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Pathology of an experimental intramammary infection with two isolates of *Streptococcus uberis* in mice

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Abstract
In the present study, *Streptococcus uberis* isolated from bovine milk was inoculated into the mice mammary gland and studied biochemical and pathological changes in time course manner. Thirty lactating Swiss albino mice were inoculated with *S. uberis* (5 x 10^3 cfu in 50µl) by intramammary route on day 7 of lactation. Experimental inoculation was done on abdominal mammary glands L4, L5, R4, R5 and control group with PBS and mice were euthanized at 2, 4, 8, 12, 24 and 48h time points. The mammary gland weight and percentage of body weight revealed significant (P<0.05) increase at initial time periods after inoculation and reduced at 48h. Serum Aspartate transaminase (AST), Alanine transaminase (ALT) and Lactate dehydrogenase (LDH) revealed initial increase indicating mammary gland tissue damage and later decreased at 48h. On gross examination, mammary gland showed reddish coloured and congestion at 12, 24h and normal pinkish appearance with milk at 48h. On histopathology, both isolates at 12, 24h showed mild to moderate infiltrations of neutrophils and alveolar macrophages in the interstitial space. At 48h showed recovery of the mammary gland to normal alveolar architecture. Thus, successfully established *S. uberis* mastitis in mice, with moderate pathological changes and biochemical changes especially LDH which may be used as an indicator for *S. uberis* mastitis in mice.

Keywords: *Streptococcus uberis*, Mice, mastitis, biochemistry, pathology

1. Introduction
India ranks first in cattle and buffalo population in the world and also ranks first in the world milk production, with total milk production of 146.3 million tonnes during 2014 and contributes to 18.6% of world milk production as per Economic Survey of India (2015-16) report. Although several diseases affect the milk productivity and economy, mastitis is regarded as one of the costliest disease confronting the dairy industry. It is estimated that mastitis accounts to about 70 per cent of all avoidable losses incurred during milk production (Sadana, 2006) [19]. The direct effects of mastitis include either temporary or permanent loss in milk production, poor milk quality, reduction in milk price, increase in treatment costs and premature culling (Krishnamoorthy *et al*., 2017a) [8]. In India, the total annual economic loss due to mastitis was calculated to be 7,165.51 crore rupees (Bansal and Gupta, 2009) [1]. Among the bacteria isolated in bovine mastitis, *Staphylococcus* species occupies an important place and next comes the *Streptococcus* species in India. The pooled prevalence estimate for the *Streptococcus* species was 14 per cent based on the meta-analysis of mastitis prevalence studies in India (Krishnamoorthy *et al.,* 2017b) [12]. The *S. uberis* is a major environmental mastitis causing pathogen, however it is important pathogen still the pathogenesis is not understood yet in detail in India. The pathogenesis of experimental intramammary infection of *S. uberis* in bovine has been studied earlier (Rambeaud *et al*., 2003) [18]. The study of mastitis in bovines is costly and involves various ethical and social issues, especially in India and also keeping the bovines in controlled environment is difficult. Hence, the pathogenesis of mastitis pathogens are mostly studied in laboratory animals like mice, rat and rabbit models. The mouse mastitis model is considered to be very good model to study mastitis compared to other laboratory animals for ease of handling, ease of keeping them in controlled environment and less cost (Krishnamoorthy *et al*., 2017) [8]. The mouse model is regarded as straight forward and suitable model for the study of bovine mastitis which provides valuable information about pathogenic mechanisms of variety of organisms involved in the intramammary infections (Notebaert and Meyer, 2006) [16]. Perusal of reported literatures revealed scanty information is

available on clinical pathology of experimentally induced mastitis in mice by using *S. uberis* especially in India. Keeping this information in background, the present study was undertaken to know the clinical pathological changes of two *S. uberis* isolates in experimental intramammary infection in mice.

