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# IN VITRO ADHERENCE OF STAPHYLOCOCCUS AUREUS TO BOVINE MAMMARY GLAND EPITHELIAL CELLS

By

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WANASINGHE, DON D.: *In vitro adherence of Staphylococcus aureus to bovine mammary gland epithelial cells.* Acta vet. scand. 1981, *22*, 99—108. — A simple in vitro test system to study the adherence of bacteria to bovine mammary gland epithelial cells is described. This test system can be used as a laboratory model to study the relative attachment of bacterial species associated with mastitis. The effects of a number of physical, chemical, enzymatic and biological factors on the adherence of a strain of Staphylococcus aureus were studied using this test system. The numbers of bacteria adhering to epithelial cells were higher when logarithmic growth phase cultures were used (4—6 h). Adherence was not affected by pretreatment of bacteria with NaCl or EDTA, or within the pH range 6—8, but was inhibited by heat (60°C for 30 min) and trypsin and papain pretreatments, whereas wheat germ lipase pretreatment enhanced adherence. Sodium lauryl sulphate, Triton X-100, and Tween 80 inhibited adherence. Adherence was also inhibited by milk from a quarter infected with S. aureus and by specific anti-staphylococcal antiserum. These findings suggest that the staphylococcal adhesin may be protein in nature.

adherence; mastitis; pathogenicity; Staphylococcus aureus; bovine mammary gland epithelial cells.

The adherence of a number of species of bacteria to various cell surfaces has been studied (*Beachey* 1980), but little work appears to have been done on the adherence of staphylococci. Adherence of bacteria to bovine mammary gland epithelium has been shown to be an important stage in the pathogenesis of bovine mastitis (*Frost* 1975, *Frost et al.* 1977). The in vitro test system used in the latter studies would be a useful laboratory model for future work in this field. Accordingly, the test system

is described herein in detail. Staphylococcus aureus is responsible for the majority of udder infections. Therefore, in this study the effects of a number of physical, chemical, enzymatic and biological factors on the adherence of a strain of S. aureus (Sa1) to bovine mammary epithelial cells were examined.

## MATERIALS AND METHODS

### *Udders*

The udders of lactating cows were examined physically when the animals were assembled for slaughter, and those udders considered clinically normal and which produced grossly normal milk were selected. The udder was excised immediately after slaughter. It was washed, shaved, and cleaned and the required quarter(s) dissected out. The epithelial lining of the ductular system from the streak canal to the ductioles was exposed by cutting with a pair of sharp-pointed scissors beginning from the teat orifice. This area was then excised from the rest of the alveolar mass with a sharp knife (Fig. 1). The epithelial surface was rinsed several times with phosphate buffered saline (PBS), pH 7.4 to wash away the milk.

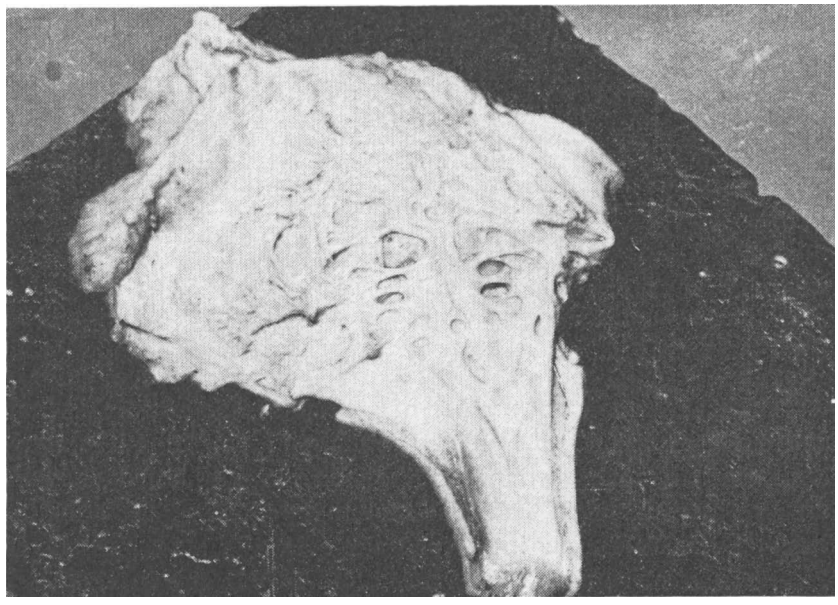

Figure 1. Bovine mammary gland quarter opened up to expose the epithelial lining.

