ISSN: 2583-9365

Mouse milk somatic cell count in coagulase negative Staphylococcus species induced mastitis

Krishnamoorthy P

P. Krishnamoorthy did his B.V.Sc., and M.V.Sc., in Veterinary Pathology from Tamilnadu Veterinary and Animal Sciences University, Chennai and Ph.D., from Veterinary College, Bangalore. He is currently holding the post of Scientist, Project Directorate on Animal Disease Monitoring and Surveillance, Bangalore. He has worked as Assistant Professor, Apollo College of Veterinary Medicine, Jaipur and Veterinary Surgeon in Madras University, Dr.ALM Post graduate Institute of Basic Medical Sciences, Chennai. He has specialized in epidemiology, nutritional pathology, molecular biology and laboratory animal management. Dr. Krishnamoorthy is a recipient of ICAR outstanding Team Research Award of ICAR as a member during 2010 and Fellow of Academy of Sciences for Animal Welfare (FASAW). He has 30 research publication including research papers, book chapters, bulletins, manuals, etc., He handled seven Institute funded projects and developed mouse mastitis model to study host pathogen interaction.

Krishnamoorthy P[1], Satyanarayana M.L[2], Shome B.R[1] and Rahman H[1].

[1] Project Directorate on Animal Disease Monitoring and Surveillance (PD_ADMAS), IVRI Campus, Hebbal, Bangalore-560024, Karnataka, India

[2]Department of Veterinary Pathology, Veterinary College, Hebbal, Bangalore-560024, Karnataka, India

Corresponding author :
Dr. Krishnamoorthy P, M.V.Sc., FASAW
Scientist, Project Directorate on Animal Disease Monitoring and Surveillance (PD_ADMAS)
IVRI Campus, Bellary Road, Hebbal, Bangalore-560024, Karnataka, India.
Phone: +918023419576, ext: 147, Fax: +918023415329, Mobile: +919480014534
Email: krishvet@gmail.com, krishnamoorthyp@pdadmas.ernet.in

Abstract

Bovine mastitis is an economically important disease of bovines and caused by multi etiological factors in which bacteria is the major cause. In the present study, milk somatic cell count (SCC) in mouse mastitis induced by *Staphylococcus epidermidis, S. chromogenes, S. haemolyticus* and *S. aureus* isolated from apparently normal bovine milk was studied. The 2 x 10^4 cfu organisms in 50 µl per teat were inoculated through intramammary route in 4th and 5th pairs of abdominal mammary gland in mice. The squeeze method, a technique was developed and standardized for milk collection from mice at 6, 12, 24, 48, 72 and 96 hr after intramammary inoculation (IMI). The milk somatic cell count was estimated using NucleoCounter SCC-100®. The mouse milk ranging from 50 to 200 µl per mice was collected successfully. The milk somatic cell count showed significant increase in *S. aureus* inoculated mice at 6, 12, 24 hr and increase was two to four folds when compared to PBS control. The three coagulase negative staphylococcus species showed increased SCC levels at 24, 48, 72 and 96 hr after IMI. Thus, Coagulase negative staphylococci (C) species can increase the mice milk somatic cell count moderately but two to four fold increase was observed in *S. aureus* which indicated the subclinical nature of CNS infections in animals. Mouse is a suitable model to study coagulase negative staphylococcus species induced bovine subclinical mastitis.

Introduction

Bovine mastitis is one of the major disease conditions affecting dairy cattle worldwide. Subclinical mastitis is difficult to detect due to the absence of any visible indications and has major cost implications. Early detection of subclinical mastitis will help in reducing the economic losses to the dairy farmers. Among the bacteria isolated in bovine mastitis, *Staphylococcus* species occupies an important place. The staphylococcal genus has been divided into coagulase positive and coagulase negative based on their ability to coagulate rabbit plasma. Coagulase negative staphylococci (CNS) have become more important as bovine mastitis causing agents during recent years. More than ten different coagulase negative staphylococcal species have been isolated from mastitis bovine milk samples and most commonly reported are *Staphylococcus chromogenes*, *Staphylococcus simulans, Staphylococcus haemolyticus, Staphylococcus hyicus* and *Staphylococcus epidermidis* (Trinidad *et al.,* 1990; Matthews *et al.,* 1992; Myllys, 1995; Thorberg *et al.,* 2006). These bacteria are sometimes referred to as environmental staphs and most frequent organisms isolated from milk samples from herds that have controlled the major mastitis pathogens (Janus, 2009). 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 bovine mastitis is mostly studied in laboratory animal models like mice, rat and rabbits. Various studies have been carried out using the animal models with major mastitis causing pathogens like *Staphylococcus aureus, Streptococcus* species and Coliforms. Simojoki *et al.,* (2009) suggested that studying of host response of CNS in experimental mastitis model is necessary to understand the host pathogen interaction. Keeping this information in background, the present study was undertaken to know milk somatic cell count in three CNS and *S. aureus* in mouse mastitis model which may help in early diagnosis of subclinical mastitis.