2. Materials and Methods

2.1 Bacteria used for inoculation

Two isolates of *Streptococcus uberis* was isolated from milk samples of apparently healthy bovines from dairy farms in Karnataka and species identification was done by using Genus, species and 16S RNA PCR by our research team as reported earlier (Shome *et al*., 2011,2012) [21,22]. The Multi-Locus Sequence Typing of the isolates using seven housekeeping genes of *S. uberis* was carried out as described earlier (Coffey *et al*., 2006) [5].

2.2 Experimental Animals and Infection

Thirty five timed pregnant (Day 12 to 15), Swiss albino mice were procured from National Centre for Laboratory Animal Science, Hyderabad. The mice were grouped in to seven groups of five mice in each group consisting of 30 for bacterial inoculation and five for PBS inoculation. The mice were housed in individually ventilated cages (IVC) and temperature and humidity were maintained at 23 ± 3 ºC and 50 to 70 per cent respectively. Mice were provided rodent pellet feed procured from M/s. Nutripet India Private Limited, Bengaluru, India and reverse osmosis purified water *ad libitum*. The animal experiments were approved by Institutional Animal Ethics Committee (IAEC) of ICAR-National Institute of Veterinary Epidemiology and Disease Informatics, Bengaluru (Registration No. 881/05/R/S/CPCSEA). The experiment was carried out as per the guidelines of Committee for the purpose of Supervision and Experiments on Animals (CPCSEA), Ministry of Environment, Forests and Climate Change, New Delhi. The *S. uberis* isolate 1 (SU1) and *S. uberis* isolate 2 (SU2) were inoculated to thirty mice each on 7th or 9th day of lactation with dose containing 5 x 10^3 cfu of bacterial suspension in PBS (50 µl) per teat of left fourth and fifth (L4, L5) and right fourth and fifth (R4, R5) teats and control mice were inoculated with sterile PBS as reported earlier (Krishnamoorthy *et al*., 2014a, b) [9, 10]. After one hour of intramammary inoculation (IMI), the pups were allowed to suckle the teat to simulate the natural field conditions of bovines in dairy farms. The mice from both bacteria inoculated and PBS control groups were euthanized at different time intervals at 2, 4, 8, 12, 24 and 48h and thorough post mortem examination was carried out.

2.3 Animal weight and mammary gland weight

The mice were weighed using weighing balance (Precisa, Switzerland) and recorded the body weight in grams. The mice mammary glands were carefully dissected out using sterile scissors and forceps. The left (L4, L5) and right (R4, R5) mammary glands were separately collected, weighed and expressed in grams. The mammary gland weight (average of left and right side) and animal body weight was used to calculate the percentage of mammary gland weight to body weight.

2.4 Serum Biochemistry

Blood was collected by intracardiac route by using sterile syringe and needle from mice in dipotassium ethylene diamine tetra acetate (K_2EDTA) vials for serum biochemistry. The serum aspartate aminotransferase (AST) was estimated by Modified UV International Federation of Clinical Chemistry (IFCC), alanine aminotransferase (ALT) by Modified UV IFCC (Bergmeyer *et al*., 1978) [2] and lactate dehydrogenase (LDH) by optimized Deutsche Gesellschaft fur Klinische Chemie methods (Buhl and Jackson, 1978) [3] by using commercially available kits (M/s. Span Diagnostics Limited, Surat, India) and Semiauto analyzer, Biosystems (BTS 320) as reported earlier (Krishnamoorthy *et al*., 2015) [11].

2.5 Pathology

The gross changes in the mammary gland and various organs was recorded and histopathological studies were carried out on representative tissue samples collected during experimental intramammary infection in mice. The mammary gland tissues and various organs like liver, kidney, spleen, heart, lung and inguinal lymph node were collected and immediately fixed in 10 per cent neutral buffered formalin. After proper fixation, the tissue samples were processed by routine paraffin embedding technique. Sections of four to five micron thickness were cut using rotary microtome with disposable blades. The sections were then stained with routine haematoxylin and eosin method (Luna, 1968) [14] and observed under bright field microscope (Nikon, Japan).