*Epithelial cells*

The epithelial cells were collected and used within 4 h of slaughter of the cows. The cells were obtained by gentle unidirectional stroking of the wet epithelial surface of the lactiferous sinus with a soft pipette brush or interdental brush with a diameter of about 4 mm (Fig. 2) to avoid the epithelial cells coming off in sheets and to cause minimal damage to the cells. After gentle stroking 10 to 15 times, the brush bearing the detached epithelial cells was twirled vigorously in 15 ml of PBS to dislogde the cells. This process was repeated several times until a sufficient quantity of cells was collected. Care was taken not to brush the same area of the epithelium more than 8 to 10 times to avoid collection of cells of the basal membrane of the epithelium. The cells were then washed 3 or 4 times in PBS by centrifugation at 1500 r.p.m. for 10 min. After the final wash, the cells were resuspended in 15 ml PBS. The cell count of the suspension was determined using a Neubaur haemocytometer. The epithelial cell suspension was diluted with PBS to contain approximately $10^4$ cells per ml for use in the tests.

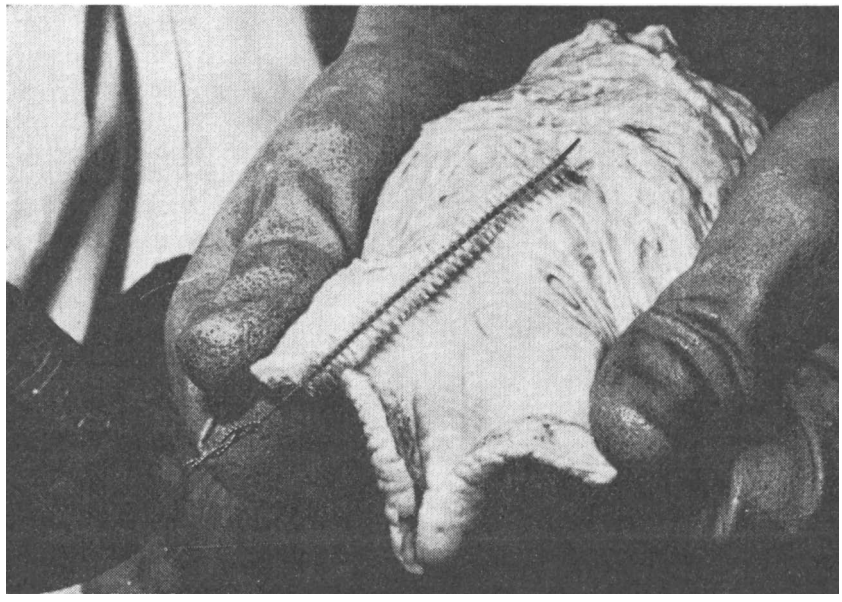

Figure 2. Collecting cells from the epithelial lining of the mammary gland by gentle stroking with a pipette brush.

*Preparation of the bacterial suspension*

The strain of S. aureus to be examined, herein strain Sa1, was routinely grown in Todd-Hewitt broth for 12—14 h. The bacteria were washed in PBS by centrifugation and resuspended in 10 ml PBS. This was called 'stock suspension' and was stored at 4°C until required (usually the following morning). In the meantime, a viable count [colony forming units (c.f.u.) per ml] of the stock suspension was made by plating out 10-fold dilutions of the suspension on 5 % sheep blood agar. Before use, the stock suspension was agitated with 10 to 15 glass beads in a vortex mixer for 30 s to break up the bacterial clumps and was then diluted in PBS to contain approximately $10^6$ c.f.u./ml. This dilution was called 'test suspension'.

