Efficacy of a Lactoferrin-Penicillin Combination to Treat β -Lactam-Resistant *Staphylococcus aureus* Mastitis¹

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ABSTRACT

The efficacy of intramammary (IM) treatments containing penicillin G (PG) alone or a combination of PG and bovine lactoferrin (bLF) was evaluated using a model of experimentally induced chronic bovine mastitis caused by a clinical isolate of *Staphylococcus aureus* highly resistant to β -lactam antibiotics. First, we confirmed that this strain could cause mastitis and infection could not be cured with PG alone. In a second trial, chronic mastitis was induced in 19 late-lactating cows by injecting a low dose of *Staph. aureus* through the teat canal of all quarters. After 15 d, cows with stable infections in their 4 quarters had their mammary quarters randomly assigned, within cow, to 1 of 4 IM treatments as follows: 1) citrate buffer, 2) 100,000 IU of PG, (3) 1 g of bLF, or 4) 1 g of bLF + 100,000 IU of PG. Treatments were repeated twice a day for 5 d. A third trial was undertaken to investigate the effect of an extended therapy on chronic mastitis acquired in a previous lactation. One month before dry-off, 20 gravid cows regrouped by dates of calving were infected in their 4 quarters. Once infections were established, cows were dried off abruptly. After calving, aseptic milk samples were collected separately from all quarters for 4 wk to monitor infection. Mammary quarters from enrolled cows were then randomly assigned, within cow, to 1 of 2 treatments as follows: 1) 100,000 IU of PG or 2) 250 mg of bLF + 100,000 IU of PG. Treatments were administered IM twice a day for 7 d. For all trials, milk samples were taken to monitor bacterial concentration and somatic cell count. Bacteriological cure rate was determined using milk samples taken 3 and 4 wk after initiation of treatments. For the second trial, cure rate was null for control quarters, 11.1% for bLF, 9.1% for PG,

and 45.5% for the bLF + PG combination. For cows infected in their previous lactation, cure rate was higher for the bLF + PG combination (33.3%) compared with PG alone (12.5%). In conclusion, bLF added to PG is an effective combination (i.e., 3- to 5-times higher cure rate) for the treatment of stable *Staph. aureus* infections highly resistant to β -lactam antibiotics.

Key words: mammary gland, mastitis, bovine lactoferrin, β -lactam antibiotic

INTRODUCTION

Staphylococcus aureus and other staphylococcal species are major mastitis pathogens. During infection, *Staph. aureus* adheres to the glandular epithelium and causes erosion, which leads to local tissue invasion (Novick, 1990). Consequently, *Staph. aureus* mastitis is difficult to control by antibiotic treatment alone and requires aggressive culling of animals with refractory infection. In past decades, the widespread use of β -lactam antibiotics to treat bovine IMI has resulted in a striking increase of *Staph. aureus* strains resistant to these antibiotics (Watts and Salmon, 1997). The poor response of *Staph. aureus* mastitis to antibiotic therapy during lactation has been reported (Owens et al., 1997a; Wilson et al., 1999). In *Staph. aureus* strains, a common mechanism of resistance is the production of β -lactamases, which are enzymes that hydrolyze the β -lactam ring of penicillin and related antibiotics (Yao and Moellering, 1995). Indeed, Jones and Heath (1985) reported that 66.1% of *Staph. aureus* isolated from bovine IMI were β -lactamase-positive. Moreover, bacteriological cure rate of mastitis caused by β -lactamase-positive *Staph. aureus* is significantly less than those caused by β -lactamase-negative *Staph. aureus* (Sol et al., 2000).

Lactoferrin is an iron-binding glycoprotein naturally found in milk, bile, saliva, tears, and polymorphonuclear cell granules (Schanbacher et al., 1997). It has been shown that lactoferrin has bacteriostatic and bactericidal properties as well as immunomodulatory and anti-inflammatory activities (Rainard, 1993; Brock,

Received September 14, 2006.

Accepted December 12, 2006.

¹Dairy and Swine Research and Development Centre contribution no. 914.

