Early-lactation extended pirlimycin therapy against naturally acquired *Staphylococcus aureus* intramammary infections in heifers: A randomized controlled trial

S. Skoulikas,* S. Dufour,†‡ D. Haine,†‡ J.-Y. Perreault,§ and J.-P. Roy^{1*‡}

*Département de Sciences Cliniques, and

†Département de Pathologie et Microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, QC, Canada J2S 2M2

‡Canadian Bovine Mastitis and Milk Quality Research Network, Saint-Hyacinthe, QC, Canada J2S 2M2

§Clinique Vétérinaire Centre-du-Québec, Notre-Dame-du-Bon-Conseil, QC, Canada J0C 1A0

ABSTRACT

The primary objective of the current study was to evaluate cure rate following an early-lactation extended intramammary pirlimycin treatment on heifers naturally infected by *Staphylococcus aureus*. The secondary objective was to assess Petrifilm Staph Express (3M Microbiology, St. Paul, MN) count plate characteristics when used in a protocol for early-lactation detection of infected quarters in heifers. Milk samples were collected from heifers ($n = 946$) in the first few days following calving (mean = 5 d). Heifers with laboratory-confirmed *S. aureus* intramammary infection ($n = 72$) were randomly allocated into 2 groups. The treatment group ($n = 54$ quarters from 38 heifers) received an intramammary infusion of 50 mg of pirlimycin once per day for 8 consecutive days in infected quarters. The control group ($n = 44$ quarters from 34 heifers) did not receive any treatment. Treatment success was defined as having negative culture results for *S. aureus* in all 3 post-treatment quarter milk samples collected on d 17, 24, and 31 post-treatment. Treatment group mammary quarters showed a statistically significant higher cure rate (64.8%) compared with the control group (34.1%). A total of 38% of quarters identified as *S. aureus*-positive using the Petrifilm Staph Express count plate were in fact identified as non-aureus staphylococci on routine laboratory-based bacteriological culture. The current study demonstrates that a higher cure rate for *S. aureus* IMI can be achieved in dairy heifers if an extended treatment protocol is put in place soon after calving. Use of Petrifilm Staph Express count plate for

identification of *S. aureus*-infected heifers could lead to unnecessary treatments because of false-positive results.

Key words: dairy heifer, intramammary infection, *Staphylococcus aureus*, extended therapy, Petrifilm

INTRODUCTION

Staphylococcus aureus is a contagious pathogen that causes persistent IMI; its prevalence in heifers around calving can range from 0 to 15% (Fox, 2009). *Staphylococcus aureus* has many virulence factors that contribute to its persistency, including immune cell evasion, hiding within phagocytic cells, and the capacity to resist oxidative bursts (Zecconi and Scali, 2013). In addition, *S. aureus* is part of the cow's natural skin flora, making this pathogen impossible to eradicate from herds. Different strains of *S. aureus* may have different shedding patterns, which makes it harder to identify and, thus, harder to control (Sears et al., 1990). Increasing the inoculum volume, using duplicate sampling, freezing milk samples, and milk incubation can all increase the probability of detecting *S. aureus* using standard bacterial culture (Zecconi, 2010).

On-farm culture methods can be used for detection of mastitis-causing pathogens. The 3M Staph Express count plate (STX; 3M, Maplewood, MN) is one of these methods. The main advantage of STX is the fact that the milk samples do not need to be sent to a diagnostic laboratory; thus, shipping delays are avoided and results can be obtained within 22 to 24 h (Silva et al., 2005). In addition, the standard inoculation volume is 1 mL as compared with 0.01 mL for a standard bacterial culture; this higher volume can increase the likelihood of identifying bacteria in the milk culture, especially when low numbers of *S. aureus* are present (Silva et al., 2005). Some authors have demonstrated, however, that this high volume may impede the proper reading of the STX because of the high number of colony-forming units present (Wallace et al., 2011). The best sensi-

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¹Corresponding author: jean-philippe.roy@umontreal.ca

tivity results for identifying *S. aureus* using STX in postpartum cows were obtained when diluting the milk in a 1:10 saline solution (92 vs. 83% in undiluted milk; Wallace et al., 2011). The disadvantage to the STX is the fact that reading and interpreting the results can be quite difficult if the reader is not properly trained or lacks experience in identifying *S. aureus* colonies. For example, the use of a weak pink zone on the STX (i.e., a weak DNase reaction) to identify *S. aureus* resulted in 22.4% false positives (Silva et al., 2005). The latter study showed that interpretation of the STX varies as a function of the readers' skill and that different readers sometimes disagree in their assessment of colony numbers, size, and the intensity of the pink zones around the colonies.

