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Development of a mouse chronic mastitis model by intramammary infusion of bovine associated *Staphylococcus aureus*

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Abstract
Mastitis, the inflammatory disease has a great impact on economy of farmer as well as on the welfare of animal. It is one of the most costly diseases in dairy industry, is an inflammation of the mammary gland caused by pathogenic infection. From last few decades its pathogenesis, curing metabolites and other alternatives are being studied using different animal models. Husbandry, management and costs included, have always been an obstacle while using large dairy animals as experimental model for the study of mastitis. A laboratory animal model for studying mastitis that is not only inexpensive but can be easily maintainer standard facilities would be of considerable importance. The present article focuses on the use of the mouse as an exploratory model of *Staphylococcus aureus*-induced mastitis for the study of bovine mastitis.

Keywords: Mastitis, mouse, *Staphylococcus aureus*, animal model, welfare

Introduction
Mastitis, the inflammatory disease has a great impact on economy of farmer as well as on the welfare of animal. It is one of the most costly diseases in dairy industry, caused by pathogenic infection which leads to lowered production and quality of milk. During the pathogenesis of bacterial induced mastitis damage and degeneration of milk-secreting alveolar cells takes place gradually resulting in necrosis of mammary tissue, which leads to a permanently reduced milk production. A number of biological and environment originated factors can cause mastitis but *Staphylococcus aureus* is known for its ability to establish
contagious and chronic disease (Sender *et al*., 2017) [7]. This *S. aureus* induced mastitis imparts a major challenge to dairy farmers due to the frequent incapacity of both the immune response and antibiotics to prevent infection and destroy the pathogen in the intramammary environment. From last few decades its pathogenesis, curing metabolites and other alternatives are being studied using different animal models. The costs associated with experimental Intramammary infection in the cow are prohibitive. Even with the use of smaller animals like goats, the cost is still a major obstacle when conducting experiments that require a minimal number of animals to get valid statistics. Consequently, since only a few hypotheses can be investigated in big animals, alternatives are needed. Here, we discuss the creation of the mouse model of *S. aureus* mastitis as such an alternative.

Material and Methods
Experimental animals
Apparently healthy adult and lactating female Swiss albino mice of 3-4 days lactation were procured from the Laboratory Animal Resource section (LAR), Indian Veterinary Research Institute. Experimental mice were acclimatized for 5days in the Experimental Animals Sheds, Division of Pharmacology, IVRI, and maintaining under standard management conditions through providing adlibitum feed and water, daily light cycle rotated between 12h of light and 12h of darkness. Their off springs were weaned 1-2h before inoculating *Staphylococcus aureus* via intramammary route.

Bacterial strain
Field strain of *S. aureus* characterized both bacteriologically and biochemically from the Division of Veterinary Medicine, ICAR- Indian Veterinary Research Institute was used for

intramammary inoculation in mice.

Preparation of inoculums of *Staphylococcus aureus* for intramammary infection

The strain was sub cultured continuously to keep it viable and in enough number for use. For preparing *S. aureus* inoculum feasible count of broth culture of *S. aureus* which is incubated overnight was determined in triplicates. Briefly, colony from Baired Parker Agar plate by scrap method was inoculated into 10 ml of nutrient broth and will be incubated for 7 hours at 37 ºC as shown in fig 1. Then the serial dilution of *S.aureus* suspension from 10^{-1} to 10^{-8} in PBS and the dose of bacteria was calculated using surface viable count method (Miles *et al*., 1938) [6].

CFU= average number of colonies ×dilution factor / volume of culture plated.

