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Efficacy of vaccination and nisin Z treatments to eliminate intramammary *Staphylococcus aureus* infection in lactating cows*

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This study evaluated the effect of a *Staphylococcus aureus* bacterin and nisin on bovine subclinical mastitis. A total of 75 Holstein subclinically mastitic cows were randomly allocated to three groups with 25 cows per group. In group I, an intramammary infusion of nisin Z at a dose of 2.50×10^6 IU was carried out once daily for three days, and an autogenous *S. aureus* bacterin was inoculated into the supramammary lymph node one week before and one week after nisin treatment. In group II, nisin was administered in the same way as in group I, but no bacterin was inoculated. Group III received no treatment and served as a control. Milk was aseptically sampled from the affected quarters before and 2, 4, and 6 weeks after treatment, for bacteriological examination and analyses of *N*-acetyl-β-D-glucosaminidase (NAGase) activity, somatic cell count (SCC), and milk protein and fat contents. Results indicated that, compared to the nisin-treated group, nisin-bacterin treatment significantly reduced intramammary *S. aureus* infections, reduced the number of quarters with milk SCCs of more than 5×10^5 cells/ml, and increased the protein and fat contents of the milk. Therefore, nisin-bacterin therapy is suggested when subclinical mastitis occurs in lactating cows.

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S. aureus is one of the bacteria most commonly isolated from intramammary infections (IMIs) in cows (Tenhagen *et al*., 2006) and is responsible for about 35% of economic losses due to mastitis (Fox and Hancock, 1989). Infections of *S. aureus* have often proven resistant to treatment and antimicrobial therapy during lactation generally results in low clinical cure rates (Luby *et al*., 2007). One reason for the poor clinical cure rates could be the intracellular location of *S. aureus*, such as in epithelial cells or macrophages, and its ability to survive antibiotic treatments (Tuchscherr *et al*., 2011). Consequently, antibiotic therapy is often ineffective in eliminating chronic udder *S. aureus* infections (Pellegrino *et al*., 2008). Furthermore, regulatory methods tend to limit the use of antibiotics in dairy cattle to reduce their residues in milk. Thus, it is important to search for novel therapeutic approaches to eliminate intramammary *S. aureus* infections.

Nisin is an antimicrobial peptide with 34 amino acids synthesized by *Lactococcus lactis* (Carr *et al*., 2002), and has been proven to have inhibitory effects on many Gram-positive bacteria such as mastitis pathogens. It has been licensed as a food preservative in 48 countries (Deegan *et al*., 2006) due to its effective antibacterial activity and safety for human consumption. We have previously shown that nisin can be successfully used to treat both clinical and subclinical mastitis in lactating cows (Cao *et al*., 2007; Wu *et al*., 2007). In addition, vaccination with *S. aureus* bacterin has improved antibiotic therapy of *S. aureus* IMIs (Steele and McDougall, 2014).

The aim of this study was to determine if vaccination against *S. aureus* potentiates nisin treatment of subclinical *S. aureus* IMI in lactating cows. Changes in IMI, SCC, NAGase activity, and protein and fat contents in milk were analyzed before and after nisin treatment.

Materials and methods

Cows

The experimental dairy farm, with about 1000 Holstein dairy cows, was situated in the suburban area of Hangzhou, Zhejiang, China. The animals were milked by machine three times per day. Cows with milk SCCs of $\geq 5\times10^5$ cells/ml were considered to have mastitis. A total of 75 cows with at least one quarter subclinically infected with *S. aureus* were used.

Nisin

The nisin Z used in this study was in the form of a purified product (1.80×10^4 IU/mg) produced by the Zhejiang Silver-Elephant Bio-Engineering Co., Ltd., Tiantai, Zhejiang, China. Before treatment, the nisin (2.50×10^6 IU) was dissolved in 20 ml of sterile physiological saline solution.

Formulation of *S. aureus* bacterin

The bacterin was prepared using *S. aureus* CQ399RP strain isolated from a mastitis case on the farm where this study was performed, and the bacteria were incubated in brain-heart infusion broth with aeration at 37 °C for 18 h. The cells were inactivated with formalin (0.4%, v/v) at 4 °C for 24 h, and re-suspended in saline (9 g/L NaCl, pH 7.0). Two washings were performed by centrifuging the inactivated bacteria in saline at 6000 r/min for 30 min at 4 °C. Each 2-ml dose contained 4×10^{10} CFU/ml of *S. aureus*. Thimerosal was added as preservative at a final concentration of 0.11 g/L. Aluminum hydroxide (35 g/L) and ginseng extract (0.25 g/L) were added as an adjuvant. Complete inactivation of the bacterin was confirmed by inoculating the bacterin on blood agar and incubating for 24 to 48 h at 37 °C.