Many studies have been conducted in dairy cows and heifers to investigate use of various antibiotics for treating *S. aureus* IMI (Owens et al., 2001; Roy and Keefe, 2012). Results from short-term antibiotic treatments are often unsatisfactory (Owens et al., 2001; Barkema et al., 2006; Oliver et al., 2007), which is why extended treatment regimens have been proposed to obtain higher cure rates (Gillespie et al., 2002; Deluyker et al., 2005; Roy and Keefe, 2012). Research suggests that the highest cure rates for *S. aureus* IMI in dairy cows in North America were obtained with pirlimycin hydrochloride and with extended treatment protocols (Roy and Keefe, 2012). To our knowledge, no study to date has evaluated the cure rates obtained from an extended antibiotic treatment protocol on recently calved heifers with *S. aureus* IMI.

We hypothesized that high cure rates could be obtained in infected primiparous cows if they were treated promptly during the early lactation period. In addition, we hypothesized that using STX in early lactation with frozen milk samples could be a valuable tool for prompt identification of *S. aureus* IMI. Specific objectives were to (1) evaluate *S. aureus* quarter and cow cure rates of animals treated with extended intramammary pirlimycin; (2) assess some test characteristics of the STX when used on quarter milk samples to identify infected quarters in early-lactating heifers; and (3) evaluate composite samples to identify *S. aureus*-infected heifers using STX.

MATERIALS AND METHODS

The chosen study design was a randomized controlled trial (**RCT**). The REFLECT statement (O'Connor et al., 2010) was used as a guideline for planning the study and throughout the manuscript for reporting. The study protocol was accepted by the Animal Ethics Committee of the Université de Montréal (15-rech-1737).

Herd and Heifer Selection

Herds constituting the source population for our study were clients of the collaborative veterinary practice involved in the research project (Clinique vétérinaire Centre-du-Québec, Notre-Dame-du-Bon-Conseil, QC, Canada). A convenient sample of dairy herds was selected from this source population. Selection criteria were (1) participating in DHI program; (2) having a historical prevalence of *S. aureus* in heifers at calving $\geq 5\%$ in the previous 12 mo; and (3) willingness to participate in the study. All recently calved heifers from the chosen herds between April 2014 and December 2015 were evaluated for enrollment in the RCT. Quarter milk samples were collected within the first few days of calving (mean = 5, minimum = 0, maximum = 17 DIM). Heifers were enrolled in the RCT when at least one quarter was diagnosed positive for *S. aureus* on the STX.

Sample size was determined a priori using a Fisher test for comparing proportions (SAS Power procedure, version 9.4, SAS Institute Inc., Cary, NC). We determined that 36 heifers (18 in each group) were needed for detecting a difference in cure rate of 65 versus 15% with a power of 80%. Clustering of heifers by herds, however, was not considered for these power calculation; therefore, an initial plan for recruitment of 50 heifers was made. However, during preliminary data analysis conducted before terminating the RCT, a higher-than-expected proportion of spontaneous cure rates (30%) was observed in untreated heifers. The sample size was adjusted to be able to detect a smaller difference in cure rate (65 vs. 30%) with a power of 80%. The RCT was pursued with the objective of enrolling 76 heifers, with 38 heifers in each group.

