Shedding Pattern of *Staphylococcus aureus* from Bovine Intramammary Infections

P. M. SEARS, B. S. SMITH, P. B. ENGLISH, P. S. HERER, and R. N. GONZALEZ
New York State College of Veterinary Medicine
Quality Milk Promotion Services
Cornell University
Ithaca, NY 14850

**ABSTRACT**

Twenty-one quarters of seven cows were experimentally infected with *Staphylococcus aureus* (ATCC 29740) to study the shedding pattern in quarter milk samples. Of 991 consecutive quarter milk samples collected from infected quarters during the trial, 745 were positive for *S. aureus* by bacteriological culture. The sensitivity of a single quarter milk sample to determine infection status of a quarter was 74.5% based on the mean of each gland’s recovery pattern. Sensitivity of bacterial culture increased to 94% and 98% by including a second and a third consecutive sample. Because *S. aureus* is shed in a cyclical manner from mammary glands, consecutive samples would be advisable for accurate diagnosis of infected quarters.

(Key words: mastitis, *Staphylococcus aureus*, sampling)

**INTRODUCTION**

The accurate determination of intramammary infection (IMI) status is essential in conducting mastitis research and herd control. Methods employed for the diagnosis of an IMI include single, duplicate, and consecutive sampling. Single and duplicate sampling involve the collection of milk samples at a single milking (2). Collection of two or more milk samples at separate milkings has been termed consecutive sampling (2).

Evaluation of the sensitivity of any sampling method should be based on a definitive diagnostic procedure. Previous research methods for identifying *Staphylococcus aureus* quarter infections have focused on percentage agreement between duplicate samples as the definitive diagnostic criteria (1, 2, 5, 7), rather than on the true shedding pattern of the organism. Whether percentage agreement is indicative of the true infection status of the quarter is questionable.

In the present study, the objectives were to observe the recovery pattern of bacteria and to determine the sensitivity of single versus consecutive sampling of bovine mammary quarters infused with *S. aureus* (ATCC 29740) and sampled consecutively. In addition, cows in a commercial herd were sampled to examine the natural infections’ shedding pattern of *S. aureus*.

**MATERIALS AND METHODS**

**Experimental Cows**

Seven Holstein-Friesian cows from the Teaching and Research Dairy Farm at Cornell University were used. Four cows were primiparous and in the first 3 mo of lactation, and three were multiparous cows in late lactation.

**Milk Samples**

Teats were cleaned and dried with individual paper towels before milking. Cows were milked using a Surge (Babson Bros., Chicago, IL) quarter milker. Fifty-milliliter quarter milk samples were collected at each milking for SCC from individual quarter milk buckets. Milk samples were delivered to the Dairy Herd Improvement Association laboratory (DHIA, Ithaca, NY) for Fossomatic SCC.

Teats were dipped in a .5% iodine teat dip after milking and dried with a single-service paper towel after 15 to 20 s exposure. Teat ends were thoroughly cleaned with gauze pledgets moistened with 70% alcohol. Individual 10-ml quarter milk samples were collected aseptically in sterile vials for bacteriological culture.

Received January 8, 1990. 
Accepted May 22, 1990.
Following sampling, all quarters were dipped in the iodine teat dip. Samples were immediately placed on ice and delivered to the laboratory (Quality Milk Promotion Services, Cornell University, Ithaca, NY).

A .1-ml aliquot of each quarter milk sample was spread onto a trypticase soy agar plate containing 5% sheep blood and .1% esculin (TBA) (BBL Microbiology Systems, Cockeysville, MD). Plates were incubated aerobically at 37°C for 48 h and examined for bacterial growth.

Experimental Challenge Procedure

Cows were sampled for culture and SCC for five consecutive milkings (2.5 d) to determine the bacterial status of the quarters. Following the fifth sampling, the teat apex of 21 quarters (left fore, right rear, right fore) of the seven cows was thoroughly recleaned with a 70% alcohol pledget. Each quarter received a 1-ml intramammary infusion of viable S. aureus. The challenge dose was delivered via a sterile 18-gauge catheter inserted into the teat sinus. A negative control quarter (left rear) was unchallenged in each cow.

The S. aureus infusions were prepared as follows. On the 1st d of preparation, 1 ml of frozen S. aureus in Trypticase Soy Broth (TSB) was thawed at room temperature, added to 6 ml of TSB, and incubated overnight at 37°C. On the 2nd d, .1 ml of the overnight culture was added to 9.9 ml TSB and incubated for 6 h at 37°C. The stock culture was titrated serially to 10^-7 dilution, and .2 ml of the 10^-5 through 10^-7 dilution was plated in triplicate on TBA and incubated overnight at 37°C. The stock culture was held under refrigeration at 8°C. On the 3rd d, the stock culture was diluted in sterile distilled water to approximate the chosen dose. Individual 3-ml plastic syringes were filled in the laboratory with a calculated dose. From each syringe, .2 ml was plated in triplicate on TBA. The challenge dose ranged from 20 to 2000 cfu/ml.

