Significance of Beta-hemolytic Staph. aureus as a Pathogen to the Bovine Mammary Gland

By

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With 7 figures and 2 tables

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Introduction

In recent years, extracellular hemolysins of Staph. aureus have not only been considered useful criteria for classification of strains into those of human and animal origin (9, 12, 13, 21, 25); the type of hemolysin produced has also been suggested to be an indication of pathogenicity of a given strain to its host (1, 3, 4, 16, 26). Strains of Staph. aureus isolated from clinical cases of bovine mastitis produce predominantly Beta-hemolysin, often in combination with other hemolysins (13, 23, 26), and its production in vitro was specifically suggested to be related to pathogenicity of a given strain to the bovine mammary gland (14). The presumed pathogenic role for hemolysins in staphylococcal mastitis is also indicated by the fact that repeated attempts had been made in the past to confer immunity against mastitis in cattle and sheep, by vaccinations consisting of toxoids of hemolysins (5, 7, 8, 17, 18, 19, 20, 22). However, despite the most frequent production of Beta-hemolysin by mastitic strains of Staph. aureus, its role in the pathogenesis of the disease does not appear to have been investigated excepting in one instance (23) where it was preliminarily reported that neither Beta-hemolytic Staph. aureus, nor its culture filtrate produced clinical infection of the bovine mammary gland. Thus, there appears to be substantial need for information concerning the biological role of Beta-hemolysin production by pathogenic staphylococci. This report deals with in vivo studies on the pathogenic significance of a Beta-hemolytic strain of Staph. aureus and its hemolysin, to bovine mammary gland.

Materials and Methods

Experimental animals: Two purebred Holstein cows from OVC Mastitis Research herd were used in these studies. Of these, one animal (No. 68) was in its first lactation, while the other (No. 3892) was in its fourth. They were kept under standard feeding and management conditions, and were neither used in
intramammary infection studies previously; nor has any ever experienced a natural infection of the mammary gland.

**Culture:** *Staph. aureus* strain 252-F, a pure Beta-hemolysin-producing organism of canine origin (24) was used for preparation of Beta-hemolysin, and for experimental intramammary inoculations.

**Beta-hemolysin:** The organism was grown for 18 hours in 20 % CO₂ in a medium containing 1.25 % Bacto yeast extract (Difco Labs., Detroit, Michigan), which was a modification of the medium used for α-hemolysin production (2). The culture supernate was saturated with (NH₄)₂SO₄·7 H₂O; and the precipitate was a dialysed against distilled water and freeze-dried. This constituted the crude Beta-hemolysin, which was partially-purified by two consecutive separations in a Sephadex G-100 column. The fractions containing Beta-hemolysin were pooled, dialysed and freeze-dried. Material similarly obtained from fractions devoid of Beta-hemolysin activity from the second separation constituted the control. Beta-hemolysin content in each preparation was assayed in 0.025 M (pH 7.4) Tris buffer in 0.85 % saline (TBS) containing traces of MgSO₄ and KCl, and the activity was expressed in Minimal Hemolytic Units (MHU)/mg. of material. One MHU was defined as the amount of Beta-hemolysin required to bring about a 50 % hemolysis of a 1 % suspension of bovine red cells, after a 60 min. incubation at 37 °C followed by refrigeration for a similar period.

**Infusion of Beta-hemolysin into bovine mammary gland:** Lyophilized preparation of crude Beta-hemolysin was reconstituted in TBS to contain known amounts of activity and was sterilized with Swinney filters, before use. Following afternoon milking, all the four teats of each of two cows were disinfected and sterile Beta-hemolysin preparation in two doses was infused into two quarters of each cow. In each animal, one of the control quarters was also infused with 0.5 ml. of TBS, while the fourth quarter was left un-infused. In a separate experiment, hemolysin-free material obtained from Sephadex G-100 column, was sterilized and infused in 0.1, 0.5 and 1.0 mg. quantities in TBS, into two quarters of each of two cows. Fore-milk samples from all quarters of experimental cows were obtained daily and their somatic cell counts were estimated electronically (15). In addition, milk samples were also routinely cultured on blood agar plates. These studies were subsequently repeated with partially-purified Beta-hemolysin.

**Phagocytosis and Chemotaxis studies on mammary leukocytes:** Following intramammary infusion of crude and partially-purified Beta-hemolysin, 200 ml. of milk was obtained daily from the quarter infused with the highest dose of hemolysin, in each cow. Approximately 100 ml. of milk, in each case, was diluted with an equal volume of 0.85 % NaCl and centrifuged at 1,800 x g, for 30 minutes, and the sedimented leukocytes were washed and resuspended in TBS. Milk serum was separated from the other half of milk, employing a technique described by HALL and LEARMOUTH (11). The leukocytes were used in phagocytosis described by HALL and LEARMOUTH (11). The leukocytes were used in phagocytosis and chemotaxis studies using methods described in detail elsewhere (NAIDU and NEWBOULD, in press), while the milk serum was tested for the presence of Beta-hemolysin by hemolysin titration, and immunogel diffusion methods.

