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# Evaluation of prophylactic and therapeutic potential of bovine recombinant Lactoferrin in MRSA associated mice mastitis model

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**Abstract**
Methicillin-resistant *Staphylococcus aureus* (MRSA), an emerging pathogen in the livestock sector, is a severe challenge to bovine mastitis therapy due to its low susceptibility to commonly used antibiotics. There is an urgent need for alternate treatment modalities, where antimicrobial peptides like Lactoferrin are gaining importance. The prophylactic and therapeutic potential of Bovine recombinant Lactoferrin was studied in the murine mastitis model by intramammary introduction of MRSA into lactating mice. The efficacy of Lactoferrin was studied and evaluated through haematological changes, IL-17, IL-10 and IgG level estimation and acute phase reaction. The CRP qualitative reaction was positive in infected, untreated mice, while CRP was negative in treated mice. IgG production was increased in all infected animals. IL-17 concentration increased at day 3 and day 6 PC in infected, untreated mice, while in treated mice, there was an increase followed by a decrease. IL-10 concentration had increased, followed by a decrease in all infected animals.

**Keywords:** MRSA, Lactoferrin, interleukin, CRP

## Introduction
India stands top in the World regarding milk production, with a total production of 221.1 million tonnes (according to NDDB, 2021-22). Even though the cattle population is more in India (193.46 million no. according to the 20th livestock census), comparative milk production is less. One of the reasons for the low performance of India is poor animal health, mainly due to mastitis, the single most significant economic problem among dairy farmers (Sharma *et al.*, 2012) [20]. Mastitis, defined as the inflammation of the parenchyma of the mammary gland, is a costly and complex disease associated with variable origin, severity and outcome depending on the agent, host and environment (Viguier *et al.*, 2009) [24]. Of all the mastitis pathogens, *Staphylococcus aureus* is recognised worldwide as a frequent cause of subclinical intramammary infections in dairy cows. *S. aureus* poorly responds to therapy with antimicrobial agents, even though antimicrobial therapy is an essential tool in mastitis control programs (Gomes and Henriques, 2015) [5]. The low cure rate in the case of staphylococcal mastitis compared to other mastitis is mainly due to the acquisition of antimicrobial resistance and biofilm formation (Taponen and Pyorala, 2009) [23]. Methicillin-resistant *S. aureus* (MRSA) is an emerging pathogen in the livestock sector that is readily transferable to humans in contact with infected livestock (Liu *et al.*, 2017) [12].
Detection of antibiotic residues in milk poses health hazards to consumers and is of increased economic importance because such milk will be unfit for human consumption (Hafez *et al.*, 2013) [7]. There is an urgent need to search for new antimicrobial therapies, namely non-antibiotic alternatives, to minimise the use of antibiotics in food-borne animals (Gomes and Henriques, 2015) [5]. Antimicrobial peptides (AMPs) are more important in treating infections caused by multidrug-resistant organisms (Mohammed *et al.*, 2016) [13]. Multiple pathogens can be targeted in one treatment by using AMPs (Jennsen *et al.*, 2006) [9]. Of all antimicrobial peptides, Lactoferrin (Lf) is gaining importance nowadays in treating bovine mastitis. Lactoferrin is an 80 KDa Iron binding glycoprotein secreted by most of the external fluids of mammals. Several physiological functions have been attributed to Lf, including broad-spectrum antimicrobial activity and anti-inflammatory and immunoregulatory activities (Guillen *et al.*, 2002) [6]. The concentration of bovine Lf in the milk is low during early lactation. This increases the udder's susceptibility to intramammary infections during early lactation (Paula, 2010) [14].

Lactoferrin alters cell membrane permeability, modifies the motility and aggregation of bacteria, inhibits the adhesion of bacteria and modulates the antibacterial activity of lysozyme and antibiotics towards bacteria (Roseanu *et al.*, 2010) [17].

