along with FIA for first injection on day zero and two boosters through s/c route. On the other hand, *S.aureus* SA16 FC protein was given with FCA for the first injection on day zero and subsequently for the two boosters with FIA to another group of rabbits. Both the groups of rabbits were boosted twice, first booster was given 15 days after first injection, second booster was after one week of first booster. Blood samples were collected from the saphenous

vein one week after the second booster; serum was separated and stored in aliquots at -20°C until further use.

3.5.6.2 Immunoblotting

SDS-PAGE followed by electroblotting of protein was carried out as described in the section 3.5.3.

1.Blocking: After electroblotting, the NC membrane was immersed in blocking buffer (0.1 ml per sq.cm. of membrane blot) and incubated overnight at 4°C. The blot was then washed thrice, each washing for five minutes using wash buffer.

2.Incubation with primary antibody: The blots were incubated for 90 min. at 37°C with 10 ml of 1:500 dilution of individual antiserum in PBS-T and then washed.

3.Probing with goat anti-rabbit IgG-HRP conjugate: The membrane blot was incubated with the secondary antibody at a dilution of 1:10,000 in PBS-T for one hour at 37°C and washed with wash buffer thrice at 5 minutes intervals.

4.Following the final wash, the blot was incubated at RT with a freshly prepared ODD substrate solution until the desired band intensity was achieved and the blot was transferred to a tray containing DW to stop further development of color. A permanent record of the Western blot was made by either photographing or scanning.

3.6 Optimization of infective dose of *S.aureus* to induce mastitis in lactating rabbits

3.6.1 *S.aureus* isolates used for inoculation

Selected isolates of *S.aureus* SA16 and SA2 were used for induction of mastitis in rabbits.

3.6.2 Preparation of bacterial suspension

Staphylococcus aureus were grown on mannitol salt agar for 18 hrs at 37°C. A loopful of culture was suspended in two mililitre PBS and suspension was adjusted to final concentration of 10^4, 10^5, 10^6 and 10^7cfu/ml after counting number of viable cells.

3.6.3 Experimental animals

Twelve female rabbits in 2^{nd} to 4^{th} day of lactation were resourced from a reputed rabbit breeder. Six animals in each group were housed in cages during the experiment. They were maintained under standard laboratory hygienic conditions, providing laboratory animal feed and water *ad libitum.* The approval of the Institutional Animal Ethics Committee was obtained prior to start of the experiment. Of these 12, six rabbits were maintained for each of the two strains of *S.aureus.*

3.6.4 Inoculation technique

Prior to infection, females were completely milked out by squeezing the mammary gland from the base of the teat. Using each of the two *S.aureus* isolates, six rabbits were infected by inoculating 0.5 ml bacterial suspension (in PBS) at the base of the teat by using 30 G needle and syringe. The bacterial count v/s mammary quarters used for induction of mastitis in rabbits are as follows:

1^{st} pair - 0.5 ml PBS only

2^{nd} pair - 0.5 ml suspension (5×10^3 cfu) i.e. 10^4 cfu/ml

3^{rd} pair - 0.5 ml suspension (5×10^4 cfu) i.e. 10^5 cfu/ml

4^{th} pair - 0.5 ml suspension (5×10^5 cfu) i.e. 10^6 cfu/ml

5^{th} pair - 0.5 ml suspension (5×10^6 cfu) i.e. 10^7 cfu/ml

3.6.5 Collection of milk from rabbits

After inoculation, gross lesions on the mammary glands were recorded and milk samples were collected from each of the mammary gland at 24 hours intervals up to 144 hours. Milk samples were collected from the lactating rabbits before and after infection, following the IM administration of 0.5 units of oxytocin. There was an engorgement of mammary glands within three minutes after injection of oxytocin compared to those of rabbits not injected with oxytocin. Maximum milk was collected by squeezing the glands from the base of the teat.

The details of collection of milk after infection are as given below:

Rabbits	Time of milk collection					
	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs	144 hrs
1st	+	+	+	+	+	+
2nd	+	+	+	+	+	+
3rd	+	+	+	+	+	+
4th	+	+	+	+	+	+
5th	+	+	+	+	+	+
6th	+	+	+	+	+	+

Milk samples collected were used for determination of SCC and for subclinical mastitis test-CMT.

3.6.6 Direct Microscopic Somatic Cell Count in Milk

The procedure followed was according to general principle of Prescott and Breed method as detailed by Schalm *et al*. (1971).

3.6.6.1 Procedure:

3.6.6.1.1 Preparation of milk films

1.The milk samples were mixed 15-25 times to obtain a uniform distribution of cells.

2.The samples were allowed to stand for 2-5 min to permit air bubbles to rise and foam to disappear.

3.Each microscopic slide was identified by a number. A level surface was selected and the slide was placed over the template to outline 1 sq.cm area.

4.Ten microlitre of milk was placed exactly in the centre of the 1 sq.cm template and was spread evenly to cover all the area delineated by the template. From each sample two films were prepared using successive areas of the slide. The films were dried at room temperature.

3.6.6.1.2 Staining:

1.The slide was placed on the slide rack and the smears were flooded with modified Newman-Lampert's stain **(**Hi-media**)** for 2 min.

2.The excess stain was drained off by keeping the slides on absorbent paper and air-dried.

3.The slide was rinsed in three changes of tap water at 42-45°C and air-dried.

3.6.6.2 Counting of cells:

Stained films were examined under oil immersion objective and the number of cells in 10-25 fields was counted. The fields were selected by moving the slide horizontally from one edge of the film through the centre to the opposite edge and then,

repeated in a vertical direction. The average number of cells per field was multiplied by the microscopic factor.

3.6.6.3 Calibration of the microscope / Calculation of microscopic factor:

The diameter of the microscopic field seen through oil immersion objective was measured using a stage micrometer slide ruled in 0.1 and 0.01 mm. The diameter of the field was measured up to two decimal points and the area of the field was calculated using the formula Πr^2.

$$\text{Microscopic factor (MF)} = \frac{\text{Area of the smear (in mm}^2\text{)}}{\text{Area of the microscopic field}}$$

The diameter was 0.16, then r = 0.08

$$\text{So, MF} = \frac{100}{3.14 \times 0.08^2}$$

$$= 4976 \approx 5000$$

Since, the milk sample taken on the slide was 0.01 ml, the total number of cells per ml of milk was given by the formula,

Cell count per ml of milk = Average no. of cells per field × MF × 100

3.6.7 California mastitis test

The test procedure recommended by Sharma and Rajani (1965) was employed for all the samples.

3.6.7.1 Composition of CMT reagent

Sodium hydroxide	1.50 g

Teepol		0.50 ml
Bromo thymol blue		0.01 g
DW	up to	100.00 ml

3.6.7.2 Procedure:

The test was performed by mixing 50 µl of CMT reagent with equal volume of milk sample on a clean, grease free microscopic slide. The results were interpreted by the presence of precipitate or gel formation.

3.7 Preparation of vaccines

Two types of vaccines were prepared using *S.aureus* SA16.

3.7.1 Biofilm vaccine

Three-day-old BF cells of *S.aureus* SA16 grown in 0.32 per cent TSB, incorporated with 0.3 per cent bentonite clay were harvested by discarding the supernatant media to remove any FC. Bentonite clay with BF growth was adjusted to a final concentration of 4×10^9 cfu / ml with PBS after counting number of viable cells. The biofilm cells were inactivated with final concentration of 0.1 per cent formalin at RT for 24 hrs and stored at 4°C until use.

3.7.2 Free cell vaccine

The *S.aureus* SA16 culture was grown in 3.0 per cent TSB for 16 hrs at 37°C and pelleted at 4000 rpm for 10 min. at 4°C. The pellet was washed thrice and finally resuspended in PBS to contain 4×10^9 cfu / ml after counting number of viable cells. The pellet was inactivated with 0.1 per cent formalin at RT for 24 hrs and stored at 4°C until use.

3.7.3 Sterility test

The vaccine was inoculated onto Nutrient agar, Mannitol salt agar, Blood agar, Brain heart infusion agar and Robertson bullock heart medium in duplicates and incubated at 37°C under aerobic and anaerobic conditions and examined periodically for any bacterial growth up to seven days.

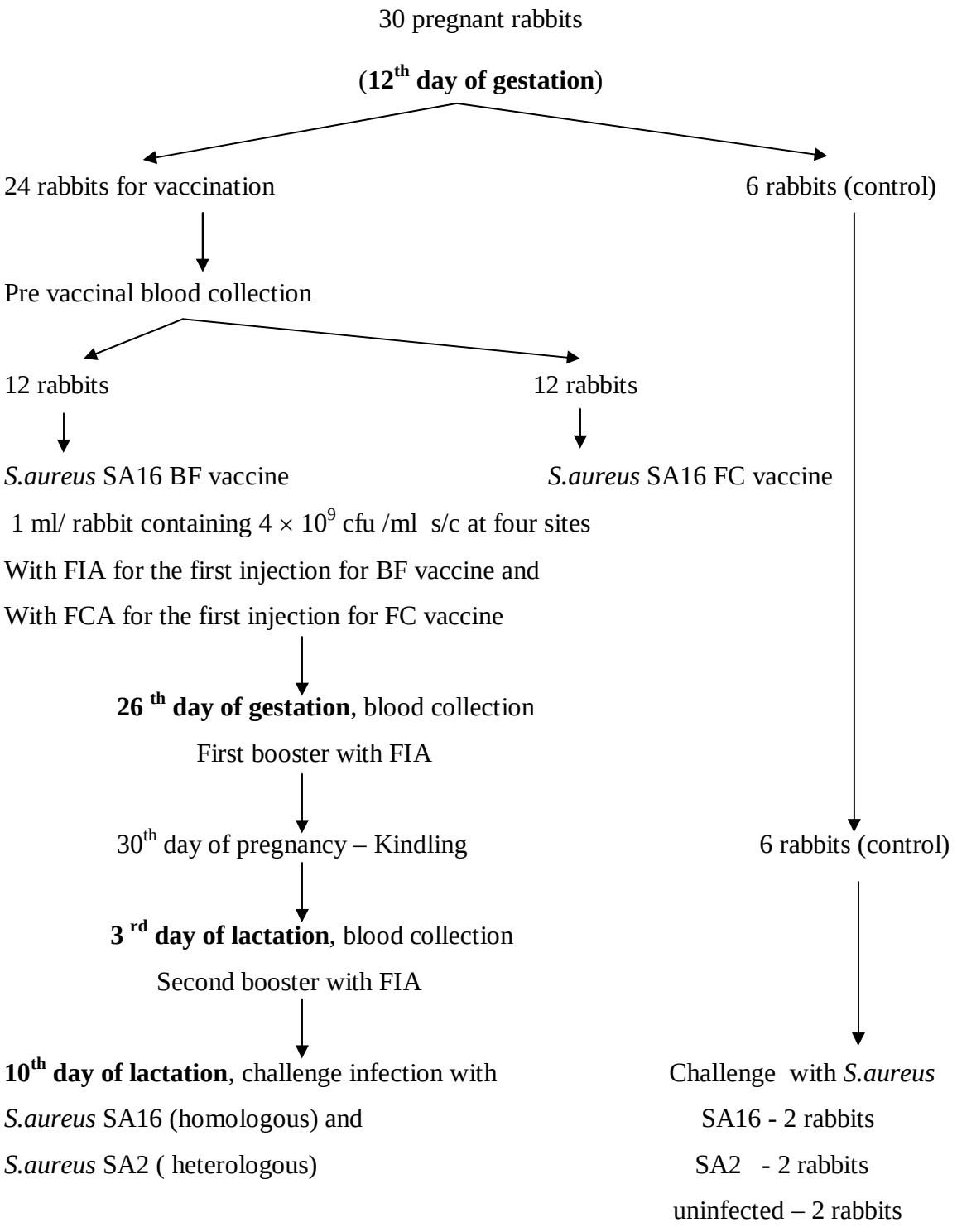
3.7.4 Immunization trials

3.7.4.1 Pregnant rabbits

Thirty pregnant rabbits procured from a reputed breeder were randomly divided into two groups of twelve each and the control group with six rabbits. Pre vaccinal sera were collected from all the rabbits.

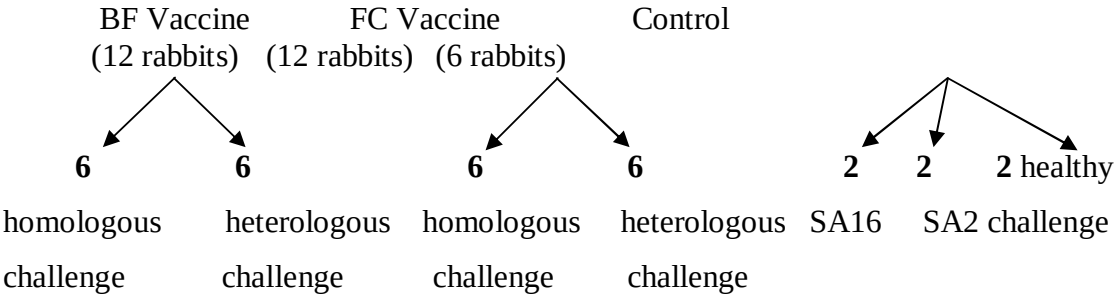
3.7.4.2 Vaccination schedule

Twelve rabbits each were immunized by BF and FC vaccines of *S.aureus* SA16. Details of vaccination are shown below in the flow chart.



3.7.5 Challenge / infection studies

Challenge was carried out on the 10th day of lactation. Pre challenge milk and blood samples were collected from all the rabbits. Six rabbits from each vaccinated group were separated and one group from BF vaccinated rabbits was challenged with *S.aureus* SA16 as homologous strain and other group with *S.aureus* SA2 as heterologous strain at the base of teat. As per the results of optimization of infection, challenge dose was fixed. Similarly, FC vaccinated group was also challenged.



The following grouping of animals were made for the purpose of analysis:

Group 1 - BF vaccinated homologous challenged

Group 2 - BF vaccinated heterologous challenged

Group 3 - FC vaccinated homologous challenged

Group 4 - FC vaccinated heterologous challenged

Group 5 - Control

Details of pre and post vaccinal collection of blood from each group of vaccinated rabbits

Rabbits	Pre immunization	Post immunization							
	Day 0 (day 12th of pregnancy)	15	22	29	30	31	35	43	50
1	+	+	+	+	+	-	-	-	-
2	+	+	+	+	+	+	-	-	-
3	+	+	+	+	+	+	+	-	-
4	+	+	+	+	+	+	+	+	-
5	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+

Details of pre and post challenge collection of milk from each group of vaccinated rabbits

Rabbits	Pre Challenge	Post Challenge				
	Day 0 (day 29 of experiment / 10th day of lactation)	1	2	6	14	21
1	+	+	+	+	+	+
2	+	+	+	+	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	+	+	+	+	+	+
6	+	+	+	+	+	+

After challenge in vaccinated and control animals, the macroscopic lesions in the mammary glands were recorded. Milk samples collected were used for determination of SCC and for subclinical mastitis test *i.e* CMT following standard procedures. Blood samples collected were used for determination of IgG response by indirect ELISA.

3.8 Enzyme Linked Immunosorbent Assay (ELISA)

3.8.1 Standardization of indirect ELISA

Reagents

a. Antigen coating buffer (Carbonate-bicarbonate buffer, pH 9.6 ± 0.05)

Stock solutions:

Solution A:

Sodium carbonate (anhydrous)	1.06 g
DW	50.00 ml

Solution B:

Sodium hydrogen carbonate	0.84 g
DW	50.00 ml

Stored at 4°C.

Working solution (0.05 M, 1X)

Solution A	3.50 ml
Solution B	8.50 ml
DW	38.00 ml

The working solution was freshly prepared before use.

b. Calcium-magnesium free phosphate buffered saline (CMF-PBS) 0.1 M, pH 7.2

Sodium chloride	8.00 g
Disodium hydrogen phosphate	1.21 g
Potassium chloride	0.20 g
Potassium dihydrogen phosphate	0.20 g
DW	1000.00ml

The solution was sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure and stored at 4°C in aliquots of 100 ml.

c. Wash buffer

CMF-PBS (pH 7.2 ± 0.2)	20.00 ml
DW	80.00 ml
Tween-20 (Hi-media Pvt. Ltd.)	0.05 ml

The solution was freshly prepared before use.

d. Bovine Gelatin-PBST (1% BG-PBST)

(Dilution buffer/ blocking buffer)

Bovine gelatin (Rallies India Ltd., Bangalore)	1.00 g
Tween-20 (Hi-media Pvt. Ltd.)	0.05 ml
CMF-PBS	100.00 ml

e. Goat anti-rabbit IgG-HRP conjugate (Sigma, U.S.A)

f. Chromogen solution: One tablet of O-phenylene diamine-dihydrochloride (OPD) of 10 mg, obtained from Sigma chemicals (USA), was dissolved in 25 ml of DW and

stored at 4°C in an amber colored glass bottle wrapped with aluminium foil. This solution was not stored for more than a week.

g. Substrate solution:

H_2O_2 (30%)	1 ml
DW	9 ml

The solution was stored at 4°C.

h. Hydrochloric acid (2.5 N) (Stopping solution)

Hydrochloric acid (35%)	22.7 ml
DW	77.3 ml

This solution was stored in amber colored bottle at RT.

i. Carrier surface: Nunc Maxisorp plates with 96 flat bottom wells.

3.8.1.1 Source of serum samples

Hyper immune sera

Hyper immune sera to *S.aureus* SA16 BF and FC proteins were raised in pregnant rabbits as described in section 3.5.5.1. After one week of the third injection, final bleeding was done; serum was separated and preserved at –20°C. This was used as positive serum control (C+) in ELISA.

Healthy serum

Pre immune serum collected from the rabbits used as negative control (C-) in ELISA.