2.6 Statistical analysis

Data obtained from mammary gland weight, percentage of mammary gland weight and biochemical parameters were analyzed by using Statistical Analysis System (SAS) software SAS Enterprise Guide version 5.1 (SAS, 2012) [20] and by one way analysis of variance (ANOVA) method (Snedecor and Cochran, 1980) [23], and obtained the significant difference between different time points. The results were expressed as the Mean ± SE (Standard error) with significant difference at $P<0.05$ and confidence interval at 95 per cent level.

3. Results

3.1 Bacteria

The two isolates used for inoculation was confirmed by genus, species specific and 16sRNA PCR and identified as *S. uberis*. The Multi-Locus Sequence Typing of the two isolates of *S. uberis* was done and found to be Sequence type (ST) 475, which is prevalent in India.

3.2 Clinical signs and Mice mammary gland weight

The clinical signs of mastitis were not apparent at 2, 4 8, 12, 24 and 48h after intramammary inoculation with SU1 and SU2 in mice. The mean ± SE mammary gland weight and percentage of mammary gland weight at different time points after IMI is presented in Table 1. No significant difference in the mammary gland weight and percentage of mammary gland weight was observed in PBS inoculated mice. The mammary gland weight showed significant ($P<0.05$) increase in SU1 and SU2 infected mice at 2h and significant ($P<0.05$) decrease at 24h for SU1 and 48h for SU2 when compared to other time points. The percentage of mammary gland weight revealed significant ($P<0.05$) increase at 2h in SU1 and 4h in SU2 and significant ($P<0.05$) decrease at 24h in SU1 and 48h in SU2.

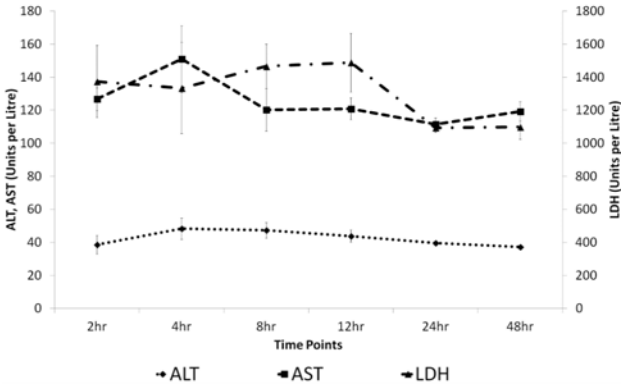
Table 1: The mice mammary gland weight (g) and percentage of mammary gland weight to body weight (%) after IMI with *Streptococcus uberis* (SU1, SU2)

Time Points	Mammary gland weight (gm)			Percentage of mammary gland weight (%)		
	PBS	SU1	SU2	PBS	SU1	SU2
2h	0.67 ± 0.07	0.81 ± 0.04[a]	0.75 ± 0.05[a]	2.07 ± 0.20	2.21 ± 0.19[a]	2.10 ± 0.13[ab]
4h	0.67 ± 0.09	0.66 ± 0.05[ab]	0.73 ± 0.04[a]	1.95 ± 0.28	2.03 ± 0.17[ab]	2.31 ± 0.12[a]
8h	0.65 ± 0.01	0.50 ± 0.04[bc]	0.60 ± 0.04[ab]	2.06 ± 0.16	1.51 ± 0.04[bc]	1.72 ± 0.13[ab]
12h	0.51 ± 0.05	0.55 ± 0.02[bc]	0.55 ± 0.10[ab]	1.51 ± 0.03	1.55 ± 0.07[bc]	1.71 ± 0.31[ab]
24h	0.57 ± 0.02	0.40 ± 0.06[c]	0.60 ± 0.04[ab]	1.92 ± 0.05	1.17 ± 0.10[c]	1.82 ± 0.13[ab]
48h	0.67 ± 0.07	0.66 ± 0.07[ab]	0.45 ± 0.006[b]	1.97 ± 0.21	1.85 ± 0.16[ab]	1.35 ± 0.02[b]
Mean	0.62 ± 0.03	0.59 ± 0.04	0.62 ± 0.03	1.91 ± 0.08	1.72 ± 0.10	1.83 ± 0.10
CI	0.58 – 0.70	0.51 – 0.67	0.56 - 0.68	1.75 – 2.07	1.52 – 1.92	1.63 - 2.03

