# Recovery of *Staphylococcus aureus* from Centrifuged Quarter Milk Samples

**A. ZECCONI,[1] R. PICCININI, A. ZEPPONI, and G. RUFFO**

Università degli Studi di Milano, Istituto Malattie Infettive Profilassi e Polizia Veterinaria, Consiglio Nazionale delle Ricerche, Centro Studio Patologia della Mammella, Via Celoria 10, 20133 Milano, Italy

## ABSTRACT

The identification of cows that are positive for mastitis caused by *Staphylococcus aureus* is difficult under field conditions. The frequency of isolation of *S. aureus* from quarter milk samples was compared with the frequency of recovery of *S. aureus* from sediment after centrifugation of those same samples. Overall, 776 quarter milk samples from 194 cows were studied. Cultures that were positive for *S. aureus* were obtained from 82 samples; 153 sediments from quarter milk samples were also positive for *S. aureus*. The results of this investigation showed that cultures of the sediment of quarter milk samples increased the number of positive outcomes up to 145.5%, depending on the herd. Using a different group of samples, including samples taken 1 to 5 d or 7 to 10 d after calving and samples taken after intramammary therapy, a 94% increase in cultures that were positive for *S. aureus* after centrifugation was found compared with cultures of the same quarter milk samples that were not centrifuged. Sedimented cultures may be useful in *S. aureus* control programs that require the segregation, selective treatment, or culling of cows that are positive for *S. aureus*.

(**Key words**: *Staphylococcus aureus*, mastitis diagnosis, quarter milk samples, centrifugation)

**Abbreviation key**: **QMS** = quarter milk sample, **SQMS** = sediment of QMS.

## INTRODUCTION

The diagnosis of IMI is mainly based on the bacteriological culture of milk. For clinical and research studies, duplicate or consecutive samples are used to enhance the probability of an accurate diagnosis (6). However, in field investigations and in programs that monitor udder health, this approach might be impractical for both technical and economic reasons (10).

When the goal of the field investigation is to diagnose mastitis caused by *S. aureus*, the problem is even more complicated; cows that are confirmed to be positive for *S. aureus* could have negative culture results for one or more consecutive samples. Different methods, including postmilking samples (10), freezing and thawing (12), and teat swabs (14), have been proposed to overcome this problem and to improve diagnosis, but none of them has gained widespread use.

The objective of this study was to compare the frequency of isolation of *S. aureus* from the direct culture of quarter milk samples (**QMS**) with the recovery of this microorganism after centrifugation and sedimentation of QMS (**SQMS**). Centrifugation is used in human medicine to enhance the sensitivity of bacteriological methods for fluids that are normally sterile, such as cerebrospinal fluids (4).

## MATERIALS AND METHODS

### Herd Sampling

The study involved 194 cows from six commercial dairy herds for which the prevalence of IMI caused by *S. aureus* was 4 to 26%. These herds were already included in the mastitis control program managed by the Istituto Malattie Infettive Profilassi e Polizia Veterinaria (Milano, Italy). Samples were collected from January to November 1995. From all of the samples that were collected during this period, a subgroup of samples was randomly chosen for each herd. The number of samples included in the subgroup was proportional to the known herd prevalence of IMI caused by *S. aureus* (Table 1). In herd 5, which had the highest prevalence of IMI caused by *S. aureus*, all samples that were collected were included in the trial.

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[1]Corresponding author.

TABLE 1. Prevalence of IMI caused by *Staphylococcus aureus* and distribution of samples in the six herds considered.

| | Herd | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| Lactating cows, no. | 120 | 150 | 80 | 180 | 85 | 140 |
| Prevalence of *S. aureus*, % | 12 | 5 | 18 | 4 | 26 | 9 |
| Cows sampled, no. | 19 | 16 | 41 | 9 | 86 | 23 |
| Quarter milk samples, no. | 76 | 64 | 164 | 36 | 344 | 92 |
| Relative frequency of samples, % | 9.8 | 8.2 | 21.1 | 4.6 | 44.3 | 11.9 |

## Milk Samples

Milk samples were collected prior to milking using aseptic procedures recommended by the National Mastitis Council (6). Teat ends were sanitized using gauze pledgets moistened with 70% alcohol. From each quarter, approximately 10 ml of milk were collected into sterile plastic vials. Samples were placed on ice and delivered immediately to the mastitis diagnostic laboratory. Samples from all lactating cows were obtained at different stages of lactation and were divided in two subsets of samples. The first subset (general sampling) included samples taken at 7 to 10 d after calving and during routine sampling (once every 3 mo); samples taken at 1 to 5 d after calving, 7 to 14 d after intramammary therapy, and repeated samples were excluded. The second subset (calving and posttherapy samplings) included samples taken at 1 to 5 and 7 to 10 d after calving and after intramammary therapy; only samples taken 7 to 10 d after calving were in common with the first subset of samples.

