III. MATERIALS AND METHODS

3.1 General considerations

The study was carried out in the Department of Pathology, Veterinary College, Bangalore. The study was conducted in two phases:

Phase I: Experimental pathology of *Staphylococcus aureus* (S.aureus) mastitis in rabbits

The study included induction of mastitis experimentally in lactating rabbits using *S. aureus* isolates from bovine mastitis.

Phase II: Immunization and Challenge Studies in rabbits

The second part of the experiment included immunization of rabbits with planktonic (Free Cell) and Biofilm vaccine of *Staphylococcus aureus* prepared at the Dept. of Microbiology, Veterinary college, Bangalore followed by challenge studies.

3.2 Experimental induction of *Staphylococcus aureus* mastitis in rabbits

3.2.1 Source of organisms used for experimental studies

The *Staphylococcus aureus* strains 06 and 50 isolated from bovine mastitis cases and maintained in the Department of Microbiology, Veterinary College, Bangalore were used to induce mastitis in rabbits. *S.aureus* 50 isolated from subclinical mastitis was used as biofilm vaccine candidate and also for homologous challenge studies. *S.aureus* 06, which was isolated from clinical mastitis was used for heterologous challenge studies.
The selection of these strains was based on possession of \textit{Bap} gene which was characterized in the present study and \textit{icaA} gene.

3.2.2 Preparation of Bacterial Suspension

\textit{Staphylococcus aureus} was grown on Mannitol salt agar for 18 hr at 37°C. A loopful of culture was suspended in 2 millilitre(ml) PBS and suspension was adjusted to final concentration of $10^4$, $10^5$, $10^6$, and $10^7$ cfu/ml after viable cell count.

3.2.3 Experimental Animals

Twelve female rabbits in 2\textsuperscript{nd} or 3\textsuperscript{rd} lactation were procured from a reputed rabbit breeder. Animals were divided into two groups and housed in cages with standard managerial condition during the experiment. They were maintained under standard laboratory hygienic conditions with standard laboratory animal feed and water \textit{ad libitum}. The approval of the Institutional Animal Ethics Committee was obtained prior to start of the experiment (Reg no.493/01/a CPCSEA dated 31-10-2001). Of the 12 rabbits, six were maintained for virulent \textit{Staphylococcus aureus} O6 and another six rabbits for \textit{Staphylococcus aureus} 50 strain.

3.2.4 Inoculation technique

Before infecting, the lactating animals were exhaustively milked by squeezing the mammary gland from the base of the teat. The two \textit{Staphylococcus aureus} (O6 and 50) strains were inoculated into six rabbits with 0.5 ml bacterial suspension (in PBS) at the base of the teat using 30 G needle attached to a syringe. The bacterial counts inoculated into different mammary glands for induction of mastitis in rabbits are as follows:
1\textsuperscript{st} pair - 0.5 ml PBS only
2\textsuperscript{nd} pair - 0.5 ml suspension (5\times10^3 cfu) i.e. 10^4 cfu/ml
3\textsuperscript{rd} pair - 0.5 ml suspension (5\times10^4 cfu) i.e. 10^5 cfu/ml
4\textsuperscript{th} pair - 0.5 ml suspension (5\times10^5 cfu) i.e. 10^6 cfu/ml
5\textsuperscript{th} pair - 0.5 ml suspension (5\times10^6 cfu) i.e. 10^7 cfu/ml

3.2.5 Sample collection

The blood and milk samples were collected from all the rabbits prior to inoculation of bacterial cultures. After inoculation, lesions in the mammary glands, if any, were recorded and blood and milk samples from each of the mammary gland at were collected at 24 hr intervals up to 144 hr for TLC, DLC and somatic cell (SCC) counts. The rabbits were carefully examined for the development of clinical signs of mastitis. At the end of every 24 hr one rabbit was euthanized by humane method. After a thorough postmortem examination, tissue samples were collected for histopathology, transmission electron microscopy and immunohistochemistry. The details of collection of blood, milk and tissue samples after infection are as below:

