ISSN 0975 - 6299 Vol 2 / Issue 3 / Jul - Sept 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE

MICROBIOLOGY

INDUCTION OF MASTITIS IN RABBIT MAMMARY GLANDS WITH BOVINE MASTITIS BACTERIAL STRAINS

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ABSTRACT

An experimental model was developed in rabbits to study bovine mastitis. Six lactating rabbits for each isolate were used to induce mastitis by using highly prevalent *E.coli* O9 and O147 isolates from bovine mastitis cases that possessed three different virulent genes. In the study, 10^4, 10^5, 10^6 and 10^7 cfu/ml of *E.coli* bacterial suspensions were inoculated in to each pair of teats via the base of the teat and to the first pair, 1 ml of PBS was administered. Gross lesions were recorded for 6 days post inoculation. Milk samples were collected from zero days, up to 144 hrs. Mastitis was induced as indicated by an increase in SCC, positivity with CMT and increase in total viable bacterial counts in milk. The macroscopic lesions were very well appreciated in all the infected quarters at 48 hrs post infection. The results in the present study clearly showed that both the serotypes induced mastitis in rabbits.

KEYWORDS

Rabbits, mastitis, *E.coli* serotypes O9 and O147, SCC, CMT.

INTRODUCTION

Mastitis remains a major challenge to the worldwide dairy industry. Based on epidemiological studies, cows were reported to be infected with a large number of *E.coli* strains from their environment and the most common etiological agent causing mastitis in cows following *Staphylococcus aureus*. Mammary gland infection studies with cattle mastitis bacterial strains have been carried out in various animal species.

Rabbits have been considered to be good models for mastitis studies as they are cheaper and have a greater number of teats. Further, they are easier to handle for intramammary infection. The study provides a convenient model for fundamental assay on mastitis, prior to final assessment in ruminant species. Earlier, mastitis studies have been carried out in rabbit model by [1,2,3,4]. The present study is undertaken to induce mastitis in rabbits using bovine *E.coli* strains.

MATERIAL AND METHODS

1. E.coli SEROTYPES USED FOR INOCULATION

E.coli O9 and O147 serotypes, which were isolated from bovine mastitis, were used. This selection of serotypes was based on their high prevalence [5], possessing maximum number of virulence genes based on earlier studies carried out by [6] in the Dept. of Veterinary Microbiology, Veterinary College, Bangalore.

2. PREPARATION OF BACTERIAL SUSPENSION

Bacteria were grown on MacConkey agar for 18 hrs at 37°C. Loopful of culture was suspended in two ml 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. EXPERIMENTAL ANIMALS

Twelve female rabbits in 2nd to 4th day of lactation were resourced from the reputed rabbit breeder. Animals were grouped into two and housed in standard cages during the experiment. They were maintained under standard laboratory hygienic conditions, providing standard 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 virulent *E. coli* O9 and O147.

4. INOCULATION TECHNIQUE

Prior to infection, females were exhaustively milked by squeezing the mammary gland from the base of the teat after the administration of 0.5 Units of Oxytocin. Using each of the two *E. coli* strains, six rabbits were infected by inoculating 0.5 ml bacterial suspension (in PBS) at the base of the teat by using 30 G needle. The bacterial count v/s mammary glands used for induction of mastitis in rabbits are as follows:

1st pair - 0.5 ml PBS only
2nd pair - 0.5 ml suspension (5×10^3 cfu) i.e. 10^4 cfu/ml
3rd pair - 0.5 ml suspension (5×10^4 cfu) i.e. 10^5 cfu/ml
4th pair - 0.5 ml suspension (5×10^5 cfu) i.e. 10^6 cfu/ml
5th pair - 0.5 ml suspension (5×10^6 cfu) i.e. 10^7 cfu/ml

5. SAMPLE COLLECTION

After inoculation lesions in the mammary glands were recorded and milk samples were collected from each of the mammary gland at 24 hrs intervals up to 144 hrs. Milk samples collected were used for determination of SCC, bacterial counts and for subclinical mastitis test-CMT.

The details of collection of milk after infection are as shown in the Table 1.