Sampling and Petrifilm Bacteriological Procedures

The dairy producer or the veterinarian of the herd collected quarter milk samples aseptically according to National Mastitis Council (2017) procedures within the first few days after calving from all first-lactation heifers with 4 functional quarters. Following collection, milk samples were kept at 4°C during transportation to the veterinary practice on the same day and then frozen at -20°C until subsequent bacteriological analysis the following day. On rare occasions, milk was immersed in liquid nitrogen for 1 min if the analysis was to be conducted on the same day. Freezing was performed before culture to increase the chances to recover *S. aureus* (Villanueva et al., 1991; Godden et al., 2002; Wallace et al., 2011). In one study, the sensitivity for *S. aureus* detection using Petrifilm (3M Microbiology, St. Paul,

MN) was approximately 5 percentage points greater when using frozen samples (Wallace et al., 2011). The milk cultures were performed the same day or the day after reception of milk samples at the veterinary practice because inclusion in the study and treatment allocation in early lactation was based on these results.

All STX bacteriological analyses were conducted at the veterinary practice by 2 trained technicians and 1 veterinarian. Prior to analyses, the quarter milk samples were thawed and a composite milk sample was produced with 1 mL of milk from each quarter milk sample. All quarter milk samples were diluted 1:10 in saline solution (0.1 mL of milk was diluted in 0.9 mL of saline). The composite sample was not further diluted in saline unless there were too many colonies to make an appropriate diagnosis. A total of 1 mL of the diluted milk (for quarter samples) or of undiluted milk (for composite samples) was inoculated on the STX, covered by the film, and gently pressed down with the Petrifilm platter to spread the milk on the entire STX surface and clear out any air bubbles. The STX were placed in an egg incubator, the Hova-Bator 1602n thermal air incubator (GQF Manufacturing Company, Savannah, GA), at 37°C and incubated for 24 h. After 24 h, if colony growth was observed, a DNase disc was added and the STX was incubated again for 1 to 4 h. A trained technician or a veterinarian read each STX; if a colony was surrounded by a pink halo, which would indicate a positive reaction to the DNase test, a presumptive diagnosis of *S. aureus* IMI was made. The veterinarian or technician identified the presumed positive colonies on the STX by encircling up to 5 colonies believed to be *S. aureus* with a pen marker. The positive STX and the frozen milk samples were then sent to the bacteriology laboratory of the Faculté de médecine vétérinaire of the Université de Montréal to perform a standard bacteriological culture on the marked colonies and, if needed, on the milk sample to confirm the *S. aureus*-positive results (see details below). All negative Petrifilm were discarded at the veterinary clinic and were not sent out to be analyzed.

In addition, the veterinarian or technician counted the colonies on the STX individually. When the count was greater than 50 colonies, they were estimated (e.g., identified as >50 or >100 cfu). The pink zone diameters were measured using a standard ruler and only the zones surrounding colonies were taken into account. Pink zones covering the entire STX were not given a numerical value and were not considered in the analysis. At the beginning of the study, pink zones were not initially measured for the first 3 mo (until July 2014) because colony characteristics on STX were not the initial focus of this study. Throughout the course of

the study, the decision to measure these pink zones was taken to better understand factors that could lead to misidentification of *S. aureus* IMI using STX.

Standard Bacteriological Culture Procedures

The bacteriological culture of the STX and milk samples was performed according to the National Mastitis Council (2004) guidelines. The STX-identified colonies were inoculated on a Columbia agar with 5% sheep's blood. The plates were incubated at $35 \pm 2^\circ\text{C}$ with 5% CO_2 for 18 to 24 h. Presence of *S. aureus* was suspected if both α - and β -hemolysis was detected on the blood agar. If *S. aureus* was not detected from the chosen colony, another of the identified colonies from the STX was inoculated. If *S. aureus* was identified from either the first or the second colony, all other subsequent identifications (choosing another colony or inoculating from a corresponding milk sample) were ceased. If *S. aureus* was, once again, not detected, 10 μL of milk from the corresponding milk sample was inoculated on Columbia agar with 5% sheep's blood. The plates were incubated at $35 \pm 2^\circ\text{C}$ with 5% CO_2 for 18 to 24 h. For identification of the organism, regardless of whether it was a colony from the STX or the milk, a coagulase test was performed if only a β -hemolytic zone was detected around the colonies; a coagulase-positive result led to classification of the organism as a *S. aureus*. If the coagulase test was negative, the organism was classified as a CNS. If the colonies were nonhemolytic, a Gram stain, a catalase test, and a DNase test were performed to classify the organism as either streptococci or other staphylococci.