3.8.1.2 Optimization of *S.aureus* SA16 biofilm and free cell antigen

An end point titration was carried out to determine the optimum single working dilution of BF protein antigen to be used to coat the Maxisorp plates. It was carried out by keeping the serum dilution constant at 1:100 against various dilutions of *S.aureus* SA16 BF and FC protein antigen.

3.8.1.3.1 *S.aureus* SA16 biofilm and free cell antigen dilutions

A serial two-fold dilution of *S.aureus* SA16 BF and FC protein antigens were prepared in carbonate-bicarbonate buffer (pH 9.6 ± 0.05) to provide dilutions of 1:10 (10μg protein /ml) to 1:320 (0.3125 μg protein/ml).

3.8.1.3.2 Serum dilution

A constant dilution of 1:100 of HIS raised against *S.aureus* SA16 grown under both BF as well as FC mode and healthy serum was prepared in one per cent BG-PBST.

3.8.1.3.3 Titration of *S.aureus* SA16 biofilm and free cell protein antigens

a. One hundred μl of antigen at various dilutions prepared in antigen coating buffer as described above were transferred to all the wells along the rows of microtitre plate and incubated at 37°C for two hours in the orbital shaker at 16 rpm.

b. The contents of the wells were discarded and the plate was washed three times with wash buffer and gently tapped over a tissue paper.

c. The unreacted sites on the carrier surface of the wells were blocked by incubating the plate with 100 µl of 1 per cent BG-PBST at 37°C for one hour in the orbital shaker at 16 rpm.

d. The content of the wells was discarded and the plate was washed three times as described in step b.

e. One hundred microlitre of 1:100 diluted control sera (HIS and healthy sera) were added to individual wells in duplicates (except conjugate control wells) and the plate was incubated at 37°C for one hour in the orbital shaker at 16 rpm. The plate was washed as described in step b.

f. One hundred microlitre of 1:25000 dilution (predetermined optimal dilution) of goat anti-rabbit IgG-HRP conjugate in one per cent BG-PBST was added to each well and incubated at 37°C for one hr in the orbital shaker at 16 rpm. The plate was washed as described in step b.

g. One hundred microlitre of freshly prepared chromogen-substrate solution containing OPD and three per cent H_2O_2 as substrate (4 µl / ml of OPD) was added to each well and the plate was kept at RT for 15 min.

h. Finally, 50 µl of 2.5 N HCl was added to each well to stop enzyme-substrate reaction.

i. Absorbance values were read at 490 nm using software based ELISA reader (Biorad Labsystems).

3.8.2 Seromonitoring of *S.aureus* antibodies by ELISA

3.8.2.1 Vaccinal and post challenge sera

Sera samples were collected from *S.aureus* BF, FC vaccinated and control rabbits at different intervals *i.e.*, pre vaccinal, 15 days after first shot, a week after first and second booster, 24, 48 hrs, 6, 14 and 21 days post-challenge with homologous *S.aureus* SA16 and heterologous *S.aureus* SA2.

3.8.2.2 Protocol of indirect ELISA

The procedure of indirect ELISA used for seromonitoring of antibodies against *S.aureus* causing experimental mastitis in rabbits is described below.

a. An optimum single working dilution of *S.aureus* SA16 BF and FC protein was prepared in coating buffer (pH 9.6 ± 0.05) and 100 µl of this was added to each well. The plate was incubated at 37°C for two hours in the orbital shaker at 16 rpm.

b. The content of the wells was discarded and the plate was washed three times with wash buffer and gently tapped over a tissue paper.

c. The unreacted sites on the carrier surface of the wells were blocked by incubating the plate with 100 µl of one per cent BG-PBST at 37°C for one hour in the orbital shaker at 16 rpm.

d. The content of the wells was discarded and the plate was washed three times as described in step b.

e. One hundred microlitre of C+ and C- was added in triplicates to the respective control wells at a final dilution of 1:100 in one per cent BG-PBST. Then, 100 µl of 1:100

dilution of each test serum sample in one per cent BG-PBST was added to each well of the plate (apart from the control wells) and incubated at 37°C for 90 min in the orbital shaker at 16 rpm. The plate was washed as described in step b.

f. One hundred microlitre of 1: 25,000 dilution (predetermined optimal dilution) of goat anti-rabbit IgG-HRP conjugate in one per cent BG-PBST was added to each well and incubated at 37°C for one hour in the orbital shaker at 16 rpm. The plate was washed as described in step b.

g. One hundred microlitre of freshly prepared chromogen-substrate solution containing OPD and three per cent H_2O_2 as substrate (4 µl / ml of OPD) was added to each well and the plate was kept at RT up to 15 min.

h. Finally, 50 µl of 2.5 N HCl was added to each well to stop enzyme-substrate reaction.

i. Absorbance values were read at 490 nm using software based ELISA reader (Biorad, Labsystems). Readings were taken after the wells with only substrate-chromogen and HCl were blanked to 'zero' at 492 nm. Optical Density (OD) values were converted into Percent Positivity (PP) values by employing the formula,

$$\text{PP value of sample sera} = \frac{\text{OD value of sample sera}}{\text{OD value of positive control sera (C+)}} \times 100$$

IV. RESULTS

4.1 Molecular Characterization of *S.aureus* isolates with reference to *'bap'* gene.

A total of 25 bovine mastitis *S. aureus* isolates (SA1 to SA25) were subjected for '*bap*' gene specific PCR. For amplification of *bap* gene, primers sasp-6m (5'-CCCTATATCGAAGGTGTAGAATTGCAC-3') and sasp-7c (5'-GCTGTTGAAGTTAATACTGTACCTGC-3') were used. The thermal cycling profile consisted of an initial denaturation at 94°C for 2 min. 40 cycles of denaturation at 94°C for 20 sec, annealing at 42°C for 20 sec and extension at 72°C for 50 sec with final extension at 72°C for 5 min. which gave consistent results. Results revealed that, out of 25 *S. aureus* isolates, 10 (40 per cent) isolates were *bap* positive that showed an amplicon of 971 bp. Among 10 '*bap*' positive isolates, three isolates (SA2, SA3 and SA4) were from clinical mastitis and seven isolates (SA7, SA8, SA10, SA15, SA16, SA17 and SA23) were from sub clinical mastitis cases All the *bap* positive isolates were also *ica*A positive (Fig.1a & 1b and Table 2).

4.2Analysis of proteins of *S.aureus* SA16 and SA2

4.2.1 Protein expression profile of *S. aureus* SA16 and SA2 grown under BF and FC mode

In the present study, the proteins of *S.aureu*s SA16 and SA2 as representative strains, grown under BF and FC mode were analysed by SDS-PAGE. The profile of proteins from both *S.aureu*s SA16 and SA2 grown under FC mode revealed polypeptides of 19.03, 22.61, 25.25, 25.43, 28.60, 33.30, 37.05, 38.09, 39.91, 44.82, 48.90, 54.39, 62.45, 79.14, 94.91 kDa. Additionally, polypeptides of 149.02 and 114.21 kDa were also

detected in *S.aureu*s SA16 and SA2 respectively. Further, proteins of *S. aureu*s SA16 and SA2 strains grown under BF mode revealed 25.43, 26.69, 30.59, 34.02, 37.05, 40.77, 44.82, 48.90, 51.77, 54.39, 57.22, 65.09, 94.91, 102.72 and 114.21 kDa peptides (Fig.2 and Table 3). The polypeptides of 19.03, 22.61, 25.25, 28.6, 33.3, 38.09, 39.91, 62.45, 79.14 and 149.02 kDa expressed in *S. aureu*s SA16 FC proteins were not detected in BF proteins, whereas the polypeptides of 26.69, 30.59, 34.02, 40.77, 51.77, 57.22, 65.09, 102.72 and 114.21 kDa expressed in *S. aureu*s SA16 BF proteins were not detected in FC proteins. Further, the polypeptides of 51.77 and 57.22 kDa were detected only in *S. aureu*s SA16 BF proteins but not in *S. aureu*s SA2 BF proteins

4.3 Western blot analysis of BF and FC proteins **of *S.aureus***

Western blot studies using hyper immune sera (HIS) against BF and FC proteins of *S.aureus* showed the evidence of antibody response against proteins of *S.aureus* SA16 and SA2 grown under BF and FC mode. On probing with hyper immune sera against *S.aureus* SA16 BF proteins, six immunogenic proteins, three in the region between 29 and 43 kDa with prominence at 30.59, 34.02 and 37.05 kDa were observed in BF proteins of *S. aureus* SA16 and SA2. Further, immunogenic proteins of 94.91 and 114.21 kDa were noticed in BF proteins of *S.aureus* SA16. Whereas, in case of BF proteins of *S.aureus* SA2, diffused immunogenic bands were noticed at this region. An additional 54.39 kDa immunogenic protein was observed in BF proteins of *S.aureus* SA16. Probing of FC proteins of *S.aureus* SA16 and SA2 with BF HIS revealed diffused bands in the region of 25.25 - 28.60, 54.39 and 62.45 kDa with an additional band at 38.09 kDa in *S.aureus* SA16 alone (Fig.3a and Table 4)

On probing with hyper immune sera against FC proteins of *S.aureus* SA16, polypeptides of 25.25, 28.60, 54.39 and 62.45 kDa in case of FC proteins of *S.aureus* SA16 and 25.25 and 28.60 kDa in case of FC proteins of *S.aureus* SA2 were observed. Furthermore, with hyper immune sera against FC proteins, a diffused band in the region of 65 kDa in case of BF proteins of *S.aureus* SA16 and 29-43 kDa in case of BF proteins of *S.aureus* SA2 were noticed (Fig.3b and Table 5).

4.4 Optimization of infective dose to induce mastitis in lactating rabbits

4.4.1 Selection of *S.aureus* isolates

S.aureus SA16 and SA2 isolates were selected for the induction of mastitis in lactating rabbits. These strains were isolated from the cases of sub clinical and clinical bovine mastitis by Rajeev (2006) and maintained in the Department of Veterinary Microbiology. *S.aureus* SA16 was selected as a biofilm and free cell vaccine candidate and *S.aureus* SA2 was selected for heterologous challenge studies. The selection of these isolates was based on possessing *bap* gene which was characterized in the present study and *ica*A gene which was based on earlier studies carried out by Rajeev (2006) in the Dept. of Veterinary Microbiology, Veterinary College, Bangalore.

4.4.2 Induction of mastitis in rabbits

Lactating rabbits (six each) were inoculated with 0.5 ml bacterial suspension of 10^4, 10^5, 10^6 and 10^7 cfu/ml of *S.aureus* SA16 and SA2 at the base of the teat by using 30 G needle and syringe for optimization of infective dose to induce mastitis in rabbits as indicated by gross lesions, SCC and CMT.

4.4.3 Gross lesions

Following inoculation with *S.aureus* SA16 and SA2, the rabbit mammary glands showed an intense tumescence, hyperemia, induration, being warmer than normal gland and painful upon palpation. All the glands that were inoculated with different concentration of bacteria showed lesions of varying degree. The lesions were well appreciated after 24 hours of inoculation, but the maximum lesions were seen after 48 hours of inoculation and then the lesions started subsiding (Figs. 4b, 4c & 4d). Mean percentage of glands showing lesions at 48 hrs post inoculation with 10^4, 10^5, 10^6 and 10^7 cfu/ml of bacterial suspension of *S.aureus* SA16 were 20, 30, 80 and 80 per cent respectively (Fig.5), whereas with *S.aureus* SA2, the lesions were 20, 20, 80 and 80 per cent respectively(Fig.6). There was a drastic reduction in the mean percentage of mammary glands infected from third day onwards that it had reduced to zero (Fig 4d). Twenty per cent of infection was seen with 10^4 cfu/ml of bacterial suspension for both the isolates (Figs.5 and 6).

4.4.4 Direct microscopic Somatic cell counts

Milk samples collected were subjected to direct microscopic SCC using Newman-Lampert stain. The SCC including leucocytes particularly heterophils, desquamated epithelial cells and macrophages stained deep blue (Figs.7a and 7b). Pre inoculation milk had SCC of 3.48×10^5 cells/ml. Mean SCC in milk at 48 hours post inoculation with 10^4, 10^5, 10^6 and 10^7 cfu/ml of bacterial suspension of *S.aureus* SA16 were 13.28×10^5 cells/ml, 12.44×10^5 cells/ml, 17.72×10^5 cells/ml and 17.68×10^5 cells/ml respectively (Fig.8), whereas with *S.aureus* SA2 based infection, it was 24.48×10^5 cells/ml, 24.46×10^5 cells/ml, 27.08×10^5 cells/ml and 30.76×10^5 cells/ml respectively (Fig.9).

Maximum SCC were obtained at 72 hours post infection compared to 24 hrs for both the strains and thereafter SCC were reduced (Figs. 8 and 9).

4.4.5 California mastitis test

Milk samples collected were subjected to CMT. Mean percentage of CMT positivity in milk of S.aureus *SA16 infused mammary glands with 10^4, 10^5, 10^6 and 10^7 cfu/ml were 30, 50, 50 and 70 per cent respectively at 48 hrs. post inoculation (Fig.10), whereas* S.aureus *SA2 infused mammary glands were 40, 50, 80 and 90 per cent respectively (Fig.11). Maximum positivity to CMT was found on day two after inoculation. It had reduced to zero on day five in the glands inoculated with both strains (Fig. 10 and 11).*

4.4.6 Selection of challenge dose

Among all the concentrations of bacterial suspensions, 10^4 cfu/ml had infected 50 per cent of the mammary glands inoculated with both *S.aureus* SA16 and SA2. Hence, this dose was found to be an optimum challenge dose. The maximum infection was seen after 48 hrs of inoculation.

4.5 Immunization

4.5.1 S.aureus *biofilm and free cell vaccine*

S.aureus *SA16 BF and FC vaccine after inactivation with 0.1 per cent formalin was found sterile as there was no growth after inoculation of the same on to Nutrient agar, Mannitol salt agar, Blood agar, Brain heart infusion agar and Robertson bullock heart medium even after seven days.*

4.5.2 Evaluation in vaccinated rabbits

Pregnant Rabbits vaccinated with *S.aureus* SA16 BF and FC vaccines were evaluated for gross lesions of mammary glands, SCC, CMT and serum IgG response by indirect ELISA after challenge. Challenge was done on day 29 of the experiment (corresponding to 10^{th} day of lactation). Sera samples were collected at different intervals (days 0, 15, 22, 29, 30, 31, 35, 43 and 50 of the experiment) and evaluated for specific IgG response by indirect ELISA. Milk samples were collected on day 0 (pre challenge), 1, 2, 6, 14 and days 21 post challenge and evaluated for SCC and CMT.

4.5.3 Gross lesions

The mean percentage of mammary glands showing lesions at 48 hrs after challenging with homologous strain (*S.aureus* SA16) was 14.55 per cent and with heterologous strain (*S.aureus* SA2) was 24.95 per cent for BF vaccinated group. For FC vaccinated group, mean percentage of mammary glands showing lesions after challenging with homologous strain (*S.aureus* SA16) was 50 per cent, whereas it was 62.5 per cent for heterologous strain (*S.aureus* SA2). After 48 hrs of challenge, it was observed that there was a drastic reduction in the mean percentage of mammary glands with lesions for BF and FC vaccinated group. But, in Group 1 (BF vaccinated - homologous challenged) and Group 2 (BF vaccinated - heterologous challenged) it had reduced to zero and 4.15 per cent respectively at 14 days post challenge, whereas in case of FC vaccinated group, it was 18.75 per cent with homologous challenge and 37.5 per cent with heterologous challenge. In Group 2 (BF vaccinated - heterologous challenged) and Group 3 (FC vaccinated - homologous challenged), it had reduced to zero at 21 days post challenge. One-way ANOVA (Tukey's multiple comparison test) was carried out to

compare different groups. There was a significant ($P<0.05$) difference between BF vaccinated groups and FC vaccinated groups and also there was a significant ($P<0.05$) difference within the BF vaccinated and FC vaccinated groups (Fig. 12).

4.5.4 Direct microscopic Somatic Cell Counts

Mean SCC in the pre challenge milk in the rabbits was very low in all the groups and the values varied from 5×10^5 cells/ml to 8×10^5 cells/ml (Fig.13). On day one post challenge, mean SCC in milk for BF vaccinated rabbits with homologous challenge was 10×10^5 cells/ml and with heterologous challenge, it was 27×10^5 cells/ml. Mean SCC for FC vaccinated group with homologous challenge was 15×10^5 cells/ml and with heterologous challenge, it was 37×10^5 cells/ml. Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparison test). A highly significant ($P < 0.01$) difference was observed within the BF vaccinated (Group 1 and 2) and FC vaccinated groups (Group 3 and 4). But, there was no significant ($P > 0.05$) difference between BF vaccinated-homologous challenged and FC vaccinated-homologous challenged groups.

On day two post challenge, mean SCC for BF vaccinated rabbits, challenged with homologous strain was 12.96×10^5 cells/ml and with heterologous strain, it was 31.5×10^5 cells/ml. For FC vaccinated group, challenged with homologous strain, SCC was 14.5×10^5 cells/ml and with heterologous strain, it was 41.15×10^5 cells/ml. Peak SCC response was obtained after two days of challenge that is on day 12 of lactation. There was a highly significant ($P<0.01$) difference within the BF and FC vaccinated groups. But, the BF and FC vaccinated groups challenged with homologous strain did not differ significantly ($P>0.05$).

Milk samples collected on days 6, 14 and 21 post challenge have also yielded same results (Fig.13). It was observed that there was a reduction in the mean percentage of SCC in BF and FC vaccinated groups after 48 hrs of challenge.