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1995). Using mastitis-causing *Staph. aureus* strains, Diarra et al. (2002) have shown in vitro that bovine lactoferrin (bLF) acted synergistically with penicillin G (PG) to decrease the MIC of PG and to reduce the growth of both β -lactam-sensitive and resistant strains of *Staph. aureus*. Additionally, bLF was reported to block the production of β -lactamase and some other exoproteins in *Staph. aureus* (Diarra et al., 2000). In fact, bLF can enhance several fold the growth inhibitory effects of numerous antibiotics including PG, ampicillin, novobiocin, erythromycin, and neomycin, especially against resistant strains (Diarra et al., 1999). Furthermore, it was also demonstrated that when combined with PG, bLF reversed the negative effect of PG on phagocytic activity of bovine polymorphonuclear leukocytes against *Staph. aureus* (Diarra et al., 2003b). Moreover, bLF alone or in combination with PG affect *Staph. aureus* aggregation and reduced invasion of mammary epithelial cells by *Staph. aureus* based on an in vitro epithelial invasion assay and an in vivo mouse model (Diarra et al., 2003a,b). Recently, Kai and coworkers (2002) demonstrated that infusion of bLF in nonlactating cows increased cure rate of staphylococcal mastitis. Hence, it is possible that exogenous infusion of bLF is useful for the treatment of bovine mastitis. The objective of this study was to investigate in dairy cattle the therapeutic potential of bLF + PG combinations to treat chronic mastitis caused by a strain of *Staph. aureus* highly resistant to β -lactam antibiotics.

MATERIALS AND METHODS

All experiments were approved by the Agriculture and Agri-Food Canada local institutional animal care committee and conducted in accordance with the guidelines of the Canadian Council on Animal Care. Animals were kept in a level 2 confinement barn at the Dairy and Swine Research and Development Centre for the entire duration of each trial.

Bacteria

The β -lactamase-producing strain, *Staph. aureus* SHY97-4320, used in the experiments presented below was a clinical isolate from a case of bovine mastitis infection previously described (Diarra et al., 2002). The MIC of antibiotics against this strain were as follows: ampicillin (8 μ g/mL), amoxicillin (64 μ g/mL), bLF (≥ 51.2 mg/mL), cefazolin (1 μ g/mL), nisin A (2.61 μ g/mL), PG (32 μ g/mL), piperacillin (64 μ g/mL), and tazobactam (32 μ g/mL).

Preparation of Bacterial Inocula

Relationship Between Turbidity and Viable Count. Before each animal trial, the relation between

optical density at 600 nm (OD_{600nm}) and measured viable count was determined for the strain *Staph. aureus* SHY97-4320. A volume of 25 μ L of frozen bacterial stock (-80°C) was plated on blood agar (tryptic soy agar + 5% sheep blood; Difco Laboratories, Detroit, MI) and incubated overnight at 37°C to verify purity. The next day, a single colony was used to inoculate 25 mL of fresh brain heart infusion broth (BHI, Difco Laboratories) and incubated overnight at 37°C . The morning of the experiment, 100 μ L of this overnight culture of *Staph. aureus* in BHI was transferred to 100 mL of fresh BHI. The culture was incubated at 37°C for 9 h without shaking. Every hour, an aliquot was aseptically removed from the culture to 1) determine culture turbidity by measuring the OD_{600nm} and 2) determine viable count. Viable count was measured by transferring 1 mL of bacteria solution in 9 mL of PBS-Tween (8.5 g/L of NaCl, 2 g/L of K_2HPO_4 , 1 g/L of KH_2PO_4 , 5 g/L of Tween 80). Solution was further serially diluted 10-fold and 100 μ L of each relevant dilution was plated on tryptic soy agar (TSA). Plates were incubated at 37°C for 24 h before determining the number of colony-forming units per milliliter. A standard curve (OD_{600nm} vs. cfu count) was drawn from the results obtained. Each experiment was repeated twice.

Bacterial Suspension for Inoculation. The morning of the challenge, 0.5 mL of an overnight culture (BHI) of *Staph. aureus* SHY97-4320 was added to 10 mL of fresh BHI and grown at 37°C until OD_{600nm} reaches 0.400 to 0.600 in the exponential growth phase. Bacteria were then centrifuged at $2,500 \times g$ for 15 min and resuspended in sterile physiological saline (Baxter Healthcare Corporation, Deerfield, IL) before being centrifuged again for 20 min at $2,500 \times g$. After 2 washes in physiological saline, the pellet was finally suspended in 5 mL of sterile physiological saline. An aliquot of the 5-mL suspension was used to determine bacterial concentration by using the relationship established between OD_{600nm} and viability count. Accordingly, the bacterial suspension was further diluted in sterile saline to obtain the desired concentration of bacteria. Syringes were filled aseptically with 3 mL of bacterial solution and kept at 4°C until needed. Intramammary (IM) infusions were performed the same day immediately after p.m. milking. Each quarter was infused with 3 mL of bacterial suspension (see experiments 2 and 3 for specific concentrations). An aliquot of the diluted suspension was taken before and after infection to confirm viability of bacteria (i.e., cfu/mL). The aliquots were 10-fold serially diluted and plated on TSA (Difco) and incubated for 24 h at 37°C .