*Adherence of bacteria to epithelial cells*

Aliquots of 2 ml each of the washed epithelial cell suspension and of the test bacterial suspension were mixed in sterile centrifuge tubes. The mixture was rotated slowly (30 r.p.m.) at 37°C for 1 h to allow contact between the bacteria and the cells. The mixture was centrifugated at 1500 r.p.m. for 10 min and the supernatant containing the unattached bacteria was carefully sucked off with a pipette and discarded. The cells were resuspended in 5 ml PBS and were washed 3 times in this manner to remove the unattached bacteria from the epithelial cell suspension. After the final wash, the cells were resuspended in 0.5 ml PBS. Smears were made on glass slides by spreading 0.2 ml of the cell suspension over approximately 1 $cm^2$.

*Staining and microscopic examination of smears*

The smears were air-dried and fixed by gentle heating. The fixed slides were stained by floding with Gram crystal violet for 10—15 s and washing away the excess stain quickly in running tap water. The stained smears were air-dried and examined under oil immersion.

*Counting the number of bacteria adhering to epithelial cells*

The number of bacteria adhering to 100 epithelial cells was counted using the following criteria. Only those bacteria which were either on the cells or directly associated with the cells and

only bacteria attached to undamaged epithelial cells which were single or together in clumps of 2 or 3 cells were counted*. Cells that did not look like typical epithelial cells were not counted. In each treatment, the number of epithelial cells to which bacteria adhered per 100 cells and the number of adhering bacteria were recorded separately.

*Pretreatment of bacteria with physical, chemical and biological agents*

To study the effect of the age of the culture, heat, pH, Tween 80, NaCl, sodium lauryl sulphate (SLS), Triton X-100 (TX-100), EDTA, trypsin, papain, wheat germ lipase (WGL), antiserum, normal rabbit serum, normal human serum, and milk on the adherence of S. aureus (Sa1), the bacteria were either subjected to these physical conditions or pretreated with the substances. Pretreatment was done by suspending the washed bacteria in the respective solutions of the substances for 1 h at room temperature (28°C), except for pretreatment with TX-100 which was done at 4°C for 14 h. After pretreatment, the bacteria were washed and resuspended in PBS (pH 7.4) prior to use in the test system.

Since all factors could not be examined in one experiment, variables were grouped into several experiments. An untreated control was included in each experiment.

*Adherence index*

The numbers of pretreated bacteria adhering to 100 epithelial cells in the treatment experiments were expressed as a percentage of the adherence of the untreated control of that experiment (adherence index). Thus, adherence index allows direct comparison among treatments within an experiment. Since epithelial cells for each experiment were obtained from a different cow, results from different experiments may not be directly comparable, but relative comparison of the adherence indices is possible.

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* In counting streptococcal chains and corynebacterial cells only those bacteria which are in direct association with the epithelial cells should be counted.

## RESULTS

### *Adherence of S. aureus to mammary gland epithelial cells*

The S. aureus (Sa1) adhered to the epithelial cells (Fig. 3). When a ratio of 1:100 of epithelial cells to bacteria ($10^4$ to $10^6$ respectively) was used, the numbers of bacteria which adhered to the cells were readily countable.