Treatment

Cows infected with *S. aureus* were randomly allocated to one of three groups (25 cows per group). In group I, cows were intramammarily infused with 2.50×10^6 IU of nisin Z once daily for three days. The dose of nisin Z was chosen based on the results of a study by Wu *et al.* (2007). They infused three different doses of nisin Z (1.25×10^6, 2.50×10^6, and 5.00×10^6 IU) into mammary glands and showed that 2.50×10^6 IU was the optimal dose. One week before and one week after nisin treatment, 2 ml of *S. aureus* bacterin was injected into the supramammary lymph node. In group II, cows received the same nisin treatment as in group I, but no bacterin. Group III was untreated and used as a control group. Before intramammary infusion, the affected quarter was completely milked out and the teat end was disinfected with a cotton swab soaked in 75% (v/v) alcohol.

Collection and analysis of milk samples

Before starting and at 2, 4, and 6 weeks after terminating treatment, milk was aseptically sampled from the affected mammary quarters for bacteriological examination and analyses of milk SCC, NAGase activity, and protein and fat contents.

Bacteriological examination

Each milk sample was inoculated on a blood agar plate and the plate was incubated at 37 °C for 24 to 48 h. After that, bacterial growth was observed for primary isolation. If three or more different colony patterns of bacteria were found, the milk sample was assumed to be contaminated and was discarded. Then a single colony was selected on the blood agar, inoculated into broth medium, and incubated at 37 °C for 18 to 24 h. Specific bacterial species, such as staphylococci, streptococci, and Gram-negative bacteria, were identified according to the procedures described by the Laboratory and Field Handbook on Bovine Mastitis, National Mastitis Council, Inc., Arlington, VA, USA.

Somatic cell count analysis

Milk samples were preserved using bronopol (2 μl/ml) and FOSSmatic™ Minor (Foss Electric, Hillerød, Denmark) was used to determine SCC.

NAGase test

Three cycles of freezing and thawing of milk samples were performed to release NAGase from cells, and the samples were then centrifuged for 20 min at 3500 r/min to get rid of the cream. Acetic acid (10%, v/v) was added to the skim milk to adjust the pH to 4.6, and the samples were centrifuged for 20 min at 3500 r/min to obtain whey. A commercial kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) was used to determine NAGase activity according to the manufacturer's protocols. The optical density (OD) value of paranitrophenol was analyzed

in triplicate at a wavelength of 400 nm during the reaction (at 37 °C) between the 4-methylumbelliferyl-*N*-acetyl-β-glucosaminide substrate and the NAGase. The amount of paranitrophenol released from 1 L of whey at 37 °C in 15 min was expressed as one unit of NAGase activity.

Analysis of milk protein and fat contents

Integrated Milk Testing MilkoScan FT6000 (Foss Electric, Hillerød, Denmark) was used to determine milk protein and fat contents.

Statistical analysis

Statistical analysis was performed using SPSS (Version 20.0, Chicago IL, USA). A χ^2 test was used to compare the number of quarters with an SCC of $\geq 5\times10^5$ cells/ml among groups at each sampling time, the number of quarters with an SCC of $\geq 5\times10^5$ cells/ml and the number of IMIs between pre- and post-treatments within a group. Analysis of variance was performed to compare NAGase activities, milk protein and fat contents among the three groups at each sampling occasion and between pre- and post-treatment times within the same group. The level of significance was set at $P<0.05$.

Results

Intramammary infections and somatic cell count

Bacteriological cure rates were 68.0%, 72.0%, and 72.0%, respectively, when milk samples were examined at 2, 4, and 6 weeks after treatment in group I (Table 1), and were 44.0%, 40.0%, and 40.0%, respectively, in group II. No change was detected in the control group throughout the experiment. Compared with the control group, both treated groups showed significantly reduced IMIs, and there were significantly fewer infected quarters in group I than in group II.

Table 1 also shows that the number of quarters with an SCC of $\geq 5\times10^5$ cells/ml significantly decreased after nisin or nisin-bacterin treatment, but was unchanged in the control group.

