Effect of Milking Without Pulsation on Teat Duct Colonization with *Streptococcus agalactiae* and Penetrability to Endotoxin

A. J. BRAMLEY

Agricultural and Food Research Council
Institute for Animal Health, Compton Laboratory
Compton, Newbury, England RG160NN

W. D. SCHULTZE

Mastitis and Milk Secretion Laboratory
Agricultural Research Service, USDA
Beltsville, MD 20705

ABSTRACT

Forty quarters of 10 cows were milked for a 9-d period with one of four treatments: 1) no liner pulsation, 2) conventional milking, 3) no pulsation for 4 d followed by conventional milking for 5 d, and 4) conventional milking for 4 d followed by milking without pulsation for 5 d. All teat orifices were inoculated with approximately .5 million cfu of *Streptococcus agalactiae* and *Streptococcus dysgalactiae* on d 1 and 5. Recoveries of *Strep. agalactiae* from the teat end were increased for teats milked without pulsation. Recoveries of *Strep. dysgalactiae* were lower than those of *Strep. agalactiae* and not increased by milking without pulsation.

In a second experiment, teats of 20 cows were milked for a 15-d period with or without liner pulsation. For 10 successive milkings, all teats were inoculated with 1.0 µg of *Escherichia coli* endotoxin either immediately or 2.5 h after each milking. The frequency of endotoxin penetration, measured by the Wisconsin Mastitis Test, in pulsated quarters and in nonpulsated quarters was similar. For quarters milked without pulsation but not for pulsated quarters, inoculation of endotoxin immediately after milking led to greater incidence of teat duct penetration than for inoculation 2.5 h after milking.

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INTRODUCTION

The teat duct forms a generally effective barrier to intramammary infection, and among its defenses are antibacterial systems associated with its keratin lining (1, 12, 9). Various anatomical and physical properties of the duct, such as duct diameter, keratin distribution, and milking rate, are related to mastitis susceptibility (14). Penetrability of the teat duct to *Escherichia coli* endotoxin has been used to assay the physical barrier of the teat duct and its interaction with machine milking (20).

The collapse of the liner on the teat during machine milking is critical to maintain the effectiveness of the teat duct barrier. Bramley et al. (6) and Woolford and Phillips (26) showed that machine milking without pulsation increased infection rates under both experimental and farm conditions. Subsequent experiments confirmed this and showed that a similar effect was observed if the duration of liner collapse was inadequate (19) or if the liners were too short relative to the length of the teat (15). Experiments (6, 19) had associated the increased infection rate of milking without pulsation with *Streptococcus agalactiae* rather than *Streptococcus dysgalactiae*, although both bacteria had been used as challenge organisms. Additionally, the teat ends of quarters milked without pulsation showed increased recoveries of streptococci compared with control quarters (6, 26). Milking without pulsation leads to increased thickness of the teat end after milking, probably due to im-
paired blood flow and edema (11). Williams and Mein (24) postulated that a function of pulsation was to protect the teat from excessive loss of keratin. Murphy (16) showed that removal of keratin from the teat duct by reaming with a plastic tube increased infection with Strep. agalactiae, and Bramley (3, 4) showed that this process increased multiplication of bacteria in the teat duct. Using the endotoxin penetrability assay, Schultze and Bramley (21) were able to show that keratin removal did not increase endotoxin penetration through the teat duct.

The objective of the study was to investigate whether the increased rates of udder infection among cows milked without pulsation are due to 1) impairment or removal of the antibacterial mechanisms of the teat duct keratin, 2) enhanced growth of bacteria at the teat end, or 3) increased penetrability of the duct to these bacteria.

MATERIALS AND METHODS

Animals

Thirty-three Friesian cows selected from an institute research herd and free of intramammary infection were used. These animals were milked twice daily in either a two-unit (Experiment 1) or six-unit (Experiment 2) tandem milking parlor fitted with recorder jars. At the times of the experiments, the cows were at pasture.

Experimental Design

Experiment 1: Effects of Pulsation on the Growth of Streptococci. Ten cows were milked for a 9-d experimental period with a different treatment applied to each teat as follows: 1) no liner pulsation throughout the experimental period, 2) liner pulsation throughout the experimental period, 3) no pulsation for 4 d followed by 5 d of pulsation milking, or 4) pulsation for 4 d followed by 5 d of milking without pulsation.

Prior to the experiment, all teats were milked with treatment 2.

Immediately following afternoon milking on d 1 and 4, all teats were inoculated with Strep. agalactiae and Strep. dysgalactiae. Numbers of these bacteria present at the teat end were assessed by swabbing on d 2 through 9.