4.5.5 California mastitis test

Mean percentage of CMT positive milk from mammary glands challenged with *S.aureus* SA16 at 48 hrs post challenge was 9.37 per cent and with *S.aureus* SA2 was 12.5 per cent for BF vaccinated rabbits. For FC vaccinated rabbits, mean percentage of CMT positive milk from mammary glands with homologous challenge was 46.87 per cent and with heterologous challenge it was 56.25 at 48 hrs post challenge. On day 6, it had reduced to 4 per cent in both the BF vaccinated groups and 29.16 and 25 per cent in FC vaccinated – homologous and heterologous challenged groups respectively. But, in case of BF vaccinated groups, there was a drastic reduction in mean percentage of CMT positivity to zero at day 14 after challenge. Statistical analysis was done using one-way ANOVA (Tukey's multiple comparison test). There was a significant difference ($P < 0.05$) between BF vaccinated and FC vaccinated group. But, there was no significant difference ($P > 0.05$) with in the BF vaccinated group (Fig.14).

4.5.6 Enzyme Linked Immunosorbent Assay

4.5.6.1 Optimization of *S.aureus* SA16 biofilm protein antigen

Indirect ELISA was standardized by optimizing antigen dilution at constant 1:100 dilution of C+ and C- sera by using goat anti-rabbit IgG-HRP conjugate at constant 1:25,000 dilution.

The mean OD values with respect to varying dilution of *S.aureus* SA16 BF protein antigen from 1:10 (10µg protein/ml) to 1:320 (0.3125µg protein/ml) against a constant 1:100 dilution of C+ and C- are presented in Table 6. It was observed that OD values suddenly dropped at 1:80 dilution of antigen as shown in Fig.15 and Table 6. Hence, 1:80 dilution (1.25 µg) of *S.aureus* biofilm protein antigen was used as optimum working dilution of antigen for monitoring *S.aureus* BF vaccinal antibodies in sera collected on days 0 (corresponding to 12th day of gestation at which first shot of vaccine was administered), 15 (26th day of gestation), 22 (3rd day of lactation), 29 (10th day of lactation at which challenge was done), 30, 31, 35, 43 and 50 from both homologous (*S.aureus* SA16) and heterologous (*S.aureus* SA2) challenged groups of rabbits vaccinated with BF and FC vaccines.

4.5.6.2 Seromonitoring of post vaccinal antibodies in vaccinated rabbits

Sera samples were collected from both *S.aureus* BF and FC vaccinated rabbits at different intervals, which included pre vaccinal (12th Day of gestation), 15 days after first immunization (26th day of gestation/on the day of first booster), a week after the first (3rd day of lactation/ on the day of second booster/ 22nd day of experiment) and second booster (29th day of experiment/ on the day of challenge), 24, 48 hrs, 6, 14 and 21 days post challenge with homologous *S.aureus* SA16 and heterologous *S.aureus* SA2. The average OD values of the sera samples collected at aforementioned periods were converted to PP values to obtain uniformity in results (Table 7 and Fig.16).

4.5.6.2.1 Comparison of homologous and heterologous challenged groups

Statistical analysis was carried out using two tailed unpaired 't' test of PP values of sera samples collected from four groups (*viz.*, 1, 2, 3 and 4) at days 0, 15, 22, 29, 30, 31, 35, 43 and 50 to find out the differences between homologous and heterologous challenged rabbits within BF and FC vaccinated groups. The comparisons were made between Group 1 & 2 (BF vaccinated - homologous challenged v/s BF vaccinated – heterologous challenged) and Groups 3 & 4 (*i.e.*, FC vaccinated - homologous challenged v/s FC vaccinated - heterologous challenged). Analysis showed that the difference in the PP values of sera collected on days 0, 15, 22, 29, 30, 31, 35, 43 and 50 from Groups 1 & 2 was 'non-significant' ($P>0.05$). Likewise, when Groups 3 & 4 were compared, the difference in the PP values of sera collected on days zero, 15, 22, 29, 30 and 31 was non-significant ($P>0.05$) as shown in Fig.16 and Table 7.

4.5.6.2.2 Comparison of biofilm, free cell vaccinated and control groups

Statistical analysis was made to compare BF vaccinated and FC vaccinated groups by two tailed't' test, based on PP values of serum samples collected at days 0, 15, 22, 29, 30, 31, 35, 43 and 50 (Fig.16 and Table 7). Analysis showed that the difference in the PP values of sera collected on days 0 and 15, from BF vaccinated and FC vaccinated groups was 'non-significant' ($P>0.01$). But, a highly significant ($P< 0.01$) difference was obtained in the PP values of sera collected from BF vaccinated and FC vaccinated groups on days 22, 29, 30, 31 and 35. The highly significant ($P<0.01$) difference was obtained in the PP values of sera collected from BF vaccinated and FC vaccinated groups on days 43 and 50.

4.5.6.2.3 IgG response in pre challenge sera

The comparison of PP values of pre vaccinal sera collected from BF, FC vaccinated and control rabbits on day '0' showed non-significant difference ($P>0.05$), Whereas on day 15 and 22, significant ($P<0.05$) difference was obtained in the PP values of sera collected from BF vaccinated and control rabbits and non-significant difference ($P>0.05$) was obtained with respect to PP values in rabbits vaccinated with free cells. Further, a highly significant difference ($P<0.001$) was noticed in BF, FC vaccinated and control rabbits sera collected on day 29 *i.e.,* pre challenge. Both FC and BF vaccinated rabbits were challenged on day 29 (corresponds to the 10^{th} day of lactation).

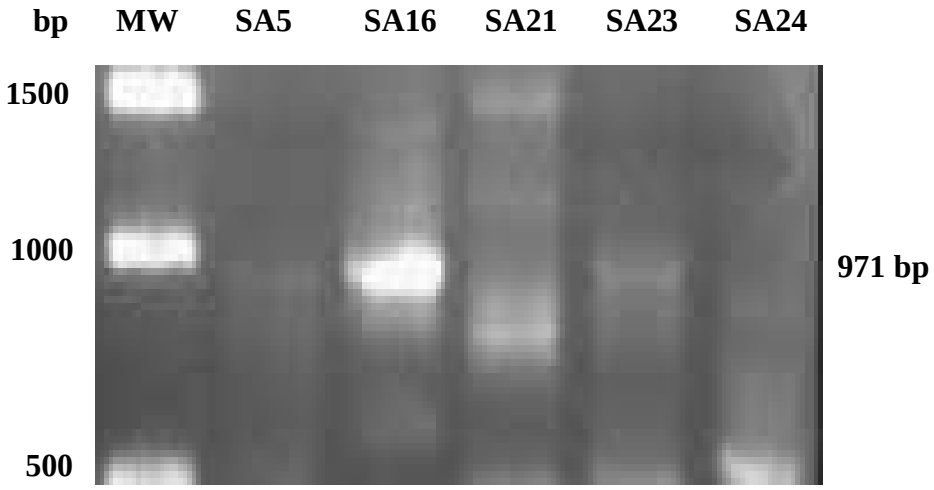
4.5.6.2.4 IgG response in post challenge sera

The comparison of PP values of sera collected from BF, FC vaccinated and control rabbits on day 30, (*i.e.*,24 hrs after challenge) showed a significant ($P < 0.01$) difference among BF, FC vaccinated and control rabbits and it was observed that there was a sudden drop in the PP values on day 30. Further, on day 31, (48 hrs after challenge), a significant difference ($P < 0.01$) was observed among BF, FC vaccinated and control rabbits and there was significant increase in the PP values from day 31 onwards in sera collected from BF vaccinated groups and reached 67.78 in Group 1 (BF vaccinated - homologous challenged) and 56.38 in Group 2 (BF vaccinated – heterologous challenged) compared to 40.23 in Group 3 (FC vaccinated - homologous challenged) and 35.97 in Group 4 (FC vaccinated - heterologous challenged) on 50^{th} day of the experiment which is clearly evident (Fig.16 and Table 7).

Table 2: '*bap*' positive and *ica*A positive *S.aureus* isolates

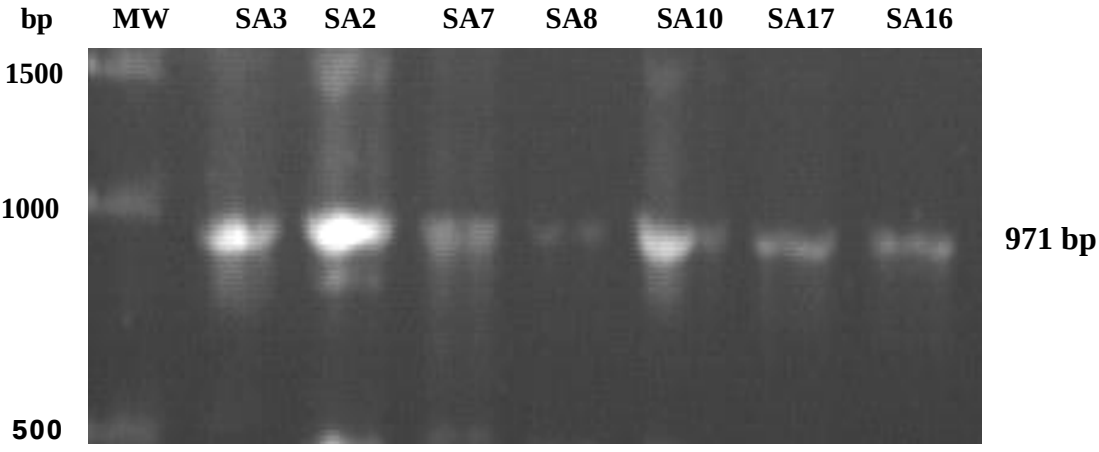
Sl. No.		***S.aureus* isolates**	**'*Bap*' gene**	***ica*A gene**
1	Clinical mastitis	SA1	-	+
2		**SA2**	**+**	**+**
3		SA3	+	+
4		SA4	+	+
5		SA5	-	-
6		SA6	-	+
7	Sub clinical mastitis	SA7	+	+
8		SA8	+	+
9		SA9	-	+
10		SA10	+	+
11		SA11	-	+
12		SA12	-	+
13		SA13	-	+
14		SA14	-	+
15		SA15	+	+
16		**SA16**	**+**	+
17		SA17	+	+
18		SA18	-	-
19		SA19	-	+
20		SA20	-	+
21		SA21	-	+
22		SA22	-	+
23		SA23	+	+
24		SA24	-	+
25		SA25	-	+

Fig. 1a: PCR profile with '*bap*' gene specific primers for *S. aureus* isolates



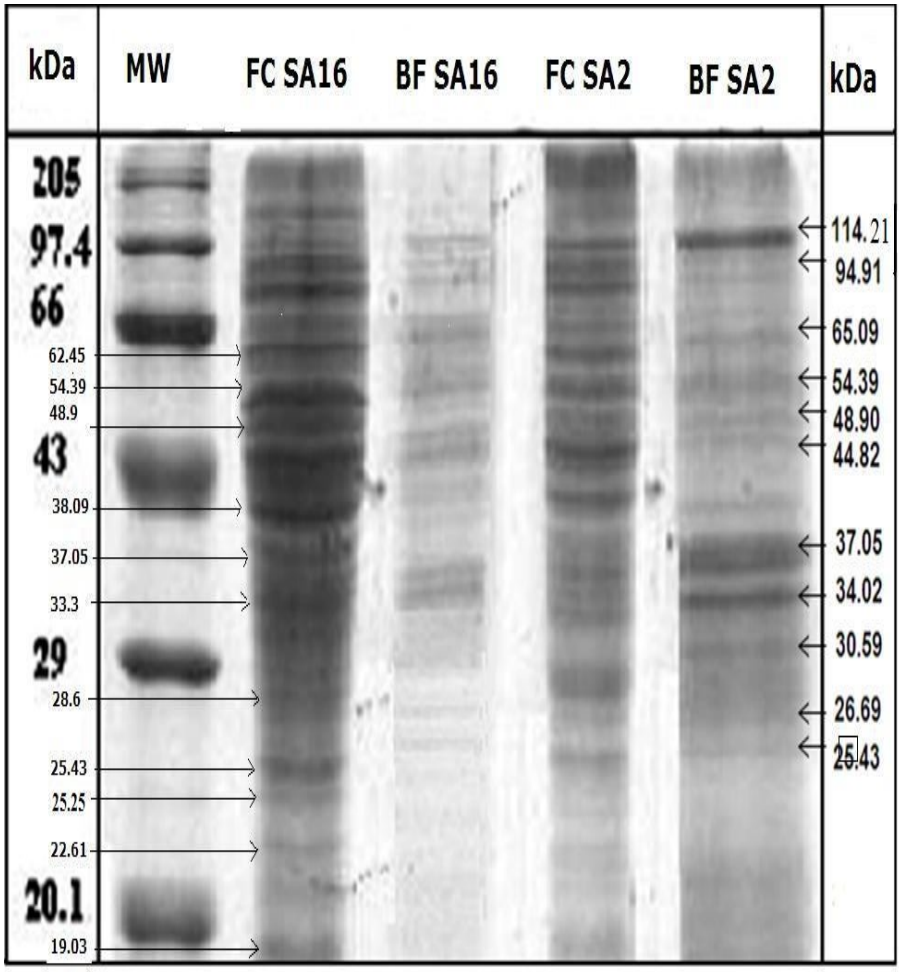
Lane 1: MW: 500 bp DNA ladder
Lane 2, 3, 4, 5 and 6: *S. aureus* SA5, SA16, SA21, SA23 and SA24

Fig. 1b: PCR profile with '*bap*' gene specific primers for *S. aureus* isolates



Lane 1: MW: 500 bp DNA ladder
Lane 2, 3, 4, 5, 6, 7 and 8: *S. aureus* SA3, SA2, SA7, SA8, SA10, SA17 and SA16

Fig. 2: Protein profile of *S. aureus* SA16 and SA2 grown under BF and FC mode



Lane 1: MW: Molecular weight markers
Lane 2: *S. aureus* SA16 FC proteins
Lane 3: *S. aureus* SA16 BF proteins
Lane 4: *S. aureus* SA2 FC proteins
Lane 5: *S. aureus* SA2 BF proteins

Table 3: Protein profile of *S. aureus* SA16 and SA2 grown under BF and FC mode

MW (kDa)	SA16 FC	SA16 BF	SA2 FC	SA2 BF
149.02	+	-	+	-
114.21	-	+	+	++
102.72	-	+	-	+
94.91	+++	+	+++	+
79.14	++	-	+++	-
65.09	-	+	-	+
62.45	++	-	+	-
57.22	-	+	-	-
54.39	+++	+	+++	+
51.77	-	+	-	-
48.90	++	+	+	+
44.82	+++	+	++	+
40.77	-	+	-	-
39.91	+++	-	++	-
38.09	+++	-	++	+
37.05	+	+	+	+++
34.02	-	+	-	+
33.30	+	-	+	-
30.59	-	+	-	+
28.60	+	-	+++	-
26.69	-	+	-	+
25.43	++	+	-	+
25.25	++	-	+	-
22.61	+	-	+	-
19.03	+	-	+	-

- Absent
++ Moderately prominent
+ Less prominent
+++ More prominent

Fig. 3a: Western blot analysis of *S. aureus* BF and FC proteins probed with *S.aureus* SA16 BF hyper immune serum

BF HIS

Fig.3a

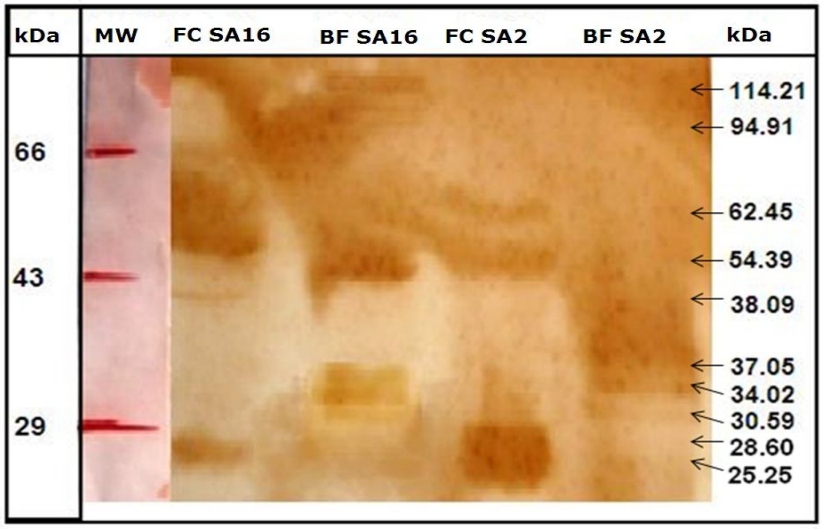


Table 4: Western blot analysis of *S. aureus* BF and FC proteins probed with *S.aureus* SA16 BF hyper immune serum

***S.aureus* SA16 FC proteins (kDa)**	***S.aureus* SA16 BF proteins (kDa)**	***S.aureus* SA2 FC proteins (kDa)**	***S.aureus* SA2 BF proteins (kDa)**
-	114.21	-	-
-	94.91	-	-
62.45(+++)	-	62.45 (++)	-
-	54.39(+++)	54.39 (++)	-
38.09 (+)	-	-	-
-	**37.05**	-	**37.05**
-	**34.02**	-	**34.02**
-	**30.59**	-	**30.59**
28.60(+++)	-	28.60	-
25.25 (+++)	-	25.25	-

Fig. 3b: Western blot analysis of *S.aureus* BF and FC proteins probed with *S.aureus* SA16 FC hyper immune serum