Intramammary Infusions

Infusion of mammary quarter with bacteria, therapeutics, or both was performed according to the proce-

cedure described by Nickerson et al. (1999) with few modifications. All infusions were performed after milking. Before inoculation, the teat end of each enrolled quarter was thoroughly wiped to remove gross contamination and dipped in a solution of iodine. After a minimum of 30 s contact time, teats were wiped dry and subsequently scrubbed with gauzes soaked in 70% ethanol. Teats were allowed to air-dry. Foremilk was then discarded and IM infusion was performed. Immediately after, all quarters were thoroughly massaged and teats dipped again with an iodine solution. Disposable gloves were worn throughout the procedure and changed between each animal.

Aseptic Milk Samples Collection and Determination of Bacterial Count

Milk samples were always aseptically collected before milking using the procedure suggested by the National Mastitis Council (1996). After foremilk was discarded, a 10-mL milk sample was collected for each individual quarter in a 15 mL sterile tube. Milk samples were kept on ice before being stored at -20°C . Milk samples were thawed and plated in triplicates on TSA plates with a spiral plating system (Autoplate 4000, Spiral Biotech Inc., Norwood, MA) using "Uniform 250 μL " and "Exponential 50 μL " modes. Plates were then incubated 24 h at 37°C before colony count was obtained using an automated colony reader (QCount, Spiral Biotech Inc.).

Preparation of Treatments

All manipulations for the preparation of active ingredients solutions were performed under a biological cabinet. Penicillin G powder was bought from Novopharm Ltd. (Toronto, Ontario, Canada), and bLF was from DMV International (Veghel, The Netherlands). The amount of endotoxin per milligram of bLF did not exceed 0.2 ng. All solutions were prepared in sterile citrate buffer (1 mM citric acid, 10 mM sodium bicarbonate, 100 mM sodium chloride, pH 7.2) using sterile and lipopolysaccharide-free vessels. Stock solutions were prepared and kept frozen until used. Syringes with active ingredients were filled with 10 mL of thawed stock solutions 1 to 2 h prior to infusion and kept at 4°C until needed.

Experiment 1: In Vivo Confirmation of Antibiotic Resistance

Animal Conditioning and Experimental Procedures. For this preliminary experiment, a total of 28 quarters with confirmed mammary infections with PG-resistant *Staph. aureus* SHY97-4320 (between 10^3 and

10^4 cfu/mL) were selected amongst 11 early lactating cows to initiate PG treatment. Mammary quarters were randomly assigned to 1 of 2 treatments to test resistance of bacteria to PG treatment: 250,000 IU of PG in 10 mL of commercial sterile saline or 500,000 IU of PG in 10 mL of sterile saline. Intramammary treatments were initiated after the morning milking on d 0 and repeated after each milking for 5 consecutive days (total of 10 doses). To determine bacterial load and response to treatment, aseptic milk samples were taken on d 0, 1, 2, 3, 4, 6, and 7.

Experiment 2: Effect of bLF-PG Combination in Late-Lactating Cows

Nineteen late-lactating cows had all 4 mammary quarters individually inoculated (185 cfu) through the teat cistern with the PG-resistant *Staph. aureus* SHY97-4320 as described above. Aseptic milk samples were collected from all mammary quarters before and regularly after bacterial inoculation to monitor infection establishment and stability. Infections were allowed to stabilize for 15 d (between 10^3 and 10^4 cfu/mL) before treatment initiation. Only cows with 4 infected quarters were allowed to enter the trial. Fifteen days after the initial infection, only 11 cows out of 19 had stable infection in their 4 quarters. Enrolled quarters were then randomly assigned, within cow, to 1 of 4 IM treatments as follows: 1) citrate buffer, 2) 100,000 IU of PG, 3) 1 g of bLF, or 4) 1 g of bLF + 100,000 IU of PG. All treatments were prepared in sterile citrate buffer to a final volume of 10 mL as described above. Intramammary treatments were initiated after the evening milking on d 0 and repeated after each milking for 5 consecutive days (total of 10 doses). To determine bacterial load (cfu count), additional aseptic milk samples were taken during treatment period (1, 2, 3, and 5 d after initial infusion of treatments) and after cessation of treatments on d 7, 13, 20, and 27 corresponding to 2, 8, 15, and 22 d after cessation of treatment, respectively. Milk samples harvested before treatment initiation as well as on d 20 and 27 d were sent for bacteriological analysis (Biovet Laboratory, St-Hyacinthe, QC, Canada). Variable number tandem repeats genotyping (selective primers kindly provided by F. Gilbert, INRA Laboratory, Tours, France) was performed as described by Gilbert et al. (2006) to ascertain the presence of specific *Staph. aureus* strain SHY97-4320. Each quarter was also monitored for SCC and milk lactose content.