Experiment 2: Effect of Pulsation on the Penetration of Endotoxin Through the Teat Duct. The experiment consisted of two parts, each of which was divided into three periods. In the first part, the right teats of 20 cows were milked with pulsation, and the left teats were milked without pulsation. In the second part, the treatments were reversed. Seventeen of the 20 cows were used in both parts of the experiment. The three other cows had to be replaced for various reasons.

In both parts, the three periods of the experiment were acclimatization, experimental, and postexperimental. The acclimatization period included 14 milkings, in which the treatments were applied and all quarters were milk-sampled and tested at each milking. The experimental period included 10 milkings, in which the treatments were applied and endotoxin inoculations were performed after each milking. In each cow, one right and one left quarter were inoculated immediately after cluster removal and the other pair after a delay of 2.5 h. The postexperimental period included 6 milkings, in which treatments and sampling continued but endotoxin inoculation ceased.

During the acclimatization and postexperimental periods, all teats were dipped after milking in sodium hypochlorite (4% available chlorine).

Milking Machine Conditions

Experiment 1. The milking cluster consisted of Alfa-Laval 20003B liners (Alfa-Laval, Kansas City, MO) fitted with stainless steel shields and joined by 11-mm i.d. clear plastic short milk tubes to a 16-mm i.d. half udder claw. Each long milk tube was 16 mm i.d. Liners were mounted in clear plastic shells fitted with Alfa-Laval buffer adaptor rings (Number 25570), and the cluster was weighted to 2885 g. Liner pulsation was 59 to 60 cycles/min 66:34 ratio controlled by a master pulsator and two Fullwood, Ltd. (Cheshire, Engl.) pulse taps at each stall. For the treatment without pulsation, the pulsation chamber and milking vacuum were interconnected. Vacuum was supplied to each half udder claw by a separate Gascoigne J vacuum pump (Kompex Pneumatics, Hitchin, Herts, Engl.) with a rated capacity.
of 153 L/min at 50 kPa. The operating vacuum was 50 kPa. Regular records of vacuum, pulsation rates, and ratio were obtained prior to and during the experiment from an Alfa-Laval portable recorder.

Experiment 2. The cluster consisted of Alfa-Laval 96000-1 liners fitted with shields and installed in Alfa-Laval metal shells (Number 24932). A half udder claw was used, as for Experiment 1. The pulsation rate was 53 cycles/min with a 60:40 pulsation ratio. No pulsation was achieved, in a similar manner to Experiment 1. Vacuum was supplied by two vacuum pumps, each with a rated capacity of 594 L/min at 50 kPa (Kompex Pneumatics). The operating vacuum was 50 kPa.

Milking Routine

In both experiments, milking was performed twice daily at 0615 and 1430 h. On entering the parlor, the cow’s teats were foremilked, washed with water, and dried using individual paper towels. The half udder cluster was then applied, and the cow was milked. Clusters were removed manually at the end of milking. Clinical signs of mastitis were recorded at each milking. After milking each cow, teat cup clusters were decontaminated by the circulation of water at 85°C for 15 s.

Bacterial Challenge

Streptococcus agalactiae NCD00349 and Strep. dysgalactiae NCD00350 were used. These strains have been used extensively in milking machine experiments (6, 19). The bacteria were grown in skim milk containing 5% glucose and 3% yeast extract (Oxoid Ltd., Basingstoke, Engl.) at 37°C for 6 h. These cultures were cooled to between 2 and 4°C, and bacterial numbers were determined by 10-fold dilution in half-strength broth (Oxoid nutrient broth 2) and by plating on 7% calf blood agar containing 1% esculin (British Drug Houses Ltd., Poole, Dorset, Engl.). The following day, these cultures were diluted in skim milk to contain approximately 500,000 cfu of each strain/0.01 ml. Using a modified microsyringe (13), 0.01 ml of this bacterial suspension was then inoculated 1 to 2 mm into each teat orifice after milking on days 1 and 4 of Experiment 1.

Test Apex Swabs

Swabs of the teat end were taken after each afternoon milking on d 2 through 9 of Experiment 1. After teat washing and drying, individual cotton-tipped swabs moistened in skim milk were rubbed across each teat orifice 5 to 6 times. The swabs were then placed into 2 ml of skim milk, well mixed, and 10-fold dilutions prepared in half strength broth prior to plating 0.1-ml aliquots on modified Edwards medium. These plates were incubated at 37°C for 48 h, and the colonies of Strep. agalactiae and Strep. dysgalactiae were enumerated.

Endotoxin Penetrability Assay

This was performed as described previously (20) using 1.0 μg of E. coli 0128:B12 lipopolysaccharide (Difco, Detroit, MI) in a volume of 2.5 μl. After scrubbing of the teat end with 70% ethanol, this volume was inoculated 3 mm into the teat duct using a Newbold-type inoculator (17). Teats were inoculated for 10 successive milkings during the experimental period of Experiment 2 as previously described.

