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Mouse milk bacterial count in coagulase negative staphylococcus species induced mastitis

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Abstract

Bovine mastitis in an economically important disease of dairy cattle and caused by multi etiological factors. In the present study, milk viable bacterial count 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 10^4CFUorganisms in 50 µl per teat were inoculated through intramammary route in 4th and 5th pairs of abdominal mammary gland in mice. The mouse milk ranging from 50 to 200 µl per mice wascollectedat 6, 12, 24, 48, 72 and 96 hrs after intramammary inoculation. The milk was diluted with sterile PBS and subjected to bacterial countby pour plate method. The viable bacterial count in mice milk showed significant ($P<0.05$) increase in bacterial colonies at 12, 24, 48 and 72hrs after *S. aureus* infection in mice. The three coagulase negative staphylococci(CNS) species showed initial increase in bacterial counts at 12 and 24 hrs but declined from 48 to 96 hrs after IMI in mice. Thus, CNS species can increase the mice viable bacterial count moderately but ten to fifteen fold increase was observed in *S. aureus* infected mice mammary gland.This indicated the subclinical nature of CNS intramammaryinfection in mice and also the host ability to overcome and eliminate the CNS infection. Mouse is a suitable model to study coagulase negative staphylococcus species induced bovine subclinical mastitis.

Key words:

Introduction

Bovine mastitis, a major disease conditions affecting bovines worldwide. Subclinical mastitis is difficult to detect due to the absence of any visible indications and has major cost implications. 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. 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). More than ten different coagulase negative staphylococcal species have been isolated from 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). 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 in view, the present study was undertaken to knowmilk bacterial count in three CNS speciesand *S. aureus* in mouse mastitis model which may help in understanding the host pathogen interaction in CNS species mastitis.

Materials and methods

Animals and housing

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 into 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 ± 3ºC and 50 to 70 per cent respectively. Mice were provided with standard pellet feed (M/s. Provimi Animal Nutrition India Private Limited, Bengaluru) 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 mastitis and milk collection

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 organisms were inoculated through intramammary route at the dose of 2 x 10^4colony forming unit (CFU) per teat in 4^{th} and 5^{th} pairs of abdominal mammary glands in mice (Fig. 1) which simulated the four quarters of bovine. The intramammary inoculation (IMI) was carried using insulin syringe with 30 G needle and volume of inoculum was 50 µl per teat. The pups were allowed to suckle after one hr of inoculation to simulate the natural conditions. The pups were separated from mother four hrs before the milk collection. The milk was collected by squeeze method as reported earlier (Krishnamoorthy *et al*., 2014) from lactating mouse at 6, 12, 24, 48, 72 and 96 hrs after intramammary inoculation with bacteria and PBS.

Viable bacterial count

Milk samples collected from the mice were subjected for estimation of total viable bacterial count of different coagulase negative staphylococcus species and *S. aureus* by pour plate method. Colony forming units of *Staphylococcus epidermidis, S. chromogenes, S. haemolyticus* and *S. aureus* in the milk secretion during infection were determined by plating the diluted milk onto the Brain Heart Infusion Agar plates. Milk was diluted using sterile PBS serially 1 in 10 dilutions and all dilutions were used for plating in triplicate. After plating, the plates were incubated at 37ºC for 24 hrs. Presence of coagulase negative and positive species was confirmed by formation of white or grey or yellow colour colonies over the agar plates. Colonies were counted in colony counter and expressed as CFU x 10^4 per ml. The CFU per ml of milk was calculated as below

$$\text{CFU/ml (\%)} = \text{Colonies per plate} \times \text{Dilution factor} \times \frac{1}{\text{Aliquot used for plating (200 µl)}}$$

Statistical analysis

The bacterial count obtained from milk samples from different groups 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 bacterial growth was observed in Brain Heart Infusion agar plates by using pour plate method indicated that more amount of bacterial colonies in milk of *S. aureus*infected mice when compared to three CNS species. The mean ± SE of viable bacterial counts in mice milk (CFU x 10^4 per ml) after intramammary inoculation with three CNS species and *S. aureus* is presented in Table 1. The mean viable bacterial counts (x 10^4 per ml of milk) were 1.24 ± 0.38, 2.05 ± 0.65, 3.57 ± 0.92 and 6.83 ± 2.36 in *S. epidermidis, S. chromogenes, S. haemolyticus*and *S. aureus* inoculated mice respectively. This indicated a significant increase in *S. aureus* when compared with three CNS species inoculated mice. The PBS inoculated mice milk showed no viable bacterial counts at different time points. There was no significant difference in the mean bacterial counts at 72 and 96 hrs after IMI with three CNS species and *S. aureus*when compared to the PBS control. The bacterial count showed rapid increase in the mean viable counts at 12, 24 and 48 hrs after IMI with *S. aureus* and peaked at 24 hrs. There was significant ($P<0.05$) difference in bacterial counts at 6, 12 and 24 hrs after IMI inoculation with *S. haemolyticus* when compared to *S. epidermidis*and *S.*

chromogenes. The mean viable counts at all the time points revealed an increase in *S. aureus*infected mice when compared to three CNS speciesand PBS control. The *S. epidermidis*and *S. chromogenes*showed no significant difference in the mean bacterial counts at different time points after IMI in mice. The bacterial counts decreased during 48 to 96 hrs after IMI with three CNS species.