<table>
<thead>
<tr>
<th>Rabbits</th>
<th>Time of sample collection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr</td>
</tr>
<tr>
<td></td>
<td>B</td>
</tr>
<tr>
<td>1\textsuperscript{st}</td>
<td>√</td>
</tr>
<tr>
<td>2\textsuperscript{nd}</td>
<td>√</td>
</tr>
<tr>
<td>3\textsuperscript{rd}</td>
<td>√</td>
</tr>
<tr>
<td>4\textsuperscript{th}</td>
<td>√</td>
</tr>
<tr>
<td>5\textsuperscript{th}</td>
<td>√</td>
</tr>
<tr>
<td>6\textsuperscript{th}</td>
<td>√</td>
</tr>
</tbody>
</table>

Note: B= Blood, M==Milk, T= Tissues.
3.2.5.1 Collection of milk and blood from rabbits

Milk samples were collected from the lactating rabbits before and after infection, following I/M administration of 0.5 units of Oxytocin. There was engorgement of mammary glands within three minutes after injection of Oxytocin (Plate.2) compared to those rabbits not injected with Oxytocin (Plate.1). Maximum milk was collected by squeezing the glands from the base of teat (Plate.3). The milk samples for SCC estimation after infection from individual pairs of mammary glands inoculated with PBS and different doses of bacterial suspension (5×10^4 cfu to 5×10^7 cfu) were collected separately in collecting tubes.

Milk samples collected were used for determination of Somatic Cell Count. The mammary gland tissues and selected parenchymatous organs were subjected for routine histopathology. Appropriate mammary gland tissues were collected in three percent of (3%) glutaraldehyde in PBS for Transmission Electron Microscopic studies.

Blood samples were collected from the saphenous vein. The blood samples were subjected for estimation of TLC and DLC as per the methods described by Benjamin (2005). Serum was separated and stored in aliquots at -20°C until further use.

3.3 Microscopic Somatic Cell Count (SCC) in Milk

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

- **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 and foam to disappear.

3. A clean, grease free microscopic slide was placed over the template to outline four 1 Sq.cm areas.

4. Ten µL 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 and then stained.

- **Staining**

1. The slides with milk smears were placed on the slide rack and were flooded with modified Newman-Lampert 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 slides were rinsed in three changes of tap water at 42-45°C and air-dried.

- **Counting of cells**

Stained films were examined under 100X magnification and the number of cells in 20 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.

### 3.3.2 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 $\pi r^2$.

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

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 $\times$ MF $\times$ 100

### 3.4 Raising of hyperimmune serum (HIS)

Four rabbits were procured from a commercial rabbit farm and randomly divided into two groups of two rabbits each and were used for raising hyper immune serum (HIS) against *S. aureus* 50 BF and FC proteins.
One group of rabbits received \textit{S.aureus} 50 BF protein along with (FIA) Freund’s Incomplete adjuvant for both first injection on day zero and two boosters through s/c route. On the other hand, \textit{S.aureus} SA50 FC protein was given with (FCA) Freund’s complete adjuvant 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. The hyperimmune sera thus collected were used for ELISA and immunohistochemistry.

3.5 Preparation of vaccines

3.5.1 Source of Vaccines

The freecell and biofilm vaccines produced at the Department of Microbiology, Veterinary College, Bangalore were used to immunize pregnant rabbits against mastitis. The strain 50 was used for the production of vaccine.

Two types of vaccines were prepared using \textit{S.aureus} 50.

3.5.2 Free cell / planktonic vaccine

The SA 50 culture was grown in 3.0 per cent TSB (Tryptose Soya Broth) for 16 hr 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 $4 \times 10^9$ cfu / ml after counting number of viable
cells. The pellet was inactivated with 0.1 per cent formalin at RT (Room Temperature) for 24 hr and stored at 4°C until use.