[a, b, c]: Means with same superscript within the column do not differ significantly (P>0.05)
CI: Confidence interval at 95 per cent level

3.3 Serum Biochemistry

The serum Aspartate transaminase (AST), Alanine transaminase (ALT) and Lactate dehydrogenase (LDH) values at different time points after IMI with *S. uberis* is presented in Graph 1. The AST showed initial increase at 4 and 8h after IMI with *S. uberis* isolates in mice. The increase was significant (P<0.05) difference at 4h when compared to the 24h in mice. ALT values revealed non-significant increase at 4h after IMI when compared to other time periods. At 8 and 12h after IMI with *S. uberis* in mice revealed non-significant increase of LDH and indicated that it might be resulted from mammary gland tissue damage caused by the infecting organisms.



Graph 1: Graph showing serum Alanine transaminase (ALT), Aspartate transaminase (AST) and Lactate dehydrogenease (LDH) values at different time points after intramammary inoculation with *Streptococcus uberis* in mice.

3.3 Gross pathology

The PBS inoculated mice revealed normal pink colour of mammary gland structure and on incision normal milk oozed out. The gross changes were not observed during 2, 4 and 8h after IMI, but reddish colour, haemorrhagic and congested mammary glands was observed at 12 and 24h (Fig. 1a). The mammary gland appeared pink colour with milk on incision and appeared normal at 48h (Fig. 1b) after IMI of SU1 and SU2 isolates in mice. On palpation, at 24h the gland was firm to palpate and upon incision thick, slightly discoloured milk oozed out from the infected mice mammary glands when compared to PBS group. There was no difference between the two isolates SU1 and SU2 in the gross changes of mammary gland in mice. There was no gross changes in liver, kidney, spleen, lung, heart and inguinal lymph nodes.

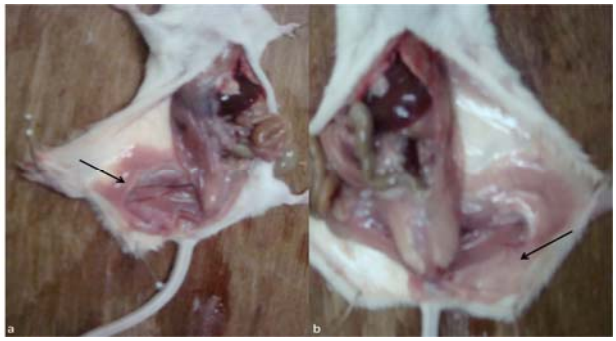


Fig 1: Gross changes observed in the abdominal mammary gland in mice at 24h showing congested and reddish colour mammary gland (arrow) indicating inflammatory reaction (1a) and at 48h mammary gland revealed pinkish colour (arrow) with milk secretion in the gland (1b).

3.4 Histopathology

The mammary glands in mice inoculated with sterile PBS as control did not reveal any histological changes at 2, 4, 8, 12, 24 and 48h. The microscopic appearance of the mammary gland was normal in which several lobules showed secretory acini and were distended with eosinophilic secretory material in the alveolar lumen and separated by interlobular septa. Mammary gland of mice infected with SU1 and SU2 revealed no observable histopathological lesions at 2, 4, 8h after IMI. The SU1 and SU2 showed histopathological lesions at 12h with mild changes with infiltration of few inflammatory cells mainly neutrophils in the interstitial space and between the lobules (Fig. 2a). The lesions at 24h included thinning of alveolar epithelial cells and loss of connective tissue with enlarged lobules, moderate degree of hyperemia with perilobular and interlobular edema. The lumen of acini was filled with varying amount of eosinophilic secretory material along with desquamated epithelial cells and moderate infiltration of inflammatory cells mainly neutrophils and macrophages (Fig. 2b). There was moderate infiltration of neutrophils with multi-lobed nucleus and mononuclear cells mainly macrophages and lymphocytes in the alveolar lumen which was shown in Fig. 2c. The mammary gland revealed proliferation of alveolar epithelial cells with increase in alveolar structure and congestion of blood vessels at 48h after IMI (Fig. 2d). No observable histological changes were observed in the liver, kidney, spleen, heart, lung and inguinal lymph node of mice after IMI with SU1 and SU2.