FC HIS

Fig.3b

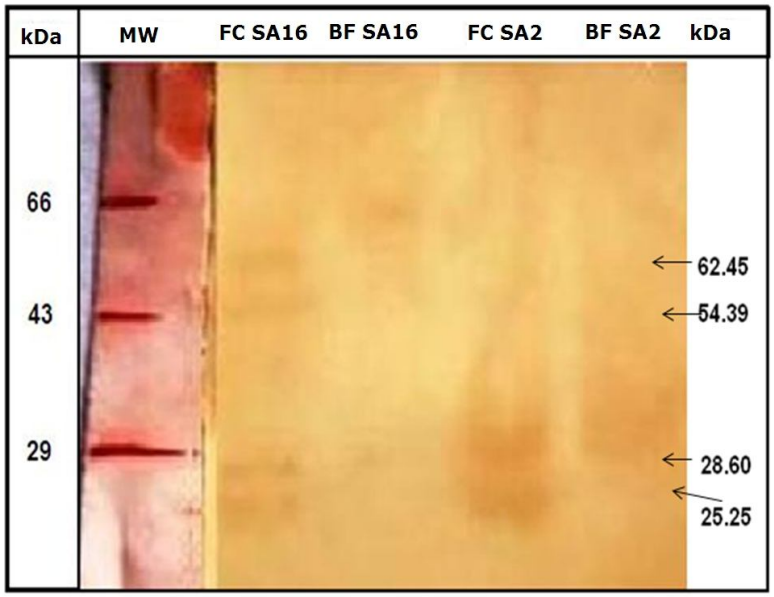


Table 5: Western blot analysis of *S.aureus* BF and FC proteins probed with *S.aureus* SA16 FC hyper immune serum

***S.aureus* SA16 FC proteins (kDa)**	***S.aureus* SA16 BF proteins (kDa)**	***S.aureus* SA2 FC proteins (kDa)**	***S.aureus* SA2 BF proteins (kDa)**
-	65	-	-
62.45	-	-	-
54.39	-	-	-
28.60	-	28.60	29 - 43
25.25	-	25.25	

Fig.4a: Mammary glands showing gross lesions in rabbits inoculated with *S. aureus*

a.Pre inoculation

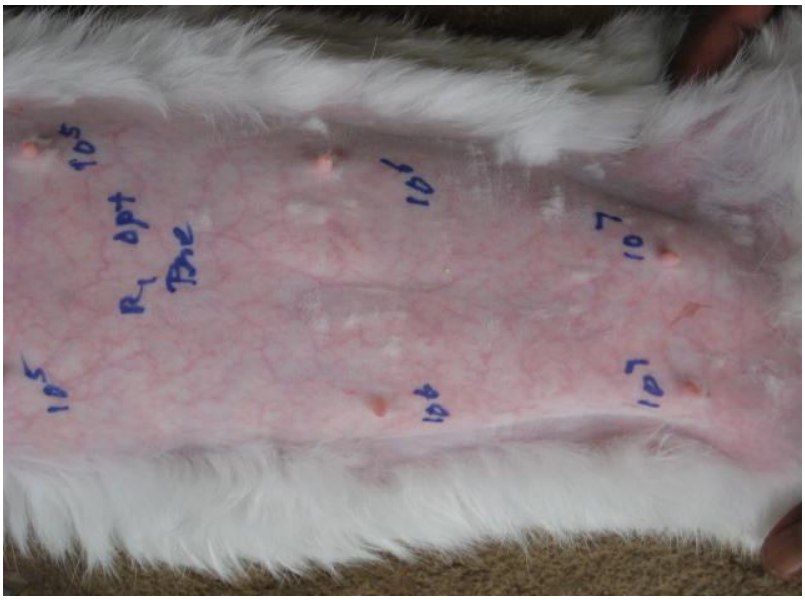


Fig.4b: Mammary glands showing gross lesions in rabbits inoculated with *S. aureus*

b. 24 hours postinoculation

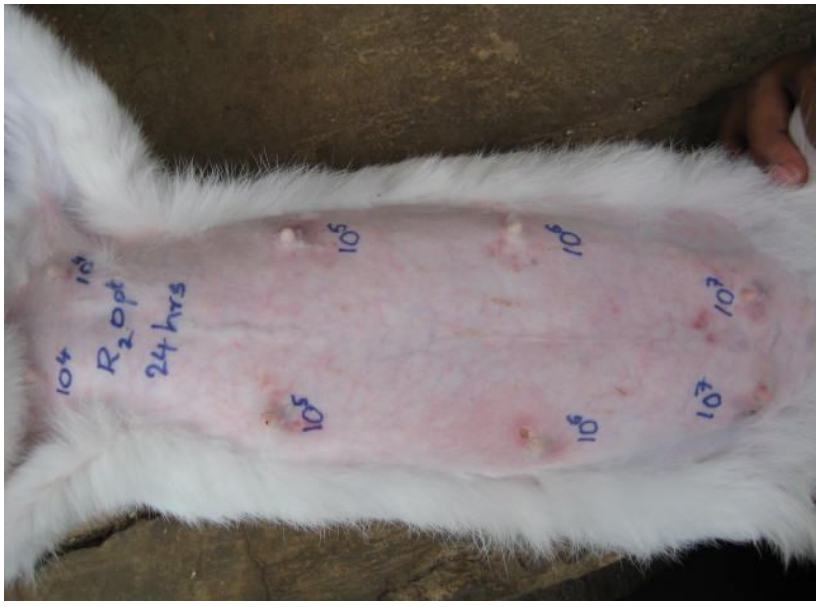


Fig.4c: Mammary glands showing gross lesions in rabbits inoculated with *S. aureus*

c.48 hours post inoculation

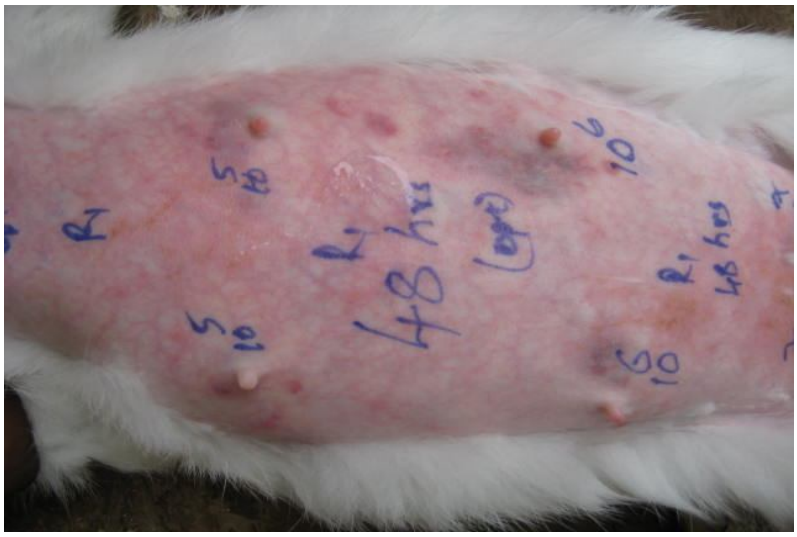


Fig.4d: Mammary glands showing gross lesions in rabbits inoculated with *S. aureus*

d.72 hours post inoculation

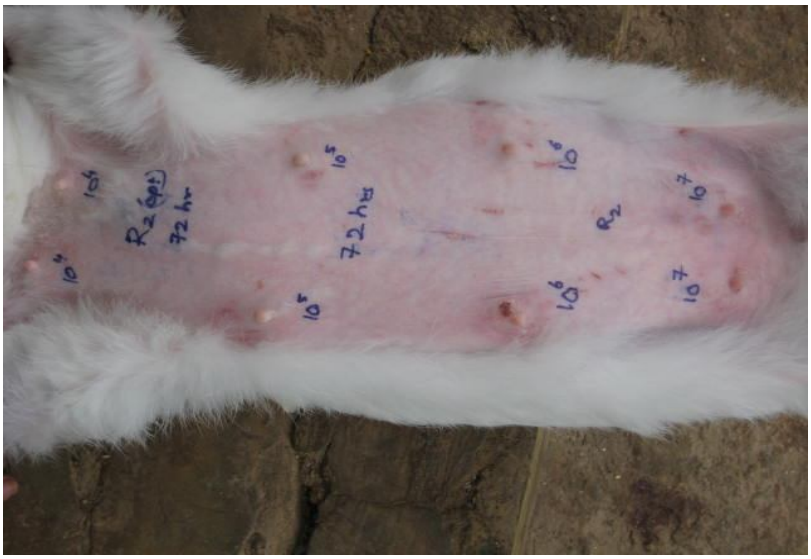


Fig. 5: Percentage of mammary glands showing lesions in rabbits inoculated with *S. aureus* SA16

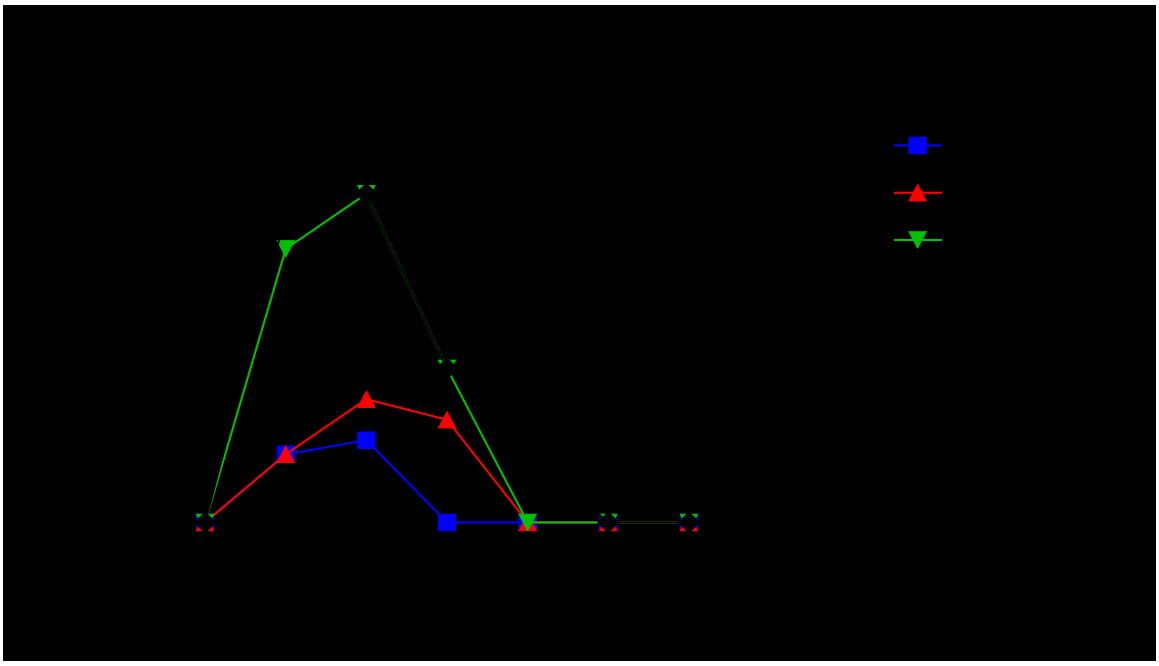


Fig. 6: Percentage of mammary glands showing lesions in rabbits inoculated with *S. aureus* SA2

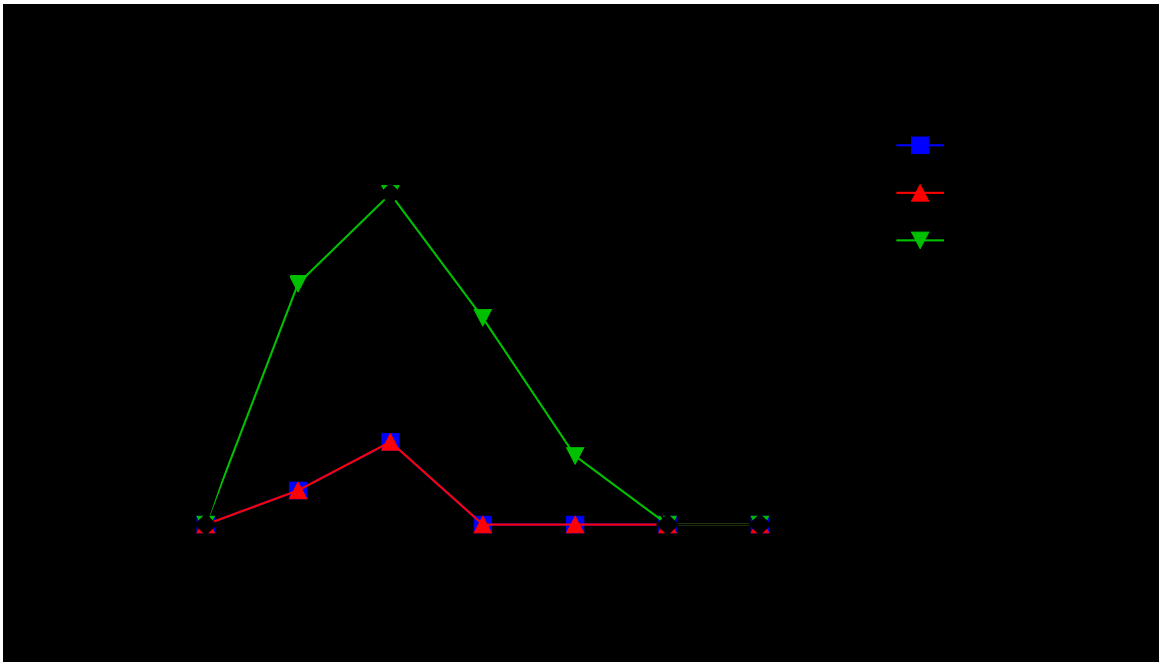


Fig. 7a: Somatic cell counts of pre inoculation rabbit milk (1000X)

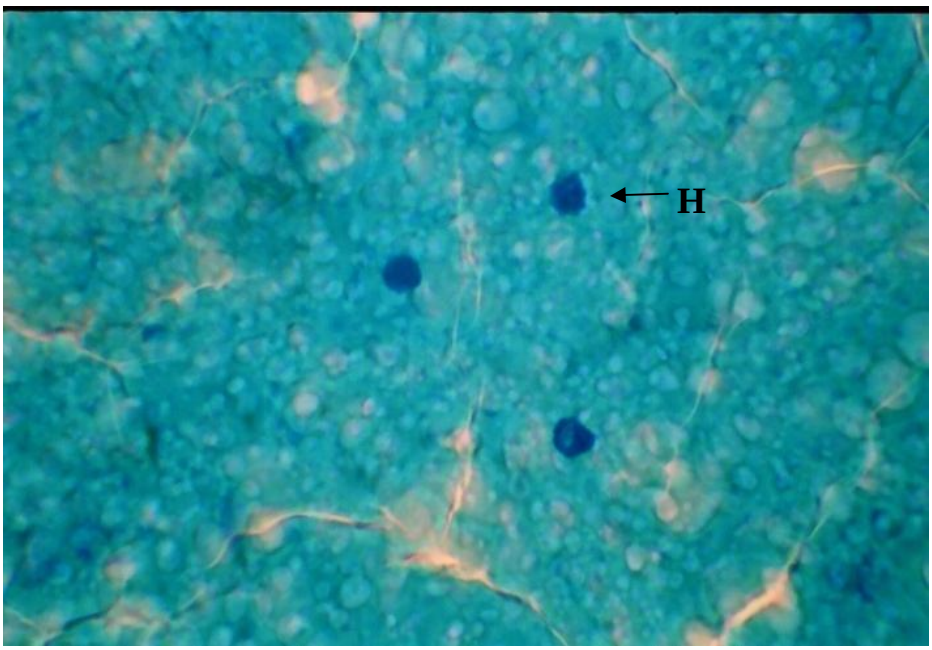


Fig. 7b: Somatic cell count in 48 hrs post inoculation rabbit milk (1000X)

E-Desquamated epithelial cells H-Heterophil

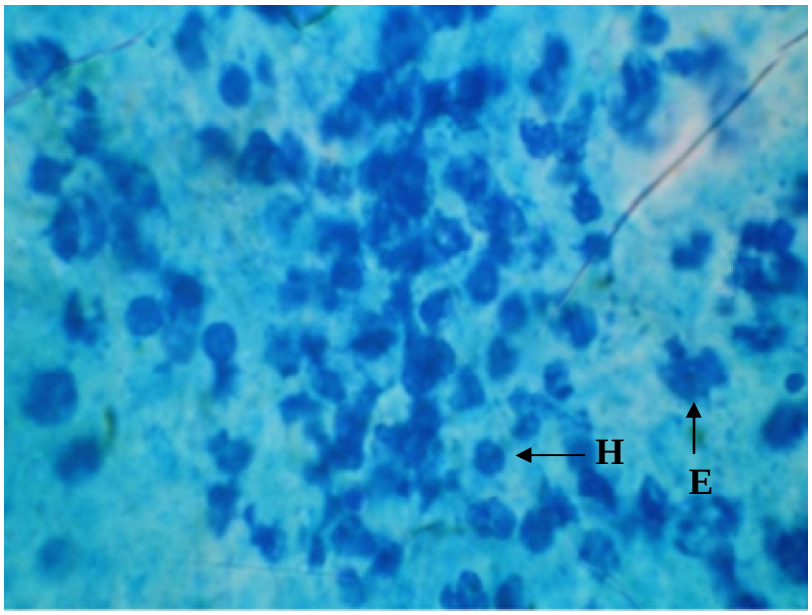


Fig. 8: Optimization of infective dose using* S. aureus *SA16 based on somatic cell counts