Experiment 3: Effect of a bLF-PG Combination in Chronically Infected Cows

Animal Selection and Experimental Procedures. Twenty healthy Holstein cows in late lactation

with bacteriologically negative milk samples and a milk SCC of less than 2×10^5 cells/mL milk per individual quarter were selected for this study. Cows were all gravid and had expected dates of calving grouped within a 2-wk interval. Each individual mammary quarter was designated as an experimental unit. Thirty days before drying off, 3 mL of saline containing 100 cfu of *Staph. aureus* SHY97-4320 were infused into the teat cistern of each quarter as described above. Aseptic milk samples were collected from all mammary quarters before and regularly after bacterial inoculation to monitor infection establishment and stability. Quarters that were not infected were reinfected 1 wk after the initial infusion. Infections were allowed to stabilize (between 10^3 and 10^4 cfu/mL) for 1 mo before cows were dried off abruptly. After all cows had calved, aseptic milk samples were collected independently from all quarters for 4 wk to monitor infection pattern. Based on these results, 14 out of the 20 cows were enrolled in this experiment: 7 cows with all 4 quarters infected, 3 cows with 3 quarters infected, 4 cows with 2 quarters infected. Mammary quarters from experimental cows were randomly assigned, within cow, to 1 of 2 treatments as follows: 1) 100,000 IU of PG (n = 20) or 2) 250 mg of bLF plus 100,000 IU of PG (n = 21); 4 quarters were treated with saline only. All treatments were prepared in sterile citrate buffer with a final volume of 10 mL as described above. Intramammary treatments were initiated after the p.m. milking on d 0 and repeated twice a day after each milking for 7 consecutive days (total of 14 doses). Aseptic milk samples were taken every day for 7 d during treatment period (d 1 to 7), and then 2, 4, 7, 14, 21, and 28 d after cessation of treatment to determine milk bacterial concentration. Milk production, gross milk composition, and SCC of each quarter was also determined.

Cure Rate Determination

Bacteriological analysis (Biovet Laboratory) and PCR genotyping (Gilbert et al., 2006) were performed on samples taken prior to initiation of treatment and during wk 3 and 4 after initiation of treatment to ascertain the presence of the specific *Staph. aureus* SHY97-4320. Cure rate was also determined using samples taken 3 and 4 wk after initiation of treatment. During routine bacteriological analysis, sterile milk samples were plated on TSA as a quality control, and 10% of these samples showed concentrations of <10 cfu/mL. Hence, based on this observation, we defined bacteriological cure rate as follows: if a cow had 0 cfu/mL in milk samples taken 3 and 4 wk after onset of treatment and SCC less than 200,000 cells/mL, she was cured; if one sample had 0 cfu/mL and the second one less than 10

Figure 1. Comparison of 2 penicillin (PG) doses on β -lactamase-producing *Staphylococcus aureus* SHY97-4320 shedding in milk from experimental infected quarters. Quarters were infused twice a day with 250,000 IU (○; n = 14) or 500,000 IU (■; n = 14) of PG for 5 consecutive days. Day 0 = time of the first intramammary infusions of treatments; data are presented as log₁₀ transformed values $\pm$ SEM.

cfu/mL with a SCC <200,000 cells/mL, she was considered cured if we could determine that she was not infected by *Staph. aureus* strain SHY97-4320 based on bacteriological evaluation and PCR analyses.

Statistical Analysis

The data were analyzed as repeated measurements using the MIXED procedure of SAS (SAS Institute, 2002) with spatial power as the covariance structure. For the second experiment, data were analyzed as a 2×2 factorial design with levels of PG and bLF as the main factors. The Tukey-Kramer posthoc test was used for pairwise comparisons. Because values were not normally distributed, statistical analysis of bacterial count was performed on the log₁₀-transformed values. Association between cure rates and the 4 treatment combinations were tested with Fisher's exact test. Cure rates were also submitted to a logistic analysis using the LOGISTIC procedure of SAS with contrasts comparing each treatment combination to the control. Effects were considered significant at $P \leq 0.05$ and to tend to be significant at $0.05 < P \leq 0.10$.