## Materials and Methods

### Chemicals and media

Molecular biology grade chemicals and media from Sigma-Aldrich (USA), Becton Dickinson (BD) and company (USA), Merck Specialities Private Limited (India), and HiMedia Laboratories Pvt. Ltd. (India) were used in the present study.

### Commercial Kits

Interleukin-10 kit (R & D systems, Inc. USA) and Interleukin 17 kit(R &D systems, Inc. USA) were used.

### Selection of pathogenic MRSA for trial

MRSA MGT strain was used as the challenge strain after its PCR confirmation.

### Determination of Mean colony forming unit (cfu) of MRSA in18 h old Broth culture

Viable counts of 18 h incubated broth cultures of MRSA MGT were determined in 3 cultures (triplicates). The average of the counts was taken as the mean viable count of 18 h incubated broth cultures of the organism (cfu/ml = Average no. of colonies x reciprocal of the dilution).

### Experimental animals

Adult healthy Swiss albino female and male mice of 6-8 weeks and weighing not less than 25 – 30 gms were used for the trial. The trial involved prophylactic feeding of Bovine recombinant Lactoferrin to intact female mice and therapeutic feeding of Bovine recombinant Lactoferrin to lactating mice. *In vivo*, studies of lactating mice infected with MRSA by intramammary route were compared with lactating healthy mice. All experiments were conducted with Institutional Animal Ethics Committee approval. Experimental animals were maintained in a laboratory animal housing facility. After ten days of acclimatisation, the female mice were divided into different groups and treated accordingly as depicted in Table 1.

**Table 1:** Experimental trial groups

| Groups (n= 6) | Particulars | Challenge | Treatment (Prophylactic) | Treatment (Therapeutic) | Reference |
|---|---|---|---|---|---|
| Group I | Normal healthy (NC) | - | - | - | - |
| Group II | Positive control (PC)- MRSA | IMI MRSA | - | - | - |
| Group III | Prophylactic group- Lf* (PL) | IMI MRSA | Lf* @ 0.3% in D/W, OD, 5 days | - | Takakura *et al.* (2003) [22] |
| Group IV | Therapeutic group-Lf* (TL) | IMI MRSA | - | Lf* @ 2% in D/W, BID, 5 days | Chen *et al.* (2013) [3] |

*Lactoferrin

### Prophylactic therapy with Bovine recombinant Lactoferrin

Bovine recombinant Lactoferrin (Himedia Laboratories, Mumbai) at a concentration of 0.3% solution in drinking water (equivalent to 0.5 g/kg/day) was administered from day one post acclimatisation for 5 consecutive days to mice in group III (Takakura *et al.*, 2003) [22]. After prophylactic therapy, these animals were kept for male and female mice cohabitation in the ratio of 1:3.

### Bacterial intramammary challenge of mice

Three days after delivery, the animals were anaesthetised with ketamine and xylazine at 85mg and 12 mg/kg body weight, respectively. The mice were infected in the R4 (fourth on the right) and L4 (fourth on the left) mammary abdominal glands after shaving the area around the gland and swabbing with cotton soaked in 70% ethanol. Briefly, aliquots of 50 µl of the bacterial suspension of MRSA MGT containing approximately $1\times10^{5}$ CFU in endotoxin-free PBS were injected into the mammary ducts of each mouse through the teats using a 1ml insulin syringe with a 31G blunt needle.

### Therapeutic feeding of Bovine recombinant Lactoferrin

After 24 hrs of bacterial challenge, a therapeutic dose of Lactoferrin (2% in drinking water) was administered to mice in the Therapeutic lactoferrin group (Group IV). Doses were repeated at 12 hr intervals for 5 days.

### C-reactive protein estimation

C-reactive protein estimation in the serum samples of experimental animals using a standard kit MICROSIDD TM– CRP (Latex Agglutination Method) at 0 hr, 72 hr and 144 hr was performed as per the manufacturer's instructions.