Table 1
Time of milk sample collection from the rabbits

Rabbits	Time of milk collection in hours					
	24	48	72	96	120	144
1st	√	-	-	-	-	-
2nd	√	√	-	-	-	-
3rd	√	√	√	-	-	-
4th	√	√	√	√	-	-
5th	√	√	√	√	√	-
6th	√	√	√	√	√	√

6. SOMATIC CELL COUNT IN MILK

The procedure followed was according to standard general principle of Prescott and Breed method as detailed by [7].

PROCEDURE:

PREPARATION OF MILK FILMS

The milk samples were mixed 15-25 times to obtain a uniform distribution of cells. The samples were allowed to stand for 2-5 min to permit air bubbles to rise and foam to disappear. Identification number of the sample was written on a clean microscopic slide. A level surface was selected and the slide was placed over the template to outline four 1 sq.cm areas. 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.

NEWMAN-LAMPERT STAINING:

The slide was placed on the slide rack and the smears were flooded with modified Newman-Lampert stain **(**Himedia**)** for 2 min. The excess stain was drained off by standing the slides on absorbent paper and air-dried. The slide was rinsed in three changes of tap water at 42-45°C and air-dried.

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.

CALIBRATION OF THE MICROSCOPE:

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 $\Pi r^{2.}$

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

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

7. AVERAGE VIABLE COUNTS OF E.coli IN MILK

Colony forming units of *E.coli* O9 and O147 in the milk secretion was determined by plating diluted and undiluted secretion onto the surface of MacConkey and EMB plates. Secretion was diluted in sterile PBS. All dilutions were done in duplicate. After plating, plates were incubated at 37°C for 24 hrs. Presence of *E.coli* was confirmed by formation of metallic sheen over the colonies on EMB. Colonies were counted and expressed as cfu × 10^6/ ml.

8. CALIFORNIA MASTITIS TEST

The test was followed according to standard procedure [8].

COMPOSITION OF CMT REAGENT

Sodium hydroxide	1.50 g
Teepol	0.50 ml
Bromo thymol blue	0.01 g
Distilled Water up to	100.00 ml

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.

RESULTS

I. GROSS LESIONS

Following inoculation with *E.coli* O9 and O147 serotypes, the rabbit mammary glands showed intense tumescence, hyperemia, induration, being warmer than normal gland and painful upon palpation. Figure 1 reflects the healthy mammary glands of a rabbit. All the glands that were inoculated with different concentration of bacteria showed lesions of varying degree as shown in the Fig. 2. The lesions were well appreciated after 24 hrs of inoculation, but the maximum lesions were seen after 48 hrs of inoculation and then the lesions started subsiding. 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 *E.coli* O9 were 50, 100, 100 and 82.5 per cent respectively, whereas with *E.coli* O147 these were 62.5, 90, 90 and 100 per cent respectively. There was a drastic reduction in the mean percentage of mammary glands infected from third day onwards and on sixth day it had reduced to zero. Fifty per cent of infection was seen with 10^4 cfu/ml of bacterial suspension for both the serotypes (Graph 1 and 2).