Experimental Design

To establish the shedding pattern of S. aureus, 56 consecutive milk samples were collected from each challenged quarter following inoculation. The consecutive sampling interval (daily a.m. and p.m. milking) was consistent throughout the trial. Bacteriological culture and SCC were performed on each quarter milk sample. All bacterial growth was identified and recorded. The challenge S. aureus was identified by a tube coagulase test and by a characteristic broad band of incomplete hemolysis following 24 h of incubation. Further incubation caused the band of hemolysis surrounding the colony to be completely lysed. The number of colony-forming units were counted and recorded. Culture plates with ≥3500 cfu/ml were considered uncountable, and all were designated at 3500 cfu/ml for the statistical analysis. The SCC from the 5th milking postinoculation to the 55th milking postinoculation were converted to linear scores (6, 8).

Of the 56 consecutive samples collected for each experimentally infected quarter, the period of infection was defined as the first positive culture sample (12 to 24 h postinfusion) to the final consecutive milking or to the last positive culture. To determine the sensitivity of identifying S. aureus, the number of positive cultures in each gland was divided by the total number of cultures for the gland obtained during the period of infection. The overall sensitivity of identifying S. aureus by the use of a single milk sample is equal to the mean sensitivity of these infected glands. Because consecutive samples are independent, the probability of obtaining at least one positive culture among multiple samples could be calculated by multiplying probabilities.

Naturally Occurring Intramammary Infections

Four Holstein-Friesian cows from a local dairy farm were consecutively quarter sampled for 16 d. In order to avoid interference with the routine dairy milking schedule, postmilking a.m. and premilking p.m. quarter samples were collected and cultured by the same procedure as outlined for the experimental challenge animals except that .05-ml inocula of milk was plated. A .05-ml aliquot of milk was plated to meet clinical culture protocol under simultaneous evaluation.

RESULTS

Experimental Challenge

Prior to S. aureus challenge, all glands of the seven experimental cows were negative to
culture for five consecutive milkings. The mean linear score (LS) of bacteria shed of these quarters was 3.12 ± 1.0. Nineteen of the 21 glands (90%) challenged with S. aureus became infected. Staphylococcus aureus was isolated and SCC remained elevated for one or more samples throughout the 56 consecutive milkings. No other individual pathogens were isolated with the exception of environmental contaminants in 98 out of 1101 (9%) cultures. Milk samples from two glands were never positive to culture. Of the 19 infected glands, 2 cleared spontaneously 14 to 16 d after challenge. For 16 glands, one or more samples were negative to culture. The 7 control glands remained negative throughout the sampling period.

Two different shedding cycles of S. aureus were identified for this isolate. A low shedding cycle was defined as shedding a mean of ≤1000 cfu/ml. Sixteen of 19 infected glands exhibited a low shedding cycle as exemplified by an individual gland in Figure 1. Half of these glands were from primiparous cows and half were from multiparous cows. The three remaining glands were glands of two primiparous cows and shed a mean of ≥2000 cfu/ml, representing a high shedding cycle (Figure 2).

The mean LS of the high and low cycle glands were not significantly different. Figure 3 shows LS from a typical low and a typical high shedding gland. The high cycle glands had a mean LS of 6.4 ± .54 and the low cycle glands had a mean LS of 6.5 ± .61.

Comparison of the multiparous cows with the primiparous cows showed that there was a significant difference in the mean LS (P<.01). The primiparous cows mean LS was 6.1 ± .4, whereas the multiparous mean LS was 6.8 ± .7.

By following the bacteriological profile of the S. aureus infections over time, the sensitivity of a single sample at one point was determined. A total of 991 consecutive samples from all 19 infected glands were cultured and 745 were positive (≥1 cfu/1 ml inoculum). The sensitivity of the testing associated with each gland ranged from 41 to 100%. The 16 low cycle glands had an average sensitivity of 70% ± 13.5%, whereas the three high cycle glands had sensitivities of 100%.

The overall sensitivity of a single sample at any one point during the infections was 74.5% ± 16.75. The probability of a false negative to culture can be determined as presented in Table 1. Although a single sampling method only yielded a probability of 74.5%, two and three consecutive samples gave 94 and 98% probabilities, respectively, of at least one true positive culture when plating using .1 ml of inoculum (Table 2).
TABLE 1. Probability of obtaining a negative culture in consecutive milk samples when a *Staphylococcus aureus* (ATCC 29740)-infected gland is sampled at intervals of 8 h.

<table>
<thead>
<tr>
<th>Number of samples</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>.255</td>
</tr>
<tr>
<td>2</td>
<td>.255 x .255</td>
</tr>
<tr>
<td>3</td>
<td>.255 x .255 x .255</td>
</tr>
</tbody>
</table>

\(^1\)Probability of a false negative.

TABLE 2. Probability of obtaining a positive culture in consecutive milk samples when a *Staphylococcus aureus* (ATCC 29740)-infected gland is sampled at intervals of 8 h.