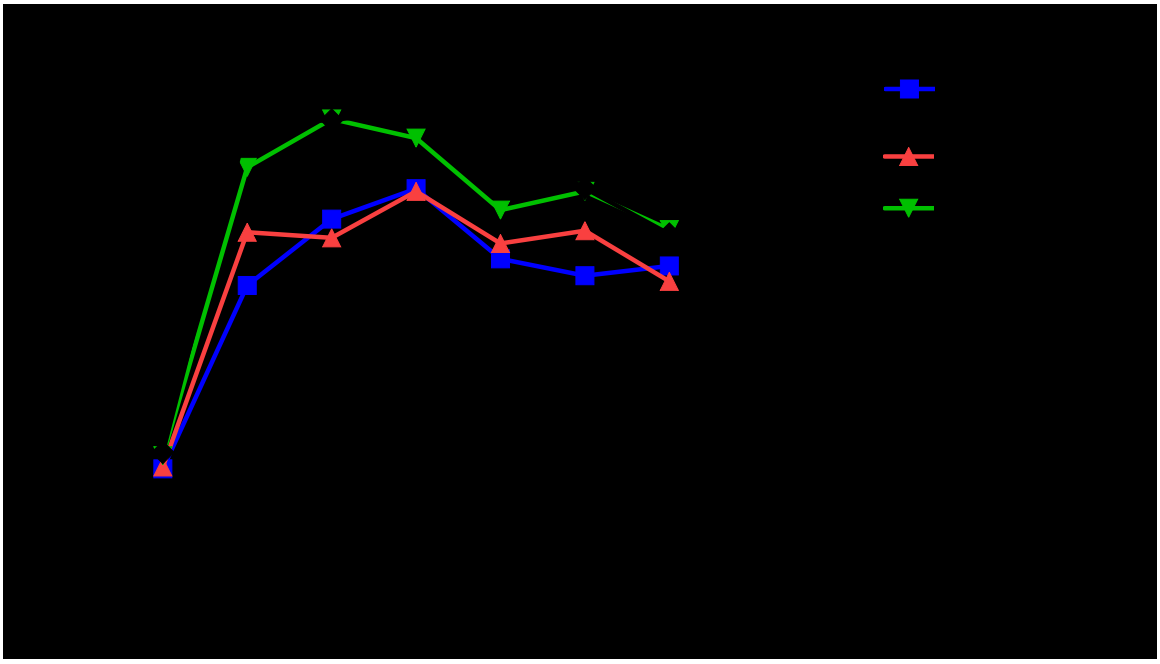


Fig. 9: Optimization of infective dose using* S. aureus *SA2 based on somatic cell counts

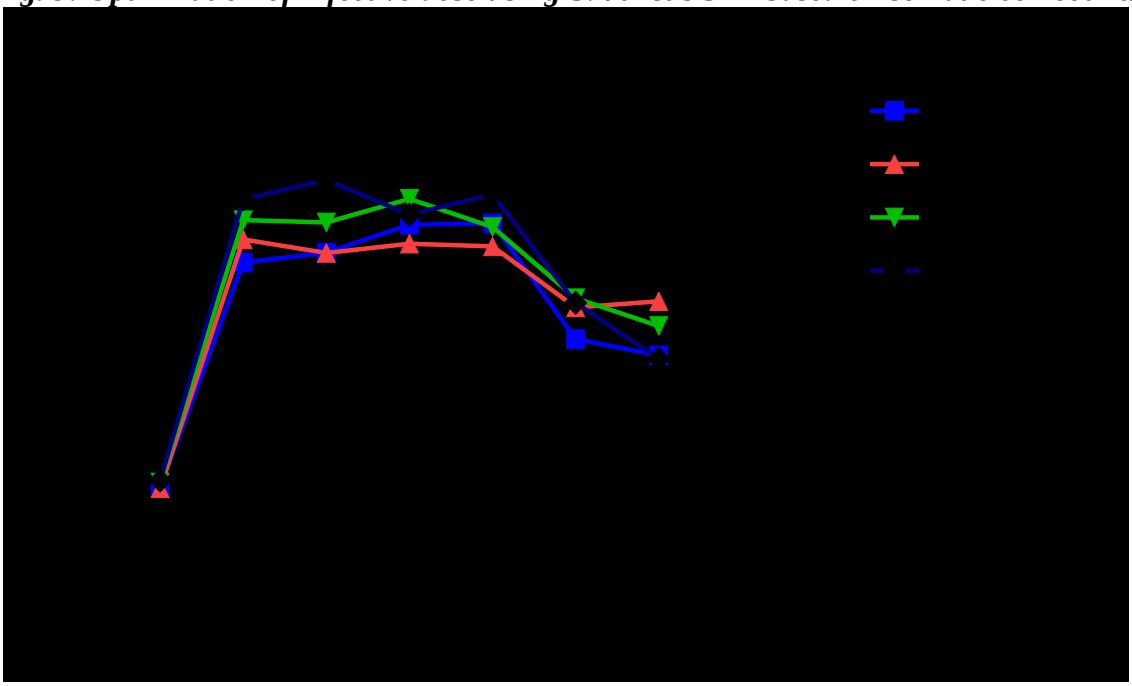


Fig. 10: Percentage of CMT positive milk from rabbits inoculated with *S. aureus* SA16

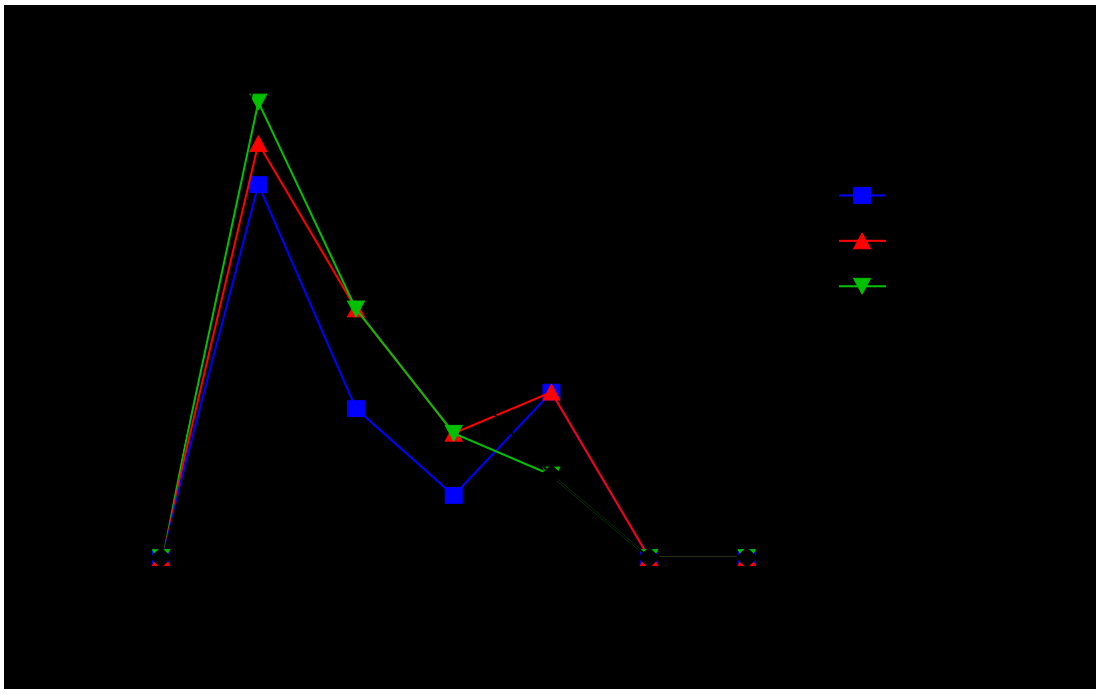


Fig.11: Percentage of CMT positive milk from rabbits inoculated with *S. aureus* SA2

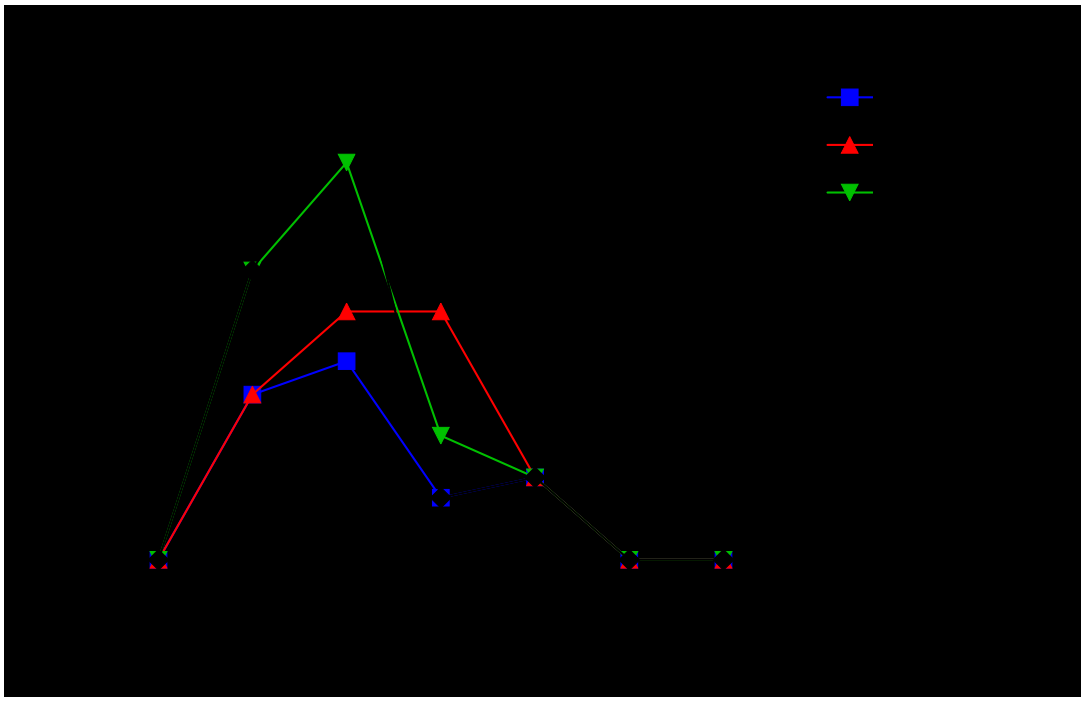


Fig. 12: Percentage of mammary glands showing lesions in *S.aureus* BF and FC vaccinated rabbits challenged with homologous and heterologous strains.

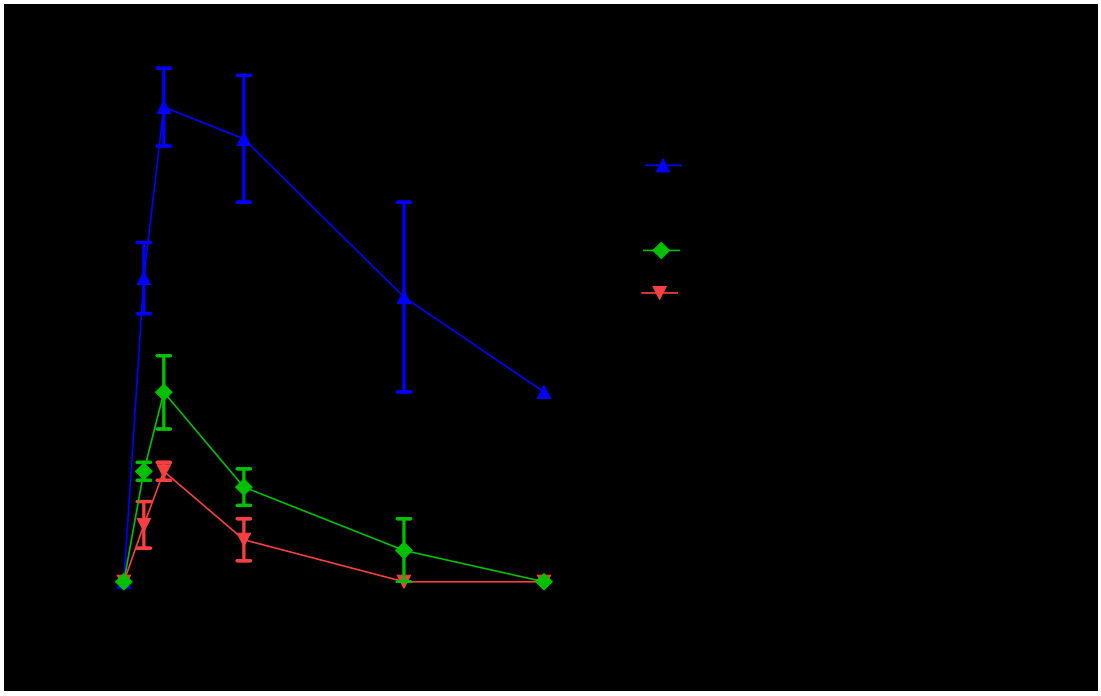


Fig. 13: Somatic cell count response in *S.aureus* vaccinated rabbits challenged with homologous and heterologous strains

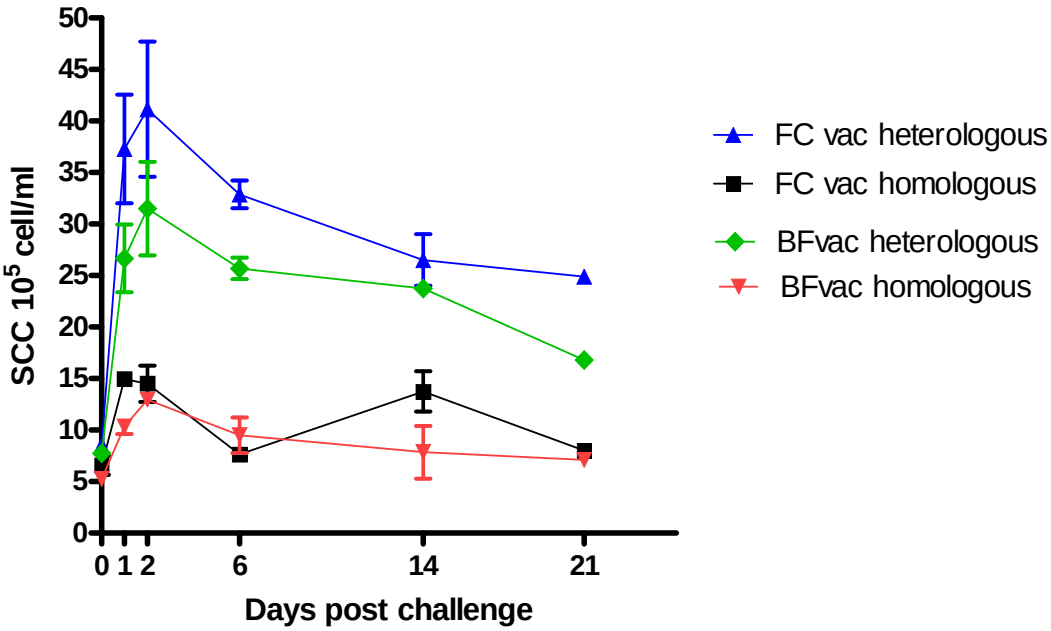


Fig. 14 : Percentage of CMT positivity in *S.aureus* vaccinated rabbits challenged with homologous and heterologous strains

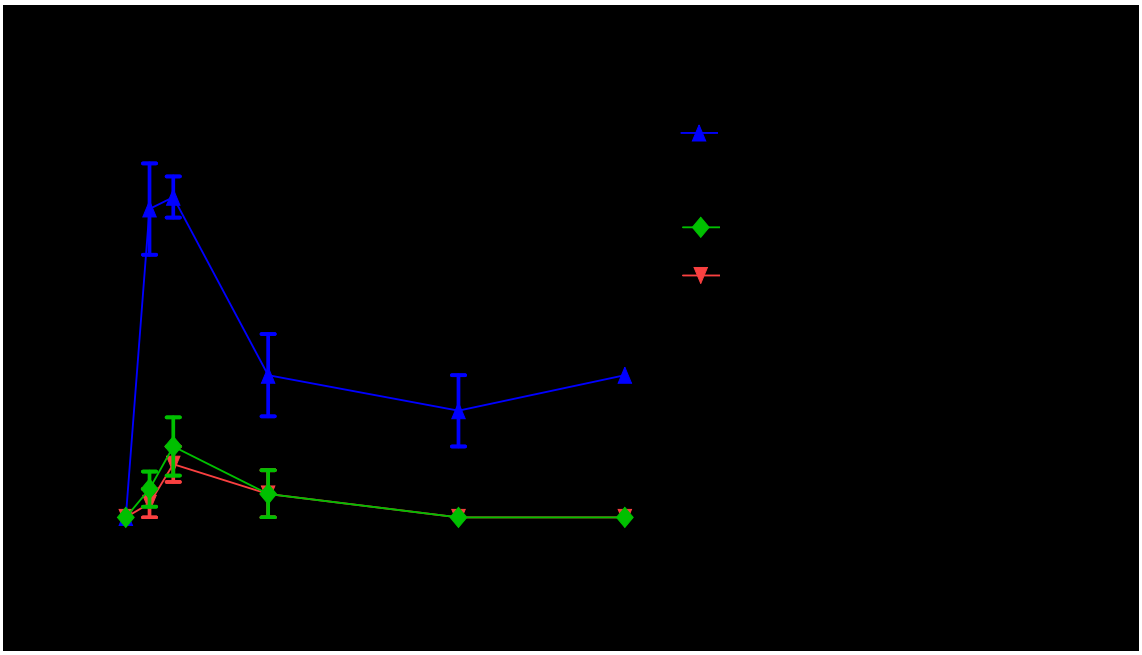


Fig. 15: Determination of optimum dilution of *S.aureus* SA16 BF antigen for use in ELISA