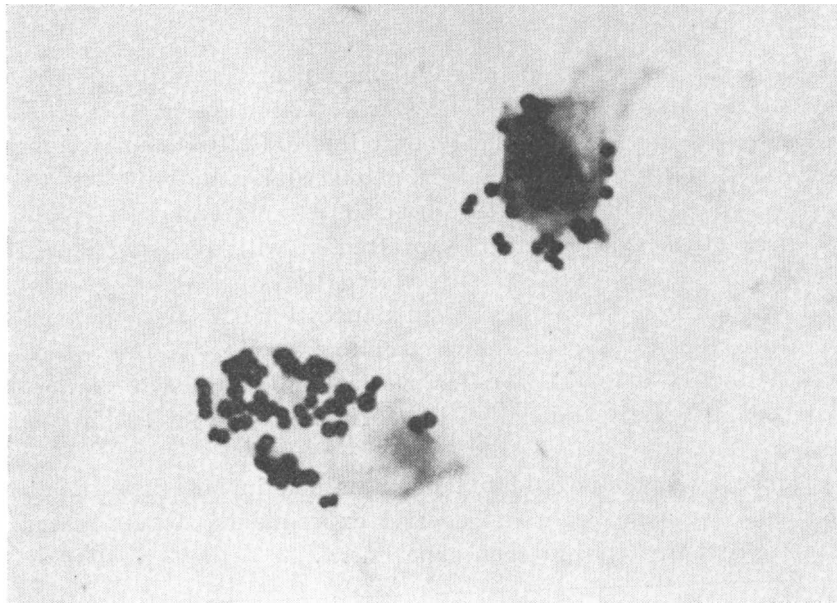

Figure 3. Staphylococcus aureus adhering to ductular epithelial cells. Stained with Gram crystal violet. Magnification, × 900.

### *Effect of age of culture and pH on adherence*

The results are presented in Table 1. The 4 and 6 h cultures adhered better than older cultures (10 h, 24 h, 72 h). The pH range tested did not influence the ability of the organism to adhere.

### *Effects of pretreatment of Sa1 with physical, chemical, enzymatic and biological substances*

The effects of pretreatment of Sa1 with heat, Tween 80, NaCl, sodium lauryl sulphate (SLS), Triton X-100 (TX-100), EDTA, trypsin, papain, wheat germ lipase (WGL), milk from an infected cow, milk from an uninfected cow, homologous antiserum,

T a b l e 1. Effect of age of culture and of pH on adherence of Staphylococcus aureus (Sa1) to bovine mammary gland epithelial cells.

| Parameter studied | Number of cells with adhering bacteria per 100 cells | Number of bacteria adhering to 100 cells |
|---|---|---|
| Age of culture | | |
| 4 h | 45 | 114 |
| 6 h | 41 | 111 |
| 10 h | 31 | 68 |
| 24 h | 27 | 72 |
| 72 h | 26 | 57 |
| pH | | |
| pH 6.0 | 58 | 146 |
| pH 7.4 | 59 | 170 |
| pH 8.0 | 67 | 146 |

normal rabbit serum, and normal human serum are presented in Table 2. These factors were studied in a number of experiments and the results are expressed as adherence indices of the untreated control of each experiment to enable comparison across a range of experiments.

NaCl and EDTA did not alter the adherence. WGL at 4 mg and 2 mg per ml increased adherence. Tween 80, SLS, and TX-100 reduced adherence. With these 3 detergents, the reduction in adherence was greater with higher concentrations. Adherence was reduced by proteolytic enzymes. Milk from an uninfected quarter reduced adherence somewhat, but the reduction was greater with milk from an infected quarter (this quarter was infected with a strain of S. aureus). Homologous rabbit antistaphylococcal antiserum reduced adherence, while there was no effect with normal rabbit serum. Normal human serum reduced adherence at low dilution but was without effect at a 20-fold higher dilution.

## DISCUSSION

In relation to the volume of research done on different aspects of mastitis, little has been done on the pathogenesis of this disease. The practical difficulties of conducting research in live animals may have discouraged such research. Therefore, a simple in vitro test system, as described in this study, could serve as a useful laboratory model for future investigations.

Table 2. Effects of pretreatment of Staphylococcus aureus (Sa1) with physical, chemical, enzymatic and biological agents on the adherence to mammary gland epithelial cells.