RESULTS

Experiment 1: In Vivo Confirmation of Antibiotic Resistance

The effect of IM infusion of PG on shedding of the β -lactamase-producing strain *Staph. aureus* SHY97-4320 is depicted in Figure 1. As early as one day after the first IM infusion, treatments with 250,000 or 500,000 IU reduced ($P < 0.001$) milk bacterial concentration

Figure 2. Effect of penicillin (PG), bovine lactoferrin (bLF), or both on bacterial concentration in milk from 14 d infected quarters by β -lactamase-producing *Staphylococcus aureus* SHY97-4320. Quarters were infused twice a day with citrate buffer (○; n = 11), 1 g of bLF (■; n = 9), 100,000 IU of PG (▲; n = 11), or bLF + PG (×; n = 11) for 5 consecutive days. Day 0 = time of the first intramammary infusions of treatments; data are presented as Log_{10} transformed values $\pm$ SEM.

down to 1.79 ± 0.27 and $1.09 \pm 0.37 \log_{10}$ cfu/mL, respectively (Figure 1). For both PG concentrations, this effect was not enhanced ($P = 0.57$) by additional PG infusions because a plateau was reached and persisted throughout the treatment period. However, milk bacterial concentration increased rapidly after the cessation of treatment ($P < 0.001$), reaching 3.19 ± 0.37 or $2.89 \pm 0.36 \log_{10}$ cfu/mL. For all time points, statistical analysis indicated no significant difference ($P > 0.1$) in efficacy of both PG treatments (250,000 vs. 500,000 IU) to reduce milk bacterial concentration.

Experiment 2: Effect of a bLF-PG Combination in Late-Lactating Cows

Intramammary infusion of *Staph. aureus* SHY97-4320 resulted in the expected inflammatory reactions as measured by standard diagnostic markers of mastitis. For all cows, IM infusion of *Staph. aureus* caused an increase in rectal temperature (from $38.5 \pm 0.3^\circ\text{C}$ to $39.4 \pm 0.5^\circ\text{C}$; $P < 0.001$) within 24 h. Fifteen days after the initial infection, only 11 of 19 cows had stable subclinical infection in their 4 quarters as demonstrated by a milk bacterial count greater than 10^3 cfu/mL in consecutive milk samples (data not shown).

The primary purpose of this trial was to compare bacterial cure rates of a traditional PG treatment with a novel approach that consisted of a combination of PG with bLF. Figure 2 depicts $\log_{10}$ bacterial number according to treatment over time. The number of bacte-

Figure 3. Cure rate of infected quarters by β -lactamase-producing *Staphylococcus aureus* SHY97-4320 in lactating cows after twice a day intramammary infusions for 5 d of citrate buffer (control; n = 11), 1 g of bovine lactoferrin (bLF; n = 9), 100,000 IU of penicillin G (PG; n = 11), or the combination of bLF and PG (n = 11).

ria shed by control quarters remained stable for the whole period. During the treatment period, all quarters that received bLF, PG, or both had a significant decrease in milk bacterial count ($P < 0.01$) when compared with the control. Five days of treatment led to a 2, 2.4, and 3 log reduction in bacterial number after treatment with PG, bLF and bLF + PG, respectively (Figure 2). However, as early as 2 d after the last treatment (d 7), the number of milk colony-forming units in quarters treated with PG or bLF alone were no longer different from their pretreatment concentrations ($P > 0.10$). In bLF + PG treated cows, the bacterial number increase gradually but remained significantly lower ($P < 0.05$) on d 20 and tended to be lower on d 27 ($P < 0.1$) when compared with the control and the 2 others treatments. A significant interaction between PG and bLF was present on d 3, 5, 7, and 20 ($P < 0.05$), whereas only a tendency was pointed out on d 13 ($P = 0.10$) and 27 ($P = 0.08$).

Cure rate was null for control quarters whereas one quarter was considered cured in either bLF (11.1%; 1 out of 9) or PG (9.1%; 1 out of 11) treatments. In contrast, 5 of 11 quarters receiving the bLF + PG combination were completely cured from infection, resulting in a 45.5% cure rate (Figure 3). Fisher's exact test detected an association between treatments and cure rate ($P = 0.04$). Even though the global effect was not significant in the logistic analysis ($P = 0.18$), comparison of each treatment to the control indicated that using both bLF and PG results in better cure rate ($P = 0.04$). In fact, odds ratios were 7.3 when using both bLF and PG and 1.45 when using only 1 of the 2 treatments.