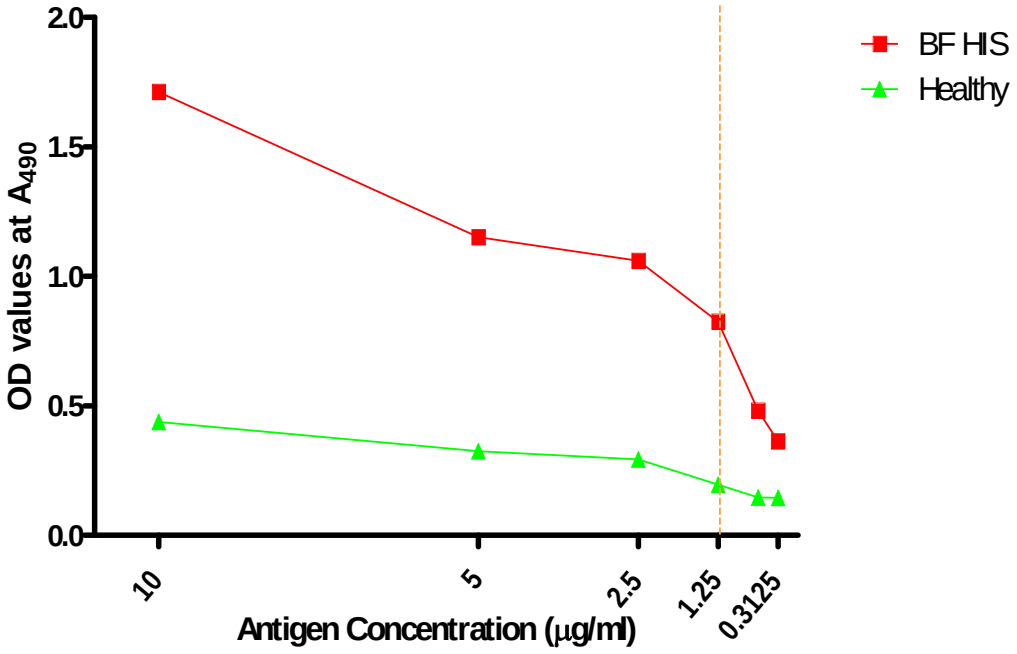


Table 6: Mean OD values of various dilutions of *S.aureus* SA16 BF antigen

Dilution of Antigen (Concentration of antigen in µg/ml)	BF HIS	Healthy serum
1:10 (10.00)	1.7115	0.4380
1:20 (5.00)	1.1510	0.3250
1:40 (2.50)	1.0605	0.2935
1:80 (1.25)	**0.8240**	**0.1960**
1:160 (0.625)	0.4810	0.1470
1:320 (0.3125)	0.3635	0.1455

Fig. 16: Serum IgG response by indirect ELISA in *S.aureus* vaccinated rabbits -Distribution of PP values of serum samples from different groups of rabbits

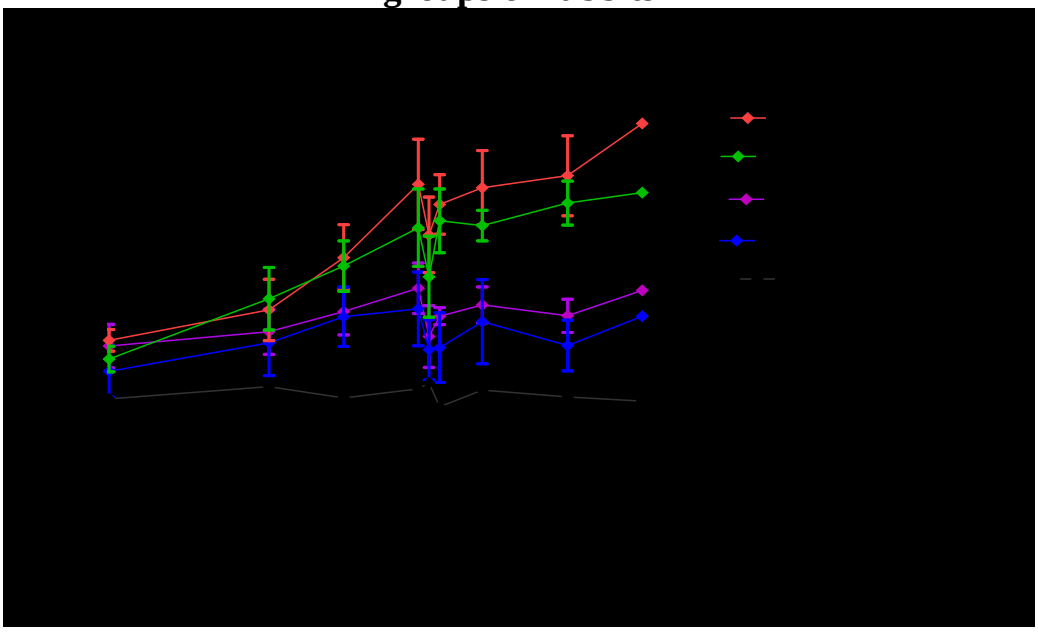


Table 7: Distribution of PP values of the sera samples collected from different groups of vaccinated and control rabbits at various intervals.

	Days serum collected								
	0	**15**	**22**	**29**	**30**	**31**	**35**	**43**	**50**
Group1– *S.aureus* BF vaccinated & homologous challenged	31.97	37.00	45.62	57.73	49.37	54.41	57.15	59.14	67.78
Group 2– *S.aureus* BF vaccinated & heterologous challenged	28.87	38.84	44.23	50.56	42.47	51.71	50.90	54.64	56.38
Group 3 – *S.aureus* FC vaccinated & homologous challenged	31.01	33.40	36.70	40.57	32.6	35.96	37.81	36.01	40.23
Group 4 – *S.aureus* FC vaccinated & heterologous challenged	26.83	31.55	35.87	37.16	30.40	30.76	35.07	31.12	35.97
Group 5 – Control	22.31	24.23	22.45	23.97	24.92	21.00	23.78	22.61	21.95

V. DISCUSSION

Mastitis remains as one of the most important diseases in dairy cattle despite the progress made in improving general udder health in recent years. It continues to be one of the economically most important diseases of dairy cattle, accounting for 38 per cent of the total direct costs of the common production diseases. Losses have been estimated at $2 billion/year,of which 70 per cent was attributed to reduced milk yield from subclinical mastitis. *Staphylococcus aureus* is one of the most frequently isolated contagious mastitis pathogens that causes either clinical or subclinical or chronic bovine mastitis with high economic losses to the farmers. In cows, intramammary infections (IMI) due to *S.aureus*, which account for 25 to 30 per cent of total IMI, are generally subclinical (Leitner *et al*., 2000 and Dego and Tareke, 2003). *Staphylococcus aureus* is a common cause of intramammary infections, which frequently become chronic, associated with the ability of the bacteria to produce biofilm and also recurrent infections are often attributable to biofilm growth of bacteria (Cucarella *et al.*, 2004 and Melchior *et al.*, 2006).

Biofilm formation is accompanied by significant genetic and subsequent physiological changes in the bacteria. A group of surface proteins sharing several structural and functional features is emerging as an important element in the biofilm formation process of diverse bacterial species. The first member of this group of proteins was identified in a *S. aureus* mastitis isolate and was named 'BAP' for biofilm-associated protein. As common structural features, Bap- related proteins: (i) are present on the bacterial surface; (ii) confer upon bacteria the capacity to form a biofilm; (iii) play a

relevant role in bacterial infectious processes (Lasa and Penades, 2006 and Latasa *et al*., 2006).

In view of an effective control of mastitis, immunization against mastitis has been a goal of researchers for many years and vaccination against mastitis pathogens is practiced in some dairy farms, especially in western countries. Research on mastitis vaccines has been conducted for at least 35 years. Mastivac I, a newly introduced vaccine designed to protect *S.aureus* mastitis, is being commercially used in Israel since 2004 (Leitner *et al.,* 2008). Many other conventional vaccines are also commercially available against *S.aureus* mastitis. The efficacy of such vaccines in reducing the severity of clinical disease has been demonstrated (Yancey, 1993; Nordhaug *et al*., 1994a; Tenhagen *et al*., 2002; Leitner *et al*., 2003b and Lee *et al*., 2005) but the vaccines were unable to prevent new intramammary infections. Further, the efficacy of conventional *S.aureus* vaccines has yet to prove their effectiveness with respect to Indian perspective. Recently, *E.coli* BF and FC based vaccines were compared by vaccination trials in rabbits (Kavitha, 2008 and Jyothi, 2009) and in lactating cows (Chandrashekhara, 2009). The study in rabbits indicated the superiority of *E.coli* BF vaccine as the CMT positivity, SCC values and percentage of mammary glands showing lesions on challenging, were significantly less in case of BF vaccinated groups. Further, specific IgG levels in serum and milk and specific IgA levels in milk as detected by ELISA were significantly high in BF vaccinated than FC vaccinated rabbits. The study in lactating cows with *E.coli* biofilm vaccine has indicated better humoral and cell mediated immune response as the serum IgG level was significantly high and percentage of CD4+ and CD8+ cells were increased in BF vaccinated than FC vaccinated and control cattle (Chandrashekhara, 2009).

In order to develop an effective vaccine to control bovine mastitis caused by *S.aureus,* the present study was undertaken to evaluate the *S.aureus* biofilm based vaccine in rabbits.

5.1 Molecular typing of *S.aureus* isolates for '*bap*'(Biofim associated protein) gene.

The ability of *S.aureus* to form biofilm *in vivo* is considered to be a major virulence factor influencing its pathogenesis in mastitis. 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 *bap* gene is a newly identified gene that encodes the biofilm-associated protein i.e. 'BAP' which is involved in biofilm formation in bovine mastitis causing *S. aureus* (Cucarella *et al*., 2001, Gotz, 2002 and Vautor *et al.,* 2008). The ability of staphylococci to form 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, polysaccharide intercellular adhesion (PIA), an exopolysaccharide 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 defences and antibiotic treatment . Although exopolysaccharides are important and often essential compounds of the biofilm matrix, recent evidences suggest that a biofilm-associated protein in a bovine mastitis causing *S. aureus* isolate plays a leading role during the development of the microbial communities. Later on, other surface proteins homologous to 'Bap' and involved in biofilm development have been described in many Gram-positive and Gram-negative bacteria such as *Esp* of *Enterococcus faecalis* and *BapA* of *Salmonella enterica ssp.enterica* serotype Enteritidis (Cucarella *et al*., 2001, Götz, 2002 and Latasa *et al*.,

2006). Polymerase chain reaction based amplification of *bap* gene not only helps us to identify the potential of *S. aureus* to produce biofilms but also its role in establishment of infection in both clinical and sub clinical mastitis.

In the present study, a PCR analysis was carried out using '*bap*' specific primers for 25 bovine mastitis *S. aureus* isolates (SA1 to SA25) which were isolated from clinical and subclinical mastitis and characterized by Rajeev (2006). Out of the 25 isolates, 10 (40 per cent) isolates were '*bap*' positive that showed an amplicon of 971 bp. Among 10 '*bap*' positive isolates, three isolates (SA2, SA3 and SA4) were from clinical mastitis and seven isolates (SA7, SA8, SA10, SA15, SA16, SA17 and SA23) were from sub clinical mastitis cases (Fig.1a & 1b and Table 2). In the previous study conducted by Rajeev (2006) who had subjected these 25 isolates of *S.aureus* for *ica*A specific PCR and found that 23 (92 per cent) isolates were positive for *ica*A gene. It was found that all the *bap* positive isolates were also *ica*A positive in this study (Table.2). These findings are in agreement with Cucarella *et al.* (2004) who analyzed 195 bovine subclinical mastitis *S.aureus* isolates by PCR using *ica*ADBC and *bap* 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 also studied the relationship between the ability to produce chronic bovine mastitis and biofilm formation and found that *bap*-positive isolates were significantly more able to colonize and persist in the bovine mammary gland *in vivo* and were less susceptible to antibiotic treatments when forming biofilms *in vitro.* In addition, analysis of the structural *Bap* gene revealed the existence of alternate forms of expression of the Bap protein in *S. aureus* isolates obtained under field conditions throughout the animal's life. 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 polysaccharide intercellular adhesin / PIA product (a biofilm matrix polysaccharide).

5.2 Analysis of proteins of *S.aureus* SA16 and SA2 grown under BF and FC mode

Bacteria do not express the same antigens *in vitro* as *in vivo* — a mechanism known as 'phase variation'. When bacteria grow *in vivo*, they must cope up with a hostile environment in which certain nutrients are not abundant, and where the host attempts to eliminate them in different ways. The biofilm form of bacteria grown *in vitro* mimic the *in vivo* antigens (Costerton *et al.*, 1999) and it is a fact that only *in vivo* derived antigens can afford protection against homologous and heterologous serotypes (Heddleston and Rebers, 1972). These studies indicated that antigens expressed in biofilm may play a role in the immunoprophylaxis against mastitis caused by *S.aureus*.

5.2.1 Protein expression profile of *S. aureus* SA16 and SA2 grown under BF and FC mode

In the present study, *S.aureu*s SA16 and SA2 strains were selected to study the immune response against proteins of these two strains grown under BF and FC mode. The selection of these two isolates was based on presence of *bap* gene which was characterized in the present study and also *ica*A gene which was based on earlier studies carried out by Rajeev (2006). Both *ica*A and *bap* participate in the biofilm formation in *S.aureus* by encoding proteins involved in the synthesis of a biofilm matrix

polysaccharide and biofilm associated protein respectively. The biofilm associated protein promotes primary attachment to living or inert surfaces and intercellular adhesion which are the two important stages in biofilm formation as reported by Cucarella *et al.* (2004). *S.aureu*s SA16 and SA2 strains were grown in BF mode according to the method standardized by Naveenkumar (2005) using 0.32 per cent TSB with 0.3 per cent bentonite clay as an inert surface for three days, whereas in FC mode, bacteria were grown in three per cent TSB for 16 hrs.

The protein profiles of *S.aureus* SA16 and SA2 grown under BF mode had differed from *S.aureus* SA16 and SA2 grown under FC mode by 56 per cent with unique expression of 26.69, 30.59, 34.02, 40.77, 51.77, 57.22, 65.09, 102.72 and 114.21 kDa and repression of 19.03, 22.61, 25.25, 28.60, 33.30, 38.09, 39.91, 62.45, 79.14 and 149.02 kDa. The unique proteins of 51.77 and 57.22 kDa were detected only in *S. aureu*s SA16 BF proteins, but not in *S. aureu*s SA2 BF proteins or in FC proteins. The polypeptides of 37.05, 54.39 and 94.91 kDa were expressed in BF and FC of both the strains with more prominent expression of 37.05 kDa protein in BF cells (Fig.2 and Table 3). Similar findings were also observed by Naveenkumar (2005) who reported that the protein profiles of bovine mastitic *S.aureus* BF cells differed from FC by 22 per cent with over expression of 79, 65, 60, 48 and 40 kDa proteins and repression of 85 kDa protein. He also found that the unique proteins of 67, 37, 26 and 20.8 kDa proteins were expressed only in BF cells. The unique expression or over expression of additional proteins or repression of proteins in bacteria grown under BF mode was also reported by Arun, (2002) , Veeregowda, (2003) , Prakash, (2004) and Lacqua *et al*. (2006).

Bacteria grown under BF mode are demonstrably and profoundly different from their FC counterparts in antigenic character. The adhesion of bacteria to surface triggers the expression of a number of genes, making the BF cells phenotypically different from the FC of the same species (Costerton *et al.*,1995). There is a mounting evidence to show that both up and down regulation of number of genes occur in the attaching cells upon initial interaction with the substratum. Combaret *et al.* (2000) found that 22 per cent of the genes were up regulated and 16 per cent were down regulated in BF forming *Pseudomonas aeruginosa.* Genes encoding for enzymes involved in glycolysis or fermentation (phosphoglycerate mutase, triosephosphate and alcohol dehydrogenase) are up regulated in BF forming *S.aureus* and opined that the up regulation of these genes could be due to oxygen limitation in the developed BF, favouring fermentation (Becker *et al.*, 2001).

5.3 Western blotting

Western blotting is an important useful technique extensively used to detect antigenic relatedness and immunogenic components in a crude antigen mixture. This technique was employed to analyse the immunogenicity of BF and FC proteins. In the present study, HIS was raised against *S.aureus* SA16 BF and FC proteins and used to identify the immunogenic proteins and their cross reactivity with heterologous strain of *S.aureus* SA2. Western blot studies using HIS against BF proteins and FC proteins of *S.aureus* showed the evidence of antibody response against proteins of *S.aureus* SA16 and SA2 grown under both planktonic as well as BF mode.

5.3.1 Western blot analysis of *S.aureus* BF and FC proteins probed with *S.aureus* SA16 BF and FC hyper immune serum

On probing with hyper immune sera against *S.aureus* SA16 BF proteins, immunogenic proteins in the region between 29 and 43 kDa with prominence at 30.59, 34.02 and 37.05 kDa were observed in BF proteins of *S. aureus* SA16 and SA2. Further, immunogenic proteins of 94.91 and 114.21 kDa were noticed in BF proteins of *S.aureus* SA16. Whereas, in case of BF proteins of *S.aureus* SA2, diffused immunogenic bands were noticed at this region. An additional 54.39 kDa immunogenic band was observed in BF proteins of *S.aureus* SA16. Probing of FC proteins of *S.aureus* SA16 and SA2 with BF HIS revealed bands in the region 25.25 - 28.60, 54.39 and 62.45 kDa with an additional protein of 38.09 kDa in *S.aureus* SA16 alone (Fig.3a and Table 4).

On probing with hyper immune sera against FC proteins of *S.aureus* SA16, polypeptides of 25.25, 28.60, 54.39 and 62.45 kDa in case of FC proteins of *S.aureus* SA16 and 25.25 and 28.60 kDa in case of FC proteins of *S.aureus* SA2 were observed. Furthermore, with hyper immune sera against FC proteins, a diffused band in the region of 65kDa in case of BF proteins of *S.aureus* SA16 and 29-43 kDa in case of BF proteins of *S.aureus* SA2 were noticed (Fig.3b and Table 5).