Materials and Methods

Animals and housing

Three coagulase negative staphylococci (CNS) namely *Staphylococcus epidermidis, Staphylococcus chromogenes* and *Staphylococcus haemolyticus* and one coagulase positive *Staphylococcus aureus* were isolated from apparently healthy bovine milk from dairy farms in Karnataka. The timed pregnant (Day 12 to 15), one hundred and sixty eight Swiss albino mice were procured from National Centre for Laboratory Animal Science, National Institute of Nutrition, Hyderabad. The mice were grouped in to seven groups (six time points and one control) of six mice each containing 42 mice per organism. The mice were housed in individually ventilated cages (IVC) during the experiment. The temperature and humidity of the animal room were maintained at 23 ± 3 ºC and 50 to 70 per cent respectively. Mice were provided with standard pellet feed and purified water *ad libitum,* and autoclaved paddy husk as bedding material. The animal experiments was approved by Institutional Animal Ethics Committee (IAEC) of Veterinary College, Bangalore and carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, New Delhi.

Mouse milk collection

The organisms were inoculated through intramammary route at the dose of 2 x 10^4 cfu per teat in 4th and 5th pairs of abdominal mammary glands in mice which simulated the four quarters of bovine. The pups were allowed to suckle after one hr of inoculation to simulate the natural conditions. The pups were separated from the mother four hours before the milk collection. The milk was collected from lactating mouse at 6, 12, 24, 48, 72 and 96 hr after intramammary inoculation with bacteria and PBS. The mouse was administered with 0.5 units of oxytocin intramuscularly in the thigh region. Maximum amount of milk was collected by squeeze method from L4, L5, R4 and R5 part of mammary glands from the base of the teat. The milk samples were collected by using micro pipette and micro tips in sterile 0.2 ml collection tubes (PCR tubes).

Milk somatic cell count

The somatic cell count of mouse milk was carried out by using somatic cell counter (NucleoCounter SCC-100, Chemometec, Denmark). The mouse milk sample was diluted since the quantity of milk obtained was very less. The diluted milk sample was mixed with equal amount of lysis buffer to form cell lysate. The lysis buffer disrupted the plasma membrane and liberated the nuclei of the cells which were stained with propidium iodide stain. The number of nuclei stained with propidium iodide was counted using fluorescence microscope in combination with automatic image analyzer inside the NucleoCounter SCC-100. Five micro litre of mouse milk was diluted in 45 µl of sterile PBS in a 1.5 ml micro centrifuge tube and mixed gently. Added 50 µl of lysis buffer to it and mixed gently. Then the SCC-cassette was kept on the tube and pressed the white plunger to suck the diluted milk in to the cassette channels. Pressed the button-Analysis in the somatic cell counter and waited for few minutes. The number of somatic cells in the milk sample was shown in the machine and computer monitor, which was recorded and multiplied with the dilution factor to know the exact number of somatic cells in the mouse milk sample. The somatic cell count in the mouse milk was calculated using Somatic View software Version 1.0 (Chemometec, Denmark).

Statistical analysis

The number of somatic cells in a mouse milk sample was expressed as x 10^5 cells per ml of milk. The data obtained were analyzed by one way analysis of variance (Snedecor and Cochran, 1989) and by using Statistical Analysis System (SAS) software version 9.3, Mumbai, India.

Results

The mouse milk was collected by squeeze method (Fig.1) which was developed and standardized successfully and the amount of milk collected ranged from 50 to 200 µl. The mean ± SE of milk somatic cell count in mice infected with three CNS and *S. aureus* has been presented in Table 1. The mean mice milk somatic cell count (x 10^5 per ml) were 3.05 ± 0.30, 10.67 ± 1.38, 7.97 ± 0.89, 5.26 ± 0.51 and 12.46 ± 2.99 in PBS control, *S. epidermidis, S. chromogenes, S. haemolyticus* and *S. aureus* inoculated mice respectively. In the present study, the SCC of PBS inoculated mice showed mean cell count which ranged from 2.46 – 3.64 x 10^5 per ml of milk. The mean SCC in mice milk samples collected from mice inoculated with *S. aureus* revealed a significant ($P<0.05$) increase at 6, 12 and 24 hr after IMI. The increase in SCC was drastic at 12 and 24 hr after IMI in the *S. aureus* infected mice. At 6 and 12 hr after IMI with three CNS, no significant difference in SCC was observed when compared to PBS control. At 48, 72 and 96 hr after inoculation, there was a significant ($P<0.05$) increase in SCC levels in *S. epidermidis* and *S. chromogenes* infected mice when compared to the PBS control. The *S. haemolyticus* showed increase in SCC at 12, 24 and 48 hr when compared to PBS control. The overall mean SCC of all the time points revealed increase in SCC of infected mice when compared to the PBS control.