Treatment Groups

Heifers identified as infected by *S. aureus* based on STX results of the veterinary practice were randomly allocated in 2 groups using the randomization function in Microsoft Excel (Microsoft Corp., Redmond, WA). The producers were then informed by a veterinarian on whether a given heifer would be treated. The producer and the veterinarian were not blind to the allocation groups, as the negative control group heifers received no antibiotic treatment. The treatment group heifers received an intramammary infusion of 50 mg of pirlimycin hydrochloride (Pirsue, Zoetis, Florham Park, NJ) once a day for 8 consecutive days. The producer administered the treatment aseptically in all infected quarters (i.e., if a heifer had more than 1 quarter infected, all infected quarters were treated). Uninfected quarters were left untreated.

Follow-Up

Quarter milk samples were aseptically collected from all initially infected quarters from all heifers of both groups on d 17, 24 and 31 following STX result (d 1). Treatment was initiated for the treatment group heifers on d 1. Samples were sent frozen to the laboratory of the Faculté de médecine vétérinaire of the Université de Montréal for bacteriological culture. Milk samples were analyzed using standard laboratory procedures as previously described. After the end of the follow-up period, control heifers could be treated if desired by the producer. The laboratory technicians analyzing the quarter milk samples were blind to the allocation group.

Cure Definitions

Presence or absence of a *S. aureus* IMI was confirmed with follow-up milk samples analyzed using the standard laboratory bacteriological methods described above. Milk samples were considered contaminated if 3 or more phenotypically different bacterial species were observed on the plate. *Staphylococcus aureus*, however, were still identified and accounted for if suspected (based on phenotypical considerations) in contaminated samples. At the quarter level, treatment failure was defined as observing *S. aureus* in any of the 3 post-treatment samples. Quarters with all 3 post-treatment milk samples negative for *S. aureus* were considered cured. Quarters with ≥ 1 post-treatment contaminated sample or with a missing post-treatment sample were considered of unknown cure status and excluded from subsequent analyses. At the heifer-level, a heifer was defined as cured if all her initially infected quarters were cured. A treatment failure was defined if any of her initially infected quarters were positive for *S. aureus* at any of the post-treatment samplings. Heifers with ≥ 1 quarter with unknown status were excluded from subsequent heifer-level analyses. Furthermore, quarters or heifers were excluded from subsequent analyses if during the follow-up period (1) they developed clinical mastitis or another sickness that required the use of other antibiotics; (2) the heifer was culled; or (3) a quarter dried.

Statistical Analysis

Odds of Cure. Effect of treatment on cure probability was analyzed both at quarter (i.e., cure of the quarter) and heifer (i.e., cure of the heifer) levels using SAS 9.4 (SAS Institute Inc.). An α value of 0.05 was chosen for significance. Odds of bacteriological cure in treatment and control groups were compared

using a logistic regression model, using the GENMOD procedure of SAS, with cure status as the outcome and treatment group as the main predictor. Only quarters and heifers confirmed positive to *S. aureus* pretreatment using standard laboratory bacteriological procedures were used for cure rate analyses (i.e., quarters or cows identified as positive pretreatment using STX, but negative by laboratory procedures, were excluded). To account for clustering of observations by heifers and herds (in the quarter-level model) or simply by herd (in the heifer-level model), robust variance was used to compute standard errors (Dohoo et al., 2009).

Petrifilm Staph Express Count Plate Characteristics. Descriptive statistics were used to determine the number of positive STX that were not in agreement with the standard laboratory bacteriological results. In addition, descriptive statistics were used for the evaluation of the phenotypical characteristics (i.e., colony shape and DNase zone) of the different bacteria leading to STX misinterpretation. A Pearson's χ^2 test was used to determine any association between the colony's phenotypical characteristics on the STX and the actual bacterial species identified using standard laboratory bacteriological culture.