Wisconsin Mastitis Test

Penetration of endotoxin through the teat duct leads to an inflammation measurable as a marked increase in Wisconsin Mastitis Test score using the method and criteria described by Schultze (20).

RESULTS

Table 1 summarizes the log mean and standard errors for recoveries of Strep. agalactiae and Strep. dysgalactiae from teat orifices milked with the four different treatments in Experiment 1. These means were compared by Student’s t test, and the probabilities of the means being different are shown. Recoveries of Strep. agalactiae were significantly higher for the teats milked throughout the experiment without pulsation compared with those milked with pulsation. Recoveries of Strep. agalactiae were higher for periods without pulsation than for periods with pulsation in the changeover treatments, but these were significant only for teats that commenced without pulsation. Recoveries of Strep. dysgalactiae were consis-
TABLE 1. Mean log recoveries and SEM of *Streptococcus agalactiae* and *Streptococcus dysgalactiae* from teat ends milked with and without pulsation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean log recoveries per swab</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Strep. agalactiae</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulsation</td>
<td>1.68</td>
<td>.61</td>
<td></td>
</tr>
<tr>
<td>No pulsation</td>
<td>3.22</td>
<td>.21</td>
<td>&lt;.008</td>
</tr>
<tr>
<td>Pulsation to no pulsation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulsation</td>
<td>2.02</td>
<td>.30</td>
<td></td>
</tr>
<tr>
<td>No pulsation</td>
<td>3.08</td>
<td>.24</td>
<td>NS</td>
</tr>
<tr>
<td>No pulsation to pulsation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulsation</td>
<td>1.56</td>
<td>.21</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>No pulsation</td>
<td>3.24</td>
<td>.27</td>
<td></td>
</tr>
<tr>
<td><em>Strep. dysgalactiae</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulsation</td>
<td>.93</td>
<td>.15</td>
<td></td>
</tr>
<tr>
<td>No pulsation</td>
<td>1.61</td>
<td>.19</td>
<td>NS</td>
</tr>
<tr>
<td>Pulsation to no pulsation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulsation</td>
<td>1.35</td>
<td>.29</td>
<td></td>
</tr>
<tr>
<td>No pulsation</td>
<td>1.56</td>
<td>.22</td>
<td>NS</td>
</tr>
</tbody>
</table>

Tentatively lower than those of *Strep. agalactiae*. There was a trend toward greater recoveries of *Strep. dysgalactiae* from teats that were not pulsed, but this was usually not significant (the exception being teats changed from no pulsation to pulsation).

The log mean recoveries of *Strep. agalactiae* are shown (Figure 1) for each daily swabbing of teats milked throughout the experiment with or without pulsation. Teats milked without pulsation showed increased recoveries of *Strep. agalactiae* after 6 d of milking without pulsation. Increased recoveries of *Strep. agalactiae* from teats milked without pulsation were observed for 8 of the 10 trial cows, as shown in Figure 2. The recoveries of *Strep. agalactiae* from teat ends prior to and following the change from milking with and without pulsation and vice versa are shown in Figure 3. Recoveries of *Strep. agalactiae* increased within 1 to 2 d of the cessation of pulsation. Similarly, when teats were switched from no pulsation to pulsation, there was a marked decline of *Strep. agalactiae* within 1 to 2 d of the changeover.

During the trial, three intramammary infections developed, all with *Strep. agalactiae*. Two of these occurred 3 and 5 d following the switch to milking without pulsation. The other infection occurred in a quarter milked with pulsation 2 d following inoculation.

The effects of milking with or without pulsation on the penetrability of the teat duct to *E. coli* endotoxin are recorded in Table 2. Inoculation of endotoxin into pulsed teats led to a detectable inflammation for 26/80 teats inoculated (32.5%) compared with 30/80 (37.5%) for nonpulsated teats. These results are not significantly different. Nonpulsated teats that were inoculated immediately after milking had ceased showed significantly greater rates of endotoxin penetration than those inoculated following a delay of 2.5 h (21/40 vs. 9/40, *P* < .05). There was no significant effect of time of...

![Figure 1](image1.png)

Figure 1. Mean log recovery of *Streptococcus agalactiae* from teat end swabs of udder quarters milked with or without pulsation for a 9-d period (n = 10/mean value). Teats were inoculated after afternoon milking on d 1 and 4.

![Figure 2](image2.png)

Figure 2. Mean recoveries of *Streptococcus agalactiae* from the teat ends of 10 cows milked with or without pulsation over 9 d (n = 10/mean value).
Figure 3. Effect of switching from pulsation to no pulsation milking on the recovery of *Streptococcus agalactiae* from the teat ends of 10 cows (n = 10/mean value). Treatments were switched between d 4 and 5, and inoculations were performed after afternoon milking on d 1 and 4.

inoculation on penetration in pulsed teats (15/40 vs. 11/40).