Before initiation of treatment, all quarters exhibited a high SCC, as showed in Figure 4A. During the treat-

Figure 4. Effect of penicillin (PG), bovine lactoferrin (bLF), or both on A) SCC and B) lactose content in milk from 14-d infected quarters by β -lactamase-producing *Staphylococcus aureus* SHY97-4320. Quarters were infused twice a day with citrate buffer (○; $n = 11$), 1 g of bovine lactoferrin (bLF; ■; $n = 9$), 100,000 IU of penicillin G (PG; ▲; $n = 11$), or bLF + PG (×; $n = 11$) for 5 consecutive days. Day 0 = time of the first intramammary infusions of treatments; data are presented as $\log_{10}$ transformed values $\pm$ SEM.

ment period (sampling d 1 to 5), infusion of PG alone did not modify average milk SCC ($P > 0.05$). However, there was a residual effect of PG to reduce SCC 2 d after cessation of treatments (d 7; $P = 0.05$), whereas only a tendency was observed on d 13 ($P = 0.07$). This effect was lost thereafter ($P > 0.1$). On the other hand, infusion of bLF alone or in combination with PG increased the average milk SCC during treatment period ($P = 0.001$ and $P = 0.003$, respectively). This effect remained 2 d after treatment interruption for either bLF- or bLF + PG-treated quarters ($P = 0.05$ and $P = 0.03$, respectively) but it was lost thereafter ($P > 0.1$).

Figure 5. Effect of penicillin G (PG) or PG + bovine lactoferrin (bLF) on A) bacterial concentration and B) SCC in milk from quarters chronically infected by β -lactamase-producing *Staphylococcus aureus* SHY97-4320. Quarters were infused twice a day for 7 consecutive days with 100,000 IU of PG (▲; $n = 20$) or 250 mg of bLF + 100,000 IU of PG (×; $n = 21$). Day 0 = time of the first intramammary infusions of treatments; data are presented as A) $\log_{10}$ transformed values $\pm$ SEM, and B) as unadjusted means $\pm$ SEM.

Milk production was not affected by treatments ($P > 0.1$; data not shown). All quarters had similar high milk lactose content before the beginning of the treatment period (Figure 4B). Intramammary infusion of PG did not affect milk lactose content ($P > 0.1$). Milk from quarters infused with bLF had smaller lactose content 3, 5, and 7 d after treatment initiation ($P < 0.04$). A significant interaction between bLF and PG on milk lactose content was present on d 3 and 5 ($P = 0.05$ and $P = 0.03$, respectively). Milk lactose was equivalent for all treatments for the rest of the trial.

Experiment 3: Effect of a bLF-PG Combination in Chronically Infected Cows

Figure 5A depicts $\log_{10}$ bacterial numbers according to treatment over time. Within 1 d of treatment, all

quarters had a significant drop in milk bacterial count ($P < 0.001$). Treatment with bLF significantly enhanced the antimicrobial activity of PG ($P < 0.01$). Milk colony-forming unit concentrations of bLF + PG-treated quarters tended to be less (2.8 log decrease) than those of solely PG-treated (2.4 log decrease) quarters on d 3 ($P = 0.09$) and 4 ($P = 0.10$), whereas this effect was more pronounced on d 5 ($P = 0.02$), 5.5 ($P = 0.007$), and 7 ($P = 0.009$). On d 5, treatment with bLF + PG led to a 3.6 log decrease in the bacterial number, whereas this decrease was 2.2 for bLF treatment. In both treatments, milk colony-forming unit concentrations started to increase ($P < 0.001$) after cessation of treatment. Thirty-five days posttreatment, 2 of the PG treated quarters were considered cured, whereas 6 quarters were cured amongst those receiving the bLF + PG combination treatment, resulting in cure rates of 12.5 and 33.3%, respectively; this difference was, however, not significant.

Before initiation of treatments, all quarters exhibited high SCC as shown in Figure 5B. Initiation of the combination treatment induced an immediate increase of SCC ($P < 0.001$) before decreasing the day after. During treatment period, SCC concentrations were higher in quarters receiving bLF + PG combination than those receiving PG alone ($P < 0.01$). This effect was totally lost 2 d after IM infusions were interrupted ($P = 0.73$).

DISCUSSION

Previous work had demonstrated that it is feasible to experimentally induce *Staph. aureus* mastitis by IM infusion (through the teat canal) of a small number of bacteria (Postle and Goodland, 1978). Accordingly, IM infusion of β -lactamase-producing *Staph. aureus* bacteria in our study was able to cause mastitis as evidenced by the recovery of viable bacteria in the milk and higher milk SCC comparable to concentrations reported by others (Haley et al., 1981; Sol et al., 2000). Furthermore, the antibiotic bacteriological response of mammary quarters infected with this clinical isolate of PG-resistant *Staph. aureus* SHY97-4320 was investigated. Our results clearly showed that PG alone (i.e., 250,000 and 500,000 IU per injection twice a day for 5 d) was not efficient to treat this type of *Staph. aureus* infection. Indeed, during treatment a bacteriological response was observed, but a plateau was attained, confirming that this strain was capable of withstanding antibiotic therapy, and infection reemerged as soon as the therapy ended. Hence, we proceeded in subsequent experiments with this specific strain of *Staph. aureus* but with a reduced dose of PG (100,000 IU) per injection with or without bLF.