Use of Composite Versus Quarter Milk Samples. The composite milk samples were made by combining 1 mL of milk from each quarter into a separate tube. One milliliter of undiluted composite milk sample was inoculated on the STX as previously described and was sent to the bacteriological laboratory along with the other samples. Descriptive statistics were used to describe the proportion of quarter milk samples, which were positive for *S. aureus*, but had a concurrent composite sample for which *S. aureus* could not be retrieved on the STX.

RESULTS

A total of 946 heifers from 27 herds were evaluated for enrollment in the RCT. A total of 248 quarters from 164 heifers were diagnosed infected by *S. aureus* using STX and were initially enrolled in the study. However, of those, only 156 quarters from 110 heifers (60 in the treatment group; 50 in the control group) were confirmed as infected by standard culture at the laboratory and were available for further analyses. A total of 38 animals were excluded from the study because of various reasons, including ≥ 1 post-treatment contaminated milk samples ($n = 8$ in treatment group; $n = 6$ in control group), missing follow-up samples ($n = 4$ in treatment group; $n = 5$ in control group), received other antibiotics ($n = 7$ in treatment group; $n = 5$ in control group), dried off quarter ($n = 2$ in treatment group), and culling ($n = 1$ in control group). Therefore,

Table 1. Heifer (n = 72) and quarter (n = 98) cure rates in a randomized controlled trial evaluating effect of an 8-d intramammary pirlimycin treatment for treating IMI due to *Staphylococcus aureus* in early lactation

Item (no., unless noted)	Heifer			Quarter		
	Treated	Control	Total	Treated	Control	Total
Total infected	38	34	72	54	44	98
Cured	23	12	35	35	15	50
Noncured	15	22	37	19	29	48
Cure rate (%)	60.5	35.2		64.8	34.1	

a total of 72 heifers (38 treated and 34 controls) with 98 infected quarters (54 treated and 44 controls) from 20 different herds were included in the analysis.

Heifer-Level Cure Rate

Fifty-nine percent (59%; 23/38) of heifers treated with pirlimycin and 33% (12/34) of heifers in the control group were cured (Table 1). The odds of eliminating the infection in the treated group were 2.8 (95% CI = 1.1, 7.3) times higher than in the control group. We noted an unequal distribution of heifers among treatment groups in regards to the number of quarters infected. Two times more heifers had more than 1 quarter infected in the treatment group (n = 18 out of 38) as compared with the control group (n = 9 out of 34). Furthermore, unconditional association between the number of infected quarters (categorized as 1 or >1 infected quarters) was evaluated using the described logistic model and robust variance estimation. Having multiple infected quarters was associated with 8.0 (95% CI = 3.2, 19.5) times lower odds of cure at the heifer level. The number of infected quarters was added as a predictor to the logistic model to account for the confounding of the association between treatment and cure by the number of infected quarters. When adjusting for confounding by numbers of infected quarters, the odds ratio obtained was greater, as expected. The odds of cure for a heifer were 3.7 (95% CI = 1.3, 10.5) times higher in the treatment group than in the control group after controlling for the number of infected quarters.

Quarter-Level Cure Rate

Sixty-four percent (64.8%; 35/54) of quarters were cured in the treatment group compared with 34.1% (15/44) in the control group (Table 1). Odds of cure were 3.6 (95% CI = 1.5, 8.2) times greater in the treatment group compared with the control group. The effect of the number of colonies (categorized as <100 cfu/mL vs. ≥100 cfu/mL) observed on the STX during diagnosis on probability of cure was assessed using the Pearson's χ^2 test. When considering all 98 quarters

together, we observed no effect of number of colonies on probability of IMI elimination. In milk samples with <100 cfu/mL, 29/60 IMI were eliminated; when ≥100 cfu/mL were observed, 19/38 IMI were eliminated. Similar results were observed in treated quarters (19/30 when <100 cfu/mL vs. 16/24 when ≥100 cfu/mL) and in untreated quarters (12/30 when <100 cfu/mL vs. 3/14 when ≥100 cfu/mL).

