Evaluation of a Nisin-Based Germicidal Formulation on Teat Skin of Live Cows

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ABSTRACT

A purified preparation of the nontoxic antimicrobial peptide, nisin (AMBICIN N®), was used in the formulation of a germicidal sanitizer suitable for use on cow teats. The germicidal activity of the formulation against mastitis pathogens was measured on teat skin of live cows. The nisin-based formulation gave a mean log reduction of 3.90 against *Staphylococcus aureus* and 4.22 log reduction against *Escherichia coli* after exposure for 1 min to the germicide. This activity was comparable with that exhibited by a 1% iodophor teat dip but was significantly greater than that exhibited by the .1 and .5% iodophors and by the .5% chlorhexidine digluconate teat dips. The nisin-based formulation showed little or no potential for skin irritation after multiple application to skin, but iodophor and chlorhexidine digluconate teat dips showed significant potential for skin irritation in comparable studies.

(**Key words**: premilking, germicide, antimicrobial peptide, nisin)

INTRODUCTION

New IMI are the consequence of bacteria gaining access to the mammary gland between milkings (2, 6) and the transfer of pathogens either between quarters within a cow or between cows at milking time (3, 6, 11). Prevalence of IMI in a herd is determined by the rate and the duration of the IMI (2, 5). Postmilking teat dipping is an effective method for reducing the prevalence of contagious organisms (*Staphylococcus aureus* and *Streptococcus agalactiae*), and premilking teat dipping is effective in reducing the incidence of IMI caused by environmental pathogens. The use of premilking teat dipping has been shown to be effective in reducing new IMI (9, 12), but the risk of milk supply contamination increases when the germicide is not completely removed before milking (9, 10).

The evaluation of a germicide's potential for use as a teat sanitizer prior to milking needs to consider before field efficacy evaluation 1) the germicide's ability to exhibit broad and rapid activity toward mastitis pathogens on the teat, 2) the formulation's potential for skin irritation, and 3) its potential for introducing hazardous residues into milk under recommended use. The purpose of this study was to evaluate the potential of a nisin-based germicidal formulation for use as a teat sanitizer.

Nisin is a nontoxic antimicrobial peptide. A preparation of nisin (Nisaplin; Aplin and Barrett, England) has been used worldwide as a preservative for cheeses and canned goods (7). Potentially, nisin is a safe alternative to more traditional chemical germicides, such as iodines and chlorhexidines. Nisin in a germicidal sanitizer that is formulated for use before milking could lower the risk of introducing poten-

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tially hazardous germicide residues in milk.

A nisin-based formulation has been developed that has rapid and broad-spectrum bactericidal activity toward a range of Gram-positive and Gram-negative pathogens (1). The nisin-based test formulation evaluated as a teat sanitizer also contained sufficient 1-propanol to promote rapid drying on teat skin without causing irritation. The germicidal activity of this formulation was evaluated on live teat skin of dairy cows against mastitis pathogens and compared with the activity of conventional iodophor-based and chlorhexidine digluconate-based teat sanitizers of accepted performance.

MATERIALS AND METHODS

A highly purified preparation of nisin was supplied as AMBICIN N® (Applied Microbiology, Inc., New York, NY) and was suitable for use in formulation of a germicide.

Germicidal Teat Dips

The following germicidal teat dips were compared for bactericidal activity on live teat skin: .1% iodophor (QUARTER MATE®; West Agro, Kansas City, MO) and .5% iodophor (THERATEC™; Babson Bros., Naperville, IL); 1% iodophor (Teat Guard™; Ecolab, Inc., St. Paul, MN) and .5% chlorhexidine digluconate (Della-Dip™; Alfa-Laval, Inc., Kansas City, MO); and AMBICIN N®, a 1-propanol 16.1% test formulation now commercially available (CONSEPT®, Babson Bros.).

Germicide Quencher Solutions

Bacto-letheen broth (Difco Laboratories, Detroit, MI), modified to contain 1% sodium thiosulfate, was used to inactivate the iodine- and chlorhexidine-based germicides as previously recommended for excised teat trials (13). Neutralizing buffer (50 m*M* Tris·HCl, pH 7.8, 5 m*M* $MgSO_4$, 20 m*M* $CaCl_2$, .1 *M* NaCl, and .1% gelatin) was used to neutralize the activity of nisin-based formulations [(1); Blackburn et al., 1989, Applied Microbiology, Inc., unpublished observation].