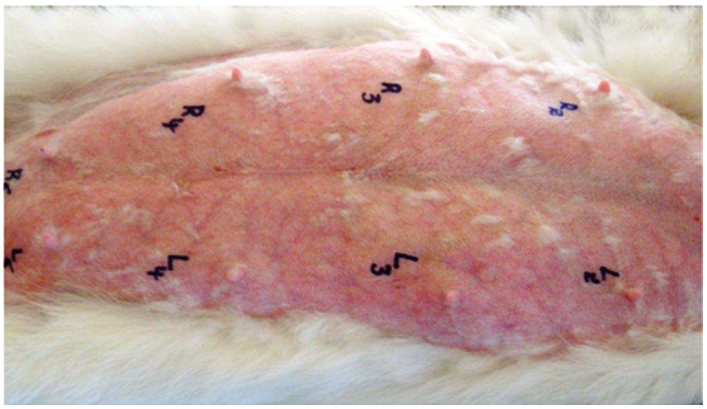


Fig. 1
Healthy mammary gland

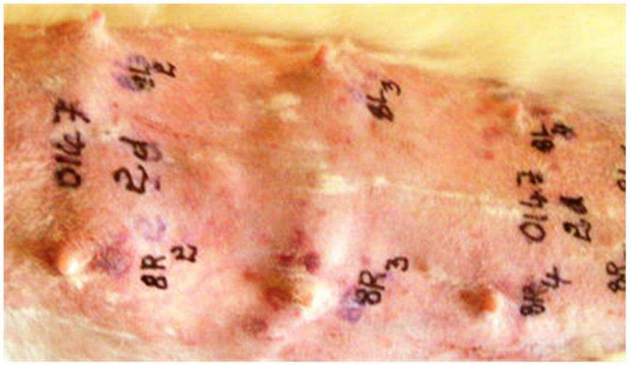
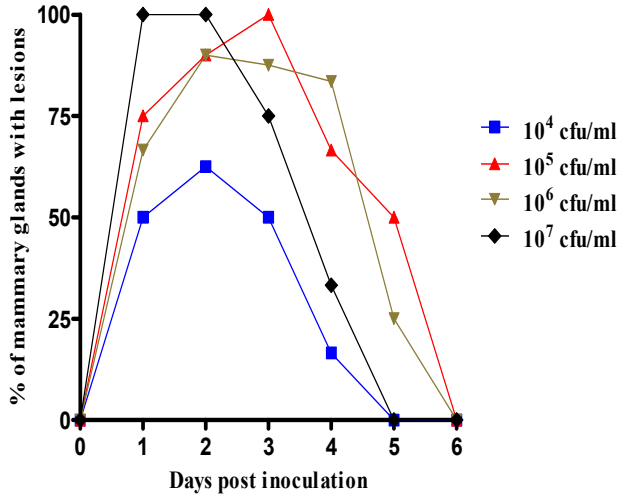


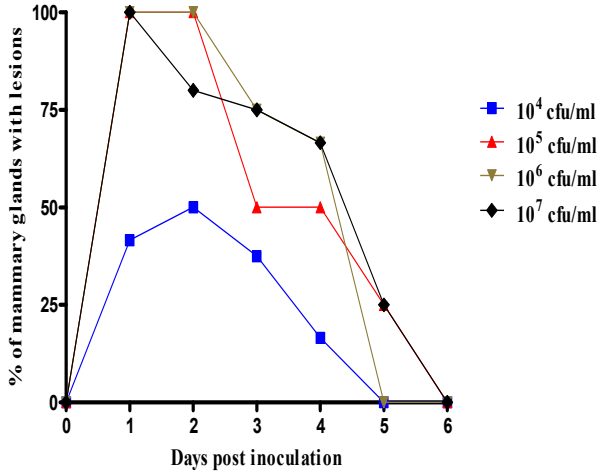
Fig. 2
Gross lesions of rabbit mammary gland after inoculation showing intense tumescence, hyperemia, induration

Graph 1
Percentage of mammary glands showing lesions in rabbits inoculated with E.coli O9



Graph 2

Percentage of mammary glands showing lesions in rabbits inoculated with E.coli O147



II. SOMATIC CELL COUNTS (SCC)

Milk samples collected were subjected for direct microscopic SCC using Newman-Lampert Stain. The SCC including leucocytes particularly heterophils, desquamated epithelial cells and macrophages stained deep blue as shown in Fig. 3 and 4. Pre inoculation milk had SCC of 0.31 × 10^6 cells/ml. Mean SCC in milk at 48 hrs post inoculation with 10^4, 10^5, 10^6 and 10^7 cfu/ml of bacterial suspension of *E.coli* O9 were 8.61 × 10^6 cells/ml, 9.02 × 10^6 cells/ml, 9.83 × 10^6 cells/ml and 7.75 × 10^6 cells/ml respectively, whereas with *E.coli* O147 based infection, it was 7.74 × 10^6 cells/ml, 6.96 × 10^6 cells/ml, 7.55 × 10^6 cells/ml and 6.77 × 10^6 cells/ml respectively. From 48 hrs onwards, counts were reduced. Maximum SCC was obtained at 48 hrs post infection compared to 24 hrs for both the serotypes (Graph 3 and 4).