Discussion

The mean mice milk SCC was increased in three CNS and *S. aureus* infected mice when compared to PBS control. However, the *S. aureus* inoculated mice showed significant increase in mice milk SCC at 6, 12 and 24 hr after intramammary inoculation. The mouse milk somatic cell count in experimental mastitis was not reported earlier. Further, the increased SCC in *S. aureus* infected mammary glands has been reported previously in dairy cows (Petzl *et al.,* 2008; Paradis *et al.,* 2010; Singh and Garg, 2011) and rabbits (Azeemullah, 2010). The *S. epidermidis* and *S. chromogenes* increased the SCC at 24, 48, 72 and 96 hr after IMI in mice moderately. The *S. haemolyticus* and *S. aureus* behaved in similar manner in different time points but SCC was increased moderately by *S. haemolyticus.* This finding concurred with previous reports which indicated the increase in geometric mean SCC in CNS infected quarters was 138,000 cells per ml and in *S. aureus* infected quarters was 357,000 cells per ml (Djabri *et al.,* 2002). Haas *et al.,* (2004) indicated the relationship between the high averages of SCC with the risk of occurrence of CNS mastitis in dairy cows. The milk SCC in dairy cows infected with CNS species has been shown to increase SCC by many researchers (De Vliegher *et al.,* 2003; Taponen *et al.,* 2007; Simojoki *et al.,* 2009; Piepers *et al.,* 2010; Malek Dos Reis *et al.,* 2011). Supre *et al.,* (2011) reported that the milk SCC in dairy cows increased due to CNS species infection which may be comparable with *S. aureus* infected cows at times. The resident macrophages and the epithelial cells of the mammary gland after detection of invading pathogen releases several chemoattractants which trigger the migration of leukocytes, mainly PMN cells, from blood towards the inflammed mammary gland (Zhao and Lacasse, 2008). The migrated PMN cells were considered as the first line of defense in the mammary gland, but the presence of functional PMN in milk was crucial to the host defense against bacterial pathogens (Paape *et al.,* 2003). The main functions of PMN cells in the infected mammary glands is to phagocyte the pathogens and destroy them via oxygen dependent and oxygen independent systems. At the same time, PMN cells can potentially harm the mammary gland by promoting tissue injury via reactive oxygen species generation and by granular enzyme release on degranulation (Zhao and Lacasse, 2008). Thus the somatic cell count can serve as indicator for diagnosis of CNS induced subclinical mastitis. The mice might be used as suitable model to study the CNS subclinical mastitis and its therapeutic intervention strategies in future.

References

1. Azeemulla HR (2010). Pathology of experimental mastitis and studies on the immune responses against *Staphylococcus aureus* biofilm vaccine against mastitis in rabbits. Ph.D. thesis, Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, India.
2. Djabri B, Bareille N, Beaudeau F, Seegers H (2002). Quarter milk somatic cell count in infected dairy cows: a meta-analysis. *Vet. Res.* 33: 335-357.
3. De Vliegher S, Laevens H, De Vriese LA *et al.,* (2003). Prepartum teat apex colonization with *Staphylococcus chromogenes* in dairy heifers is associated with low somatic cell counts in early lactation. *Vet. Microbiol*. 92: 245-252.
4. Haas YD, Veerkamp RF, Barkema HW, Grohn YT, Schukken YH (2004). Associations between pathogen specific cases of clinical mastitis and somatic cell count patterns. *J. Dairy Sci.* 87: 95-105.
5. Janus A. 2009. Emerging mastitis pathogens. *Vet. World.* 2(1): 38-39.
6. Malek Dos Reis CB, Barreiro JR, Moreno JFG, Procionato MAF, Santos MV (2011). Evaluation of somatic cell count thresholds to detect subclinical mastitis in Gyr cows. *J. Dairy Sci*. 94: 4406-4412.
7. Matthews KR, Harmon RJ, Langlois DBE. 1992. Prevalence of *Staphylococcus* species during the periparturient period in primiparous and multiparous cows. *J. Dairy Sci.,* **75**: 1835-1839.
8. Myllys V. 1995. Staphylococci in heifer mastitis before and after parturition. *J. Dairy Res.,* **62**: 51-60.
9. Paape MJ, Bannerman DD, Zhao X, Lee JW (2003). The bovine neutrophil: structure and function in blood and milk. *Vet. Res.* 34: 597-627.
10. Paradis ME, Bouchard E, Scholl DT, Miglior F, Roy JP (2010). Effect of nonclinical *Staphylococcus aureus*

or coagulase negative staphylococci intramammary infection during the first month of lactation on somatic cell count and milk yield in heifers. *J. Dairy Sci*. 93: 2989-2997.