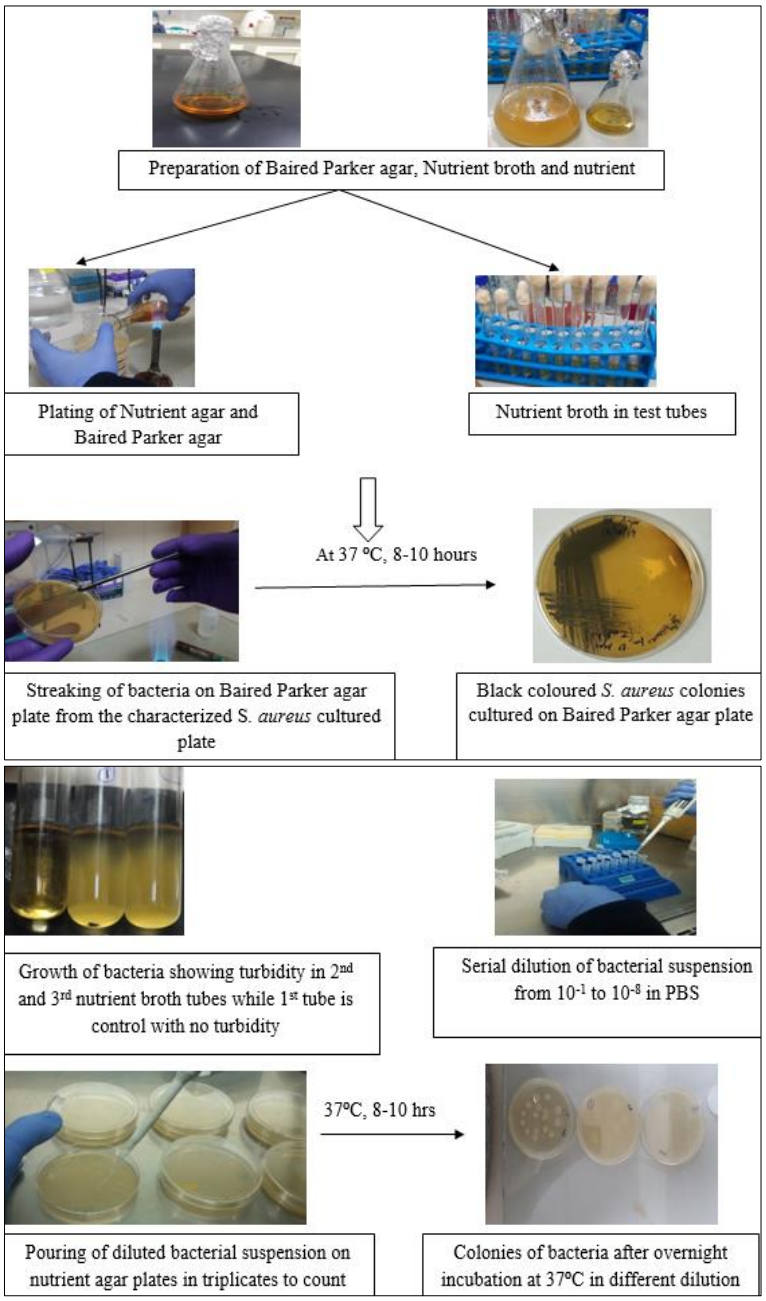

Fig 1: Figures in form of flow picture showing the preparation of *Staphylococcus aureus* for Intramammary infection

Experimental inoculation of *S. aureus* to create chronic fibrotic mouse mammary gland model

The experimental trial was conducted in 10-15th days of post parturient lactating mice. The mice were divided into 2 groups *viz*. Group A (Healthy control), Group B (Fibrotic control). For intramammary challenge, 8-12 days post parturient lactating mice were anaesthetized with ketamine (87 mg/kg b.wt.) and xylazine (23 mg/kg b.wt.) combination. Proper shaving and disinfection of abdominal region was done. The 4th or 5th left mammary gland was selected. Each teat orifice was exposed by a small cut under dissecting microscope and intramammary inoculation of bacteria was done through teat canal in left was inoculated using 31 G blunt needle through teat canal. The 4th and 5th mammary gland was secured by forceps and inoculated with 50 µl of inoculums in PBS containing 3×10^{2}c.f.u. of field strain of *Staphylococcus aureus* isolated from bovine mastitis as shown in fig 2. In group A mice, 50 µl of PBS was inoculated in both left 4th and 5th mammary gland

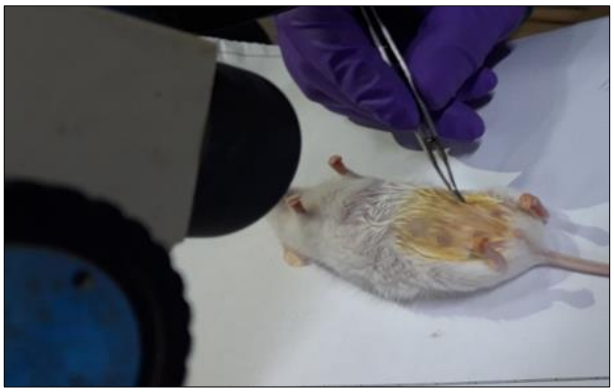

Fig 2: Inoculation of *Staphylococcus aureus* in mammary gland of a lactating

Necropsy and gross pathology

Following bacterial inoculation, gross examination of mice and its mammary gland was done for development of mastitis and fibrotic changes. At the time of necropsy, gross appearance of mammary glands *viz*. size, colour, consistency were also observed.