NAGase activity

Table 2 shows that NAGase activity significantly decreased after nisin or nisin-bacterin treatment while no significant changes were found in the control group.

Protein and fat contents

In group I, milk protein increased significantly from (2.86±0.14)% to (3.08±0.18)%, (3.08±0.19)%, and (3.07±0.19)% at 2, 4, and 6 weeks after treatment, respectively, and milk fat significantly increased from (2.99±0.11)% to (3.18±0.15)%, (3.25±0.19)%, and (3.26±0.18)%, respectively. In group II, milk protein significantly increased from (2.84±0.18)% to (2.98±0.25)%, (3.02±0.23)%, and (3.01±0.21)%, respectively, at 2, 4, and 6 weeks after treatment, and milk fat increased significantly from (2.99±0.16)% to (3.13±0.21)%, (3.17±0.24)%, and (3.21±0.27)%, respectively. No changes were found for milk protein

Table 1 Results of milk bacteriological and SCC examination before and after treatment

Group	Quarters before treatment	Quarters with *S. aureus* IMI			Quarters with SCC $\geq 5\times10^5$ cells/ml		
		2 weeks	4 weeks	6 weeks	2 weeks	4 weeks	6 weeks
Nisin Z+vaccine	25	$8^{\dagger *}$	$7^{\dagger \# *}$	$7^{\dagger \# *}$	11 (44.0%)$^{\dagger *}$	9 (36.0%)$^{\dagger *}$	10 (40.0%)$^{\dagger *}$
Nisin Z	25	$14^{\dagger *}$	$15^{\dagger *}$	$15^{\dagger *}$	13 (52.0%)$^{\dagger *}$	12 (48.0%)$^{\dagger *}$	13 (52.0%)$^{\dagger *}$
Control	25	25	25	25	25 (100.0%)	25 (100.0%)	25 (100.0%)

Within a column, $^{\dagger}P<0.01$, compared with the control; $^{\#}P<0.01$, compared with the nisin-treated group. Within a row, $^{*}P<0.01$, compared with quarters before treatment

Table 2 NAGase activity in milk before and after treatment

Group	NAGase activity before treatment (U/L)	NAGase activity after treatment (U/L)		
		2 weeks	4 weeks	6 weeks
Nisin Z+vaccine	54.02±9.75	34.16±9.83$^{\dagger *}$	30.25±11.96$^{\dagger *}$	29.88±14.15$^{\dagger *}$
Nisin Z	55.65±21.13	35.53±12.51$^{\dagger *}$	31.69±13.53$^{\dagger *}$	32.22±15.37$^{\dagger *}$
Control	57.31±22.18	56.45±17.84	56.03±16.26	57.04±17.29

Data are expressed as mean±standard deviation (SD), with n=25 per group. Within a column, $^{\dagger}P<0.01$, compared with the control. Within a row, $^{*}P<0.01$, compared with NAGase activity before treatment

((2.83±0.09)%, (2.80±0.10)%, (2.81±0.09)%, and (2.80±0.10)%, respectively) or milk fat ((2.96±0.12)%, (2.98±0.12)%, (3.01±0.11)%, and (3.01±0.10)%, respectively) before treatment and 2, 4, and 6 weeks after treatment in the control group.

Discussion

Because of the failure of chemotherapy for treating *S. aureus* mastitis and the problems associated with antibiotic residues in milk, antibiotic treatment is usually not recommended during the lactating period. We previously successfully treated subclinical IMIs in lactating cows using nisin Z, resulting in a cure rate of 50% for *S. aureus* infection (Wu *et al*., 2007). Similar results were obtained in the present study in which the bacteriological cure rate for *S. aureus* was about 40%–44%. The main advantage of using nisin as an antimicrobial agent to treat mastitis is its safety compared with conventional antibiotics. In the present study, nisin treatment was improved by immunization with *S. aureus* bacterin in the supramammary lymph node. The bacteriological cure rate was significantly higher in the nisin-bacterin group (68%–72%) than in the nisin group (40%–44%).

IMI is the major factor inducing high milk SCCs (González-RodríGuez *et al*., 1995). A high SCC is usually taken as an indicator of an IMI caused by bacteria, and a low SCC ($<1.50\times10^5$ cells/ml) in general implies freedom from such infections (Sordillo *et al*., 1997). We previously demonstrated that the number of quarters with high milk SCCs ($\geq5\times10^5$ cells/ml) was significantly reduced by nisin treatment. In the present study, fewer quarters with high milk SCCs ($\geq5\times10^5$ cells/ml) were found in nisin-bacterin-treated cows than in the nisin-only group. Fewer quarters of high milk SCCs paralleled significantly reduced milk NAGase activity after treatment in both treated groups compared with the control or pre-treatment values.

