

THE POTENTIAL ROLE OF ANTIMICROBIAL PROTEINS IN THE TREATMENT OF BOVINE MASTITIS

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INTRODUCTION

The intramammary administration of antibiotics is the most common method used to treat bovine mastitis (1). Antibiotic treatment during lactation has a low cure rate for many mastitis pathogens, and the loss of milk due to drug residues results in a poor cost-benefit ratio for most antibiotic therapy during lactation (2). In addition, there is an increasing concern over the presence of drug residues in milk (3), which has led to the search for alternatives the classical antibiotic approach (4,5,6,7,8). The bacteriocins, lysostaphin (9) and nisin (10), are effective toward mastitis pathogens. These are nontoxic proteins, digested and inactivated by intestinal enzymes, and thus, are potentially less hazardous than the antibiotics currently available for use in dairy cattle (6).

This study examined the efficacy of several lysostaphin and nisin combinations for the treatment of experimental bovine mastitis infections by *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus uberis*.

MATERIALS AND METHODS

Holstein cows at 60 to 120 days lactation were infected either by intramammary inoculation with the target organisms or by selecting infected cows from teat dip trials based on isolation of these organisms from IMI. In all cases, cows were infected for a mean of 30 days with a range of 14 to 105 days prior to treatment.

Treatment and Sampling Protocol

Cows were sorted according to infectious organisms and randomly placed in treatment groups. In cases of multiple infections within a cow, all quarters were treated with the same dosage. Intramammary infusions of experimental formulations were made following each of 3 consecutive milkings (PM, AM, PM) and milk samples were collected for culture after each milking starting 48 hours prior to treatment and post-treatment through day 7, once daily through day 14, and 3 samplings around day 21. Milk was collected daily for the first 14 days and at day 21 for evaluation of somatic cell counts.

The antimicrobial peptide intramammary infusion used a highly purified preparation of nisin (AMBICIN N[®]: Applied Microbiology, Inc, New York, NY) in combination with a preparation of recombinant lysostaphin, (AMBICIN L[®]: Applied Microbiology, Inc). Each treatment was administered by intramammary infusions in a 60 ml aqueous solution. Cows were observed for changes in milk and gland appearance at each milking for the first 48 hours following treatment.

RESULTS

The cows did not exhibit any abnormal physiological reactions following the treatments. Intramammary infections were characterized as subclinical and remained subclinical throughout the

trials.

Table 1. *In vitro* bactericidal activity of lysostaphin and nisin toward *Staphylococcus aureus*

Lysostaphin (ug/ml)	Nisin (ug/ml)					
	0	0.2	0.5	1.0	2.0	4.0
0	100	45	33	9.0	2.5	0.5
0.1	43	0.7	2.6	0.15	0.04	0.004
1.0	<10 ⁻³	<10 ⁻⁴	-	-	<10 ⁻⁴	-

* Initial viable count: 5x10⁷; incubation in milk for 2h at 37°C

Lysostaphin combined with nisin shows synergistic *in vitro* bacteriocidal activity toward *S. aureus* (Table 1). Intramammary infusion of combinations of lysostaphin with nisin produced rapid reduction of *S. aureus* titers in all treated quarters with a return of some refractory infections by 5 days post-treatment (Figure 1). Not all quarter milk samples cultured positive for *S. aureus* after treatment in the cases where treatment failed. However, true cures could be determined by multiple cultures over the observed period.

Combinations of lysostaphin with nisin infused into the infected glands also showed a marked reduction of *Str. agalactiae* resulting in the elimination of the infection (Figure 2).

Figure 1. The percentage of glands cultured positive for *Staphylococcus aureus* after intramammary treatment with aqueous lysostaphin combined with nisin