Histopathology

The representative tissue pieces from mammary glands were collected and fixed in 10% neutral buffered formalin for histopathological studies. All the tissues were processed routinely to obtain 4-5 µm thick paraffin embedded sections, stained with haematoxylin and eosin (H&E) for histopathology. Duplicate sections were used Masson's Trichrome stain for assessment of fibrosis in mammary gland tissue (Luna, 1968) [5].

Masson's trichrome staining

Deparaffinized mammary gland tissue sections were hydrated through descending grades of ethyl alcohol to distilled water. The mammary gland tissues sections were incubated in Bouin's solution for 1 h at 56 degree Celsius or overnight at room temperature to improve staining quality. The slides were rinsed in running tap water for 5-10 min to remove the yellow colour, stained with Weigert's iron haematoxylin working solution for 10 min, rinsed in running warm tap water for 10 min and washed in distilled water for 5 min. The sections were then stained by Biebrich scarlet-acid fuchsin solution for 15 min and washed in distilled water for 5 min. To observe differentiation, the sections were kept in (1:1) phosphomolybdic-phosphotungstic acid solution for 15 min, and subsequently were transferred directly (without rinse) to aniline blue solution for 10 min. Next, the slides were rinsed in distilled water to remove the excess stain then the slides were kept in 1% acetic acid solution for 1 min. After that the slides were washed in distilled water, air dried, cleared in xylene and mounted with DPX mounting medium for examination

Results and Discussion

All the animals were examined for their general demeanour and gross pathological changes in *S. aureus* inoculated mammary gland. After 24 h of infection animals were found to be dull, anorectic; however, after 6-7 days they were found to be active and regained their appetite. This observation is in contrary to findings of other workers (Chandler, 1970; Bramley *et al*., 1989; Brouillette *et al.*, 2004) [3, 2, 1] in which mice showed profound dullness, anorexia and ruffled fur coat. However, present study concurred with study of Singh *et al*., 1997 [8] in which there was no systemic disturbances on candidal induced mastitis in goats. This could be due to bacterial dose and localized inoculation which might not be capable to produce significant effect systemically whereas enough to produce structural damage in mammary gland. Gross examination of infected mammary gland of group B mice showed very slight swelling, hardness with no remarkable change in colour as compared to control group. This may be due to low dose of bacterial inoculation to create chronic mastitis. Mice were healthy with no pathological changes in mammary gland of healthy group A.

Gross pathological changes in mammary gland of both A and B group were recorded by sacrificing animals after 35th and 50th day of PBS and bacteria inoculation, respectively. The mammary gland of mice in group A was found normal without any swelling and discoloration both after 35th and 50th day of PBS inoculation as shown in fig.3 (a and b) while the left 4th and 5th mammary glands of mice in group B were showing moderate swelling, firmer consistency with reddish discoloration on 35thday of bacteria inoculation as shown in fig.3 (c) while on 50thday of bacteria inoculation mammary glands were showing mild swelling with slight reddish discoloration as shown in fig. 3 (d). These observations concurred with the study of Hingade, (2017) [4] in which mice mammary gland were showing suppurative foci, were swollen and soft after 25th day of infection but with time interval the fibrotic mammary gland became atrophied and firm to touch.

Microscopic examination of the tissue sections of the mammary glands of the mice of two groups i.e., group A (healthy control) and group B (fibrotic animal group) was carried out. In the group A (healthy control) with no infection, after 35th and 50th day of PBS inoculation shown in fig.4 (a-d), no inflammatory changes were observed in the tissue sections of the mice mammary gland. Glandular structure was found to be normal with intact myoepithelial cells containing milk in some of alveoli, while after 50th day of PBS inoculation most of the alveoli were atrophied or involuted with abundant adipocytes and mature blood vessels. Collagen fibers were minimum and found to be associated with the supporting framework of the mammary gland structure like around blood vessels. On the contrary, in the group B (fibrotic group) 35th day of bacteria inoculation, severe degree of inflammatory changes were observed in the tissues sections of the mammary glands. The changes included intense infiltration of inflammatory cells including neutrophils, macrophages, lymphocytes and fibroblasts. Moderate to high degree of degeneration and necrosis was observed with presence of micro abscesses, and moderate amount of collagen fibers was observed in the parenchyma. Adipocytes were minimal with disrupted acinar structures as shown in fig.4 (e-f). Similar changes like moderate to severe degree of inflammation with intense infiltration of inflammatory cells, degeneration, necrosis and atrophy of the glandular epithelium and associated tissues with no remaining milk secretion were observed after 50th day of bacteria inoculation. Deposition of collagen fibers was intense as shown in fig.4 (g-h)