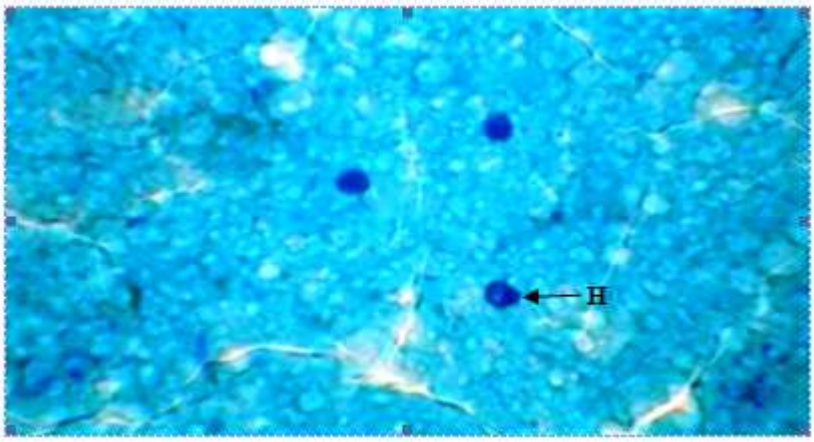


Fig. 3

SCC of pre inoculation rabbit milk H – Heterophils

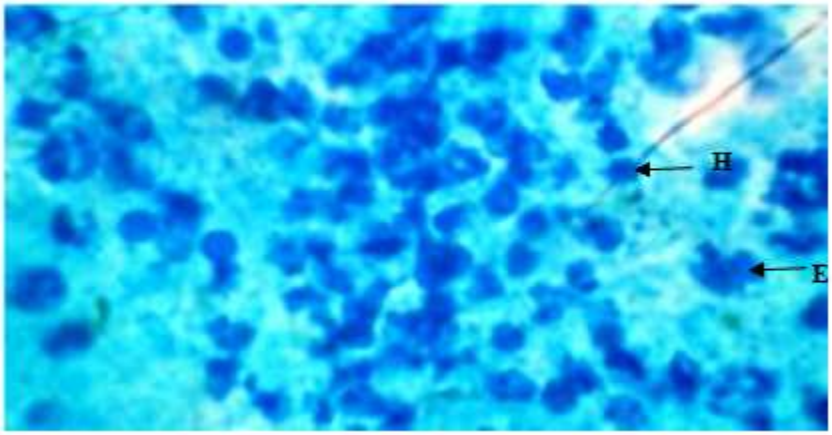
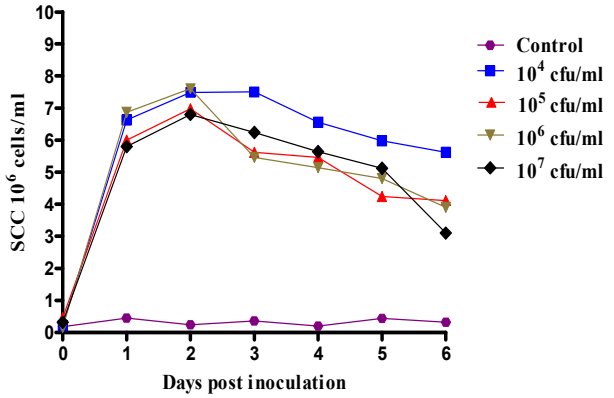


Fig. 4

SCC in 48 hrs post inoculation rabbit milk H – Heterophils, E – Desquamated epithelial cells