3.5.3 **Biofilm vaccine**

Three-day-old BF cells of SA 50 culture grown in 0.32 per cent TSB, incorporated with 0.3 per cent bentonite clay were harvested by discarding the supernatant medium to remove any FC. Bentonite clay with BF growth was adjusted to a final concentration of $4 \times 10^9$ cfu / ml with PBS after viable cell count. The biofilm cells were inactivated with 0.1 per cent formalin at RT for 24 hr and stored at 4°C until use.

3.5.4 **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.

3.6 **Immunization and Challenge Studies in rabbits**

3.6.1 **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.

3.6.2 **Vaccination schedule**

Twelve rabbits each were immunized by FC and BF vaccines of SA 50.
Details of vaccination are shown below in the flow chart.

30 pregnant rabbits
\(\text{(12}^{\text{th}} \text{ day of gestation)}\)

24 rabbits for vaccination
6 rabbits (control)

Pre vaccinal blood collection

12 rabbits
12 rabbits

\(S.\text{aureus 50 BF vaccine}\)
\(S.\text{aureus 50 FC vaccine}\)

1 ml/ rabbit containing \(4 \times 10^9\) cfu /ml s/c at four sites

With FIA for the first injection for BF vaccine and
With FCA for the first injection for FC vaccine

\(26^{\text{Th}} \text{ day of gestation}, \text{ blood collection}\)

First booster with FIA

30\(^{\text{th}}\) day of pregnancy – Kindling
6 rabbits (control)

\(3^{\text{rd}} \text{ day of lactation}, \text{ blood collection}\)

Second booster with FIA

\(10^{\text{th}} \text{ day of lactation}, \text{ challenge infection with}\)

\(S.\text{aureus 50 (homologous) and}\)

\(S.\text{aureus 06 (heterologous)}\)

Challenge with \(S.\text{aureus}\)

SA 50 - 2 rabbits
SA 06 - 2 rabbits
uninfected – 2 rabbits
3.7. Challenge studies

3.7.1 Experimental animals

Thirty rabbits procured from a reputed breeder were used for the challenge studies. Twelve rabbits were immunized with the planktonic vaccine of *S. aureus* 50 and another twelve rabbits were immunized with biofilm vaccine of *S. aureus* 50 and the remaining 6 rabbits were used as controls. The recommended dose of the vaccine was administered to the pregnant rabbits by subcutaneous route on 12<sup>th</sup>, 26<sup>th</sup> day of pregnancy and 3<sup>rd</sup> day of lactation.

The animals were challenged on the 10<sup>th</sup> 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 FC vaccinated rabbits was challenged with SA 50 and other group with SA 06 at the base of teat. As per the results of experimental induction of mastitis, challenge dose was fixed. Similarly, BF vaccinated group was challenged.

<table>
<thead>
<tr>
<th>BF Vaccine</th>
<th>FC Vaccine</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>(12 rabbits)</td>
<td>(12 rabbits)</td>
<td>(6 rabbits)</td>
</tr>
</tbody>
</table>

- 6 homologous challenge
- 6 heterologous challenge
- 6 homologous challenge
- 6 heterologous challenge
- 2 SA50 healthy
- 2 SA06 healthy
Details of post challenge collection of blood (B), milk (M) and tissues (T) from each group of vaccinated rabbits.

<table>
<thead>
<tr>
<th>Rabbits</th>
<th>24 hr</th>
<th>The day of optimum infection</th>
<th>144 hr (6th day)</th>
<th>14th day</th>
<th>21st day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>M</td>
<td>T</td>
<td>B</td>
<td>M</td>
</tr>
<tr>
<td>1st</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
<td>2nd</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>3rd</td>
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<td>5th</td>
<td>+</td>
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<td>-</td>
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<td>+</td>
</tr>
</tbody>
</table>

6th rabbit was used as a standby.