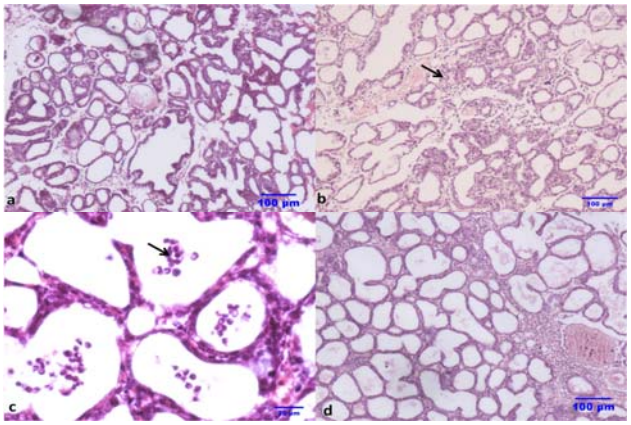


Fig 2: Histopathological changes observed at different time points in mice after IMI with *S. uberis,* at 12h few inflammatory cells mainly neutrophils are seen in the interstitial space (2a). At 24h, thinning of alveolar lining epithelial cells, dilatation of alveolar lumen and moderate infiltration of inflammatory cells (arrow) with congestion of blood vessels (2b). Higher magnification showing inflammatory cells neutrophils and macrophages (arrow) with dilatation of alveolar lumen (2c). Normal architecture of alveolar lumen with congestion of blood vessels (2d). Haematoxylin and Eosin stain, Scale bar = 100µm and 20 µm.

4. Discussion

There was no clinical signs reported in mice infected with two isolates of *S. uberis* SU1 and SU2 in the present study. The Lasagno *et al.,* (2012) [13] reported no signs or systemic symptoms evoked by *S. uberis* inoculation in experimental goats and concurred with the present study. The absence of clinical signs in this study may be due to low dose of organisms as compared to other reported studies in which the dose used was above 10^5 cfu. In the present study, the mammary gland weight showed significant increase at 2 and 4 h after IMI in SU1 and SU2, respectively. The slight increase in the mammary gland weight was observed when compared to the PBS control mice. The increased mammary gland weight may be due to the inflammatory processes, congestion and infiltration of inflammatory cells at early time points. However, the slight reduction in mammary gland weight at 24h was observed, may be due to loss of milk in mammary gland and compensated by the infiltration of inflammatory cells.

Aspartate aminotransferase (AST) has been used to detect the extent of liver cell damage during disease conditions and recent studies have shown the relationship between the AST values and development of clinical mastitis. The AST showed non-significant increase at 4 and 8h after IMI with *S. uberis* isolates in mice and concurred with previous reports with other mastitis pathogens (Krishnamoorthy *et al*., 2015, Zaki *et al.,*2008, Moyes *et al*., 2009) [11, 24, 15]. However, Zaki *et al.,* (2008) [24] reported a highly significant increase in AST values after infection with *Streptococcus agalactia* in buffaloes. The aspartate aminotransferase may be potential marker for the risk of mastitis in early lactation in dairy cattle (Krishnamoorthy *et al*., 2015, Moyes *et al.*, 2009) [11, 15]. The increase of AST values was attributed to the stressful conditions after IMI in mice (Lasagno *et al.*, 2012) [13]. ALT values revealed non-significant increase at 4h after IMI when compared to other time periods. The results of the present study concurred with the previous report (Zaki *et al*., 2008) [24]. At 8 and 12h after IMI with *S. uberis* in mice revealed non-significant increase of LDH activity and concurred with previous studies with various mastitis causing organisms (Krishnamoorthy *et al*., 2015, Zaki *et al.,* 2008, Chagunda *et al*., 2006, Kermori *et al.,* 1989) [11, 24, 4, 7]. The increase can be attributed to the participation of leukocytes which have LDH activity at the 1,000 U/mg protein level in mastitic milk. This indicated that it might be resulted from mammary gland tissue damage caused by the infecting SU1 and SU2 in mice.