## Follow-up

To evaluate whether the isolation of *S. aureus* from SQMS was a random result or a true IMI, 8 cows were randomly selected from herd 5 and were sampled five times with 7-d intervals between samplings (Table 2).

## Sample Analysis

At the laboratory, a 0.01-ml aliquot of each QMS was thoroughly mixed, spread on a 5% blood agar plate (Merck-Bracco, Milan, Italy), and incubated at 37°C for 24 h. Afterward, the milk sample was centrifuged at 2000 × *g* for 15 min, the supernatant was

TABLE 2. Results of cultures of three consecutive quarter milk samples (QMS) and sediments from QMS (SQMS) of 8 cows in herd 5. Cultures were performed weekly for 5 wk.

| Cow no. | Type of culture | Number of quarters infected | | | | | Infection status |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| 1 | QMS | 0 | 0 | 1 | 0 | 0 | UC IMI[1] |
| | SQMS | 0 | 0 | 1 | 0 | 0 | UC IMI |
| 2 | QMS | 0 | 0 | 0 | 0 | 0 | Negative[2] |
| | SQMS | 0 | 0 | 0 | 0 | 0 | Negative |
| 3 | QMS | 0 | 0 | 2 | 0 | 0 | UC IMI |
| | SQMS | 0 | 0 | 2 | 0 | 0 | UC IMI |
| 4 | QMS | 0 | 0 | 0 | 0 | 0 | Negative |
| | SQMS | 1 | 0 | 3 | 1 | 0 | IMI[3] |
| 5 | QMS | 0 | 0 | 1 | 0 | 0 | UC IMI |
| | SQMS | 1 | 0 | 2 | 0 | 0 | IMI |
| 6 | QMS | 1 | 4 | 3 | 0 | 0 | IMI |
| | SQMS | 3 | 4 | 3 | 0 | 3 | IMI |
| 7 | QMS | 1 | 0 | 0 | 0 | 0 | UC IMI |
| | SQMS | 1 | 1 | 0 | 0 | 0 | IMI |
| 8 | QMS | 0 | 1 | 1 | 1 | 1 | IMI |
| | SQMS | 0 | 1 | 2 | 1 | 1 | IMI |

[1]UC = Unconfirmed: one of three consecutive samples was positive.

[2]All three consecutive samples were negative.

[3]Two of three consecutive samples were positive.

discarded, and the bottom layer was resuspended in 0.05 ml of sterile saline. The entire amount was spread on a 5% blood agar plate and incubated at 37°C for 24 h.

Isolated colonies of *S. aureus* were identified by a positive tube test for free coagulase, a positive agglutination test (Slidex Staph-kit™; BioMerieux, Florence, Italy), and by the API-Staph ID 32™ system (BioMerieux).

### Definitions

The recovery of one or more colonies of *S. aureus* from a QMS or from SQMS was defined as a positive result. Therefore, the lower detection limit for cultures from QMS was 100 cfu/ml, and, for a culture from the SQMS, the lower detection limit was 1 cfu/10 ml. In the follow-up study, a cow was defined as persistently positive when, in two out of three samples, at least one quarter was positive for *S. aureus*.

### Statistical Analysis

The frequencies of cultures that were positive for *S. aureus* from QMS and from SQMS were compared using McNemar's test on each pair of QMS and SQMS (3). The calculations were performed using SigmaStat software (11).

## RESULTS

### Results for the First Subset (General Sampling)

Overall, 776 QMS from 194 cows were studied. A culture that was positive for *S. aureus* was obtained from 82 QMS; 71 sediments from 694 QMS that were negative for the microorganism were found to be positive for *S. aureus* (Table 3), and the two frequencies were different (McNemar's test, $P < 0.001$).

The number of cultures that were positive for *S. aureus* in the SQMS was higher than the number of cultures that were positive for *S. aureus* in the QMS in herds 4 ($P < 0.013$), 5 ($P < 0.001$), and 6 ($P < 0.001$). An increase was observed also in herds 1, 2, and 3; however, the differences between SQMS and QMS were not significant.

### Results for the Second Subset (Calving and Therapy Sampling)

Overall, 508 QMS from 87 cows were studied. The distribution of cultures that were positive for *S. aureus* was related to time of sampling. Significantly higher frequencies of cultures from SQMS that were positive for the microorganism were observed in samples that were taken after calving. For example, an increase of 57.1% was observed for samples taken at 1 to 5 d after calving, and an increase of 103.8% was observed for samples taken at 7 to 10 d after calving. A significant increase (120.0%) in cultures from SQMS that were positive for *S. aureus* was also observed after antibiotic treatment (Table 4).