Petrifilm Staph Express Count Plate Diagnostic Characteristics

Out of the 946 heifers sampled, 164 heifers were positive on the STX for *S. aureus*, which resulted in an apparent prevalence of 17%. Out of the positive STX, 110 were confirmed positive by standard bacteriological culture, which resulted in an apparent prevalence of 12%. The proportion of false-positive heifers (when compared with laboratory-based bacteriological culture) observed on the STX was 33% (54/164). At the quarter level, we found 248 positive for STX, and only 154 of these were confirmed with bacterial culture, which resulted in 38% false-positives (again, when compared with laboratory-based culture). Other staphylococci species were isolated from all the false-positive STX.

To evaluate whether there was an association between the shape of the colony (i.e., star-shaped vs. round-shaped) on the STX and bacteria species, information that could possibly be used to improve diagnostic characteristics, a Pearson's χ^2 test was performed using all milk samples harboring either *S. aureus* or other staphylococci (n = 399). We observed a significant association between actual bacterial species (as identified by the laboratory) and shape observed on the STX ($P < 0.01$). We found 7% of *S. aureus* isolates compared with 25% for other staphylococci that formed a star-shaped colony on the STX (Table 2).

The association between the bacterial species (as identified by the laboratory) and the size of the DNase zone on the STX (categorized as ≤2 or >2 mm) was also assessed using a Pearson's χ^2 test, and a significant association was observed ($P < 0.01$). We noted 67% of *S. aureus* isolates producing a zone of lysis greater

Table 2. Colony shapes and final identification by standard bacteriology of 399 isolates coming from milk samples of dairy heifers in early lactation and identified as *Staphylococcus aureus* on Petrifilm Staph Express (3M Microbiology, St. Paul, MN) count plate [no. (%)]

Colony shape on Petrifilm Staph Express count plate	<i>S. aureus</i>	Other staphylococci
Round	226 (92.6)	116 (74.8)
Star	18 (7.4)	39 (25.1)
Total	244	155

than 2 mm; this proportion was only 29% for other staphylococci. (Table 3).

Quarter Versus Composite Samples

The possibility of using only composite milk samples, rather than individual quarter milk samples, for detection of *S. aureus*-infected heifers was assessed. There were a total of 164 heifer composite samples of STX sent to the bacteriological laboratory in the current study. We found 9 composite samples that were negative on the STX but had at least 1 *S. aureus*-positive quarter. We noted 95% (155/164; 95% CI: 89.9, 97.1) agreement between composite samples and quarter-positive samples when using the STX (Table 4).

DISCUSSION

The apparent *S. aureus* prevalence obtained in the current study of 12% was in the upper range of 0 to 15% reported around calving in other studies across North America (Fox, 2009). We observed an absolute quarter cure rate difference of 30.7% between the treatment group and control group. The quarter cure rate observed in the current study (64.8%) was numerically higher, but similar to another study using a standard 2-d postpartum pirlimycin treatment in heifer quarters (57.1%; Oliver et al., 2007). When comparing to a study looking at extended 8-d pirlimycin treatment on lactating dairy cows (which observed cure rates of 36 to 88%), we observed a cure rate that lies within the same range in our study (Deluyker et al., 2005).

Antimicrobial resistance for microbial pathogens is a great concern for public health. The emergence of resistance has coincided with the decrease in new antimicrobial development from pharmaceutical companies, which means we have fewer tools available to combat microorganisms that have tremendous powers of adaptability in any environment (Spellberg et al., 2008). Future aims should be to minimize nonrational use of antibiotics in agriculture by using an appropriate antibiotic for a specific pathogen, prescribing a dose and a time interval that has the best efficacy against

Table 3. Size of DNase reaction zones and final identification by standard bacteriology of 210 isolates coming from milk samples of dairy heifers in early lactation and identified as *Staphylococcus aureus* on Petrifilm Staph Express (3M Microbiology, St. Paul, MN) count plate [n (%)]