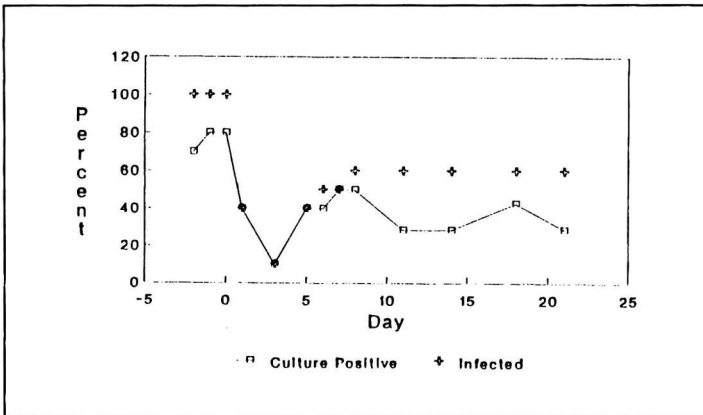
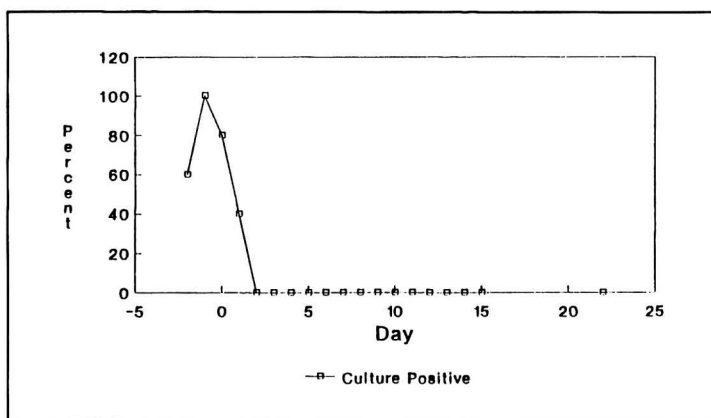


Figure 2. The percentage of glands cultured positive for *Streptococcus agalactiae* after intramammary treatment with aqueous lysostaphin combined with nisin



Spontaneous elimination of experimentally infected *S. aureus* in the first 14 days of the established infections were 2/26 quarters (7.7%). The cure rates for lysostaphin combined with nisin are shown in Table 2 with the elimination of 19/29 quarter infections (66%) for *S. aureus*. The experimental treatment was successful in eliminating 35/37 infected quarters (95%) for *Str. agalactiae*. Of the two quarters which did not respond, infections were eliminated on subsequent retreatment. Although the number of experimental *Str. uberis* infections were limited, response to lysostaphin combined with nisin resulted in the elimination of 6/6 quarter infections.

Table 2. Treatment of bovine mastitis by intramammary infusion of combination of nisin and lysostaphin in aqueous solution

Target	Glands	Cures	Percent cures
<i>Staphylococcus aureus</i>	29	19	66
<i>Streptococcus agalactiae</i>	37	35	95
<i>Streptococcus uberis</i>	6	6	100

DISCUSSION

These studies using aqueous infusions of lysostaphin with nisin have demonstrated efficacy toward *S. aureus*, *Str. agalactiae*, and *Str. uberis* experimental infections. All quarters were infected for a mean of 30 days (range of 14 to 105 days) prior to treatment. There was no correlation between the number of days infected and the cure rate. Following treatment, a significant reduction of somatic cells was observed in glands cured by microbiological criteria.

Nisin and lysostaphin have complimentary and synergistic bactericidal activities towards major mastitis pathogens. These bacteriocins are active against both dividing and nondividing bacteria. They

are nontoxic proteins inactivated by digestive enzymes in the intestine and should pose no hazard in the milk supply. The clinical evaluations indicate that, appropriately formulated, these antimicrobial agents have significant potential for use in the treatment of mastitis infections.

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SUMMARY

Two antimicrobial proteins were used in the treatment of *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus uberis* intramammary infections of lactating dairy cows. AMBICIN N[®], a highly purified preparation of the antimicrobial peptide Nisin, and AMBICIN L[®], a recombinant lysostaphin used in aqueous infusion, were effective in reducing intramammary infections. Intramammary infections were confirmed by consecutive daily isolation of the microorganism from milk cultures. All treated quarters were confirmed infected within 48 hours of treatment. Each infected gland received 3 intramammary treatments at 12 hour intervals with combinations of lysostaphin with nisin. Cure was defined as consecutive daily negative culture of milk samples collected through day 14 and at day 21. Isolation of the target organism between day 3 - 14 or at day 21 defined scored as a treatment failure. For quarter infections, cure rates were 19/29 (66%) for *S. aureus*, 35/37 (95%) for *Str. agalactiae*, and 6/6 (100%) quarters for *Str. uberis*. Nisin and lysostaphin are nontoxic proteins inactivated by digestive enzymes in the gut and their residues should pose no hazard in the milk supply. This experimental trial suggests that, appropriately formulated, these antimicrobial proteins could be useful in the treatment of bovine intramammary infections.