### Identification of Infected Cows

Positive cultures were more frequently observed in SQMS, but this increased frequency did not directly mean that more cows were identified as being positive for *S. aureus*. If the sediment method increased only the frequency of positive quarters in cows that were known to be infected, the overall number of cows that were positive for *S. aureus* would remain the same.

Data were then analyzed by cow instead of quarter. Cows were identified as being positive for *S. aureus*

TABLE 3. Distribution of cultures that were positive for *Staphylococcus aureus* from quarter milk samples (QMS) and sediments from QMS (SQMS) in the six herds considered.

| | Herd | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | Total |
| QMS, no. | 7 | 20 | 9 | 0 | 35 | 11 | 82 |
| Positive samples, % | 9.2 | 31.2 | 5.9 | 0 | 10.2 | 12.0 | 10.5 |
| SQMS, no. | 11 | 21 | 11 | 8 | 75 | 27 | 153 |
| Positive samples, % | 14.5 | 32.8 | 6.7 | 22.2 | 21.8 | 29.3 | 19.7 |
| SQMS – QMS | 4 | 1 | 2 | 8 | 40 | 16 | 71 |
| Increase,[1] % | 57.1 | 5.0 | 22.2 | NE[2] | 114.3 | 145.5 | 86.6 |
| $P<$[3] | NS[4] | NS | NS | 0.13 | 0.001 | 0.001 | 0.001 |

[1]Increase = (SQMS – QMS)/QMS.

[2]Not able to be evaluated.

[3]McNemar's test.

[4]$P < 0.05$.

TABLE 4. Distribution of cultures that were positive for *Staphylococcus aureus* from quarter milk samples (QMS) and sediments from QMS (SQMS) in relation to time of sampling.

| | Time of sampling | | |
|---|---|---|---|
| | 1 to 5 d After calving | 7 to 10 d After calving | 7 to 14 d After therapy |
| Total samples, no. | 160 | 276 | 72 |
| QMS, no. | 14 | 26 | 10 |
| Positive samples, % | 8.8 | 9.4 | 13.9 |
| SQMS, no. | 22 | 53 | 22 |
| Positive samples, % | 13.8 | 19.2 | 30.6 |
| SQMS – QMS | 8 | 27 | 12 |
| Increase,[1] % | 57.1 | 103.8 | 120.0 |
| $P<$[2] | 0.013 | 0.001 | 0.001 |

[1]Increase = (SQMS – QMS)/QMS.

[2]McNemar's test.

when at least one quarter was positive for the microorganism. This analysis confirmed that the sediment technique identified greater numbers of cows that were positive for the microorganism (Table 5). Data showed a significant increase in the number of cows that were positive for *S. aureus* in herds 3 (120.0%) and 5 (130.0%); in all of the other herds, a nonsignificant increase was observed. Overall, 80.5% more cows were identified from cultures of SQMS.

### Follow-up

Overall, the results of the follow-up study confirmed results of the general study; there was an 82.3% increase in positive cultures from SQMS versus the number of positive cultures from QMS. Particularly, the follow-up study showed that only 2 cows were persistently positive for *S. aureus* in both cultures from QMS and SQMS. Four cows produced only 1 positive culture from the QMS out of the five samplings, 2 of them had identical results for the SQMS assays, and the other 2 cows had confirmed *S. aureus* cultures from SQMS. Finally, 2 cows had negative cultures from QMS for five consecutive samplings. One of those cows also had negative results from the culture of SQMS, but the remaining cow had 3 positive cultures from SQMS of five total cultures (Table 2). By defining the infection status of cows based on the presence of positive results for 2 of 3 samples (5), overall cultures from QMS identified only 2 infected cows, but cultures from SQMS identified 5 infected cows.

## DISCUSSION

The diagnosis of IMI caused by *S. aureus* is essential to mastitis control programs because a cow that is positive for the bacteria can be treated, segregated, or culled. The failure to identify these cows leads to a source of infection in the herd. The perpetual discussion of the necessity of single or consecutive samplings to identify infected quarters has not generated definitive answers, and arguments for both single or consecutive samplings have been made (5, 8). For practical reasons, single sampling is commonly used during herd monitoring programs (1, 10), and consecutive samplings are used in clinical trials. For single sampling, even adoption of 100 cfu/ml as the lower threshold limit to define a positive culture in a QMS leads to a 25% probability of a false-negative result (12). This frequency could be higher for IMI caused by *S. aureus* because of the shedding pattern of the microorganism (9). To decrease the number of