Bacterial Strains

Bacterial strains used as challenge organisms during the trial were *Staph. aureus* (ATCC 29740), *Strep. agalactiae* (Cornell 48), *Streptococcus uberis* (ATCC 27958), *Klebsiella pneumoniae* (field strain), and *Escherichia coli* (field strain). Pure suspensions were prepared for all organisms as previously reported (13) except that the final challenge suspensions were made in .1% proteose-peptone broth (Difco Laboratories). A sufficient number of frozen ampules were prepared so that a new culture was available for each trial. A frozen 1-ml ampule was thawed, added to a 6-ml tube of trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD), and incubated aerobically for 5 to 7 h at 37°C. A purity check of the 6-ml tube was made by streaking .01 ml onto trypticase soy agar containing 5% sheep blood and .1% esculin (BBL Microbiology Systems) and examined after incubation for 18 h at 37°C. The 6-ml culture was used to inoculate 200 ml of trypticase soy broth, which was then incubated aerobically overnight (16 to 18 h) at 37°C. The cells were harvested by centrifugation, washed twice with .1% proteose-peptone broth (Difco Laboratories), and resuspended to the original volume in .1% proteose-peptone broth. A purity check was made of the final challenge suspensions, and the suspensions were refrigerated overnight at 4°C. On the following morning, the challenge suspensions were used to inoculate teats for evaluation of germicidal activity of test solutions.

Evaluation of Germicidal Activity on Live Teat Skin

Lactating dairy cows at the Teaching and Research Center, Cornell University, were used for the study. Udder hairs were clipped when necessary. Iodine udder wash (Iodophor II®; Ecolab, Inc.) was diluted to 25 ppm of iodine in warm water and applied with disposable paper towels to the underside of the udder and to the entire teat surface until all debris was removed. Once washed clean, the teats were dried with single-use paper towels. Gauze pledgets moistened in 70% alcohol were used to encircle the teat until the pledget remained white after application to ensure that the surface was free of iodine residues. Each teat was submerged in 70% isopropanol and allowed to air dry for 5 min.

All teats were dipped with a suspension (10^8 cfu/ml) of the test organism approxi-

mately 15 mm above the teat orifice and allowed to air dry for 10 min. Following this exposure to the mastitis pathogen, the two front teats were dipped up to 30 mm from the teat orifice with either the experimental nisin-based formulation or with a positive control germicidal teat dip. A timer was set for 1 min as the first front teat was dipped with germicidal solution, a 15-s lag followed, and then the second front teat was dipped. The two hind teats were undipped negative controls. After the 1-min exposure to a germicide, the treated teats were rinsed with the quencher appropriate for the germicide. The rinsing was performed by applying 10 ml of the quencher from a 12-ml syringe, starting 15 to 20 mm up from the teat orifice and angling the syringe to allow the quencher to cover the teat surface and to run downward, covering the teat surface. The rinse was collected into a sterile container held directly under the teat. The same quencher and procedure were used on the untreated teats to provide negative controls. Immediately following sample collection, the teats were immersed in .5% iodophor teat dip.

The recovered rinse was placed on ice and returned to the laboratory. One hundred microliters of rinse (undiluted and 10^1, 10^2, and 10^3 dilutions) were plated in duplicate on trypticase soy blood agar containing esculin. Plates were incubated aerobically at 37°C for 24 to 48 h prior to determination of colony-forming units. *Escherichia coli* and *K. pneumoniae* cultures were identified by colony morphology on MacConkey agar (BBL Microbiological Systems) and by the API20E System (Analytab Products, Plainview, NY). Streptococci were identified by colony morphology, hemolytic patterns, CAMP reaction, and presence or absence of esculin hydrolysis. *Staphylococcus aureus* was identified by colony morphology, hemolytic patterns, and the tube test for free coagulase.

Evaluation of Potential for Skin Irritation Test

Studies were performed with the test formulation of nisin equal to and 12 times the concentration intended for use in the germicidal evaluation and with commercial teat dips of 1% iodophor (Teat Guard™; Ecolab, Inc.) and .5% chlorhexidine digluconate (Della-Dip™; Alfa-Laval, Inc.) to determine their potential for skin irritation on intact and abraded rabbit skin according to Draize score irritation (4). Six rabbits were used for each product tested. A sample of .5 ml of each test solution was applied to each 2.5-cm square, shaved, intact or abraded skin site, and the rabbit trunk was encased with impermeable occlusive wrapping between applications. The wrapping was removed after 16 h of exposure, and the sites were scored for presence of erythema and edema. After scoring, the rabbits were treated again and rewrapped. Scoring for irritation and application of product were performed daily for 6 d except in cases in which irritation was severe; in those cases, for humane reasons, the study was terminated for those products. The final examination for irritation was conducted 72 h after the last treatment. The criteria for scoring dermal irritation were the degree of erythema or eschar (redness or scab formation) and edema (swelling) as directed in Title 16, Consumer Product Safety Commission, *Code of Federal Regulations* 1500.41 (4).