11. Petzl W, Zerbe H, Gunther, J *et al.,* (2008). *Escherichia coli*, but not *Staphylococcus aureus* triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. *Vet. Res*. 39: 18.
12. Piepers S, Opsomer G, Barkema HW, De Kruif A, De Vilegher S (2010). Heifers infected with coagulase negative staphylococci in early lactation have fewer cases of clinical mastitis and higher milk production in their first lactation than non infected heifers. *J. Dairy Sci.* 93: 2014-2024.
13. Simojoki H, Orro T, Taponen S, Pyorala S (2009). Host response in bovine mastitis experimentally induced with *Staphylococcus chromogenes*. *Vet. Microbiol*. 134: 95-99.
14. Singh M, Garg A (2011). Incidence of different pathogens and milk compositional changes in subclinical and clinical milk samples of cows. *Indian J. Anim. Res.* 45(1): 51-55.
15. Snedecor GW, Cochran WG. 1989. Statistical Methods. The Iowa State University Press, Ames, Iowa, USA. pp 196-252.
16. Supre K, Haesebrouck F, Zadoks RN, Vaneechoutte M, Piepers S, De Vilegher S (2011). Some coagulase negative Staphylococcus species affect udder health more than others. *J. Dairy Sci*. 94: 2329-2340.
17. Taponen S, Koort J, Bjorkroth J, Saloniemi H, Pyorala S (2007). Bovine intramammary infections caused by coagulase-negative staphylococci may persist throughout lactation according to amplified fragment length polymorphism-based analysis. *J. Dairy Sci*. 90: 3301–3307.
18. Thorberg BM, Kuhn I, Aarestrup FM, Brandstrom B, Jonsson P, Danielsson-Tham ML, 2006. Phenotyping and genotyping of Staphylococcus epidermidis isolated from bovine milk and human skin. *Vet. Microbiol.,* 115:163-172.
19. Trinidad P, Nickerson SC, Alley TK. 1990. Prevalence of intramammary infection and teat canal colonization in unbred and primigravid dairy heifers. *J. Dairy Sci.,* 73: 107-114.
20. Zhao X, Lacasse P (2008). Mammary tissue damage during bovine mastitis: causes and control. *J. Anim. Sci*. 86: 57-65.

Table 1: The mean mice milk somatic cell count (x 10^5 per ml) after intramammary inoculation with three CNS species and S. aureus at different time points

Time points	PBS Control	*Staphylococcus epidermidis*	*Staphylococcus chromogenes*	*Staphylococcus haemolyticus*	*Staphylococcus aureus*
6 hr	1.70 ± 0.70 ^{b}	2.80 ± 0.40 ^{b}	3.15 ± 0.25 ^{b}	3.30 ± 0.40 ^{b}	8.45 ± 0.85 ^{a}
12 hr	3.88 ± 0.47 ^{b}	7.75 ± 0.35 ^{b}	6.85 ± 0.35 ^{b}	6.65 ± 0.55 ^{b}	25.85 ± 1.25 ^{a}
24 hr	3.15 ± 0.35 ^{d}	15.80 ± 1.60 ^{b}	9.65 ± 0.55 ^{c}	6.25 ± 0.25 ^{cd}	26.75 ± 1.15 ^{a}
48 hr	3.20 ± 0.30 ^{c}	11.05 ± 0.55 ^{a}	7.65 ± 0.45 ^{ab}	7.25 ± 0.85 ^{b}	3.64 ± 0.70 ^{c}
72 hr	3.15 ± 1.45 ^{b}	12.15 ± 0.25 ^{a}	7.85 ± 1.25 ^{ab}	5.00 ± 0.20 ^{b}	5.20 ± 0.40 ^{b}
96 hr	3.20 ± 0.70 ^{b}	15.00 ± 3.00 ^{a}	12.70 ± 0.20 ^{a}	3.10 ± 0.40 ^{b}	5.05 ± 1.55 ^{b}
Mean	3.05 ± 0.30	10.67 ± 1.38	7.97 ± 0.89	5.26 ± 0.51	12.49 ± 2.99
CI	2.46 – 3.64	7.97 – 13.37	6.23 – 9.71	4.27 – 6.25	6.63 – 18.35

Values expressed in cells x 10^5 per ml in milk as Mean ± SE

a, b, c, d : Means with same superscript within the row do not differ significantly (P>0.05)

CI: Confidence interval at 95 per cent level

Fig. 1 Photograph to show collection of milk from mice mammary glands by squeeze method.