DNase reaction zone (mm) on Petrifilm Staph Express count plate	<i>S. aureus</i>	Other staphylococci
≤2	38 (33.0)	67 (70.5)
>2	77 (67.0)	28 (29.4)
Total	115	95

a pathogen (i.e., limiting sub-lethal concentrations), and limiting their use when necessary. In the United States and Canada, around 80% of dairy herds treat all quarters of all cows during the dry period with an intramammary antibiotic regardless of the bacteriological status of the animal (Dufour et al., 2012; USDA, 2016). Perhaps this type of practice for managing mastitis is not the best use of antibacterial products. To date, a limited number of studies have investigated the links between the use of antimicrobials in dry cow therapy and bacterial resistance, thus not providing us with enough evidence to make a definitive conclusion on the matter. Nevertheless, in one study conducted on Canadian dairies, administration of penicillin-novobiocin combination, cloxacillin, penicillin-novobiocin combination, and cephalixin for dry cow therapy was associated with penicillin and ampicillin resistance in *S. aureus* and with ampicillin resistance in *Escherichia coli* isolates retrieved from milk (Saini et al., 2012, 2013). In that same study, administration of pirlimycin and of ceftiofur for clinical mastitis treatment was associated with pirlimycin resistance in *S. aureus* isolates and with ampicillin resistance in *E. coli* isolates, respectively. Moreover, a study conducted in Wisconsin demonstrated that prior exposure to antibiotics, more specifically pirlimycin for the treatment of IMI, increased the antibiotic's MIC of *S. aureus*, CNS, and streptococci (Pol and Ruegg, 2007). In our study, we implemented a treatment protocol on infected quarters only based on

Table 4. Comparison of the agreement between composite and individual quarter milk samples coming from dairy heifers in early lactation for the detection of *Staphylococcus aureus* using Petrifilm Staph Express (3M Microbiology, St. Paul, MN) count plate

Individual quarter sample	Composite sample		
	Positive	Negative	Total
Positive	155	9	164
Negative	0	782	782
Total	155	791	946

culture results. The dose used kept a sufficiently high concentration of pirlimycin in the mammary gland for a period of 8 d and was proven previously to be effective at eliminating *S. aureus* in infected quarters (as compared with a standard 2-d treatment or with other antibiotics). Applying this to young animals, early in lactation, can potentially decrease the prevalence of *S. aureus* within a herd and, in addition, decrease the overall use of antibiotics.

Researchers studying prepartum treatment of *S. aureus* IMI in dairy heifers obtained spontaneous quarter cure rates that ranged between 10.5 and 28% (Trinidad et al., 1990; Owens and Ray, 1996; Owens et al., 2001). In regards to postpartum treatment in dairy cows, one group observed 6% of spontaneous cure rate for *S. aureus* infected quarters for first-parity cows, having between 1 and 10 cfu/mL and >200 DIM (Deluyker et al., 2005). Deluyker et al. (2005) observed a spontaneous cure rate in first-parity cows >200 DIM of 3.4 and 1.7% for samples containing 11 to 100 and >100 cfu/mL, respectively. One potential hypothesis for the high spontaneous cure rate observed in our study is that heifers may be infected with strains that are less adapted to the host and are not as persistent as strains usually observed in adult cows, thus making self-elimination easier. One study looking at different *S. aureus* strains using multilocus sequence typing observed that the majority of IMI on a given herd are usually caused by a limited number of *S. aureus* strains that belong to a specific clonal complex (Smith et al., 2005). However, to identify a strain as being part of a specific clonal complex, we would have needed to type the strains identified in the milk culture, which was beyond the scope of the current study.

Moreover, evidence suggests that higher colony forming units per milliliter of milk plays a role in cure rate as well (Deluyker et al., 2005; Barkema et al., 2006). In the current study, no significant association between number of colony forming units and the outcome was observed. The colony count in our study, however, was performed using the STX samples included in the study ($n = 98$) instead of bacteriological culture.