**Inoculation of Staph. aureus strain 252-F into mammary glands:** An isolated colony of the organism was inoculated into 20 ml. of nutrient broth, and cultured at 37 °C for 12 hours. The culture was initially diluted 1 in 100 in half strength nutrient broth, which was further diluted three more times in TBS to a final concentration of 10⁻⁷, just before intramammary inoculation. Following afternoon milking, 0.1 and 0.2 ml. of the diluted culture was inoculat-
ed respectively into two quarters of a cow; while one of the control quarters was infused with 0.2 ml. of TBS and a fourth quarter left un-inoculated. Duplicate blood agar plates were inoculated with 0.1 ml. of the final dilution and cultured overnight to determine average bacterial numbers. As in the previous experiment, the somatic cell counts were routinely monitored, and milk serum from inoculated quarters was examined for presence of Beta-hemolysin.

**Results**

Infusion of crude staphylococcal Beta-hemolysin, even in minute quantities, resulted in marked irritation of mammary glands in both cows, as suggested by elevation of somatic cell counts in fore-milk of infused quarters (Figs. 1 to 4). In a total of six tests on two cows, all but one resulted in significant elevations of milk cell counts starting from the very first milking following intramammary infusions. Control quarters infused with TBS, as well as those left un-infused, in no case revealed demonstrable elevations of milk cell counts throughout the duration of observation. The general observations common to both cows were summarized as follows: (i) Infusion of Beta-hemolysin in quantities of 0.5 MHU or more regularly resulted in elevated somatic cell counts in milk, by first milking following intramammary infusion; (ii) During the height of intramammary reactions, which was between 15 and 40 hrs. post-infusion, the quarters were swollen, warm and tender on palpation and their secretions frequently contained visible clots. The milk yields were markedly reduced and the animals were off-feed and showed general signs of discomfort. The intensity of reaction, in each case, was directly related to the amount of Beta-hemolysin infused; (iii) There was a five to seven-fold difference in the peak cell counts of fore-milk samples of two cows.

![Fig. 1. Somatic cell counts in fore-milk from control quarters and those infused with crude β-hemolysin. (Cow 68; Experiment I)](image1)

![Fig. 2. Somatic cell counts in fore-milk from control quarters and those infused with crude β-hemolysin. (Cow 68; Experiment II)](image2)
receiving identical amounts of Beta-hemolysin into comparable quarters, and this difference was partly attributable to the stages of lactation of the two cows; (iv) A second infusion three weeks later into the same quarters with

Fig. 3. Somatic cell counts in fore-milk from control quarters and those infused with crude $\beta$-hemolysin. (Cow 3892; Experiment I) • — R. F — 3.0 MHU; ▲ — L. F — 1.5 MHU; △ — L. H — 0.5 ml. of TBS; ○ — R. H — control

Fig. 4. Somatic cell counts in fore-milk from control quarters and those infused with crude $\beta$-hemolysin. (Cow 3892; Experiment II) • — R. F — 3.0 MHU; ▲ — L. F — 1.5 MHU; △ — L. H — 0.5 ml. of TBS; ○ — R. H — control

Fig. 5. Somatic cell counts in fore-milk from quarters and those infused with lyophilized, hemolysin-free material (Cow 68) • — R. F — 0.1 mg. in 0.1 ml. of TBS; △ — L. H — 0.5 mg. in 0.5 ml. of TBS

Fig. 6. Somatic cell counts in fore-milk from quarters infused with lyophilized, hemolysin-free material (Cow 3892) ○ — R. H — 1.0 mg. in 1.0 ml. of TBS; ▲ — L. F — 0.5 mg. in 0.5 ml. of TBS
Table 1

Phagocytosis of Staph. aureus strain 252-F in the presence of added β-hemolysin by PMNs obtained from mammary glands of two cows each infused with 3.0 MHU of β-hemolysin