RESUMEN

Dos proteínas antimicrobianas fueron usadas en vacas lecheras lactantes para el tratamiento de infecciones intramamarias causadas por *Estafilococo aureus*, *Estreptococo agalactiae* y *Estreptococo uberis*. AMBICIN N, una preparación altamente purificada del péptido antimicrobiano Nisina, y

AMBICIN L, una lisostafina recombinante usada en infusión acuosa, fueron efectivas en la reducción de infecciones intramamarias. Estas infecciones fueron confirmadas mediante el aislamiento diario y consecutivo de los citados microorganismos de las muestras de leche. Todos los cuartos mamarios infectados fueron confirmados como tales dentro de las 48 horas posteriores a la exposición. Combinaciones de lisostafina y nisina fueron usadas en el tratamiento de las infecciones, y cada glándula infectada recibió 3 infusiones intramamarias administradas a intervalos de 12 horas. La cura debida al tratamiento fue definida como cultivos negativos obtenidos de muestras de leche tomadas diariamente hasta el día 14, y posteriormente en el día 21. El aislamiento de los organismos utilizados en la producción de infecciones entre los días 3 y 14 ó en el día 21 fue definido como fracaso del tratamiento. Al considerar las infecciones en los cuartos mamarios, las tasas de curación fueron 19/29 (66%) para *Staf. aureus*, 35/37 (95%) para *Estrep. agalactiae*, y 6/6 (100%) para *Estrep. uberis*. Lisostafina y nisina son proteínas atóxicas inactivadas por enzimas digestivas en los intestinos y sus residuos no representan peligro alguno en la provisión de leche. Este estudio experimental sugiere que, formuladas apropiadamente, estas proteínas antimicrobianas podrían ser útiles para el tratamiento de infecciones intramamarias en los bovinos.

ZUSAMMENFASSUNG

Für die Behandlung von Euterinfektionen laktierender Kühe durch *Staphylococcus aureus*, *Streptococcus agalactinae* und *Streptococcus uberis* wurden zwei antimikrobielle Peptide, Bacteriozine, verwendet. AMBICIN N[®], ein Reinpräparat des antimikrobiellen Peptides Nisin, und AMBICIN L[®], ein rekombinantes Lysostaphin, welches in wässriger Lösung verwendet wird, führten zu verminderter intramammärer Infektion. Die Euterinfektionen, welche durch intramammäre Infusion oder durch Zitzenbehandlung mit den pathogenen Erregern erzielt wurden, konnten durch Isolation der Erreger aus der Milch täglich zwischen 14 und 105 Tagen nach der Infektion (Mittelwert: 30 Tage) nachgewiesen werden. In allen infizierten Vierteln konnten innerhalb von 48 Stunden nach der experimentellen Infektion pathogene Erreger nachgewiesen werden. Jedes infizierte Euter wurde dreimal im Abstand von 12 Stunden intramammär mit einer Kombination von Lysostaphin und Nisin behandelt. Die verschiedenen Behandlungen wurden nachfolgend auf ihren Effekt geprüft. Heilung wurde als mehrere täglich aufeinanderfolgende negative bakterielle Untersuchungen der Milch bis zum 14. Tage p.i. so wie ein negatives Resultat am 21. Tage p.i. definiert. Die Isolation der gesuchten Bakterien während der Zeit vom 3. bis zum 14. Tage oder am 21. Tage wurden als misslungene Behandlung angesehen. In Bezug auf Euterviertel war die Heilungsrate 19/26 (66%) für *Staph. aureus*, 35/37 (95%) für *Str. agalactinae*, und 6/6 (100%) für *Str. uberis*. Diese Bacteriozine sind nicht-toxische Proteine, welche durch Verdauungsenzyme im Darm inaktiviert werden. Ihre Residuen in der Milch sind als harmlos zu betrachten. Diese Experimente zeigen, dass die erwähnten antimikrobiellen Proteine, mit fachgemässer Anwendung, in der Behandlung boviner infektiöser Euterentzündungen eingesetzt werden können.