In the present study, the important findings of western blot analysis of *S.aureus* proteins was that a total of six immunogenic proteins *viz.* 30.59, 34.02, 37.05, 54.39, 94.91 and 114.21 kDa were detected in *S.aureus* SA16 BF proteins when probed with *S.aureus* SA16 BF HIS. These proteins were not detected either in BF proteins or FC proteins when probed by FC HIS. This indicates the superiority of BF proteins which are

capable of inducing better antibody response compared to FC proteins. Further, the immunogenic proteins of 30.59, 34.02, 37.05 kDa were observed in *S.aureus* SA2 BF proteins, 25.25 , 28.60, 54.39 and 62.45 kDa in FC proteins with an additional protein of 38.09 kDa in *S.aureus* SA16 FC proteins alone when probed with *S.aureus* SA16 BF HIS indicating the cross reactivity of BF proteins. But, such immunogenic cross reactivity was not observed when blot was probed with FC HIS. These findings are in line with observations of Naveenkumar (2005) who analysed bovine mastitic *S.aureus* BF and FC proteins by western blotting. The proteins of BF and FC when probed with BF hyper immune serum showed thirteen immunogenic proteins including over expressed 79, 65, 60, 48 and 40kDa proteins along with the unique proteins of BF cell 67, 37, 26 and 20.8 kDa were found to be immunogenic. Similar observations were also made by Arun (2002) who reported that a maximum of eight and five BF OMPs of *Pasteurella multocida* homologous and heterologous strains respectively were detected by *Pasteurella multocida* A:1 BF hyper immune serum indicating immunogenicity and cross reactivity of BF OMPs.

Antibody response detected against proteins of both the strains with recognition of extra proteins as immunogens confirmed the superiority of BF-based antigen with respect to their cross protection. Additional immunogenic proteins were recognized by *S.aureus* SA16 BF protein HIS in antigen grown under BF mode compared to FC protein HIS probing. Similar findings were also observed in BF of *S*. Gallinarum (Prakash and Krishnappa, 2002), *P. multocida* A: 1 (Arun, 2002), *E. coli* (Veeregowda, 2003) and bovine mastitic *E. coli* (Sumathi (2005) BF cells. Kavitha (2008) studied the western blot analysis of bovine mastitic *E.coli* BF and FC OMPs. She reported that additional

24.4, 28.5 kDa polypeptides in the case of OMPs of both *E.coli* O9 and O147 grown under BF mode and 34.5 kDa polypeptide in the case of *E.coli* O147 grown under BF mode when probed with *E.coli* O9 BF HIS indicating the immunogenicity and cross reactivity of novel proteins expressed when *E.coli* was grown under BF mode.

These observations proved that the BF cells expressing some unique proteins which are highly immunogenic but are absent in FC and may play an important role in protection. To conclude, BF antigens may be the suitable vaccine candidates against mastitis. The current work, supporting the earlier findings of Naveenkumar (2005) on immunoblot analysis indicated that the protein expression profile of *S. aureus* isolated from bovine mastitis cases and then grown under BF mode differed from their FC counterparts. It can be concluded that antigen grown under BF mode expressed cross-reactive proteins, which can be subsequently incorporated in the bovine mastitis vaccine to confer cross protection.

5.4 Experimental studies on mastitis in rabbits

Rabbits have been considered to be good animal models for mastitis studies as the lactating mammary gland of the rabbit is susceptible to natural infection by staphylococci and the disease could be reproduced in the laboratory by injecting low numbers of organisms into the mammary tissue. It is easier to handle them for intramammary injections or infusions (Adlam *et al.* 1977 and Adlam *et al*. 1980). Bovine and ovine mastitis studies have been carried out in rabbit model by Amorena *et al.* (1991), Reinoso *et al.* (2002) and Kavita (2008).

In the present study, rabbits were used as an experimental model to study bovine mastitis with respect to induction of mastitis as well as protection against challenge infection by biofilm based vaccine. The preliminary study was carried out in the lactating rabbits to optimize the infective dose of *S. aureus* to induce mastitis by bovine mastitis isolates, *S.aureus* SA16 and *S.aureus* SA2 possessing both *bap* and *ica*A genes. In the study, inoculation was done with 10^4, 10^5, 10^6 and 10^7 cfu/ml of bacterial suspension at the base of the teat. The mammary glands were observed for gross lesions. Milk samples collected from zero day up to 144 hrs were used for the determination of SCC and CMT.

Milk SCC are considered to be an important parameter for assessing mammary health status in lactating animals and milk yield decreases as SCC and incidence of mastitis increase. Thus, SCC in heifer mammary gland secretions were analyzed to measure the degree of inflammation and potential reductions of future milk yield. The SCC of milk samples collected during the study were determined. California mastitis test is an effective cow side proxy for SCC useful to predict IMI in cows (Batra and McAllister, 1984 and Vianni and Filho, 1989). Somatic cell count determination has been widely accepted as a screening test to identify intramammary infections in lactating cows. The higher prevalence of *S.aureus* IMI is associated with higher milk SCC (Schalm and Noorlander 1957, Miljkovic and Milojevic 1962, Sharma and Rajani 1965, Chakraborthy and Hazarika 1977, Okello, 1992, Mohinikumari and Janakiramguptha, 2002, Middleton *et al.*, 2002 and Mdegala *et al*., 2004).

In the present study, mastitis was induced in rabbits by both *S.aureus* SA16 and *S.aureus* SA2 strains as indicated by gross lesions (Figs.4b, 4c, 4d and 5), increase in

SCC (Figs.8 and 9) and CMT positivity (Figs.10 and 11). The macroscopic lesions were very well appreciated in all the quarters of mammary glands infected with varying concentration of bacterial suspension of both *S.aureus* SA16 and *S.aureus* SA2 strains at 48 hrs post infection (Fig 4c). There was a drastic increase in SCC in the milk samples collected from all the quarters at 48 hours post infection. Also, maximum quarters of mammary glands were found positive for CMT on day two after inoculation with different concentration of bacterial suspension of both *S.aureus* SA16 and *S.aureus* SA2 strains. These findings are in agreement with Reinoso *et al*. (2002) who reported the varying degrees of macroscopic lesions *viz.*, swelling, necrosis of the quarters in rabbits inoculated with 10^8 cfu/ml of virulent and avirulent strains of *S. aureus* isolated from the bovine mastitis cases. Amorena *et al*.(1991) reported that macroscopic lesions such as swelling and induration were observed at 24 and 48 hrs after inoculating with $5x10^5$cfu/0.5 ml of *S.aureus* bacterial suspension into the mammary gland of lactating rabbits. Craven and Anderson (1982) reported that acute mastitis could be produced by inoculating 10^6cfu of bovine mastitis causing *S.aureus* into normal lactating mouse mammary glands. They observed rapid multiplication of bacteria with production of alpha toxin, necrosis and death of the inoculated mice. Reinoso *et al*. (2002) reported increased SCC in milk samples collected from mastitis induced rabbits. The results in the present study clearly show that both the strains were able to induce mastitis in rabbits, which may be used for challenging in vaccinated animals. Based on these indicators of mastitis, 10^4cfu / ml of bacterial suspension was found to be an optimum infective dose for challenging the vaccinated animals.

5.5 Immunization and challenge studies in rabbits

In the present study, *S.aureus* grown under BF mode was exploited as potential vaccine candidate against mastitis caused by *S.aureus* in bovines. Rabbit model was used to test the efficacy of this vaccine. Experimental trials in pregnant rabbits using these vaccines showed very promising results necessitating further evaluation in the bovine system under both experimental and field conditions.

Immunization was carried out with *S.aureus* SA16 BF as well as FC vaccine. Comparative evaluation was done by challenging both the groups with *S.aureus* SA16 (homologous strain) and *S.aureus* SA2 (heterologous strain). Rabbits vaccinated with *S.aureus* SA16 BF and FC vaccines were evaluated for gross lesions of mammary glands, SCC, CMT and serum IgG response by ELISA after challenge.

The mean percentage of mammary glands showing lesions, mean SCC and CMT positivity after challenging with homologous strain (*S.aureus* SA16) and with heterologous strain (*S.aureus* SA2) were considered. Milk samples collected at different intervals (days 0, 1, 2, 6, 14 and 21 post challenge) were evaluated for SCC and CMT. Sera samples collected at different intervals (days 0, 15, 22, 29, 30, 31, 35, 43 and day 50) were evaluated for IgG response by indirect ELISA.

5.5.1 Gross lesions

Statistical analysis was carried out using one way ANOVA (Tukey's multiple comparison test). There was significant difference ($P<0.05$) between BF vaccinated groups and FC vaccinated groups and also there was a significant difference ($P<0.05$)

within the BF vaccinated (Group 1 and Group 2) and FC vaccinated groups (Group 3 and Group 4) with respect to mean percentage of mammary glands showing lesions(Fig. 12). The mean percentage of mammary glands showing lesions at 48 hrs after challenging with homologous strain (*S.aureus* SA16) and heterologous strain (*S.aureus* SA2) was less (14.55 per cent in Group 1 and 24.95 per cent in Group 2) in BF vaccinated rabbits compared to FC vaccinated (50 and 62.5 per cent in Group 3 and 4 respectively) animals indicating BF vaccine had conferred better protection and cross protection against challenge compared to FC vaccine. The results are in accordance with Kavitha (2008) who reported bovine mastitis *E.coli* BF vaccine had conferred higher cross protection upon challenge studies using homologous (*E.coli* O9) and heterologous (*E.coli* O147) serotypes in rabbits wherein mean percentage of mammary glands showing lesions at 48 hrs after challenging with homologous serotype (*E.coli* O9) was 12.5 per cent and 11 per cent with heterologous serotype (*E.coli* O147) in BF vaccinated rabbits compared to 41.46 per cent with homologous and 65.62 per cent with heterologous serotype for FC vaccinated group.

In another study by Prakash (2006) observed that progeny from *E.coli* BF vaccinated broiler parents had only 16.66 per cent mortality upon homologous (*E.coli* O78) challenge and 16.66, 33.32 and 16.66 per cent mortality upon three heterologous (*E.coli* O2) challenges on days 15, 29 and 39 respectively whereas 100 per cent mortality was observed in all the three challenges upon heterologous challenge and 66.64, 50 and 66.64 per cents upon homologous challenge in progeny from unvaccinates. Similar findings were also observed by Shivaraj and Krishnappa (2002) and Veeregowda (2003). These studies supported the hypothesis that BF vaccine is superior to the FC

vaccine against experimentally induced mastitis in rabbits using *S.aureus* isolates from bovine mastitis.

5.5.2 Somatic Cell Count

In the case of mastitis, an enhanced immune response is not always considered beneficial. One important component of the immune response is the migration of large numbers of white blood cells (in the udder called somatic cells) to the infected gland. The presence of somatic cells in the milk is not considered a positive outcome as somatic cells are evidence of mastitis and reduce the quality of milk. An increase in SCC in milk leads to the release of lipolytic (lipases) and proteolytic (plasmin) enzymes which can degrade the triglycerides of milk fat and casein contents of the milk. This leads to poor quality milk in the mastitis affected animals (Saeman *et al.*,1988). Unless a vaccine can prevent new infections throughout lactation and dramatically reduce the SCC of affected animals, it may be difficult for a producer to recognize the benefit of using a *S. aureus* vaccine (Ruegg, 2001). However, it is crucial to obtain a recruitment of activated polymorphonuclear leukocytes (PMNL) into the milk of vaccinated animals as early as possible after the entry of *S.aureus* into the mammary gland. This is very much essential for the effective phagocytosis of invading pathogens by the PMNL (Sutra and Poutrel, 1994).

In the present study, statistical analysis was carried out using one way ANOVA (Tukey's Test) to compare SCC of Group 1 (*S.aureus* SA16 BF vaccinated rabbits challenged with homologous strain *i.e., S.aureus* SA16) and Group 2 (*S.aureus* SA16 BF vaccinated rabbits challenged with heterologous strain *i.e., S.aureus* SA2). Comparisons

were also made between SCC of Group 3 (*S.aureus* SA16 FC vaccinated rabbits challenged with homologous strain) and Group 4 (*S.aureus* SA16 FC vaccinated rabbits challenged with heterologous strain).

Analysis showed that the difference in the SCC of milk collected on day '0' *i.e.*, before challenge was 'non-significant' in all the groups. The values were varied from 5×10^5 cells/ml to 8×10^5 cells/ml (Fig.13). Meanwhile, on day one *i.e.*, 24 hrs post challenge, there was sudden increase in SCC in all the four groups (10×10^5 in Group1, 27×10^5 in Group 2, 15×10^5 in Group 3 and 37×10^5 cells/ml in Group 4) indicated the recruitment of activated PMNL into milk of vaccinated animals. Similar observations were made by Kavitha (2008) who also reported a sudden increase in SCC in milk samples from rabbits vaccinated with *E.coli* BF vaccine and challenged with homologous and heterologous serotypes. These findings were in accordance with Sutra and Poutrel (1994) who reported that it is very much essential to obtain a recruitment of activated PMNL into the milk of vaccinated animals as early as possible after the entry of *S.aureus* into the mammary gland to prevent the establishment of the infection. They also opined that the migration of large number of PMNL into the mammary gland must coincide with the presence of opsonising antibodies in milk for effective phagocytic killing of the invading pathogens. In a study conducted by Jyothi (2009) who analysed the milk samples collected from the same rabbits for *S.aureus* specific IgG response and *E.coli* specific IgG response from rabbits vaccinated with *E.coli* BF vaccine and reported that a significant increase in the specific IgG antibodies to *E.coli* and *S.aureus* BF vaccine in milk samples collected from four groups of rabbits supported this hypothesis. When SCC were compared between different groups, a highly significant difference ($P < 0.001$) was

observed within the BF vaccinated (Group 1 and 2) and FC vaccinated groups (Group 3 and 4). But, there was no significant difference ($P>0.05$) between BF vaccinated-homologous challenged and FC vaccinated-heterologous challenged groups. On day two post challenge, peak SCC response was obtained with a highly significant difference ($P<0.001$) within the BF and FC vaccinated group. But, the BF and FC vaccinated groups challenged with homologous strain did not differ significantly ($P>0.05$).

Milk samples collected on days 6, 14 and 21 post challenge have also yielded similar results (Fig.13). It was observed that there was a reduction in the SCC in BF and FC vaccinated groups after 48 hrs of challenge. Similar observations were made by Pankey *et al.* (1985), Leitner *et al.* (2003a) and Shakoor *et al.* (2006). Pankey *et al.* (1985) reported that the somatic cell counts were significantly lower for cows vaccinated with protein A and a commercial staphylococcal bacterin(Somatostaph®) and challenged with *S.aureus.* Leitner *et al.* (2003a) also found that the SCC were very low in cows vaccinated with *S. aureus* vaccine composed of three field isolates of bovine mastitis and challenged with a highly virulent *S. aureus* strain. Shakoor *et al.* (2006) observed that *S.aureus* vaccines (live attenuated, simple bacterin, dextran sulphate adjuvanted and oil adjuvanted) reduced the somatic cell count 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. However, in Group1 *i.e., S.aureus* BF vaccinated rabbits challenged with homologous strain, the mean SCC were reduced to below 10×10^5 cells per ml compared to other groups indicating the superiority of BF vaccine over FC vaccine in reducing the SCC in milk thereby protecting the mammary gland against challenge.

5.5.3 California Mastitis Test

California mastitis test is an effective cow side proxy for determination of SCC in milk and useful to predict IMI in cows . It has been widely implemented as a screening test to identify intramammary infections in lactating cows (Okello, 1992 , Mohinikumari and Janakiramguptha, 2002 and Mdegala *et al*., 2004). In the present study, statistical analysis was carried out using one way ANOVA (Tukey's Test) to compare percentage of CMT positive mammary glands of Group 1 and 2 at 48 hrs post challenge. Comparisons were also made between Group 3 and 4. Analysis indicated that there was a significant difference ($P < 0.05$) between BF vaccinated and FC vaccinated groups. But, there was no significant difference ($P > 0.05$) within the BF vaccinated groups with respect to mean percentage of CMT positive mammary glands (Fig.14) at 48 hrs post challenge. Mean percentage of CMT positive mammary glands was 9.37 and 12.5 per cent in Group 1 and 2 (BF vaccinated) respectively as against 46.87 and 56.25 per cent in Group 3 and 4 (FC vaccinated) respectively. Further, on day 6 , it had reduced to 4 per cent in both the BF vaccinated groups and 29.16 and 25 per cent in FC vaccinated – homologous and heterologous challenged groups respectively. But in case of BF vaccinated groups, there was a drastic reduction in mean percentage of CMT positivity to zero at 14 days after challenge as against 20 per cent in FC vaccinated groups. These findings were in agreement with Kavitha (2008) who also analysed the mean percentage of CMT positive mammary glands in rabbits vaccinated with bovine mastitis causing *E.coli* BF vaccine and observed a significant difference ($P < 0.05$) between BF vaccinated and FC vaccinated group and no significant difference ($P > 0.05$) within the BF vaccinated group with respect to mean percentage of CMT positive mammary glands indicating cross protection conferred by BF vaccine unlike FC vaccine. This analysis

supported the hypothesis that BF vaccine is superior to FC vaccine with respect to protection and cross protection against *S.aureus* challenge infection.

5.5.4 Enzyme Linked Immuno Sorbent Assay

Seromonitoring of animals for the presence of antibodies against mastitis causing pathogens has an immense value as a measure of level of protection. Further, periodical monitoring of *S.aureus* antibodies has a distinct advantage of providing an insight into the immune status against mastitis. Enzyme-linked immunosorbent assay is one of the sensitive methods for the detection of *S.aureus* antibodies in the serum of vaccinated as well as mastitis affected animals (Matsushita *et al*., 1990; Grove and Jones, 1992 ; Herbeline *et al*., 1997 and Leitner *et al.,* 2000).