<table>
<thead>
<tr>
<th>Amount of β-hemolysin added to leukocytes</th>
<th>Percent phagocytosing PMNs after two hr. incubation&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>Cells from cow # 68</td>
</tr>
<tr>
<td>58.7 ± 6.2</td>
<td>63.8 ± 5.9</td>
</tr>
<tr>
<td>1.0 MHU</td>
<td>33.5 ± 7.1</td>
</tr>
<tr>
<td>10.0 MHU</td>
<td>16.0 ± 2.6</td>
</tr>
<tr>
<td>1.0 MHU</td>
<td>38.0 ± 6.2</td>
</tr>
<tr>
<td>10.0 MHU</td>
<td>19.2 ± 4.1</td>
</tr>
</tbody>
</table>

<sup>1</sup> Mean and S. D of six tests

Table 2

Effect of pre-incubation with β-hemolysin on the chemotactic response<sup>1</sup> of PMNs obtained from mammary glands of two cows each infused with 3.0 MHU of β-hemolysin

<table>
<thead>
<tr>
<th>Amount of β-hemolysin added to leukocytes&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Chemotactic Indices&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>Cells from cow # 68</td>
</tr>
<tr>
<td>15.78 ± 1.99</td>
<td>16.40 ± 2.42</td>
</tr>
<tr>
<td>1.0 MHU</td>
<td>2.18 ± 0.61</td>
</tr>
<tr>
<td>0.19 ± 0.13</td>
<td>3.07 ± 0.61</td>
</tr>
<tr>
<td>10.0 MHU</td>
<td>0.48 ± 0.37</td>
</tr>
</tbody>
</table>

<sup>1</sup> Chemotactic agent used was new-born colostrum-deprived calf serum
<sup>2</sup> Leukocytes pre-incubated with β-hemolysin before testing for chemotactic response
<sup>3</sup> Mean and S. D of six tests

identical amounts of Beta-hemolysin, always resulted in more pronounced intramammary reactions, as seen by higher peak cell counts, and this was possibly due to the priming effect of the first infusion; (v) Infusion of 0.1 MHU of Beta-hemolysin resulted in demonstrable increase in cell counts in one cow and not in the other; (vi) Intramammary infusions of partially-purified Beta-hemolysin essentially confirmed the observations with crude hemolysin; (vii) Infusion of material from fractions devoid of Beta-hemolysin activity, failed to produce any demonstrable intramammary reaction, even in quantities up to 1.0 mg. (Fig. 5, 6); and finally (viii) All control quarters remained clinically normal throughout the experimental duration, excepting one quarter of one cow which spontaneously contracted an acute infection with an organism tentatively identified as E. coli. This quarter was subsequently treated.

Fig. 7. Somatic cell counts in fore-milk from control quarters and those inoculated with Staph. aureus strain 252-F (Cow 68). ○ — R. H. — 90 CFU; ▲ — L. F. — 45 CFU; ● — R. F. — 0.2 ml. of TBS; △ — L. H. — control; † — Organism isolated in morning milk; ‡ — Organism isolated in afternoon milk
Neutrophils from milk of mammary glands infused with Beta-hemolysin phagocytosed *Staph. aureus* strain 252-F actively, and this activity was significantly reduced ($P = > 0.05$) when they were first incubated with Beta-hemolysin (Table 1). Similarly, milk neutrophils responded vigorously to the chemotactic effect of new-born colostrum-deprived calf serum; but were susceptible to the effects of Beta-hemolysin (Table 2).

Inoculation of 45 and 90 colony forming units (CFU) of *Staph. aureus* strain 252-F into two quarters of a cow resulted in establishment of the organism in the mammary glands (Fig. 7). The organism could be isolated first from the foremilk of one quarter at 24 hrs., and from the other quarter at 36 hrs. post inoculation, and persisted in the mammary gland until the sixth day, after which the organism appeared to have been spontaneously eliminated. Somatic cell reaction in milk roughly coincided with the initial appearance and subsequent presence of the organism in the milk of inoculated quarters. The first demonstrable elevation in somatic cell counts occurred subsequent to the isolation of the organism in milk of one quarter, and simultaneously with it in another. In both the quarters, peak cell counts were reached on the third day post-inoculation (60 hrs.) when the peak bacterial numbers were also found in milk; and from then on the cell counts showed a sudden decline. Elevated somatic cell counts in milk were maintained for 14 days, after which they reached and remained at control levels. During the height of infection, there was intense mammary reaction in the form of drop of milk yield, swelling and tenderness; and these changes were more pronounced in the quarter inoculated with 90 CFU. The intramammary reaction subsided following the return of somatic cell counts to normal levels. The quarter inoculated with 90 CFU, however, remained swollen and firm for nearly 12 weeks, even though the somatic cell reaction receded and the milk yields appeared normal. Infection did not spread to control quarters, and there was no recidivation or recurrence in inoculated quarters during the period of observation. Serum from milk of inoculated quarters during the height of infection, as well as sera from milk of quarters infused with varying doses of Beta-hemolysin failed to reveal the presence of detectable amounts of Beta-hemolysin, both by titration against bovine red cells and immunogel diffusion employing rabbit antiserum against crude Beta-hemolysin preparation.