Statistical Analyses

The raw data consisted of the mean of the duplicate plate counts for each collection expressed as bacterial colony-forming units per milliliter of rinse recovered from live cow teats. Raw data were transformed to logarithm base 10 prior to data manipulation or analysis. Logarithm counts for negative control quarters were averaged within each cow as were log counts for the experimental or positive control quarters. The difference between these two values (log reduction resulting after application of germicide) was calculated for each cow.

Log reduction data from 13 treatment combinations were analyzed with SAS GLM (14) in two separate groups. The nisin-based germicide was evaluated against the five mastitis pathogens previously described. *Staphylococcus aureus* and *E. coli* were used as target organisms to test the four commercial products.

Treatment means were separated using the Waller-Duncan Bayesian k-ratio test (15). This test is a multiple comparison procedure that is based on minimizing Bayesian risk rather than controlling Type I error. The selected value of k, where k = seriousness of Type I error divided by seriousness of Type II error, determines the level of conservativeness of the test.

A k ratio of 100 corresponds roughly to $P = .05$. Significant differences are also dependent on ANOVA main effect F values; lower F values result in more conservative mean separation.

RESULTS

The potential for skin irritation of teat sanitizers was evaluated on intact and abraded rabbit skin according to Draize test scores, as indicated in Table 1. The nisin-based formulations showed little or no potential for irritation. By contrast, irritation potential was shown for the 1% iodophor and the .5% chlorhexidine digluconate preparations. The evaluation of the skin irritation by 1% iodophor was terminated prematurely because of severe irritation.

Data demonstrating the germicidal activity of the nisin-based formulation on live cow teat skin against five major mastitis pathogens are summarized in Table 2. The mean log reduction for bacteria on intact skin was greater than 3 logs against *Staph. aureus, Strep. agalactiae, Strep. uberis, K. pneumoniae,* and *E. coli* after a 1-min exposure to the nisin formulation. The germicidal activity of the nisin-based formulation was similar among pathogens as shown by a nonsignificant ANOVA F test ($P = .42$), resulting in a single Waller-Duncan grouping (Table 2).

The germicidal activity of the nisin-based formulation against *Staph. aureus* and *E. coli* was compared with each of four commercial products: three different iodophor concentrations and .5% chlorhexidine digluconate. The difference in performance between the germicidal treatments was highly significant (ANOVA F test, $P = .0001$). The log reduction data for the nisin-based formulation and the commercial teat dips toward *Staph. aureus* and *E. coli* are summarized in Tables 3 and 4. Germicidal activity was significantly less ($P < .05$) with the lower iodophor concentrations of .1 and .5% against *Staph. aureus* and *E. coli.* The performance of .5% chlorhexidine digluconate was more effective ($P < .05$) than the .1% and .5% iodophors but significantly less effective ($P < .05$) than the 1% iodophor and the nisin-based formulation against *Staph. aureus.* Against *E. coli,* chlorhexidine digluconate was equally effective as the .1% iodophor but significantly ($P < .05$) less effective than all the other formulations. The 1% iodophor teat dip provided germicidal activity against *Staph. aureus* and *E. coli* equivalent to that of the nisin-based formulation.

TABLE 1. Comparative skin irritation to rabbits caused by exposure to teat dips.

	Dermal irritation scores[1]	
	Single application[2]	Multiple application[3]
AMBICIN N® (1×)[4]	.21	.3
AMBICIN N® (12×)[5]	.09	.04
1% Iodophor	.5	3.34[6]
.5% Chlorhexidine digluconate	.38	2.34

[1]Interpretation of irritation scores: <1.0, the product has little or no potential for irritation; 1.0 to 1.9, the product has the potential for mild irritation; 2.0 to 2.9, the product has the potential for moderate irritation; 3.0 to 4.9, the product has potential for severe irritation: >5.0, the product is considered to be highly dangerous.

[2]Mean irritation scores from intact and abraded sites of six rabbits at 72 h after the application of teat dip.

[3]Mean irritation scores from intact and abraded sites of six rabbits at 72 h after the last of seven daily teat dip applications.

[4]1× indicates test formulation equal to the concentration intended for use in germicidal evaluation.

[5]12× indicates test formulation 12 times the concentration intended for use in germicidal evaluation.

[6]Skin flaking and peeling observed.

