Intramammary Challenge of the Bovine Mammary Gland with Coliform Bacteria During Early Involution

DEBORAH TODHUNTER, K. Larry SMITH, and JOSEPH S. HOGAN
Department of Dairy Science
The Ohio State University
Ohio Agricultural Research and Development Center
Wooster 44691

ABSTRACT

Isolates of Escherichia coli (n = 12), Klebsiella pneumoniae (n = 20), and Klebsiella oxytoca (n = 10) were used to challenge involuting mammary glands at 7 d of the dry period. Bacteria were selected for challenge on the basis of their ability to grow in a pooled source of dry cow secretion obtained at 21 d of involution. Challenge bacteria were classified as highly adapted (in vitro growth $> 7 \text{ cfu} 10^{9}/\text{ml}$) or poorly adapted (growth $< 2 \text{ cfu} 10^{9}/\text{ml}$) for growth in dry cow secretion. Intramammary infusion of Escherichia coli, K. pneumoniae, and K. oxytoca resulted in 0, 40, and 30%, respectively, of quarters infected. Isolates highly adapted for growth in dry cow secretion caused 75% of K. pneumoniae and 67% of K. oxytoca experimental intramammary infections. Results indicated that the ability to overcome inhibitory properties of dry cow secretion was related to the establishment of K. pneumoniae and K. oxytoca intramammary infections in the dry gland. There was no evidence that growth of E. coli in dry cow secretion related to pathogenicity in the dry gland. Experimental challenge using multiple isolates did confirm the resistance of the involuting mammary gland to E. coli infection.

(Key words: coliform bacteria, intramammary challenge, mammary gland involution)

INTRODUCTION

Gram-negative bacteria require iron for growth, and the ability of an invading pathogen to obtain iron from the host is considered a virulence factor (12). Cell-free, fat-free, dry cow secretion has been shown to inhibit the growth of many gram-negative bacteria (10, 19). Inhibitory properties of dry cow secretion have been partially attributed to lactoferrin and antibody, which prevent iron acquisition by many gram-negative bacteria. In vitro studies have demonstrated that growth of Klebsiella pneumoniae in dry cow secretion was greater than Escherichia coli and Klebsiella oxytoca (18). Additionally, natural infection data have indicated that origin of K. pneumoniae intramammary infections (IMI) is equally divided between the first and last halves of the dry period. In contrast, origin of E. coli IMI was highly associated with calving and early lactation (16; Todhunter et al., unpublished data). The ability of a gram-negative bacterium to infect the bovine mammary gland during involution appears to depend in part on its ability to overcome the inhibitory properties of the secretion. The objective of this study was to compare the abilities of E. coli, K. pneumoniae, and K. oxytoca to infect experimentally the bovine mammary gland during the early dry period and to persist to lactation.

MATERIALS AND METHODS

Animals

Holstein dairy cattle were dried off by abrupt cessation of milking approximately 60 d prior to anticipated calving. Following the final milking, all mammary quarters of all cows were infused with a commercially available dry cow antibiotic preparation (300 mg cephepirin benzathine, Tomorrow, Agricultural Products Division of Bristol-Myers Co., Dewitt, NY). Housing and management of cows were previously described (15, 16).
Quarter foremilk samples were aseptically obtained at 14 and 7 d prior to drying off, the day of drying off, and 5 and 7 d of involution to determine the microbiological status and total SCC of all quarters prior to bacterial challenge. Sampling of mammary quarters, microbiological procedures, and milk SCC were as previously described (15). Somatic cell counts were expressed as SCC (log10) per milliliter of secretion.

Challenge Bacteria

Gram-negative bacteria were originally isolated from naturally occurring IMI in the Ohio Agricultural Research and Development Center dairy research herd. Isolates of \textit{E. coli}, \textit{K. pneumoniae}, and \textit{K. oxytoca} were tested for growth in a pooled source of dry cow secretion (SDCS) obtained from 11 cows at 21 d of involution. Secretion was centrifuged at 48,000 × g at 4°C for 60 min and the fat layer removed. The cell-free, fat-free supernatant was sterilized by filtration through a series of membrane filters (Millipore Corporation, Bedford, MA) and stored at -20°C prior to use. In vitro growth assays consisted of 250 μl of SDCS inoculated with approximately 10^2 cfu in a 10-μl volume. Cultures were incubated in an humidified atmosphere for 18 h at 37°C. Following incubation, cultures were serially diluted in phosphate-buffered saline (PBS) and four 10-μl spots of diluted and undiluted bacterial cultures were plated onto the surface of a MacConkey agar (Difco Laboratories, Detroit, MI) plate. In vitro growth was expressed as colony-forming units (log10) per milliliter. Gram-negative bacteria were selected for challenge on the basis of growth in SDCS and divided into two groups: 1) poorly adapted or growth < 2 cfu log10/ml; and 2) highly adapted or growth > 7 cfu log10/ml.