**Discussion**

SLANETZ and BARTLEY (23) have reported that, a strain of *Staph. aureus* which formed only Beta-hemolysin did become established, when $3 \times 10^8$ organisms were injected into quarters of a test cow, but produced no clinical evidence of infection. They have also observed that Beta-hemolysin was essentially nontoxic to the bovine mammary gland, except when 75 ml. of culture filtrate (titre 1:64 of Beta-hemolysin) was infused which resulted in some swelling, with the quarter secreting watery milk containing a few clots. In the present investigations, the findings that even 45 to 90 CFU of a Beta-hemolytic strain produced clinical mastitis; and that Beta-hemolysin infusions in doses as low as 0.5 MHU consistently resulted in intense intramammary reaction, are at variance with the above (23). The discrepancies between our findings and those of SLANETZ and BARTLEY are difficult to explain. Consistent results obtained in eight infusions of Beta-hemolysin, and two infections with the organism under defined conditions, lead us to believe that our observations are significant. The finding that neutrophils from milk of mammary glands infused with Beta-hemolysin behaved normally in their phagocytosis and chemotactic properties, which were significantly inhibited on incubation with
Beta-hemolysin, is in complete conformity with the observations reported on bovine circulating neutrophils (NAIDU and NEWBOULD, in press). The normal behaviour of milk cells also suggests that Beta-hemolysin was not present in significant concentrations in experimentally infused mammary glands, to act upon and affect the physiological activities of milk neutrophils.

In these studies, it was presumed that the observed effects in bovine mammary gland, were indeed brought about by \textit{Staph. aureus} Beta-hemolysin, rather than by any other unknown substance in the hemolysin preparation. Even though the Beta-hemolysin preparations used were crude and were possibly associated with impurities, the evidence available from all the experiments put together suggests the improbability that the observed effects were caused by a substance other than Beta-hemolysin, though such a possibility cannot be ruled out. The following considerations strongly incriminate Beta-hemolysin as being responsible for the observed effects: (i) All the effects on mammary gland and its neutrophils were brought about only by preparations containing demonstrable amounts of Beta-hemolysin, and not by hemolysin-free material obtained during purification of crude hemolysin; (ii) The extent of change in the physiological properties of milk neutrophils or the severity of intramammary reaction, was roughly proportional to the amount of Beta-hemolysin; (iii) In the cow inoculated intramammarily with \textit{Staph. aureus} strain 252-F, visible glandular reaction and elevation of somatic cell counts in milk, occurred simultaneously or subsequently to indications of multiplication of the organism in the infected mammary glands. The height of reaction, likewise, was reached following the isolation of the organism in greatest numbers in milk, and the severity of reaction began to subside when the bacterial numbers in milk showed a decline. Elevated somatic cell reaction in infected quarters continued for a period of eight to ten days subsequent to the last isolation of the organism from these quarters, and this corresponds closely with the normal duration of cell reaction observed in Beta-hemolysin infusions. These events suggest the probability of Beta-hemolysin production in the infected mammary glands by the organism.

The theory that \textit{Staph. aureus} may produce intramammary reaction by elaborating Beta-hemolysin, is based on the assumption that the organism does produce the hemolysin in the mammary gland. Preliminary studies made to examine this possibility failed to reveal detectable amounts of free Beta-hemolysin in the milk samples from infected quarters, both by hemolysin titration and gel diffusion methods. The antiserum used in gel diffusion studies was weak, which may have been a factor in failure to detect presence of Beta-hemolysin in milk. It is equally possible that Beta-hemolysin may have been present in minute quantities due to dilution, undetectable by the methods used. Staphylococcal \textit{a}-hemolysin has been reported to undergo polymerization on contact with animal membranes \textit{in vitro} and \textit{in vivo} (10). If Beta-hemolysin too underwent such a change, it would have been difficult to demonstrate its presence in milk. The possibility also exists, that the Beta-hemolysin is adsorbed onto the glandular epithelium of the mammary gland, thus resulting in its absence in detectable amounts in milk. The observation that milk from quarters infused with Beta-hemolysin, failed to reveal presence of Beta-hemolysin on subsequent examination may point to such a possibility. Thus, it appears that even if Beta-hemolysin is produced by \textit{Staph. aureus} in the infecting mammary gland, demonstration of its presence would be difficult.