The rabbits were subjected to a thorough clinical evaluation throughout the experiment. The blood and milk samples were collected from individual rabbits as per the experimental protocol. The blood samples were analysed for TLC and DLC. The milk samples were subjected for SCC.

The sacrificed rabbits were subjected for thorough postmortem examination and the gross lesions, if any, were recorded. The mammary tissues and parenchymatous organs were collected in appropriate manner and subjected to histopathological examination. The mammary tissue sections were subjected for immunohistochemistry to demonstrate the presence of bacterial antigens and immune cells. Transmission electron microscopic studies were carried out on selected samples to note ultrastructural changes. The results were correlated with the results of ELISA and Western Blot studies.
3.8 Clinical Evaluation

All the rabbits used in the experiment were monitored carefully throughout the course of the experiment. The body temperature of all the rabbits used in the experiment was recorded after proper acclimatization. During the experiment, the body temperature of individual rabbit was recorded at intervals of 24 hr. The rabbits were observed for the development of clinical signs of mastitis after inoculation with *S. aureus* cultures as per the experimental protocol. The number of mammary glands showing visible gross lesions suggestive of mastitis under each infective dose was recorded and the mean number of mammary glands with lesions was determined accordingly.

3.8.1 Determination of the mean number of mammary glands showing visible gross lesions of mastitis

The mammary glands of infected/challenged rabbits were carefully examined at the interval of 24 hr and the mean number of glands showing visible gross abnormalities was recorded. During the infection trials, the number of glands showing visible gross changes out of the total number of glands inoculated with the respective dose of bacterial suspension viz., $10^4$, $10^5$, $10^6$ and $10^7$ cfu were recorded separately and the percentage of mammary glands showing gross lesions at these different infective doses was determined accordingly. During the challenge trials, since the challenge dose was fixed at $10^6$ cfu, the percentage of mammary glands showing gross changes was determined for the challenge dose only.
3.9 Blood Parameters

The blood samples collected from the rabbits used in the experiment were subjected for the estimation of Total Leukocyte Count (TLC) and Differential Leukocyte Count (DLC) as per the methods described by Benjamin (2005).

3.10 Histopathology

The histopathological studies were carried out on representative tissue samples collected during both infection trials and challenge trials.

The mammary gland tissues and parenchymatous organs collected from the rabbits were immediately fixed in 10% neutral buffered formalin. After proper fixation the representative samples from the mammary gland tissues and parenchymatous organs were processed by routine paraffin embedding technique. Sections of four to five micron thickness were cut using rotary microtome with disposable blades. These sections were then stained with routine haematoxylin and eosin (Luna, 1968). The histological lesions observed in sections of various organs were systematically recorded.

Wherever necessary, the sections were stained with special stains like Kossa’s, Mason’s Trichrome as per the methods described by Luna (1968).

3.11 Ultrastructural Studies: Transmission Electron Microscopy

Mammary gland tissue samples measuring 1-2 mm in thickness were collected from all the rabbits during both the phases of the experiment and fixed in 3% glutaraldehyde for electron microscopic studies. Transmission electron microscopic
studies were carried out on representative mammary gland tissues collected from both infection trials and challenge trials.

3.11.1 Transmission Electron Microscopy protocol

For electron microscopic studies, tissue samples collected were transferred to vials and fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 hr at 4°C and post fixed with 2% aqueous osmium tetroxide in the same buffer for 2 hr. After the fixation, the samples were dehydrated in a series of graded alcohols and infiltrated and embedded in Araldite 6005 resin or spur resin (spur, 1969). Ultra thin sections (50-70 nm) were cut with a glass knife on a Leica Ultra cut (UCT-GA-D/E-1/00) microtome. Ultra thin sections were mounted on copper grids and stained with saturated aqueous Uranyl acetate and counter stained with Reunols lead citrate. The sections were observed under transmission electron microscope (Model: Hitachi, H-7500 from JAPAN) at required magnifications and photographs were taken at RUSKA Labs, College of Veterinary Science, SVVU, Rajendra Nagar, Hyderabad, India.