5.5.4.1 Optimization of *S.aureus* BF antigen

An Indirect ELISA was standardized by optimizing *S.aureus* BF antigen dilution at 1.25 µg/ml against a constant 1:100 dilution of positive serum control (C+) and negative serum control (C-) by using anti-rabbit IgG-HRP conjugate at a constant 1:25,000 dilution. The end point titration of antigen showed a sudden drop in the OD values at 1:80 dilution of antigen (Fig.15 and Table 6). At this concentration of antigen, the OD value of strong positive at 490 nm was 0.824. Hence, 1.25 µg/ml (1:80) dilution of *S.aureus* BF protein antigen was used as optimum working dilution of antigen for monitoring *S.aureus* BF and FC vaccinal antibodies in sera samples collected on days 0, 15, 22, 29, 30, 31, 35, 43 and 50 from different groups of rabbits. Kavitha (2008) used higher concentration of antigen *i.e.,* 5 µg/ml of *E.coli* BF OMP antigen and a constant serum dilution of 1:100 as optimum working dilution for monitoring *E.coli* BF vaccinal

IgG antibodies in sera collected from the *E.coli* BF vaccinated rabbits. Herbeline *et al*. (1997) used 4 µg/ml of purified *S.aureus* α - toxin (alpha toxin) and test sera samples diluted at 1:2000 and 1: 4,000 were used as optimum dilutions for indirect ELISA to measure antibodies against *S.aureus* α - toxin in cows vaccinated with staphylococcal α - toxin. Gilbert *et al.* (1994) used 1 µg/ml of purified capsular polysaccharide type 5 of *S.aureus* to measure the specific antibodies in lactating cows immunized with purified capsular polysaccharide type 5 of *S.aureus.* The lower concentration of antigen used in the present study was probably an indication of better quality of antigen employed in the test and which was also economical.

5.5.4.2 Monitoring of pre and post challenged specific IgG antibodies in *S.aureus* BF, FC vaccinated and control rabbits

Sera samples collected on days '0' (corresponded to 12^{th} day of gestation/pre immunization) 15, 22, 29, 30, 31, 35, 43 and 50 from *S.aureus* BF, FC vaccinated challenged with homologous and heterologous strains and control rabbits were subjected to indirect IgG ELISA. In the present study, it was observed that there was a gradual increase in the PP values of sera samples collected from different/ four groups (*viz.*, 1, 2, 3 and 4 and 5) at days 15 and 22 of the experiment compared to control *i.e.*, OD values were increased after immunization and after each booster and remained elevated till the end of the experiment at 50 day (Fig.16 and Table 7). These findings are supported by Guidry *et al*. (1991) who reported that serum agglutination and ELISA titers of cows immunized with two different strains of mastitis causing *S. aureus* were increased after immunization and after each booster and maintained till the end of the experiment at 112 day.

At the same time, no significant difference among Group 1 and 2 and also Group 3 and 4 was noticed in the PP values of sera samples collected at the same periods. Meanwhile, there was a significant increase in the PP values of sera samples collected at day 29 of the experiment *i.e.,* a week after 2nd booster dose given to the animals indicating the boosting of humoral response in immunized animals. However, there was a sudden drop in the PP values on day '30' of the experiment *i.e.,* on the first day of post challenge and then increased gradually after 2nd day till 21st day post challenge. The sudden drop in the level of IgG antibodies in the sera samples collected on day one after challenge could be due to the selective passive transfer of IgG from blood circulation into the mammary gland and subsequent involvement of specific IgG in phagocytic killing of bacteria at the site of infection. Immunoglobulin G is a very good opsonin and a primary component of immune system responsible for promoting phagocytosis by PMNL as the polymorphonuclear neutrophil phagocytosis is the most effective defense against *S.aureus* intramammary infection (Leitner *et al.*, 2000). In the present study, the sudden drop in the level of specific IgG antibodies in the sera samples was coincided with the significant increase in the SCC in milk of vaccinated rabbits after 24 hrs of challenge with *S.aureus* (Fig.13 and 16). These findings were in agreement with Gilbert *et al.* (1994) who opined that the concurrent presence of specific antibodies and early recruitment of PMNL in the milk is required for efficient phagocytosis of *S.aureus* during intramammary infections. Sutra and Poutrel (1994) also opined that the migration of large number of PMNL into the mammary gland must coincide with the presence of opsonising antibodies in milk for effective phagocytic killing of the invading pathogens. Similar observations were also made by Miller *et al.* (1988); Watson (1989) and Leitner *et al.* (2000) who reported the enhancement of neutrophil phagocytosis by specific antibodies

of IgG isotype is responsible for protection of ruminant mammary gland against challenge exposure after immunization with staphylococcal vaccines. Subsequently, a significant increase in the antibody levels after 48 hrs post challenge may be because of intra mammary antigenic stimulation which served as intra mammary booster to increase antibody response and remained elevated till the end of the experiment up to 50 days.

5.5.4.3 Comparison of homologous v/s heterologous challenged groups

The statistical analysis was carried out to find out differences between homologous and heterologous challenged rabbits within BF and FC vaccinated groups using two tailed unpaired 't' test.The differences among PP values of sera samples collected from four groups (*viz.*, 1, 2, 3 and 4) at days 0, 15, 22, 29, 30, 31, 35, 43 and 50 (Fig.16 and Table 7). It was observed that the difference in the level of specific IgG antibodies in the sera collected from BF vaccinated group challenged with homologous strain v/s BF vaccinated group challenged with heterologous strain, on days 0, 15, 22 , 29, 30, 31, 35, 43 and 50 was 'non-significant' ($P>0.05$). This confirms the ability of BF vaccine to confer cross protection against heterologous strain. Further, when FC vaccinated group challenged with homologous and heterologous strain were compared on days 43 and 50, the difference was 'significant' ($P<0.05$), indicating inability of FC vaccine to confer cross protection against heterologous strain. Thus, the hypothesis of cross protection conferred by BF vaccines is supported by this analysis. These findings are in agreement with Veeregowda (2003) who reported that experimental vaccination trials with *E.coli* BF vaccine against colibacillosis conferred 100 per cent cross protection compared to 58 per cent with FC vaccine against IM. challenge infection in poultry. Field trials conducted with BF vaccine against colibacillosis indicated 100 per cent cross

protection in poultry. Using a live attenuated strain of *S.aureus* (W 79*)* as a vaccine, Watson and Colditz, (1985) were able to provide moderate protection in ewes and heifers from challenge with homologous (W 79) and heterologous *S.aureus* strains.

5.5.4.4 Comparison of biofilm v/s free cell vaccinated groups

Statistical analysis was also performed to compare BF vaccinated and FC vaccinated groups by two tailed 't' test, based on PP values of sera samples collected at days 0, 15, 22 , 29, 30, 31, 35, 43 and 50. The highly significant ($P< 0.01$) difference was seen in the PP values of sera collected from BF vaccinated and FC vaccinated groups on days 22, 29, 30, 31 and 35. The highly significant ($P<0.001$) difference was obtained in the PP values of sera collected from BF vaccinated and FC vaccinated groups on days 43 and 50 (Fig.16 and Table 7). The significantly high level of antibodies in BF vaccinated groups than FC vaccinated groups emphasized the supremacy of BF vaccine.

Further, in post challenge period, although there was a sudden drop in detectable antibody levels immediately after challenge during first two days (48 hrs), followed by a sudden significant rise in the antibody levels, especially in the BF vaccinated group (Fig.16). The sudden drop in the level of IgG antibodies within 48 hours of challenge could be due to the influx of IgG antibodies from blood into the mammary gland to opsonize the invading *S.aureus* organisms for effective phagocytic killing of bacteria at the site of infection. However, subsequent highly significant increase in the antibody level from 31st day onwards, till the day 50 of experiment induced by BF vaccine is probably due to the enhanced uptake and longer retention of the BF antigens compared to that of FC vaccine. These findings are in agreement with Azad *et al*., (1999) who reported a progressive improvement in serum antibody titer and protective response with

time following *Aeromonas hydrophila* biofilm oral vaccination in common carps. They also opined that the possibility of BF antigens being available continuously for the immune system with minimally altered immunogenic epitopes could have contributed the observed higher antibody titre and subsequent protection. The glycocalyx of BF, is a polymer of neutral hexoses (Costerton *et al.,* 1981) which encapsulates and possibly protects the bacterial surface antigens from any destruction or alteration of immunogenic epitopes. The progressive increase in IgG response till day 50 of experiment may also be due to the peripheral antigenic stimulation resulting in the seeding of a proportion of lymphoid cells into mammary tissues, due to challenge which would have served as intramammary booster. This hypothesis is supported by the findings of Guidry *et al*. (1991) who obtained an elevated serum anticapsular antibodies till the end of the experiment at 112 days in cows immunized against *S.aureus* mastitis wherein the cows were immunized in the area of the supramammary lymph node and intramuscularly and were boosted on days 14, 42, and 70. On elevation of serum IgG titers for intra mammarily immunized cows, present results support the suggestions of Saif *et al.* (1984), that an intramammary booster following systemic immunization with viral antigens could elicit an enhanced systemic antibody response in addition to local immunity. Lymphoid cells stimulated locally in the gland following an intra mammary sensitization may traffic to local lymphatic tissues, bolstering systemic antibody responses. Thus, the relationship between IgG titers in serum and intra mammary sensitization can possibly be explained by the selective transfer of IgG across secretary cells (Saif *et al.*, 1984).

The mechanism of action by which immunization with BF vaccine provides protection appears to be related to the enhanced uptake, longer retention and slow release

of BF antigens (Azad *et al*., 1999) and subsequent production of antibodies to novel immunogenic proteins expressed by *S.aureus* when grown under BF mode. Further, the cross reactivity of antisera from rabbits immunized with BF proteins with heterologous strain was due to antibodies directed against such novel proteins expressed and shared by both homologous as well as heterologous strains of *S.aureus.* Enhanced immunoglobulin level of sera may lead to increased opsonization of homologous as well as heterologous strains of *S.aureus,* and thereby its elimination. Similar cross reactivity to heterologous strains was also reported by Watson and Colditz, (1985) and Hogan *et al*.(1992) on using conventional *S.aureus* and *E. coli* J5 vaccine for bovine mastitis respectively.

Earlier studies by Kavitha (2008) and Jyothi (2009) have shown that vaccination of pregnant rabbits with bovine mastitis causing *E.coli* BF vaccine was superior than FC vaccine in terms of serum IgG response as well as milk IgA and IgG response and higher cross protection against homologous and heterologous challenge. Similarly, Chandrashekhara (2009) has reported that vaccination of lactating cows with *E.coli* BF vaccine was superior to FC vaccine as serum IgG level was significantly high in BF vaccinated than FC vaccinated and control cattle. The ability of the *S.aureus* BF vaccine to induce a significant serum IgG response and cross protection against homologous and heterologous challenge infection in rabbits as demonstrated in this study, further confirm the earlier findings.

5.6Conclusion

In conclusion, analysis using *bap* specific PCR for bovine mastitis causing *S.aureus* isolates indicated 40 per cent of isolates were positive for the '*bap'* gene which

encodes biofilm associated protein responsible for the development of the biofilms. The ability of *S.aureus* to form biofilm is considered to be a major virulence factor influencing its pathogenesis in mastitis.

Immunoblot analysis indicated that proteins extracted from *S.aureus* grown under BF mode expressed immunogenic cross reactive proteins compared to FC proteins and that BF cells could be most valuable immunogens for prophylaxis and diagnosis.

In the current trials, comparison between BF and FC vaccinated rabbits indicated the superiority of BF vaccine as the CMT positivity, SCC values and percentage of mammary glands showing lesions upon challenging, were significantly less in case of BF vaccinated groups. Further, specific serum IgG levels detected by ELISA was significantly high in BF vaccinated than FC vaccinated and control rabbits.

Analysis of data with respect to CMT positivity, SCC values, Percentage of mammary glands showing lesions and specific serum IgG levels obtained from BF and FC vaccinated and challenged with homologous and heterologous strains indicated cross protection against challenge infection by BF vaccine.

The evaluation of BF vaccine in pregnant animals indicated that the animals immunized with *S.aureus* BF vaccine in their last few weeks of pregnancy are better protected against new *S.aureus* intramammary infection during their subsequent lactation period.

VI. SUMMARY

A study was undertaken to characterize 25 bovine mastitis *S. aureus* (SA1 to SA25) isolates for *bap* (Biofilm associated protein) gene, analyze proteins of *S.aureus* SA16 and *S. aureu*s SA2 grown under BF and FC mode with reference to their immunogenicity and cross reactivity, and for experimental evaluation of *S.aureus* BF based vaccine in rabbits.

The *bap* specific PCR analysis of 25 bovine mastitis *S. aureus* isolates indicated 40 per cent of isolates were '*bap'* positive that showed an amplicon of 971 bp.

The SDS-PAGE analysis of protein profiles of *S.aureus* SA16 (BF vaccine and homologous challenge strain) and SA2 (heterologous challenge strain) grown under BF and FC mode indicated that the protein profiles of *S.aureus* SA16 and SA2 grown under BF mode had differed from *S.aureus* SA16 and SA2 grown under FC mode by 56 per cent with unique expression of 26.69, 30.59, 34.02, 40.77, 51.77, 57.22, 65.09, 102.72 and 114.21 kDa and repression of 19.03, 22.61, 25.25, 28.6, 33.3, 38.09, 39.91, 62.45, 79.14 and 149.02 kDa. The unique proteins of 51.77 and 57.22 kDa were detected only in *S. aureu*s SA16 BF cells, but not in *S. aureu*s SA2 BF or free cell proteins. The polypeptides of 37.05, 54.39 and 94.91 kDa were expressed in BF and FC of both the strains with more prominent expression of 37.05 kDa protein in BF cells.

The western blot analysis of proteins with reference to their immunogenicity and cross reactivity revealed that six immunogenic proteins of 30.59, 34.02, 37.05, 54.39, 94.91 and 114.21 kDa and three immunogenic proteins of 30.59, 34.02 and 37.05 kDa

were detected in *S.aureus* SA16 BF and *S.aureus* SA2 BF respectively upon probing with hyper immune sera raised against *S.aureus* SA16 BF. These proteins were not detected either in BF proteins or in FC proteins when probed by FC HIS. This indicates the superiority of BF proteins which are capable of inducing better antibody response compared to FC proteins. Further, the immunogenic proteins of 30.59, 34.02, 37.05 kDa were observed in *S.aureus* SA2 BF proteins, indicating the cross reactivity of BF proteins. But, such immunogenic cross reactivity was not observed when blot was probed with FC HIS.

Mastitis was induced in lactating rabbits by inoculating both *S.aureus* SA16 and SA2 strains with 10^4, 10^5, 10^6 and 10^7 cfu/ml of bacterial suspension at the base of the teat. Induction of mastitis was indicated by gross lesions of mammary glands, increased SCC and CMT positivity. All these relevant indicators of mastitis showed maximum values at 48 hrs after inoculation. These results showed that both the strains had induced mastitis in rabbits and that 10^4 cfu/ml of bacterial load was found optimum for challenge infection in vaccinated rabbits.

Pregnant rabbits immunized with *S.aureus* SA16 BF and FC vaccines were evaluated for the gross lesions of mammary glands, SCC, CMT and serum IgG level by ELISA after homologous and heterologous challenge.

The statistical analysis to evaluate BF and FC vaccines with respect to development of mastitis indicated that the mean percentage of mammary glands showing lesions at 48 hrs after challenging and mean SCC and percentage of CMT positive

mammary glands after challenge was less in BF vaccinated compared to FC vaccinated rabbits.

The indirect ELISA was standardized for the detection of post-vaccinal IgG antibodies in sera samples from rabbits vaccinated with *S.aureus* BF and FC vaccines by optimizing *S.aureus* BF antigen concentration at 1.25 µg/ml and a serum dilution of 1:100. Further, serum IgG level detected by ELISA was significantly higher in BF vaccinated rabbits than FC vaccinated ones. Cross protection conferred by BF vaccine was noticed based on challenge studies using homologous (*S.aureus* SA16) and heterologous (*S.aureus* SA2) strains. In conclusion, the experimental vaccination trials in pregnant rabbits with *S.aureus* BF and FC based vaccines indicated the superiority of *S.aureus* BF vaccine to the FC vaccine against experimentally induced mastitis using *S.aureus* isolates from bovine mastitis.

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VIII. ABSTRACT

The present study was undertaken to evaluate bovine mastitis causing *S.aureus* BF based vaccine in pregnant rabbits. The '*bap*' specific PCR analysis of 25 bovine mastitis *S.aureus* isolates indicated 40 per cent of isolates were '*bap*' positive. Analysis of protein profiles of BF and FC of *S.aureus* SA16 and SA2 indicated that the protein profiles of BF had differed from FC by 56 per cent with expression of unique proteins. Western blot analysis revealed six and three immunogenic proteins respectively in *S.aureus* SA16 and *S.aureus* SA2 BF upon probing with *S.aureus* SA16 BF HIS indicating the immunogenicity and cross-reactivity of novel proteins expressed when grown under BF mode. These proteins were not detected when probed by FC HIS. Mastitis was induced in lactating rabbits by inoculating 10^4, 10^5, 10^6 and 10^7 cfu/ml of bacterial suspension at the base of the teat which was indicated by gross lesions of mammary glands, increased SCC and CMT positivity. All these relevant indicators of mastitis showed maximum values at 48 hrs post inoculation. These results showed that both the strains had induced mastitis in rabbits and that 10^4 cfu/ml of bacterial suspension could be used for challenging. Pregnant rabbits immunized with *S.aureus* SA16 BF and FC vaccines were evaluated for the gross lesions of mammary glands, SCC, CMT and serum IgG level by ELISA after challenge with homologous and heterologous strains. Statistical analysis was made to evaluate BF and FC vaccines with respect to development of mastitis indicated that the mean percentage of mammary glands showing lesions at 48 hrs after challenging and mean SCC and percentage of CMT positive mammary glands after challenge was less in BF vaccinated compared to FC vaccinated rabbits. Further, serum IgG level detected by ELISA was significantly higher in BF vaccinated than FC vaccinated rabbits. Higher cross protection conferred by BF vaccine was noticed based on challenge studies. Vaccination trials in rabbits with *S.aureus* BF and FC vaccines indicated the superiority of *S.aureus* BF vaccine to the FC vaccine.