DISCUSSION

These experiments confirm earlier observations (6) that milking without pulsation increases recoveries of bacteria from the teat end or teat duct. Although it is recognized that bacterial colonization or growth at the teat end is an important factor in the pathogenesis of mastitis (10), the precise events involved and the location of the bacteria remain undescribed. These experiments show that bacteria have differing capabilities to capitalize on the effects of milking without pulsation. The recoveries of *Strep. agalactiae* were significantly increased, whereas those of *Strep. dysgalactiae* were essentially unaffected. This difference between mastitis pathogens in their colonizing ability has been shown previously when it was demonstrated that low numbers of *Staphylococcus aureus* could readily colonize the teat orifice, but *E. coli* were unable to do so (7).

The studies of Williams (25) have also shown that bacteria may interact differently with teat canal keratin, and this may be a feature in the results found here. However, the nature of the changes to the keratin as a consequence of milking without pulsation remains unclear. Williams and Mein (24) suggested that a role of pulsation was to retain keratin within the teat duct and that rates of keratin loss increased if pulsation was absent. Some indirect experimental evidence has been generated to support this finding (25), although no histological data have been published. Certainly the mechanical removal of keratin from the teat duct increases susceptibility to infection with *Strep. agalactiae* (16, 23). The extent of changes may be slight, because Schultze and Bramley (21) demonstrated that even extreme mistreatment of the keratin lining by reaming with a metal twist drill bit led to small histological changes in the keratin lining. The experiments reported here also indicate that the changes encouraging or discouraging bacterial growth occur rapidly and are reversible. The treatments in which teats were switched from or to pulsation milking showed the characteristic change in bacterial numbers in 1 to 2 d. This delay coincides with the estimation of 1 to 2.5 d for teat canal keratin to be regenerated following physical removal (8). The mechanisms involved are probably subtle and may involve differential antibacterial or stimulatory factors in different ages of keratin. Antibacterial lipids and proteins exist within the keratin lining of the teat and have been shown to affect bacterial growth (17, 18, 19).

TABLE 2. Endotoxin penetration of the teat duct following milking with or without liner pulsation.

<table>
<thead>
<tr>
<th>Pulsation</th>
<th>Inoculation timing</th>
<th>No. positive/no. inoculated</th>
<th>Total (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>Immediately</td>
<td>6/20</td>
<td>15/40</td>
<td>NS</td>
</tr>
<tr>
<td>+</td>
<td>Immediately</td>
<td>9/20</td>
<td>11/40</td>
<td>NS</td>
</tr>
<tr>
<td>+</td>
<td>2.5 h</td>
<td>6/20</td>
<td>21/40</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>+</td>
<td>Immediately</td>
<td>10/20</td>
<td>21/40</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>-</td>
<td>Immediately</td>
<td>5/20</td>
<td>9/40</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>-</td>
<td>2.5 h</td>
<td>4/20</td>
<td></td>
<td>&lt;.05</td>
</tr>
</tbody>
</table>
duct and penetration of a solute are distinct.

The second part of the study reinforces the need to search for a biochemical explanation of the effect of milking without pulsation because it reveals that the effects do not extend to *E. coli* endotoxin. This is consistent with previous studies (21) and with results of Schultz and others (23), who showed that removal of keratin by reaming increased the growth of *Strep. agalactiae* but not the penetrability to *E. coli* endotoxin. However, although the mechanisms involved in the penetration of endotoxin and colonization of *Strep. agalactiae* appear to be different, endotoxin penetrability does seem to be a good predictor of likely susceptibility to *Strep. agalactiae* penetration (23). The lower penetrability seen for nonpulsated teats when endotoxin was inoculated 2.5 h after milking has been previously reported (22). This is probably associated with changes in edema or muscle contraction that occur after milking (11, 18). Although such changes in the milking interval may not influence multiplication of bacteria in the teat duct, they may be relevant to the movement of bacteria to the teat sinus. Previous work has demonstrated that increased penetration with *E. coli* occurs following contamination of the teat end immediately after milking (5), although the pathogen does not colonize the teat duct of the lactating cow (7).

**CONCLUSIONS**

Growth of *Strep. agalactiae*, but not *Strep. dysgalactiae*, at the teat end is influenced by pulsation. Failure of liner collapse significantly increases recoveries of *Strep. agalactiae* from teat end swabs. This effect could be reversed by the reapplication of pulsation for 1 to 2 d. These effects were seen in the majority of cows tested. Milking without pulsation did not significantly increase penetrability of the teat duct to endotoxin, indicating that the mechanisms involved in bacterial growth in the teat duct and penetration of a solute are distinct.

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