| Treatment | Adherence index | Treatment | Adherence index |
|---|---|---|---|
| Heat | | Proteolytic enzymes | |
| 37°C control | 100 | Control | 100 |
| 60°C for 30 min | 36 | Trypsin, 10 mg/ml | 11 |
| 100°C for 30 min | 38 | Papain, 10 mg/ml | 13 |
| 121°C for 15 min | 18 | | |
| | | Lipolytic enzyme | |
| Detergent, NaCl | | Control | 100 |
| Control | 100 | Wheat germ lipase, 4 mg/ml | 231 |
| Tween 80, 1.5 % (v/v) | 38 | WGL, 2 mg/ml | 118 |
| Tween 80, 0.15 % (v/v) | 75 | WGL, 0.2 mg/ml | 94 |
| NaCl, 1 mol/l | 104 | | |
| | | Milk* | |
| Sodium lauryl sulphate | | Control | 100 |
| Control | 100 | From uninfected quarter | 83 |
| SLS, 1.0 % (w/v) | 23 | From infected quarter | 56 |
| SLS, 0.1 % (w/v) | 69 | | |
| SLS, 0.01 % (w/v) | 67 | Anti-staphylococcal antiserum | |
| | | Control | 100 |
| Triton X-100, EDTA | | Homologous antiserum 1/320 | 47 |
| Control | 100 | Homologous antiserum 1/640 | 56 |
| TX-100 1.5 % (v/v) | 44 | Homologous antiserum 1/1280 | 91 |
| TX-100 0.15 % (v/v) | 65 | | |
| EDTA, 5 mol/l | 98 | Normal serum | |
| | | Control | 100 |
| | | Normal rabbit serum 1/320 | 103 |
| | | Normal human serum 1/320 | 107 |
| | | Normal human serum 1/16 | 59 |

* Supernatant fractions obtained by centrifugation at 4500 r.p.m. for 1 h.

The factor responsible for adherence (adhesin) of S. aureus appears to be better expressed in logarithmic phase cultures. The reduction in adherence upon treatment with proteolytic enzymes and heat suggests that the adhesin may be protein in nature. Pretreatment of bacteria with the detergents SLS, Triton X-100 and Tween 80 reduced adherence. This reduction in adherence may be due either to the removal of the adhesin by these substances or due to coating of the organism with these substances, despite the fact that the bacteria were washed with PBS after pretreatment. The enhanced adherence obtained upon pretreat-

ment with WGL may have resulted from removal of a putative lipid surface component, thereby exposing more adhesin on the bacterial surface. The reduction in adherence by anti-staphylococcal antiserum may be specific as the normal rabbit and human sera at the same dilutions did not reduce adherence. Indeed, the reduction in adherence by infected milk could also have been due to the presence of antibodies as the staphylococcal strain isolated from this milk was agglutinated to a titre of 1:10 by the milk supernatant fraction.

Bacterial adherence to mammary gland epithelial cells needs further investigation. This approach may be a useful means not only of studying the pathogenesis of mastitis, but also of investigating some aspects of biological and biochemical properties of bacteria in relation to adherence. Present knowledge of the pathogenesis of mastitis is limited to what takes place after the tissues are invaded by bacteria. The evidence of bacterial adherence to mammary gland epithelial cells suggests a step prior to tissue invasion, knowledge of which could prove important in prevention of mastitis. A clear understanding of the factors mediating adherence, both in cells and in bacteria, may suggest new approaches to mastitis control.

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SAMMANFATTNING

*In vitro studier av Staphylococcus aureus adherens till bovina juverepitelceller.*

En enkel in vitro test för att studera bakteriers adherens (vidhäftning) till bovina juverepitelceller beskrives. Metoden kan användas för att pröva olika mastitframkallande bakteriers adhesionsförmåga. Undersökningar har gjorts för att klarlägga hur fysikaliska, kemiska, enzymatiska och biologiska faktorer påverkar vidhäftningsförmågan hos en Staphylococcus aureus stam. Antalet bakterier som „fastnade" på epitelcellerna var högre när celler från aktivt växande bakteriekulturer (log-fas) användes. Vidhäftningen påverkades inte av en förbehandling av bakterierna med NaCl eller EDTA. Däremot minskades den om mikroberna upphettades till 60°C under 30 min eller om de behandlades med trypsin eller papain. Vetegroddolja stimulerade adhesionen. Natrium lauryl sulfat, Triton X-100 och Tween 80 inhiberade. Specifikt antiserum inhiberade vidhäftningen liksom mjölk från juver infekterat med Staph. aureus.

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