### Hematological examinations

Total leucocyte count and Differential leucocyte count (DLC) of the blood samples of mice (Jain, 1986) [8] was performed. About 0.25 ml of blood was collected from each mouse by puncturing the retrobulbar venous plexus through the inner eye canthus using heparinised microcapillary tubes and collected in tubes containing EDTA.

### Determination of Ig G using Indirect ELISA

Indirect ELISA was standardised for whole-cell sonicated antigens using the checkerboard titration method. After standardising, ELISA was repeated with samples collected on 0, 3 and 7 days of intramammary inoculation with MRSA. The absorbance was obtained at 495 nm using an ELISA reader and OD values were plotted against days.

### Determination of IL-17 and IL-10 using Sandwich ELISA

Estimation of Interleukin-17 and Interleukin-10 concentration in the serum samples of experimental animals using a standard kit (R &D systems. Inc. USA) at 0 hr, 72 hr and 144 hr was performed as per the manufacturer's instructions. Sandwich ELISA was standardised for standard antigens using the checkerboard titration method. The absorbance was measured at 570 nm and 450 nm using ELISA reader(Thermo Scientific, USA). The value obtained at 570 nm was subtracted from that obtained at 450 nm to avoid error. Optical density values are then plotted against a standard curve to obtain unknown concentrations of IL-17 and IL-10 in

serum samples.

## Results and Discussion

### Total leukocyte count (TLC)

The Total Leukocyte Count significantly increased in Group II, III and IV. However, TLC values decreased on day 6 PC compared to day 3 PC in Group III and IV animals (Table 2).

**Table 2:** Total leukocyte count (Mean±SD) of mice on Days 0, 3 and 6 post-challenge

| Group | Day 0 | Day 3 PC | Day 6 PC |
|---|---|---|---|
| Grp 1 | 4500±282 | 4175±35$^{a}$ | 4250±70$^{a}$ |
| Grp 2 | 4750±424$^{x}$ | 7725±247$^{by}$ | 10550±1060$^{bz}$ |
| Grp 3 | 4087±746$^{x}$ | 5862±615 $^{cdy}$ | 5112±1058$^{acz}$ |
| Grp 4 | 4400±575 | 5000±216$^{bd}$ | 4012±1162$^{ad}$ |

*Values with similar superscripts do not differ significantly ($p$<0.05) in columns and rows **Values with different superscripts differ significantly ($p$<0.05) in columns and rows

### Differential leukocyte count (DLC)

Under normal conditions, lymphocyte outnumbers neutrophils in all groups. After the intramammary challenge, the reverse condition was observed in Group II, infected untreated mice. There was a significant reduction in the percentage of lymphocytes at day three and day 6 PC compared to day 0. This was similar to the previous reports (Brouillette *et al.*, 2005). Contrary to this, all infected treated groups and healthy mice lymphocytes were more when compared to neutrophils (Table 3, Table 4). In Group III and IV animals, which were prophylactically and therapeutically treated with bovine Lactoferrin, there was a significant increase in neutrophil percent at day 3 followed by a significant decrease at day 6 compared to day 0. However, both values were in normal range due to Lactoferrin's anti-inflammatory and immunomodulatory action (Kutila, 2004) [11].

**Table 3:** The relative percentage of lymphocytes (Mean±SD) in response to treatment on Day 0, 3 and 6 post-challenge

| Group | Day 0 | Day 3 PC | Day 6 PC |
|---|---|---|---|
| Grp 1 | 79±1.41 | 76±5.65$^{a}$ | 77±1.41$^{a}$ |
| Grp 2 | 76±8.48$^{x}$ | 38±14.84$^{by}$ | 31.5±2.12$^{bz}$ |
| Grp 3 | 73±11.9$^{x}$ | 64±9.66$^{acy}$ | 74±9.88$^{ac}$ |
| Grp 4 | 66±5.65 | 65.25±4.11$^{ad}$ | 82±5.88$^{ad}$ |