The efficacy of the bLF-PG combination has been demonstrated in vitro against resistant strains of *Staph. aureus* (Diarra et al., 2002), but no such evidence existed in vivo. Following treatment initiation, the significant drop in milk colony-forming units of quarters treated with bLF (1 g per injection in experiment 2 or 250 mg per injection in experiment 3) or PG (100,000 IU per injection in both experiments) unlike in control quarters confirmed that the 2 antimicrobial agents tested in this study had a certain antimicrobial effect on their own. On the other hand, when bLF and PG were combined, the drop in milk colony-forming units during treatment period was more pronounced, demonstrating a synergistic effect of bLF and PG as initially reported in vitro by Diarra et al. (2002). Moreover, after cessation of treatment, average bacterial concentrations of quarters treated with bLF or PG alone returned to pretreatment concentrations within 2 d, whereas those of quarters treated with the combination remained smaller, indicating that quarters receiving this treatment combination were indeed cured from infection.

In staphylococcal mastitis, the ability of *Staph. aureus* to escape antibiotic therapy has been associated with the sequestration of bacteria in abscesses of mammary tissue (Gudding et al., 1983) as well as intracellular survival within the phagocytes (Craven and Anderson, 1980). To consider a quarter cured, it is recommended to have double-negative milk samples (Schukken and Deluyker, 1995) because *Staph. aureus* mastitis is known to show a cyclic rise and fall of detectable bacteria in the milk of infected glands (Daley et al., 1991). In these studies, bacteriological cure from infection was determined using samples taken 3 and 4 wk after initiation of treatments. Based on these samples, we observed, in experiments 2 and 3, a 3- to 5-times higher cure rate for quarters treated with the combination of bLF and PG compared with quarters receiving PG alone. Moreover, in experiment 2, one quarter was not included in our cure rate because it had 0 cfu/mL on d 20 but 3 cfu/mL on d 27 with an SCC of 367,000 cells/mL. If this quarter were included, our cure rate for the bLF + PG combination would have been 55% for chronic IMI caused by a highly resistant β -lactamase-producing strain of *Staph. aureus*.

Overall bacteriological cure rates achieved in these experiments are highly significant. Indeed, Owens et al. (1997b) reported a cure rate of 35% for chronic mastitis caused by PG-sensitive *Staph. aureus* and treated with a combination of PG procaine (100,000 IU) and novobiocin (150 mg), which is currently approved commercially for the treatment of bovine IMI. Moreover, Oliver et al. (2004) used a new broad-spectrum, third-generation cephalosporin antibiotic (Ceftiofur) for veterinary use

to treat subclinical mastitis and reported a cure rate of 36% for *Staph. aureus* after an extended therapy of 8 d. Overall, although we have used a limited number of animals, our experiments have been performed with a highly resistant strain of *Staph. aureus*, and our results clearly demonstrate the efficacy of a bLF + PG combination, which is comparable to, if not better than, currently available products on the market. Furthermore, results obtained in experiment 3 with cows that had been infected in their previous lactation suggest that it is worthwhile to treat persistently infected cows, even in early lactation, instead of culling them.

The persistency of high SCC in milk as observed in our study was previously reported when mastitis was caused by β -lactamase-producing strains, which are associated with more chronic mastitis (Sol et al., 2000). Results from experiments 2 and 3 showed that during the treatment period, SCC in milk from quarters receiving bLF with or without PG exhibited an increase that was not visible in controls. It has been reported that 10 μ g of endotoxin infused into a mammary quarter could cause a mild to moderate inflammatory reaction (Anderson and Hunt, 1989). In our study, 1 g of bLF contained 0.2 μ g of endotoxin. Consequently, although it cannot be totally excluded that the low endotoxin content of bLF could be responsible for the increase of milk SCC, we think it is highly unlikely that this low amount of endotoxin would cause such a large rise in SCC. In fact, our observations would support the view that bLF may be active in modulation and regulation of macrophages, lymphocytes, and neutrophil function (Brock, 1995; Sordillo et al., 1997), thus attracting more neutrophils into the mammary gland.

Our results also showed a lesser milk lactose content in quarters infused with bLF compared to those not receiving bLF. However, no difference was observed in average milk production, even though lactose plays a key role as an osmotic regulator of milk volume (Pyörälä, 2003). On the other hand, the association between lactose and SCC is well known because neutrophil diapedesis into the mammary gland contributes to rupturing the blood–milk barrier, resulting in leakage of milk proteins and lactose toward the bloodstream (reviewed by Kitchen, 1981). Hence, the lesser lactose content observed in bLF-treated quarters may be associated with the important increase in SCC that occurred concomitantly in these quarters rather than being associated with a direct effect of bLF on lactose synthesis.