A total of 21 cows, or 42 mammary quarters, were challenged with gram-negative bacteria at 7 d of involution. Two uninfected quarters of each cow were infused with either \textit{K. pneumoniae}, \textit{K. oxytoca}, or \textit{E. coli} in 1 ml of PBS. Quarters within a cow received different isolates of the same gram-negative species. One mammary quarter was infused with a highly adapted strain and the other quarter with a poorly adapted strain. Numbers of cows and mammary quarters infused per bacterial species were: \textit{K. pneumoniae}, 10 cows or 20 quarters; \textit{K. oxytoca}, 5 cows or 10 quarters; and \textit{E. coli}, 6 cows or 12 quarters. Mammary quarters that had a preexisting natural IMI were not used for challenge.

Bacteria were grown in brain-heart infusion broth (Difco Laboratories) at 37°C for 6 h and were diluted in PBS to achieve a final concentration of approximately 1000 cfu/ml PBS. Teats were dipped in 5.25% sodium hypochlorite (The Clorox Co., Oakland, CA), dried, and cleansed with cotton swabs soaked in 70% ethanol. Approximately 1 ml of diluted bacterial suspension was infused into the mammary quarter using a sterile 1-ml syringe fitted with a sterile teat cannula (Jorgensen Laboratories, Inc., Loveland, CO). Teats were dipped in 5.25% sodium hypochlorite after infusion. Colony-forming units infused were determined by plating 1 ml of inoculum in duplicate onto the surface of a MacConkey agar plate.

Postchallenge Sampling Schedule

Quarter foremilk samples were obtained aseptically from challenged quarters at the following times after bacterial infusion: 1, 3, 7, 14, 21, 42, and 56 d (if calving had not occurred). Samples were also obtained at calving and at 3, 7, 14, and 30 d postcalving. Detection of gram-negative bacteria in challenged quarters was by duplicate 1 ml MacConkey agar pour plates. Total colony-forming units isolated from challenge quarters were determined from pour plates or diluted secretion that was plated onto the surface of MacConkey agar plates. Plates were aerobically incubated at 37°C for 18 to 24 h prior to counting. Total bacterial numbers in secretion were expressed as colony-forming units (log10) per milliliter. Somatic cell counts were determined on all quarter samples by Coulter counter (Coulter Electronics, Hialeah, FL) (15). Secretion obtained during the dry period was diluted 10-fold in PBS prior to processing for cell counting.

Identification of Gram-Negative Bacteria

Gram-negative bacteria isolated from mammary secretion postinfusion were stored on trypticase soy agar slants (BBL Microbiological
Systems, Becton Dickinson Co. Cockeyesville, MD) and identified with the API 20E (Analytab Products, Plainview, NY) classification system. Antibiograms of gram-negative bacteria used for challenge and gram-negative bacteria reisolated from challenge quarters were determined using the Bauer-Kirby disc diffusion procedure (3).

**Diagnosis of Experimental Gram-Negative Intramammary Infection**

A gram-negative IMI was diagnosed in a challenged quarter on the basis of two out of three consecutive isolations with 30 d or less between isolations. Biochemical identification and antibiograms were used to determine if the bacteria isolated from the quarter were similar to the challenge strain. Biochemical tests had to agree at the genera level to be considered similar. Antibiotic susceptibility or resistance had to agree on 95% of antibiotics tested to be considered similar. Experimental dry period IMI were classified as: 1) short duration dry period IMI or those IMI that did not persist to calving or 2) dry period IMI that persisted to calving. Challenged quarters were classified as having an IMI caused by a different genus of bacteria when a gram-negative bacterial infection was diagnosed, but isolates differed biochemically or on the basis of antibiogram from the infused strain. Quarters in which gram-negative bacteria were isolated once and the biochemical tests or antibiograms differed from the challenge strain were classified as no isolation. Quarters with a single isolation of a gram-negative bacteria that had similar biochemical tests and antibiograms to the challenge strain were classified as a single isolation of the challenge strain.