TABLE 5. Distribution of cows that were positive for *Staphylococcus aureus* as defined by results from cultures of quarter milk samples (QMS) and sediments from QMS (SQMS) in the six herds considered.

| | Herd | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | Total |
| Positive cows from QMS, no. | 5 | 8 | 5 | 0 | 10 | 8 | 36 |
| % of All positive cows | 26.3 | 50.0 | 12.2 | 0 | 11.6 | 34.8 | 18.5 |
| Positive cows from SQMS, no. | 7 | 9 | 11 | 4 | 23 | 11 | 65 |
| % of All positive cows | 36.8 | 56.2 | 26.8 | 44.4 | 26.7 | 47.8 | 33.5 |
| SQMS – QMS | 2 | 1 | 6 | 4 | 13 | 3 | 29 |
| Increase,[1] % | 40.0 | 12.5 | 120.0 | NE[2] | 130.0 | 37.5 | 80.5 |
| $P<$[3] | NS[4] | NS | 0.041 | NS | 0.001 | NS | 0.001 |

[1]Increase = (SQMS – QMS)/QMS.

[2]Not able to be evaluated.

[3]McNemar's test.

[4]$P < 0.05$.

false-negative results, different methods have been proposed (i.e., freezing and thawing); however, the results are questionable. In human medicine, centrifugation of samples is used to increase the sensitivity of microbiological assays, and this process might be an alternative method (3). When this technique was applied to milk samples, it was shown to be practical and reliable; the only caution is that samples have to be collected properly to avoid contamination. The results of this study showed that the inoculation of plates with SQMS increased the detection of quarters that were positive for *S. aureus* by 86.6% (range, 5 to 145.5%) compared with the results obtained from QMS.

When data were analyzed for each herd, the frequency of positive cultures from SQMS was different. The difference between cultures from QMS and SQMS was significant for three herds (herds 4, 5, and 6), but, in the other three herds (herds 1, 2, and 3), differences were not significant. When the results were analyzed by time of sampling (1 to 5 or 7 to 10 d after calving and after intramammary therapy), some differences were observed. The large increase in positive cultures from SQMS that was observed for samples taken at 7 to 10 d after calving has a practical value and shows the possibility of earlier identification of quarters that are infected with *S. aureus*. The significant increase in positive cultures from SQMS after intramammary therapy could explain the observed increase in therapy failures when cows were sampled at 30 to 60 d (2) and suggests that efficacy of therapy should be carefully defined.

The differences between cultures from QMS and SQMS observed among herds suggested that different *S. aureus* strains were responsible for infections. Therefore, differences could be explained by the irregular shedding pattern of *S. aureus* and particularly by the existence of different shedding cycles, depending on the presence of different strains (9). The shedding pattern in herds 1, 2, and 3 could be defined as a high shedding cycle, which means that the possibility of detecting *S. aureus* from cultures from QMS was high. In herds 4, 5, and 6, the shedding pattern could be defined as a low shedding cycle (9); therefore, a more sensitive diagnostic method is needed. In these latter herds, the concentration of *S. aureus* in QMS was frequently below the detection limit for the bacteriological method (100 cfu/ml); therefore, the bacteria could be detected more effectively with a method such as the culturing of SQMS, which increases the sensitivity up to 1 cfu/10 ml. These results were indirectly confirmed by the numbers of colonies observed on SQMS plates, which were never higher than five.

Finally, the analysis of data regarding the consecutive culturing of QMS and SQMS performed on 8 cows confirmed that results obtained from QMS were positive less frequently; 2 cows had confirmed IMI that were caused by *S. aureus*, and 4 cows had unconfirmed IMI that were caused by *S. aureus*. Results from SQMS were more consistent: 5 out of 8 cows were confirmed positive. These results, if confirmed by a larger study, could have particular value in *S. aureus* control programs because the segregation of positive cows is beneficial (13).

## CONCLUSIONS

The peculiar shedding pattern of *S. aureus* and interaction with the immune system of the mammary gland increases the difficulties of properly identifying cows that are infected, particularly when bacteria have a low shedding pattern. In this latter case, concentrations of bacteria could be below the detection limit of the bacteriological method or the bacteria could be internalized in leukocytes and, therefore, unable to grow in culture. The use of simple centrifugation was able to overcome the inconsistency of results observed for cultures of quarters infected with *S. aureus* that had a low cycle shedding pattern.

These results suggest that sampling cows 7 to 10 d after calving and using the proposed centrifugation technique could increase the sensitivity of the bacteriological assay, identifying infected cows and heifers at the beginning of lactation, which has a clear advantage for mastitis management. This technique could be applied in herds that show inconsistent QMS results and have frequently recurrent cases of mastitis 30 d after antibiotic treatment.

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