DISCUSSION

Germicides have been formulated to kill bacterial pathogens on teat skin surfaces to reduce the number of IMI. Low concentration iodophors are recommended for use prior to milking to reduce the chances of the milk supply contamination with germicide residues left on the teat (8).

An excised-teat protocol was developed as a screening method to assess the germicidal activity of postmilking teat dips (13). Historically, a 1% iodophor teat dip and 10 to 15 min of exposure to the germicide have been considered as an appropriate positive control for evaluation of the germicidal activity of postmilking teat dips. The studies reported herein attempt to assess the germicidal activity of a range of commercially available teat dips and an experimental nisin-based formulation after

TABLE 2. Evaluation of AMBICIN N® germicidal activity after 1 min of contact with five major mastitis pathogens when used as a premilking teat dip on live teat skin.

Pathogen	Teats treated	Log negative control[1]	Log reduction (LR)[2]	Percentage of LR[3]	Waller-Duncan grouping[4]
	(n)				
Staphylococcus aureus (ATCC 29740)	20	6.32	3.90	61.77	a
Streptococcus agalactiae (Cornell 48)	15	4.50	4.43	98.60	a
Streptococcus uberis (McDonald ATCC 27958)	20	5.50	3.68	67.10	a
Klebsiella pneumoniae (field strain)	10	5.22	4.00	76.49	a
Escherichia coli (field strain)	18	4.94	4.22	85.51	a

[1]Log negative control = Mean log colony-forming units per milliliter recovered from negative control teats and averaged over cow.

[2]LR = Log colony-forming units per milliliter negative control minus log colony-forming units per milliliter treated teats.

[3]Percentage LR = 100 (LR) + log negative control.

[4]Waller-Duncan grouping = Waller-Duncan Bayesian k ratio test with k = 100.

1 min of exposure to the germicides on teat skin of live cows.

Reproducible results are difficult to obtain in a germicidal evaluation on live cows with less than 1 min of exposure time. The evaluation of germicidal activity on teat skin after 1 min of contact time provides a rapid method for screening the germicidal activity of test formulations that are potentially useful as teat sanitizers prior to milking.

Because cows are exposed to a germicide two to six times daily, a skin irritation evaluation should be considered in the evaluation of the performance of a potential teat sanitizer. The nisin-based formulation produced extremely low skin irritation scores but had a germicidal activity on teats equivalent to a commercial 1% iodophor teat dip. The performance of the nisin-based test formulation indicated that it should be evaluated further for efficacy as a teat sanitizer to reduce new IMI in field trials.

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TABLE 3. Evaluation of the germicidal activity of AMBICIN N® and four other commercial teat dips after 1 min of contact with *Staphylococcus aureus* (ATCC 29740).

Teat dips	Teats treated	Log negative control[1]	Log reduction (LR)[2]	Percentage of LR[3]	Waller-Duncan grouping[4]
	(n)				
AMBICIN N®	20	6.32	3.90	61.77	a
1% Iodophor	20	6.11	3.32	54.31	a
.5% Iodophor	20	6.50	1.24	19.10	c
.1% Iodophor	19	6.32	1.14	18.10	c
.5% Chlorhexidine digluconate	10	6.08	2.14	35.20	b

[1]Log negative control = Mean log colony-forming units per milliliter recovered from negative control teats and averaged over cow.

[2]LR = Log colony-forming units per milliliter negative control minus log colony-forming units per milliliter treated teats.

[3]Percentage LR = 100 (LR + log negative control).

[4]Waller-Duncan grouping = Waller-Duncan Bayesian k ratio test with k = 100.

TABLE 4. Evaluation of the germicidal activity of AMBICIN N® and four other commercial teat dips after 1 min of contact with *Escherichia coli* (field strain) on live cow teat skin.

Teat dips	Teats treated	Log negative control[1]	Log reduction (LR)[2]	Percentage of LR[3]	Waller-Duncan grouping[4]
	(n)				
AMBICIN N®	18	4.94	4.22	85.51	a
1% Iodophor	20	5.01	3.76	74.97	ab
.5% Iodophor	18	5.37	3.37	62.83	bc
.1% Iodophor	18	5.99	2.83	47.31	cd
.5% Chlorhexidine digluconate	10	5.81	2.35	40.99	d

[1]Log negative control = Mean log colony-forming units per milliliter recovered from negative control teats and averaged over cow.

[2]LR = Log colony-forming units per milliliter negative control minus log colony-forming units per milliliter treated teats.

[3]Percentage LR = 100 (LR + log negative control).

[4]Waller-Duncan grouping = Waller-Duncan Bayesian k ratio test with k = 100.

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