In the present study, the gross lesions were not observed in liver, kidney, spleen, lung, heart and inguinal lymph nodes at different time points. The reason for the same might be due to low dose of organisms (5×10^3) inoculated in mice. Thus the dose and species of the organisms plays an important role in establishment of infection and generation of lesions in natural infections even in dairy cattle. However, mammary gland showed the reddish discoloration of mammary gland at 12 and 24h after IMI with SU1 and SU2 which indicated the congestion and infiltration of blood cells in the mammary gland. The severity of histopathological lesions revealed no variation between the two isolates of SU1 and SU2 in mice model of mastitis in the present study. The histological changes observed in this study was supported by the gross lesions in the *S. uberis* infected mice mammary gland. The histological changes observed were moderate infiltration of inflammatory cells mainly neutrophils and mononuclear cells in the interstitial space and alveolar lumen at 12 and 24h after IMI and concurred with previous study (Krishnamoorthy *et al*., 2017a, Lasagno *et al.*,2012) [8, 13]. However, the SU1 and SU2 could not resist phagocytosis and eventually got cleared from mammary gland through phagocytosis by neutrophils and macrophages at 48h after IMI. The observations in the present study clearly indicated that there is mammary tissue damage in the mice infected with *S. uberis*. The mammary tissue damage has been shown to be induced by either apoptosis or necrosis and both host and bacterial factors contribute to epithelial tissue damage. During infection of mammary glands, the tissue damage can be initially caused by bacteria and their products. Mastitis is characterized by an influx of somatic cells, primarily neutrophils into the mammary gland which leads to break down of blood milk barrier; damage to the alveolar epithelium worsens. Polymorphonuclear neutrophils can harm the mammary tissue by releasing reactive oxygen intermediates and proteolytic enzymes (Zhao *et al*., 2008) [25]. The oxidative stress can damage all types of biomolecules like DNA, proteins, lipids and carbohydrates which perpetuates tissue injury. The PMN cells have primary, secondary and tertiary granules which contain bactericidal peptides, proteins and enzymes such as elastase, proteinases and myeloperoxidases which are released into the extracellular environment and cause tissue destruction during mastitis (Paape *et al*., 2003) [17]. The mammary tissue damage could also be caused by the proteinases and collagenolytic enzymes which degrade the extracellular matrix compounds (Haddadi *et al*., 2005) [6]. The tissue damage was induced either by apoptosis or necrosis in the mammary epithelial cells indirectly through induction of proteases or proinflammatory cytokines (Zhao *et al*., 2008) [25]. Peptidoglycan fraction of cell wall of bacteria is involved in hypersensitivity reaction of the mammary gland. The cytokines also promote a wide variety of function of PMN cells, including adhesion, surface receptor expression, free radical production and release of lysosomal constituents (Paape *et al*., 2003) [17].

5. Conclusions

In the present study, successfully induced mastitis by using two isolates of *Streptococcus uberis* from cattle in organized

dairy farms in mice model. Histopathological changes in mammary gland indicated that the *S. uberis* caused moderate mastitis in mice. *S. uberis* mastitis in mice was overcome by host factors, but still it is considered as emerging mastitis pathogen and very important in subclinical mastitis in dairy cattle. Further, studies on host pathogen interaction at sub cellular level is required to derive the mechanism of damage caused by these bacteria in mice.

6. Acknowledgement

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7. Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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