3.12 Immunohistochemical detection of the bacterial antigens in tissue.

The mammary gland sections from both infection and challenge trials were subjected for immunohistochemistry for demonstration of bacterial antigens (Staphylococcus aureus).
3.12.1 Materials

- **Immunochemicals**
  - **Primary antibody**: The hyperimmune sera raised against both FC & BF vaccines of *S. aureus* 50 strain served as the polyclonal antibody for detection of bacterial antigens in mammary tissues.
  - **Secondary antibody**: Anti-rabbit IgG raised in goat conjugated with HRPO (Horse Radish Peroxidase) was obtained from Sigma Chemicals, USA and was used at 1:200 dilution.

- **Section Adhesive-Organosilane (APES). Sigma Chemicals, USA.**

- **Hydrogen peroxide (H₂O₂) in methanol(3%)**
  Three per cent H₂O₂ was prepared by adding one ml of 30% H₂O₂ in 9 ml of methanol.

- **Protease solution (0.1%)**
  0.1% Protease solution was prepared by dissolving 10 mg of protease type IV (Sigma Chemicals, USA) in 10 ml of 0.01M PBS and stored at -20°C until used in 1ml aliquots.

- **Substrate**
  3,3-diamine benzidine tetrahydrochloride substrate was prepared freshly at the time of use by addition of 1 mg of 3,3-diamine benzidine tetrahydrochloride in 1 ml of 0.01 M PBS to which 12 µl of 3 per cent H₂O₂ was added.

- **0.01M Phosphate buffer saline – PBS (pH – 7.4)**

- **Harris haematoxylin for nuclear staining Luna, 1968)**
3.12.2 Preparation of organosilane (APES) treated slides for IHC

The slides were placed on racks, washed thoroughly in soap water, rinsed in tap water and finally rinsed in distilled water.

- The slides were allowed to dry completely.
- A two percent solution of 3-aminopropyl triethoxy-silane (APES) in acetone was prepared in a dry staining dish.
- The slides were immersed in APES solution for 5-15 minutes.
- The slides were rinsed in acetone and then rinsed in two changes of distilled water.
- Slides were allowed to dry at 37°C for two hours and then stored at room temperature until used.

3.12.3 Method

- Tissue sections were mounted on APES coated slides and dried at 37°C for three hours.
- The paraffin sections were deparaffinized and rehydrated.
- Endogenous peroxidase was blocked by covering the whole section with three percent hydrogen peroxide in methanol (100 µl), incubated at room temperature for 15-20 minutes and washed thrice in 0.01 M PBS.
- Digestion was done to expose the antigens in cells by covering the whole section with 0.1% protease solution and incubated at room temperature for 20 minutes.
The slides were rinsed in PBS and placed in PBS wash bath for two minutes. Excess fluid from the tissue was drained.

- Addition of primary antibody – The hyperimmune serum was added directly to cover the section. Subsequently the section was incubated at 37°C in a humid chamber for one hour and washed with PBS as mentioned earlier.

- Addition of secondary antibody (Anti rabbit IgG conjugated with HRPO) – The whole section was covered with the secondary antibody at the dilution of 1:300 and incubated at 37°C in a humidified chamber for one hour. After incubation, it was washed with PBS as mentioned earlier.

- Addition of substrate – freshly prepared 3,3-diamine benzidine tetrahydrochloride (DAB) with 3 % H2O2 was poured to cover the sections. Incubated for 5-10 minutes or until the desired color developed at room temperature. The section was washed again with PBS as mentioned earlier.

- Nuclear staining with Harris haematoxylin was done for three minutes. Washed in distilled water, dehydrated and cleared in xylene and the sections were mounted using DPX.