<table>
<thead>
<tr>
<th>Sample outcome</th>
<th>Number of samples collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>At least one positive</td>
<td>1 .745 (^1) 2 .935 3 .983</td>
</tr>
<tr>
<td>At least two positive</td>
<td>1 .555 2 .833</td>
</tr>
<tr>
<td>At least three positive</td>
<td>1 .414</td>
</tr>
</tbody>
</table>

\(^1\)Probability of a true positive.

Naturally Occurring Intramammary Infections

One quarter of each of four cows with a naturally occurring *S. aureus* infection was studied. Thirty consecutive milk samples were collected from each quarter, totaling 120 milk samples. In one quarter, the first milk sample was bacteriologically negative. This point was excluded since no previous positive milk sample was available to verify the infection status of the gland. Only one other point was excluded due to a missing culture. *Staphylococcus aureus* was isolated from 105 of 118 milk samples (>1 cfu/0.05 ml inoculum). The sensitivity of the assay among glands ranged from 63 to 100%. The mean sensitivity of a single sample to identify *S. aureus* at any point during an infection was 89% ± 17.5%.

Two glands exhibited the high shedding cycle, while two exhibited the low shedding cycle of *S. aureus* as defined in the experimental trial. The two glands in the high shedding cycle had associated sensitivities of 100%, whereas the two glands with the low shedding cycles had an associated mean sensitivity of 78%.

DISCUSSION

Recent recommendations of the National Mastitis Council (Smith et al., 1988) on sampling procedures for evaluation of drugs used in dairy cattle have endorsed the use of duplicate sampling before treatment and during the experimental period. Duplicate sampling is also recommended during trial exit, drying off, calving, and trial reentry.

Previous research has also advocated the use of duplicate or consecutive sampling. Jasper et al. (2) analyzed over 3000 quarter samples taken in duplicate at the same milking and found 96.2% overall agreement between the pairs. They concluded that single samples were sufficient in most situations. Postle (5), after analyzing over 11,000 duplicate or single samples, recommended that duplicate sampling be used in efficacy studies. Stolper et al. (7) calculated the duplicate quarter percent agreement for *S. aureus* for the above two studies among positive samples only. They reported 92.8 and 81.5% agreement for Jasper and Postle, respectively, and 82.5% agreement for their own study. Erskine and Eberhart (1), after finding 94.2% agreement between duplicate samples for *S. aureus*, also concluded that single samples were adequate to identify quarters infected with *S. aureus*.

In the present study, single sampling may not be an accurate method for determining the infection status of a *S. aureus* quarter infected experimentally. Although the studies cited have examined the percentage of agreement and disagreement between samples, they have failed to address the issue of the sensitivity of the sampling method (the probability of a true positive). Glands exhibiting a low shedding cycle have higher risk of a false negative when single sampling is used as the sole means of evaluating infection status of a quarter. Previous studies have shown a large proportion of duplicate samples identified as negative to culture (1, 2, 5). However, the results obtained in this study suggest the negative to culture category might have included an undetermined number of false negatives. The risk of obtaining a false negative result for *S. aureus* using a single 0.1-ml sample is estimated to be approximately 25% for experimental challenge infections. If the standard field inocula of 0.01 ml had been
used, the percentage of false negatives would have increased to 40%. This is based on the probability that every time S. aureus shed 1 to 9 cfu/0.1 ml, 68% of these samples would not have cultured positive.

The sensitivity of consecutive sampling has been evaluated in other research. Neave (3) cultured 206 S. aureus infected quarters using 0.05-ml inoculum. The quarters were consecutively sampled for 29 wk and had a 6% false negative rate. He stated that consecutive sampling of S. aureus infected glands was necessary to detect sufficient numbers of the organism. The difference between the experimental findings in this study for the rate of false negatives and those of Neave may be explained by the difference in strains of S. aureus, different number of glands, and different lengths of consecutive sampling intervals. The 11.1% false negative rate obtained with the small number of naturally infected quarters compares more favorably with the rate reported by Neave (3). Neave's findings give support to this study's contention that, due to the cyclical nature of the shedding pattern of S. aureus, a consecutive sampling method appears to improve the probability of an accurate diagnosis.

Following S. aureus consecutively has shown that a negative culture can occur repeatedly throughout the cycle. Disruptions in this cycle could be encountered when therapeutic agents are administered. Newbould (4) found that it took up to 28 d for a S. aureus infection to reappear after treatment. A single, duplicate, or consecutive sample taken at any point before the reappearance of the pathogen could be mistaken as a cure. This consideration should be taken into account when evaluating the efficacy of antimicrobial drugs for the treatment of S. aureus mastitis.

In conclusion, without the knowledge of the shedding pattern of the organism, the accuracy of a single or duplicate sample is doubtful in the diagnosis of S. aureus IMI.

ACKNOWLEDGMENTS

The authors wish to acknowledge the assistance of the Cornell Research Barn staff for the care of the animals used in the trial. Field and technical assistance of A. Cornetta and the Quality Milk Promotion Services staff was greatly appreciated. This trial was funded by American Cyanamid Co., Princeton, NJ.

REFERENCES