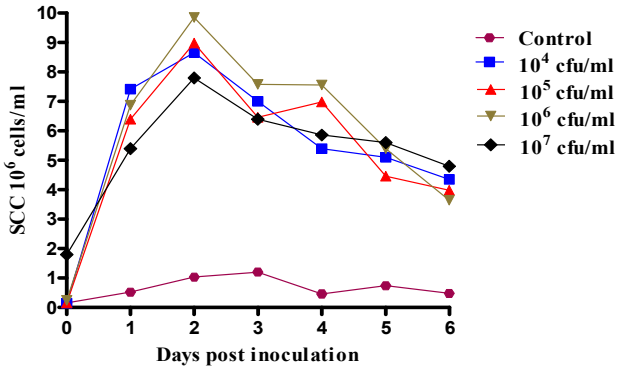
Graph 3

Optimization of infective dose using E.coli O9 based on SCC



Graph 4

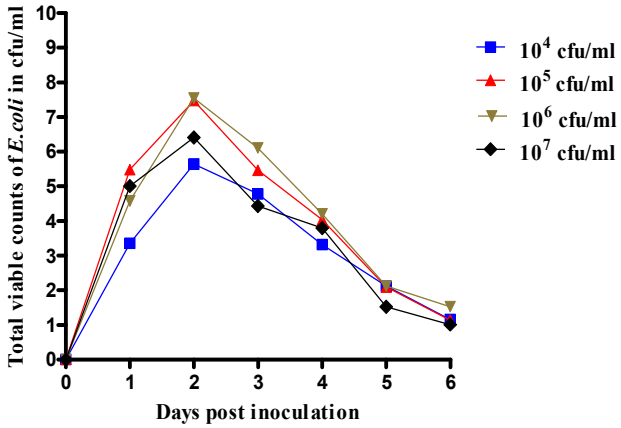
Optimization of infective dose using E.coli O147 based on SCC



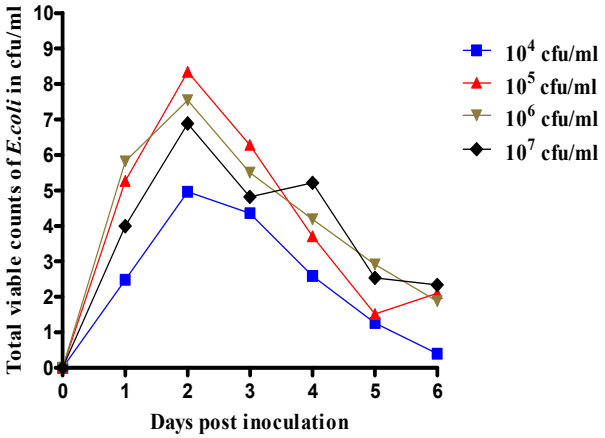
III. TOTAL VIABLE COUNTS OF E.coli IN MILK

Milk samples collected were subjected for estimation of total viable counts of *E.coli* by spread plate method. Mean number of cfu of *E.coli* O9 in milk collected from mammary glands infused with 10^4, 10^5, 10^6 and 10^7 cfu/ml were elevated over pre inoculation mammary glands from zero to 5.64 × 10^6 cells/ml, 7.47 × 10^6 cells/ml, 7.54 × 10^6 cells/ml and 6.40 × 10^6 cells/ml respectively at 48 hrs post inoculation. Similar counts were obtained from mammary glands inoculated with *E.coli* O147. Mean number of cfu of *E.coli* had reached peak at 48 hrs of post inoculation. Among all the concentration of bacterial suspension of *E.coli* O9 and O147, 10^5 cfu/ml and 10^6 cfu/ml bacterial suspensions showed maximum counts as shown in the Graph 5 and6.

Graph 5
Average viable counts of *E.coli* from milk of rabbits inoculated with *E.coli* O9



Graph 6
Average viable counts of E.coli from milk of rabbits inoculated with E.coli O147



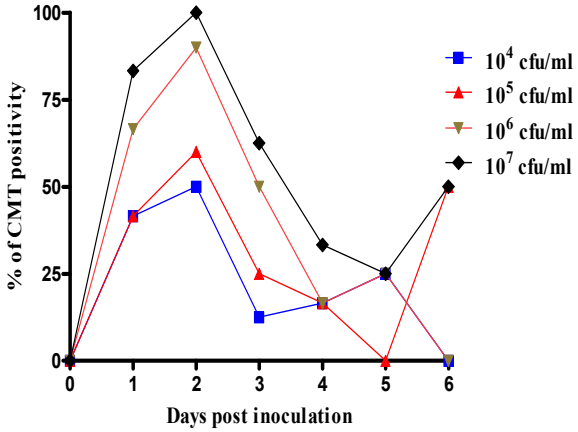
IV. CALIFORNIA MASTITIS TEST

Milk samples collected were subjected to CMT. Mean percentage of CMT positivity in milk of *E.coli* O9 infused mammary glands with 10^4, 10^5, 10^6 and 10^7 cfu/ml were 50 per cent, 60 per cent, 90 per cent and 100 per cent respectively at 48 hrs post inoculation, whereas *E.coli* O147 infused mammary glands

were 50 per cent, 90 per cent, 80 per cent and 90 per cent respectively. Maximum positivity to CMT was found on day two after inoculation. It had reduced to zero on day six and on day five in the glands inoculated with *E.coli* O9 and *E.coli* O147 respectively as shown in the Graph 7 and 8.