*Values with similar superscripts do not differ significantly ($p$<0.05) in columns and rows **Values with different superscripts differ significantly ($p$<0.05) in columns and rows

**Table 4:** The relative percentage of neutrophils (Mean±SD) in response to treatment on Day 0, 3 and 6 post-challenge

| Group | Day 0 | Day 3 PC | Day 6 PC |
|---|---|---|---|
| Grp 1 | 21±1.41 | 24±5.65$^{ac}$ | 23±1.41$^{a}$ |
| Grp 2 | 17±7.07$^{x}$ | 59.5±12.02$^{by}$ | 68.5±2.12$^{bz}$ |
| Grp 3 | 24.25±10.4$^{x}$ | 34.5±9.14$^{cdy}$ | 25.5±9.88 $^{ac}$ |
| Grp 4 | 31.5±5.97 | 32.5±4.79$^{ad}$ | 16.5±7.72$^{ad}$ |

*Values with similar superscripts do not differ significantly ($p$<0.05) in columns and rows **Values with different superscripts differ significantly ($p$<0.05) in columns and rows

The relative percentage of monocytes had not shown any significant changes in any group except Group II during the treatment period, where there was a decrease on days 3 and 6 PC. There was no significant difference in the eosinophil counts of all infected animals at days 3 and 6 PC.

### C-reactive protein (CRP)

The serum samples of Group I animals were CRP negative throughout the study at 0, 72 and 144 h PC. Acute phase protein CRP was evident in the serum samples of the infected control group at 72 h. Group III animals prophylactically treated with Lactoferrin and Group IV therapeutic lactoferrin groups revealed the absence of CRP at 72 h and 144 h PC.CRP concentration was shown to increase in bovine serum and milk during mastitis (Schroedl *et al.*, 2003) [18]. Lactoferrin has anti-inflammatory action that suppresses the inflammatory responses (Shimazaki, 2017; Kutila, 2004) [21, 11].

### Determination of total IgG antibody in serum using ELISA

There was a significant increase in OD values in all infected groups at day 3 and day 6 PC (Table 5). According to previous reports by Sfeir *et al.* (2004) [19], oral administration of bovine Lactoferrin will influence mucosal and systemic immune responses. They observed that Lactoferrin will increase IgG production, similar to observations obtained in our study.

**Table 5:** Optical Density values of Immunoglobulin G in response to therapy in mice(Mean±SD)

| Group | Day 0 | Day 3 PC | Day 6 PC |
|---|---|---|---|
| Grp 1 | 0.0867±0.0002 | 0.0842±0.0005$^{a}$ | 0.0874±0.0001$^{a}$ |
| Grp 2 | 0.0842±0.0001$^{x}$ | 0.1284±0.0045$^{by}$ | 0.2106±0.0004$^{bz}$ |
| Grp 3 | 0.0857±0.0036$^{x}$ | 0.1176±0.0028$^{cby}$ | 0.2328±0.0052$^{cbz}$ |
| Grp 4 | 0.0827±0.002$^{x}$ | 0.1123±0.00429$^{cby}$ | 0.2543±0.0081$^{cz}$ |

*Values with similar superscripts do not differ significantly ($p$<0.05) in columns and rows **Values with different superscripts differ significantly ($p$<0.05) in columns and rows

### Determination of Interleukin-17 in serum using ELISA

A significant increase in IL-17 concentration was noticed in all infected groups at day 3 PC. Eventhough a significant decrease was noticed in IL-17 concentration in all infected treated groups at day 6 PC, a significant increase in IL-17 concentration was noticed in the infected untreated group (Table 6). The magnitude of inflammatory reaction in the udder is assessed by milk leukocytosis, which is directly related to the production of Interleukin-17 (Rainard *et al.*, 2015) [16]. Rainard *et al.* (2013) [15] observed that after a challenge, IL-17 could be detected as soon as eight h post challenge and concentration will reach peak by 24 h post challenge.