Discussion

In the present study, the mean viable bacterial counts of *S. aureus* in mice milk revealed a steep increase of 10 to 15 folds at 12, 24 and 48 hrs when compared to control, which reached peak at 24 hrs and tend to decrease slightly at 72 and 96 hrs after IMI. There was no significant difference in viable bacterial counts at 72 and 96 hrs between the three CNS species and *S. aureus* when compared to control mice. The *S. haemolyticus* inoculated mice revealed significant increase in viable counts when compared to other CNS species at 6, 12 and 24 hrs after IMI. Reid *et al*., (1976) showed that the number of viable *S. aureus*recovered from mammary gland after 6 and 12 hrs were expressed as geometric mean (log10) 7.46 ± 0.31 and 8.18 ± 0.71 respectively.Bramley *et al*., (1989) studied the role of alpha or beta toxin on the recovery of *S. aureus* after intramammary inoculation of mice by culture method. They found that the recoveries of *S. aureus*ranged from $\log_{10}$ 3.6 to 8.4 CFU per mammary gland of mice. Brouillette *et al.,* (2005) reported that after inoculation of 100µl of bacterial suspension containing 1.4 to 3 x 10^2CFU in mice, recovered10^5CFU per gram of mammary gland tissue at 6hrs post infection by a CFU plate count of tissue homogenates.Notebaert *et al*., (2008) indicated that significantly large number of bacteria 3.3 ± 1.2 x 109CFU per gland were recovered after 24 and 48 hrs post inoculation with *Escherichia coli* in mouse mastitis model.Trigo *et al.,*(2009) reported induction of experimental mastitis in mouse model using *Streptococcus agalactiae* and the bacteria replicated efficiently in mammary gland and increased the viable counts by 100 fold after 24 hrs of intramammary inoculation.This may be true for major mastitis pathogens like *S. aureus, Streptococcus sp., Escherichia coli* and concurred with *S. aureus* infection in this study. Simojoki *et al*., (2009) revealed that bacterial growth increased at 8 hrs after *S. chromogenes*inoculation in dairy cows and the bacteria was eliminated fast and no bacteria could be isolated at 46 hrs from milk samples. In the present study,*S. chromogenes* infected mice showed less bacterial after 48 hrs of inoculation and concurred with the previous report (Simojoki *et al.,* 2009).

The microorganisms entering the mammary gland might escape the natural defense mechanisms of the host by rapid multiplication along the streak canal or in the teat cisternae and establish the infection upon profound proliferation during early hours of infection (Zaho and Lacasse, 2008). The number of organisms in milk has been reported to be dramatically reduced in the milk samples upon recruitment of PMNs in large numbers which are known to eliminate the organisms by several mechanisms (Heyneman *et al.*, 1990 and Paape *et al*., 2003). This clearly indicates the reason for the decreasing trend in the mean viable counts from 48to 96 hrs after IMI with CNS, which was observed in the present study. The host has the potential to eliminate the CNS species more effectively compared to *S. aureus* suggesting the subclinical nature of CNS species infection. The host defense mechanisms are effective in eliminating CNS infection indicating the subclinical infection in mammary glands of bovines also. Further, mice might be used as suitable model to study the CNS subclinical mastitis and its therapeutic intervention strategies in future.

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Table 1: The mean bacterial count (CFU x 104 per ml) in mouse milk 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 hrs	0.00 ± 0.00 ^{b}	0.47 ± 0.08 ^{b}	0.56 ± 0.04 ^{b}	1.45 ± 0.25 ^{a}	1.47 ± 0.13 ^{a}
12 hrs	0.00 ± 0.00 ^{c}	1.50 ± 0.20 ^{c}	2.10 ± 0.30 ^{c}	4.90 ± 0.36 ^{b}	11.50 ± 0.90 ^{a}
24 hrs	0.00 ± 0.00 ^{d}	2.95 ± 0.15 ^{c}	3.15 ± 0.25 ^{c}	7.21 ± 0.65 ^{b}	15.25 ± 0.35 ^{a}
48 hrs	0.00 ± 0.00 ^{c}	1.30 ± 0.10 ^{bc}	4.65 ± 0.05 ^{ab}	4.00 ± 0.30 ^{bc}	8.35 ± 1.65 ^{a}
72 hrs	0.00 ± 0.00	0.83 ± 0.07	0.75 ± 0.15	2.39 ± 0.20	3.00 ± 1.40
96 hrs	0.00 ± 0.00	0.40 ± 0.08	1.10 ± 0.30	1.45 ± 0.35	1.40 ± 0.40
Mean	0.00 ± 0.00	1.24 ± 0.38	2.05 ± 0.65	3.57 ± 0.92	6.83 ± 2.36
CI	0.00 - 0.00	0.50 – 1.98	0.78 – 3.32	1.77 – 5.37	2.21 – 11.45

Values expressed in colony forming units x 104 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 intramammaryinoculation of bacterial culture in to mice mammary gland