In the present study, we evaluated early detection methods to identify *S. aureus*-infected heifers, and these methods included using STX and assessing if a composite sample could be used as screening test. The specificity of STX conducted in one study was 98.5%, and this was associated with distinct pink zone around the colony (Silva et al., 2005). If there was a weak pink zone, the rate of false-positives observed was 22.4%. Silva et al. (2005) stated that colonies surrounded by a distinct pink zone were 120 times more likely to be *S. aureus*. We found a relatively high number of false-positive quarters associated with the STX (38%). At

the beginning of the study, all STX that had a pink zone surrounding a colony were sent to the laboratory as positive for *S. aureus*. The distinction between strong or weak pink zones was not taken into consideration. During the course of the study, an adjustment was made to better identify *S. aureus*-positive STX. The colonies with pink zones that had a diameter of ≤ 2 mm were less likely to be classified as positive for *S. aureus*. In addition, colonies that were star-shaped were also less likely to be classified as *S. aureus*. After the adjustment was made, 27% (26/98) of STX sent to the laboratory were in fact not *S. aureus* based on bacteriological results. Therefore, identifying certain characteristics on the STX, such as colony shape and DNase reaction diameter, could possibly aid in the proper diagnosis of *S. aureus*-infected animals and decreases the chances of over diagnosing *S. aureus* in heifers. Analyzing the sensitivity, specificity, and positive and negative predictive values was not part of our original study design, as it was not the main objective of our study and because this was already reported in the literature (Silva et al., 2005; Wallace et al., 2011). The negative STX plates were discarded at the clinic and evaluation of Petrifilm diagnosis accuracy was not possible. However, we observed some challenges when using the STX plates in a field setting in our study, and perhaps re-evaluation of the Petrifilm characteristics in a clinical setting is still warranted.

Five percent of positive heifers would have been missed if only the composite milk sample was used as a screening test using the STX in our study. The question remains whether missing 5% of infected heifers is enough to warrant screening for all 4 quarters instead of simply using a composite sample. Many factors must be considered, especially because our results showed that low numbers of *S. aureus* in the milk was associated with high self-cure rates; likewise, *S. aureus* is a contagious pathogen and very difficult to treat once established in the mammary gland. Perhaps herds that have a high prevalence of *S. aureus* and have difficulties in eliminating the disease should consider testing all 4 quarters in order not to miss any infected animals. Herds in which the prevalence is relatively low could rely more on composite samples and colony forming units to determine whether the animal should be treated. If an animal has low numbers of *S. aureus* on the STX, the veterinarian could potentially monitor the cow's subsequent SCC and, if needed, collect additional samples before instigating a treatment. We did not evaluate whether it is economically beneficial to treat, neither did we assess the subsequent changes in SCC or milk production, as the producers treated some of the infected heifers in the control group after completion of the follow-up period.

We did not stratify the randomization by herds in our study. Occasionally, we had a small number of infected animals per herd; hence, in some herds, the majority or all of the animals fell into 1 treatment group. In addition, we had some herds with only 1 animal being used in the analyses due to the fact that the other heifers were excluded from the study. These issues could potentially bias our results, as pathogenic strains can differ from farm to farm. Some strains may be easier to treat in some herds, and if there were more treated animals in these herds it could potentially affect the cure rates observed. Furthermore, this complete separation of data precluded the use of generalized linear mixed models for estimating odds of cure in treated and untreated heifers. To evaluate statistical significance, we had to opt for a robust variance estimation instead, a very valid, but less efficient method (i.e., in terms of power) that does not require specific assumptions regarding the exact clustering of observations (Dohoo et al., 2009). It is important to understand that, although this methodology can be used to provide valid and accurate standard error estimates for assessing statistical significance using 95% confidence intervals or *P*-values, it does not, however, correct the odds ratio point estimate obtained.

CONCLUSIONS

An extended treatment protocol (8-d treatment) on quarters of dairy heifer infected with naturally occurring *S. aureus* in the postpartum period resulted in greater odds of cure compared with untreated quarters. We also found a high spontaneous cure rate in untreated quarters; further research in that area is required. Proper training for technicians or veterinarians is needed to interpret the STX and reduce the amount of false-positives observed. Composite milk samples may miss up to 5% of positive *S. aureus* cases, which is something to consider when dealing with *S. aureus* problematic herds.

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