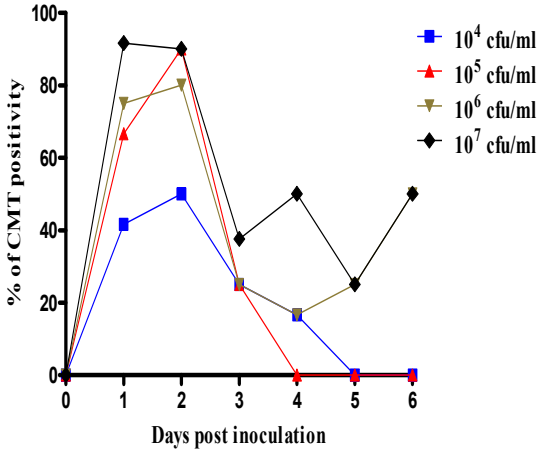
Graph 7

Percentage of CMT positive milk from rabbits inoculated with E.coli O9



Graph 8

Percentage of CMT positive milk from rabbits inoculated with E.coli O147



DISCUSSION

In the present study, an experimental model was developed in rabbits to study bovine mastitis. They help to minimize the cost and the number of animals required in the experiments. Further, they are cheaper and have a greater number of teats. In addition, they are easier to handle for intramammary infections. Earlier mastitis studies have been carried out in rabbit model by [1,2,3,4].

The preliminary study was carried out in the lactating rabbits to optimize the infective dose of *E.coli* to induce mastitis by bovine mastitis isolates *E.coli* O9 and O147 that have high

prevalence [5], possess three different virulent genes [6] and further, the virulence was confirmed by CR dye binding assay.

In the study, inoculation was done with 10^4, 10^5, 10^6 and 10^7 cfu/ml of bacterial suspension via the base of the teat. Milk samples were collected from day zero up to day six. Then, these samples were used for the determination of SCC, total viable counts of *E.coli* and CMT. California mastitis test is an effective cow side proxy for SCC useful to predict IMI in cows [9, 10]. Somatic cell count has been widely implemented as a screening test to identify intramammary infections in lactating cows [11, 12,8,13,14,15,16]. Total viable count is the standard method for the confirmation of presence of infection. All these parameters can be effectively used for the testing the efficacy of vaccine against coliform mastitis.

In the present study, Mastitis was induced in rabbits by both *E.coli* O9 and O147 serotypes as indicated by gross lesions (Fig 1, Graph 1 and2), increase in SCC (Fig 4, Graph 3 and 4), increase in total bacterial counts in milk (Graph 5 and 6) and positivity with CMT (Graph 7 and 8). The macroscopic lesions were very well appreciated in all the quarters of mammary glands infected with varying concentration of bacterial suspension of both *E.coli* O9 and O147 serotypes at 48 hrs after post infection. 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 2 after inoculation with different concentration of bacterial suspension of both *E.coli* O9 and O147 serotypes. These findings are in agreement with [4] 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 *Staphylococcus aureus* isolated from the bovine mastitis cases. They also reported increased SCC and bacterial numbers in milk samples collected from mastitis induced rabbits. The results in the present study clearly show that both the serotypes are able to induce mastitis in rabbits. Fifty per cent of infection was seen with 10^4 cfu/ml of bacterial suspension for both the serotypes which may be used for challenging in vaccinated animals.

CONCLUSION

Mastitis was induced in lactating rabbits by using both *E.coli* O9 and O147 serotypes with 10^4, 10^5, 10^6 and 10^7 cfu/ml of bacterial suspension via the base of the teat. Induction of mastitis was indicated by gross lesions of mammary glands, increased SCC, CMT positivity and increased bacterial count in milk. All these relevant indicators of mastitis showed maximum values at 48 hrs after inoculation. In conclusion, the results showed that both the serotypes induced mastitis in rabbits and 10^4 cfu/ml of bacterial load was found to be optimum for induction of mastitis and for challenging under vaccination trial studies.

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