These investigations strongly suggest that strains of \textit{Staph. aureus} producing only Beta-hemolysin could be potential mammary pathogens. However, since most mastitic strains also produce other hemolysins, the possible
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interactions of all these hemolysins would appear to determine the overall pathogenicity of the strains to the mammary gland; and such studies have not been reported. The assumption hitherto held by most workers, that Beta-hemolytic Staph. aureus and its hemolysin are of little pathogenic significance to the bovine mammary gland, does not appear to be valid.

Summary

Intramammary infusions of crude Beta-hemolysin from Staph. aureus strain 252-F, in amounts of 0.5 MHU or greater, consistently resulted in intense intramammary reaction leading to inflammation. The somatic cell counts in milk of infused glands were significantly elevated by first milking following infusion and usually lasted seven to ten days before dropping to normal levels. The severity of inflammatory reaction and somatic cell responses were roughly proportional to the amounts of Beta-hemolysin infused. Neutrophils obtained from Beta-hemolysin infused mammary glands appeared normal in their physiological properties, which however were significantly inhibited on subsequent incubation with Beta-hemolysin. These observations were confirmed by studies employing partially-purified Beta-hemolysin. Intramammary inoculations of Staph. aureus strain 252-F in small numbers (45 and 90 CFU) resulted in moderate to acute inflammation of the infected glands. Milk from infected glands, as well as those infused with Beta-hemolysin, failed to reveal presence of Beta-hemolysin, on preliminary examination. These studies strongly contradict the view held by most workers, that Beta-hemolytic Staph. aureus and its hemolysin are of little pathogenic importance to the bovine mammary gland.

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Zusammenfassung

Signifikanz von Beta-hämolytischem Staph. aureus als pathogener Keim für das Rindereuter


Résumé

Rôle de Staph. aureus β-hémolytique en tant que germe pathogène pour la mamelle chez le bovin

Des infusions intramammaires d’au moins une demi dose hémolysante de β-hémolysine grossière à partir d’une souche de *Staphylococcus aureus* (252-F) provoquent une mastite très aiguë. Le nombre de cellules somatiques dans le lait augmenta de façon significative déjà après la première traite et dura la plupart du temps sept à dix jours avant que le nombre de cellules fut à nouveau normal. L’intensité de l’inflammation et l’augmentation des cellules somatiques fut à peu près proportionnelle à la quantité d’hémolysine administrée. Les propriétés chimiotactiques et phagocytantes des neutrophiles infusés avec l’hémolysine β furent inchangées. Ces propriétés furent cependant considérablement réduites du moment où l’on a incubé les neutrophiles avec l’hémolysine. Ces résultats se confirment lors d’essais faits avec une β-hémolysine partiellement purifiée. Des infusions intramammaires faites avec une très petite quantité de staphylocoques 252-F provoquèrent des mastites moyennes mais également aiguës. Les recherches ont montré que ni le lait des glandes enflammées ni celui des mamelles injectées d’hémolysine β ne contenait d’hémolysine β. Les résultats contredisent fortement le point de vue de la plupart des auteurs qui estiment que l’hémolysine β joue un rôle mineur dans la pathogénèse des mastites chez le bovin.

Resumen

Significación del estafilococo dorado betahemolítico como germen patógeno para la glándula mamaria de la vaca

Las infusiones mamarias de un mínimo de media dosis hemolizante de hemolisina β cruda, procedente de la estirpe *Staphylococcus aureus* 252-F, ocasionaban una mastitis hiperaguda. Aumentaba considerablemente la cantidad de células somáticas en la leche, lo cual ya se evidenciaba después del primer ordena, durando casi siempre de siete a diez días, antes de que la cantidad de células volviese a alcanzar su valor normal. La intensidad inflamatoria y el aumento de las células somáticas era casi proporcional a las cantidades administradas de hemolisina infundida. Las propiedades quimiotácticas y fagocitarias de los neutrófilos en las mamas que habían sido infundidas con hemolisina β no sufrieron alteración alguna. Sin embargo, estas propiedades amenguaron bastante tan pronto como se incubaron los neutrófilos con la hemolisina β. Estos resultados se confirmaron mediante ensayos con hemolisina β purificada en parte. Las infusiones mamarias con cantidades escasas de la cepa estafilocócica 252-F ocasionaron mamitis medianas, aunque también agudas. Los ensayos provisionales establecieron que ni la leche de cuarterones inflamados ni la leche de mamas inyectadas con hemolisina β contenían la hemolisina mencionada. Los trabajos presentes se hallan en contradicción extrema con el punto de vista de la mayoría de los otros autores, los cuales opinan que las hemolisinas β apenas tienen importancia en la patogenia de la mamitis de vacas.
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### References


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