The exact mechanisms behind the superiority of the combination of bLF and PG over the use of PG alone are not fully elucidated and are probably multifactorial. However, we have reported that bLF inhibits mRNA expression of the gene responsible for the production of β -lactamase in *Staph. aureus* (Diarra et al., 1999),

hence preventing degradation of PG. In addition, the ability of bLF to revert the negative effect of PG on phagocytosis has been previously demonstrated in vitro (Miyachi et al., 1998) and also in vivo using a mouse mastitis model (Diarra et al., 2003b). Survival of *Staph. aureus* leading to therapy failure when PG is used has also been associated with a poor antibiotic penetration into the deep parenchymal tissue of the mammary gland (Owens and Nickerson, 1990). Whether bLF improves antibiotic distribution has not been investigated in this study. On the other hand, using an epithelial invasion assay, bLF alone or in combination with PG was shown to reduce invasion of mammary epithelial cells by *Staph. aureus* (Diarra et al., 2003b). Therefore, it is plausible that the relative efficacy of the bLF + PG combination used in this study results from a combination of these mechanisms, including the increase in neutrophil migration into the milk following bLF treatment in vivo. Furthermore, using a mouse infection model, we reported that 2 d of systemic treatments with bLF affected the morphology and aggregation of *Staph. aureus*, which may facilitate its killing by PG (Diarra et al., 2003a).

The bactericidal effect of β -lactam antibiotic is time-dependent. Hence, for these antibiotics, the frequency of drug administration is an important determinant of the outcome of the IMI (Craig, 1993; Pyörälä, 2002) because the duration of time that serum concentrations of antibiotics exceed MIC is the major determinant of efficacy. Our results of bacterial disappearance support this view, because the lowest milk bacterial concentration was found after 5 d (experiment 2) and 7 d (experiment 3) of treatments. Accordingly, several authors suggested that a 3-d treatment might be too short a period to treat mastitis caused by β -lactamase-producing *Staph. aureus* and, therefore, will associate an extended therapy with an increased cure rate (Pyörälä and Mattila, 1987; Sol et al., 2000; Oliver et al., 2004).

Efficacy of treatment might be obtained by the use of commercial long-acting emulsions that improve the dispersion of bLF and PG into the mammary gland or by simultaneous systemic and IM therapy. Kuttila et al. (2002) studied the disposition kinetics of bLF after IM administration of 1 g of bLF in aqueous solution; they observed that high bLF concentrations were maintained for several hours with a mean elimination half-life of 2.2 h and mean maximal concentration of 6,300 μ g/mL. However, concentrations of bLF measured in milk exceeded the infusion concentration; Kuttila et al. (2002) suggested that bLF being water-soluble, exogenous bLF did not spread throughout the infused quarter. Higher cure rates might also be achieved by a slightly higher dose of PG, inasmuch as it was demonstrated that the MIC of β -lactamase-positive strains in

milk were 10- to 100-fold higher than those in broth (Ali-Vehmas et al., 1997). Moreover, Haley et al. (1981) reported that the maximum PG concentration reached in the milk when 100,000 IU was injected IM was 36.75 IU/mL. Knowing that the strain used in this study has a MIC for PG (in broth) of 51.2 IU/mL (32 µg/mL), it is clear that the concentration of PG reached in our experiments was under MIC. Moreover, the cows used in our studies had between 2 and 4 infected quarters, which made them more immunocompromised than cows with only one infected quarter. Finally, Sol et al. (1997) showed that the probability of cure with 2 or more infected quarters with *Staph. aureus* was smaller (22.2%) than that of cows with only one infected quarter (37.1%).

Treatment of chronic staphylococcal mastitis is clearly a problem. Once established, *Staph. aureus* mastitis usually does not respond to antibiotic treatment alone, especially if the strain involved produces β -lactamases. We have shown that IM therapy using a combination of bLF and PG could enhance significantly bacteriological cure rate compared with standard PG treatment. Moreover, our results clearly showed that an extended therapy for 5 to 7 d could contribute to bacterial clearance. Interestingly, bLF appeared to have a positive impact on neutrophil diapedesis toward the mammary gland.

In conclusion, bLF added to PG is an effective combination (i.e., 3- to 5-times higher cure rate) for the treatment of stable *Staph. aureus* infections highly resistant to β -lactam antibiotics. Still, the efficacy of this bLF + PG treatment combination could probably be enhanced by the use of a higher dose of PG. Furthermore, an optimized drug formulation would improve drug dispersion in milk and tissue, and thereby efficacy of this combination, following infusion into the mammary gland of infected cows.

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