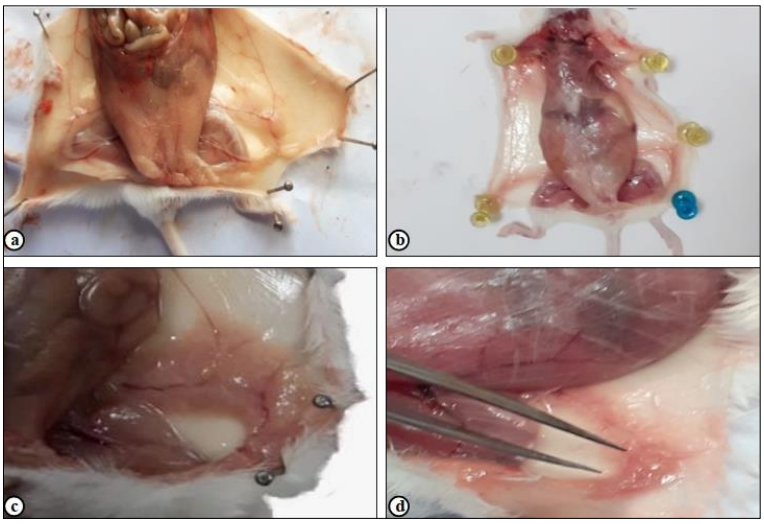

Fig 3: Comparative gross pathology of mice mammary glands of group A and B: (a) and (b) 4th mammary gland of group A (healthy control mice) with no pathological lesion after 35th day and 50th day of PBS inoculation, (c) 4th mammary gland of group B (fibrotic group) with moderate swelling, firmer consistency with reddish discoloration after 35th day of bacterial inoculation, (d) 4th mammary gland of group B (fibrotic group) with mild swelling, firmer consistency with slight reddish discoloration after 50th day of bacterial inoculation.

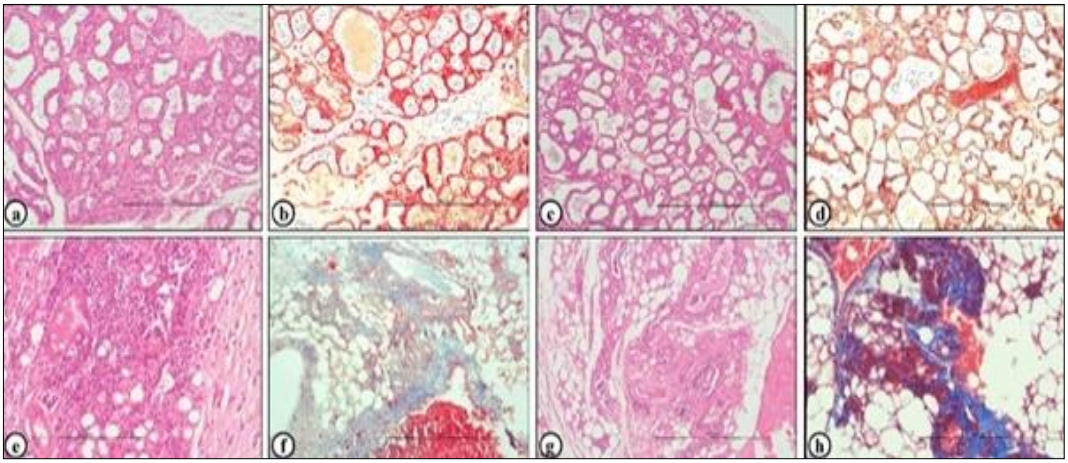

Fig 4: Comparative histopathology of mice mammary glands of group A and B: Group A (Healthy control): a-d representing closely packed adipocytes with little interstitial tissue and atrophic acini containing milk secretions in some acini both after 35th and 50th day of PBS inoculation (a &c, 10x H&E; b & d, 10x MST); Group B (Fibrotic control): e & f representing degeneration and necrosis of the glandular epithelium, intense infiltration of PMNCs, fibroblasts and collagen fibers in the tissue parenchyma after 35th day of bacterial inoculation, g & h representing degeneration and necrosis of the glandular epithelium, intense infiltration of MNCs, fibroblasts and more collagen fibers in the tissue parenchyma after 50th day of bacteria inoculation (e &g, 10x H&E; f & h 10x MST); (Arrow head: Blood vessel; Asterisks (*): Inflammatory cells)

Conclusion

This experiment proved that chronic mastitis could be readily and easily produced utilizing a comparatively less number of microorganisms. This also suggests that
the model would be very suitable for mastitis related and its therapeutic relevant studies.

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Conflict of Interest

There is no conflict of interest among the authors.

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