**In Vitro Growth Studies**

Mammary secretion was obtained prior to bacterial challenge at 7 d of the dry period. In vitro growth of each strain was determined in secretion from the mammary quarter into which it was infused. Preparation of secretion and in vitro growth determination was as described for growth in SDCS.

**Statistical Analysis**

Comparison of mean in vitro growth among the gram-negative bacterial genera in mammary secretion obtained prior to challenge was by least squares analysis of variance (17). Comparisons of mean SCC in mammary secretion was by least squares analysis of variance. Multiple comparisons was by the Tukey-Kramer method.

**RESULTS**

The mean length of the dry period for the 21 challenge cows was 64.5 d (range 56 to 79 d). Mean number of colony-forming units infused into mammary quarters at 7 d of involution was

<table>
<thead>
<tr>
<th>Infection status</th>
<th><strong>Klebsiella pneumoniae</strong></th>
<th><em>Klebsiella oxytoca</em></th>
<th><em>Escherichia coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n)</td>
<td>(%)</td>
<td>(n)</td>
</tr>
<tr>
<td>Dry period IMI</td>
<td>8 (40)</td>
<td>3 (30)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Short duration</td>
<td>5 (25)</td>
<td>3 (30)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Persist to calving</td>
<td>15 (75)</td>
<td>7 (70)</td>
<td>9 (75)</td>
</tr>
<tr>
<td>Single isolation</td>
<td>1 (5)</td>
<td>0 (0)</td>
<td>3 (25)</td>
</tr>
<tr>
<td>No isolation</td>
<td>9 (45)</td>
<td>7 (70)</td>
<td>9 (75)</td>
</tr>
<tr>
<td>Different genera</td>
<td>2 (10)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

1 Number of quarters.  
2 Percentage of *K. pneumoniae* quarters challenged (n = 20).  
3 Percentage of *K. oxytoca* quarters challenged (n = 10).  
4 Percentage of *E. coli* quarters challenged (n = 12).  
5 Dry period intramammary infections that did not persist to calving.  
6 Gram-negative bacteria isolated following challenge differed from the infused strain biochemically or on the basis of antibiograms.
Infusion of *K. pneumoniae* into mammary quarters at 7 d of involution resulted in 8 IMI or 40% of quarters challenged (Table 1). Five of 8 *K. pneumoniae* dry period IMI were of short duration and 3 persisted to calving and into lactation. Gram-negative bacteria were not detected at any time postchallenge in 9 or 45% of all quarters infused with *K. pneumoniae*. Infections caused by bacterial genera not infused (*Serratia marcescens* and *E. coli*) were detected in 2 of the *K. pneumoniae* challenged quarters. Single isolation of *K. pneumoniae* occurred in 1 or 5% of challenged quarters. Infusion of *K. oxytoca* into mammary quarters resulted in 3 IMI. None of the *K. oxytoca* dry period IMI persisted to calving. *Klebsiella oxytoca* was not detected at any time postchallenge in 70% of quarters. No confirmed IMI were detected in *E. coli* challenged quarters. *Escherichia coli* were isolated from 25% of quarters postchallenge, and all were single isolations.

Numbers of quarters from which gram-negative bacteria were isolated following intramammary infusion for each time point is shown in Table 2. Two of three *K. pneumoniae* IMI that persisted to calving were spontaneously eliminated between 14 and 30 d postcalving. One *K. pneumoniae* IMI persisted beyond 30 d of lactation. Bacteria were not isolated at all times for the duration of experimental *K. pneumoniae* IMI, particularly at 21 and 42 d postchallenge. The last isolation of *K. oxytoca* from dry period IMI occurred 42 d postchallenge. Single isolations of *E. coli* were within 3 d after bacterial infusion.

Numbers of *K. pneumoniae* in secretion from infected quarters were less than 1.5 cfu log_{10}/ml for the first 14 d postchallenge (Figure 1). Mean colony-forming units (log_{10}) per milliliter increased as calving approached and highest numbers of bacteria (2.90 cfu log_{10}/ml) were at 3 d postcalving. Mean colony-forming units (log_{10})/per milliliter in *K. oxytoca* experimental IMI was less than .15 at all times except 7 d postchallenge (data not shown). At 7 d postchallenge, mean number of bacteria was approximately 3.00 cfu log_{10}/ml and due primarily to a large increase in bacterial numbers in one quarter. *Escherichia coli* single isolations were all less than 1.0 cfu log_{10}/ml.