### 3.13 Immunohistochemical detection of immune cells in mammary gland sections

The influx of CD4 and CD8 positive T- Lymphocytes to the mammary gland tissues after vaccination was determined by direct Fluorescent Antibody Technique performed on the tissue sections taken on APES coated slides.
3.13.1 Materials

- **Immunochemicals**

**Primary antibody:** The mouse monoclonal CD4 antibodies conjugated with Fluorescein-isothiocyanate (FITC) and CD8 antibodies conjugated with Rhodamine Red (RR) were used as the primary antibodies. These antibodies were procured from Serotech Laboratories, New Delhi.

- **Section Adhesive-Organosilane (APES). Sigma chemicals, USA.**

- **Protease solution (0.1%)**

  0.1% Protease solution was prepared by dissolving 10 mg of protease type IV (Sigma Chemicals, USA) in 10 ml of 0.01M PBS and stored at -20°C until used in 1ml aliquots.

- **0.01M Phosphate buffer saline – PBS (pH – 7.4)**

- **Clean Coupling jars**

- **Glass troughs for washing**

- **Stainless steel humid chambers**

- **Incubator at 37°C**

- **Chilled Acetone**
3.13.2 Method

- Tissue sections were mounted on APES coated slides and dried at 37\(^0\) C for three hours.

- The sections were deparaffinized and rehydrated.

- Digestion was done to expose the antigens in cells by covering the whole section with 0.1% protease solution and incubated at room temperature for 20 minutes. The slides were rinsed in PBS and placed in PBS wash bath for two minutes. Excess fluid from the tissue was drained.

- The sections were dried and immersed in a coupling jar containing chilled acetone. The smears were fixed in acetone for two to three hours at -20\(^0\) C.

- **Addition of Primary Antibody:** the acetone fixed slides were kept in a humid chamber and the sections were covered with the working dilution of the primary antibody conjugate. Four microgram of the conjugate diluted in 50 µl of PBS was used as the working dilution. The conjugate was spread evenly on the section.

- The slides were kept at 37\(^0\) C in a CO\(_2\) incubator for 30 minutes. Care was taken to see that the sections would not dry out during the incubation.

- The slides were removed from the incubator and washed thoroughly in PBS. For this purpose, the slides were kept in washing troughs with PBS pH 7.4 and gently rotated for three to five minutes. The process was repeated with three changes of fresh PBS.

- Finally the slides were washed in a similar manner in distilled water, air dried, mounted using buffered glycerol and observed under fluorescent microscope.
3.14 Enzyme Linked Immuno Sorbent Assay (ELISA)

An Indirect ELISA was standardized in the Department of Microbiology, Veterinary College, Hebbal, Bangalore as per the standard protocols using suitable reagents.

3.14.1 Source of serum samples

Hyper immune sera

Hyper immune sera to *S.aureus* 50 BF and FC proteins were raised in pregnant rabbits. After one week of the third booster injection, final bleeding was done; serum was separated and preserved at −20°C. This was used as positive serum control (C+) in ELISA.

Control serum

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

3.14.2 Serum dilution

A constant dilution of 1:100 of HIS raised against *S.aureus* SA50 grown under both BF and FC mode and healthy serum was prepared in one per cent BG-PBST.

3.14.3 Seromonitoring of *S.aureus* antibodies by ELISA

3.14.4 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 hr, 48 hr, 6, 14 and 21 days post-challenge with homologous *S. aureus* SA50 and heterologous *S. aureus* SA06.

### 3.14.5 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* SA50 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 hr in an 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 an 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 H₂O₂ 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, Lab systems). 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 Per cent Positivity (PP) values by employing the formula,

\[
\text{PP value of sample sera} = \frac{\text{OD value of sample sera} \times 100}{\text{OD value of C++}}
\]

Statistical Analysis

The results of the present study were statistically analyzed by two-way ANOVA with Bonferron’s multivariate analysis using Prism pad software.