Mastitis causes injury to milk secretory cells in the mammary gland and decreases the synthesis of fat and protein (Ogola *et al*., 2007). Therefore, the ability of the mammary epithelium to synthesize and secrete the major specific milk constituents is reduced (Fox *et al*., 1985). In this study, the contents of protein and fat increased significantly after nisin or nisin-bacterin treatment compared to the control or pre-treatment values. This may be attributed to accelerated regeneration of the mammary epithelium after treatment. Milk production might also benefit from the regenerated mammary epithelium. However, the quantity of milk produced was not recorded because the milking machine lacked the capability to record milk production from individual cows.

The use of a vaccine in combination with an antimicrobial agent to treat IMIs in lactating cows is not new. Smith *et al.* (2006) treated 20 dairy cows with chronic intramammary *S. aureus* infections by vaccination with a polyvalent *S. aureus* bacterin along with intramammary administration of pirlimycin. Significantly more *S. aureus* infections were eliminated from treated cows, compared with control cows. Recently, Czernomysy-Furowicz *et al.* (2014) treated 15 cows with *S. aureus* IMI by vaccination with an anti-*S. aureus* herd-specific autovaccine combined with intramammary infusion of cefuroxime and obtained prolonged udder protection against bacterial infection. In the present study, nisin was used as an antibacterial agent. Nisin has been reported to destroy bacteria by interaction with anionic lipids and formation of pore complexes on the cytoplasmic membrane of the bacterial cells (Tong *et al.*, 2014). Immunization of mastitis vaccine at this site may trigger the synthesis of large amounts of opsonizing IgG for phagocytosis of *S. aureus* by neutrophils (Nordhaug *et al.*, 1994). The results found in group I could be due to the combined effects of nisin treatment and bacterin vaccination.

In conclusion, vaccination with *S. aureus* bacterin in the supramammary lymph node potentiated intramammary infusion of nisin for the treatment of subclinical intramammary *S. aureus* infections in lactating cows. When compared to the nisin-treated group, nisin-bacterin treatment significantly reduced intramammary *S. aureus* infections, reduced the number of quarters with high milk SCCs, and increased milk protein and fat contents. The major limitation of this study is that it was carried out on a medium-sized herd. To prove the effectiveness of nisin-bacterin treatment, the method should be tested on a large herd in which subclinical mastitis gives rise to high bulk milk SCCs over the upper limit.

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Compliance with ethics guidelines

Ran GUAN, Jun-qiang WU, Wei XU, Xiao-yan SU, and Song-hua HU declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：疫苗接种与乳酸链球菌素 Z 治疗对泌乳期奶牛乳腺感染金黄色葡萄球菌的疗效研究

目　的：评价联合应用金黄色葡萄球菌疫苗接种和乳酸链球菌素 Z 治疗对乳腺隐性感染金黄色葡萄球菌奶牛的治疗效果。

创新点：首次证明了金黄色葡萄球菌疫苗接种和乳酸链球菌 Z 治疗对泌乳期奶牛乳腺金黄色葡萄球菌感染的治疗效果。

方　法：将 75 头乳腺隐性感染金黄色葡萄球菌的泌乳期黑白花奶牛随机分为 3 组：金黄色葡萄球菌疫苗接种+乳酸链球菌素治疗组、乳酸链球菌素治疗组和不治疗对照组。在治疗前和治疗后 2、4 和 6 周，对无菌采集感染乳区奶样进行细菌学检查、牛奶体细胞计数、*N*-乙酰-β-D-氨基葡萄糖苷酶（NAGase）活性检测和牛奶品质（乳脂率和蛋白率）检测。

结　论：和乳酸链球菌素单独治疗比较，金黄色葡萄球菌疫苗接种联合乳酸链球菌素治疗显著降低了感染乳腺和高体细胞数乳腺的数量。因此，金黄色葡萄球菌疫苗接种联合乳酸链球菌素治疗对乳腺感染金黄色葡萄球菌奶牛的治疗效果优于乳酸链球菌素单独治疗。

关键词：隐性乳房炎；乳腺感染；金黄色葡萄球菌；菌苗；乳酸链球菌素