Experimental IMI on the basis of highly or poorly adapted strains is summarized in Table 3. Although number of infections was low, highly adapted strains accounted for 75% of *K. pneumoniae* dry period IMI and 67% of *K. oxytoca* IMI. For *K. pneumoniae* IMI that persisted to calving, approximately 67% were highly adapted strains.

### Table 2. Isolation of gram-negative bacteria following intramammary infusion on d 7 of the dry period.

<table>
<thead>
<tr>
<th>Days postchallenge</th>
<th>Total isolations&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Other genera&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Total isolations</th>
<th>Total isolations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>42</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>56</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Calving (C)</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C+3</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C+7</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C+14</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C+30</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>1</sup>Quarters from which challenge gram-negative bacteria were isolated.

<sup>2</sup>Number of challenged quarters from which a gram-negative bacterium was isolated that differed from the infused strain biochemically or on the basis of antibiograms.

<sup>3</sup>C + number = Number of days postcalving.
Figure 1. Numbers of *Klebsiella pneumoniae* in mammary secretion following bacterial challenge. Values are mean ± standard error of colony-forming units (log10) per milliliter from quarters with confirmed infections. Number of quarters from which isolations were made varied from 1 on d 42 postchallenge and 30 d postcalving to 6 on d 1 postchallenge.

Figure 2. Somatic cell counts in gram-negative, bacteria-challenged mammary quarters. Challenged quarters in which intramammary infections (IMI) were established are shown relative to challenged quarters in which no IMI could be diagnosed (uninfected). Asterisk indicates differences (P<.05) in SCC (log10) per milliliter between the two values at a given time point.

In vitro growth of challenge strains in secretion obtained immediately prior to challenge at 7 d of involution demonstrated differences (P<.05) among *K. pneumoniae*, *E. coli*, and *K. oxytoca* (Table 4). In vitro growth of *K. oxytoca* was 8.05 cfu log10/ml compared with 6.38 cfu log10/ml for *K. pneumoniae* and 4.49 cfu log10/ml for *E. coli*. There were no differences in in vitro growth between highly and poorly adapted strains within a species (data not shown).

A comparison of somatic cell counts in uninfected challenged quarters and in quarters in which an experimental IMI was established are shown in Figure 2. Somatic cell counts in all quarters at 7 d of the dry period prior to challenge were approximately 7.2 cells log10/ml. Somatic cell counts of dry period IMI that did not persist to calving were included in the gram-negative bacterial IMI group only for the duration of the IMI. Beyond 3 d postchallenge, SCC were elevated in infected quarters compared with counts in uninfected quarters at all times except 42 d and calving. Somatic cell counts were greater (P<.05) in infected than uninfected quarters at 21 d postchallenge and at

| TABLE 3. Intramammary infection status of *Klebsiella pneumoniae* and *Klebsiella oxytoca* challenged mammary quarters: comparison between highly and poorly adapted bacterial strains. |
|-------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|
|                                    | Highly adapted                      | Poorly adapted                      | Highly adapted                      | Poorly adapted                      |
|                                    | (n)² (%)                            | (n) (%)                             | (n) (%)                             | (n) (%)                             |
| Dry period IMI                     | 6 (75.0)                            | 2 (25.0)                            | 2 (66.7)                            | 1 (33.3)                            |
| Short duration                     | 4 (80.0)                            | 1 (20.0)                            | 2 (66.7)                            | 1 (33.3)                            |
| Persist to calving                 | 2 (66.7)                            | 1 (33.3)                            | 0 (0)                               | 0 (0)                               |

¹Strains were classified as highly adapted or poorly adapted based on growth in dry cow secretion obtained at 21 d of involution.
²Number of intramammary infections for highly or poorly adapted strains.
³Percentage of intramammary infections for infection type.

Journal of Dairy Science Vol. 73, No. 5, 1990
TABLE 4. In vitro growth of gram-negative bacteria in mammary secretion obtained immediately prior to intramammary challenge.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>(n)</th>
<th>$X^2$</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsiella pneumoniae</td>
<td>20</td>
<td>6.38a</td>
<td>.41</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>9</td>
<td>4.49b</td>
<td>.97</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>10</td>
<td>8.05c</td>
<td>.19</td>
</tr>
</tbody>
</table>

a,b,c Means with different superscripts differ (P<.05).

1 Each strain was grown in secretion from the mammary quarter challenged with that strain.
2 Values expressed as mean (cfu $\log_{10}$/ml) ± standard error of the mean.