**Table 6:** The concentration of Interleukin-17 in response to therapy in mice (Mean±SD)

| Group | Day 0 | Day 3 PC | Day 6 PC |
|---|---|---|---|
| Grp 1 | 46.102±0.00014 | 42.616±0.716$^{a}$ | 45.604±0.711$^{a}$ |
| Grp 2 | 43.109±4.24$^{x}$ | 169.161±2.113$^{by}$ | 252.537±7.07$^{bz}$ |
| Grp 3 | 44.602±2.120$^{x}$ | 94.168±2.128$^{cy}$ | 84.61±3.535$^{cz}$ |
| Grp 4 | 43.604±2.116$^{x}$ | 106.395±1.829$^{dy}$ | 94.704±3.641$^{cdz}$ |

*Values with similar superscripts do not differ significantly ($p$<0.05) in columns and rows **Values with different superscripts differ significantly ($p$<0.05) in columns and rows

### Determination of Interleukin-10 in serum using ELISA

A significant increase in IL-10 concentration was noticed in all infected groups at day 3 and day 6 PC compared to day 0. Eventhough a significant increase was noticed in IL-10 concentration in all infected treated and untreated groups at day 3 PC, a significant decrease in IL-10 concentration was

noticed at day 6 PC in infected groups except group II (Table 7). Bruni *et al.* (2016) [2] reported that Lactoferrin will suppress inflammatory effects caused by bacteria. There were previous reports that Lactoferrin inhibits the formation of interleukins concerned with inflammatory responses, and Lactoferrin, if provided during non-lactating periods, showed profound protective effects against bacterial infection (Shimazaki, 2017) [21].

**Table 7:** Concentration of Interleukin-10 in response to therapy in mice (Mean±SD)

| Group | Day 0 | Day 3 PC | Day 6 PC |
|---|---|---|---|
| Grp 1 | 42.17±1.55 | 39.19±1.87$^{a}$ | 41.93±3.36 |
| Grp 2 | 39.68±0.62$^{x}$ | 199.18±2.63$^{by}$ | 57.06±0.37$^{z}$ |
| Grp 3 | 45.30±0.278$^{x}$ | 70.60±7.46$^{cy}$ | 61.07±1.72 |
| Grp 4 | 43.30±4.28$^{x}$ | 105.61±5.1$^{d\,cy}$ | 67.29±8.69 |

*Values with similar superscripts do not differ significantly ($p$<0.05) in columns and rows **Values with different superscripts differ significantly ($p$<0.05) in columns and rows

The mice prophylactically treated with bovine recombinant Lactoferrin showed reduced rates of mastitis compared with other infected groups. This study observed significant changes in CRP, DLC, enhanced IgG levels and better cytokine response in mice of Group III and IV. Prophylactic feeding of bovine Lactoferrin in a dry period prevents the establishment and progression of MRSA-induced intramammary infection. This may be due to the creation of an iron-deficient environment by Lactoferrin, which limits bacterial growth and alters bacterial cell membrane permeability by Lactoferrin. Many workers previously gave similar reports (Roseanu *et al.*, 2010; Diarra *et al.*, 2003) [17, 4]. Previous reports by Kai *et al.* (2002) [10] prove the therapeutic use of bovine Lactoferrin in treating and controlling S. aureus-induced mastitis, but the efficacy in MRSA associated mastitis has not yet been proved. In this study, even though mice treated prophylactically are giving better results, Group IV mice therapeutically treated with Lactoferrin are not giving promising results.

## Conclusion

MRSA is resistant to almost all antibiotics, so alternate treatment modalities should be considered for treating MRSA infection. Prophylactic treatment using Lactoferrin during the dry period will prevent bacterial invasion and reduce inflammatory reactions. Lactoferrin inhibits the formation of interleukins concerned with inflammatory responses and has anti-inflammatory, antibacterial and immunomodulatory action. The mouse model of mastitis proposed can be effectively used in studying the pathogenesis and therapy for bovine mastitis.

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