Highly adapted strains accounted for 67% of K. pneumoniae IMI that persisted to calving. Although the actual number of infections established was small, ability to overcome the inhibitory components of dry cow secretion may have been a contributing factor to establishment of a K. oxytoca and K. pneumoniae IMI. Some poorly adapted strains were able to establish experimental IMI. Dry cow secretion was obtained from several cows, and variation in growth inhibition of secretion from individual animals as involution progresses may have been an important factor. The ability of the polymorphonuclear neutrophil (PMN) to infiltrate and phagocytize invading bacteria is considered the major factor in elimination of infection (4, 13). Differences in phagocytic and bactericidal properties of PMN among individual cows has been demonstrated (20) and may account for experimental IMI caused by gram-negative bacteria poorly adapted for growth in SDCS in the current study. Ability of E. coli to grow in SDCS was not related to establishment of an experimental IMI during the early dry period. However, there was a relationship between K. pneumoniae and K. oxytoca growth in SDCS and the establishment of IMI.

Bacterial numbers isolated from experimental K. pneumoniae and K. oxytoca infections were low (<1.5 cfu $\log_{10}$/ml secretion) during the first half of the dry period. Klebsiella pneumoniae numbers in secretion gradually increased as parturition approached, and highest bacterial numbers were observed during the 1st wk of lactation. Previous studies on growth of E. coli and Klebsiella spp. in cell-free mammary secretions obtained at various stages of the dry period demonstrated that mammary secretion became more inhibitory as involution progressed (2, 10, 19). Growth inhibition was markedly decreased in prepartum secretion and colostrum and lost in milk (2, 10, 19). The increase in numbers of K. pneumoniae could be attributed to loss of inhibitory properties of secretion in the prepartum and lactating gland that coincides with a decrease in lactoferrin concentration and an increase in citrate concentration.

Growth of gram-negative bacteria in secretion obtained from the challenged mammary quarter at 7 d of involution demonstrated no differences between highly and poorly adapted strains. The highly and poorly adapted classifi-
cation of bacteria was based on secretion obtained at 21 d of the dry period and appeared not to be related to growth of bacteria in secretion obtained at 7 d of the dry period. However, growth inhibition of K. pneumoniae was not as great in secretion obtained from the fully involuted gland. Ability to continue to grow in secretion as involution progresses may be a more important factor for establishment of experimental IMI and persistence of IMI to calving. In addition, SDCS was a pool obtained from 11 cows, whereas secretion at 7 d was from a single quarter. Antibody, lactoferrin, and citrate components may have been different. However, growth of all bacteria was inhibited in dry cow secretion obtained at 7 d of the dry period compared with control medium.

Elevated SCC have been shown to protect the lactating gland from experimental coliform infections (14). Somatic cell counts in mammary quarters at challenge and throughout the dry period were in excess of 7.00 log_{10}/ml secretion and similar to reports of total cells in nonlactating glands (5, 6, 8, 9). Bacteria were not isolated at any time postinfusion in 60% of challenged quarters. In addition, all K. oxytoca dry period IMI and 63% of K. pneumoniae experimental IMI were rapidly eliminated. Growth of challenge bacteria in SDCS was in the absence of phagocytic cells. The elimination of gram-negative bacteria by phagocytic cells may have been a significant factor in prevention of experimental IMI. Failure of phagocytic cells to eliminate K. pneumoniae IMI could be attributed to decreased phagocytic efficiency of milk PMN (11) and to a possible lack of opsonic activity of dry cow secretion and milk for different strains of K. pneumoniae. The capsule of K. pneumoniae has antiphagocytic properties (21) and may be an important factor in establishment of IMI. However, phagocytosis of bacteria was not an adequate explanation for the observation of the susceptibility of the prepartum gland to E. coli IMI. Experimental E. coli IMI were readily established in quarters with cell counts in excess of 6.0 log_{10}/ml (1, 7).

The ability to overcome the inhibitory properties of dry cow secretion was related to, but was not an absolute indicator of, the ability of K. pneumoniae and K. oxytoca to establish an IMI in the dry gland. No relationship was evident between growth of E. coli in dry cow secretion and pathogenicity of E. coli for the dry gland. However, current studies did confirm the resistance of the mammary gland during early involution to E. coli infection.

ACKNOWLEDGMENTS

Research was supported in part by USDA Special Grant Number 85-CRSR-2-2616. The authors thank Pamela Schoenberger, Sue Romig, Tina Bowman, Heidi Rennecker, and Lucinda Shock for technical assistance.

REFERENCES