Mastitis: II. Evaluation of Antimicrobial Amines for Use as Teat Dips

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ABSTRACT

Recent proposals by the Food and Drug Administration to regulate teat dips as drugs have led to a search for safer teat dip ingredients. Primary, secondary, and tertiary alkyl amines (carbon-10 to -18 chain length) inhibit growth of mastitic bacteria (Streptococcus agalactiae, Streptococcus uberis, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae) in a broth tube culture assay. Since carbon-13 compounds were active, a carbon-13 primary (tridecanamine hydrochloride), secondary methyl (N-methyltridecanamine), secondary ethyl (N-ethyltridecanamine), tertiary dimethyl amine (N, N-dimethyltridecanamine), and carbon-12 quaternary amine (N, N, -trimethyl-dodecaneammonium chloride) were tested for their ability to reduce experimentally applied populations of S. agalactiae or E. coli on the bovine teat surface. The five compounds were compared at concentrations of 100, 500, 1,000, 3,000, 7,000, and 10,000 ppm. Activity was greater against the gram-positive S. agalactiae than against the gram-negative E. coli. The tertiary amine was most active, producing a log reduction of 4 (reduction of bacterial number from $10^6$ to $10^2$) at a concentration of 3,000 ppm in the teat dip. The relative order of effectiveness for the amines was: dimethyl tertiary > methyl secondary > ethyl secondary > primary = quaternary. The results suggest that these amines may be useful as potent, effective antibacterial agents for incorporation into teat dips.

INTRODUCTION

Economic losses from mastitis in the United States have recently been estimated to cost $1.294 billion annually (2). Routine postmilking teat dipping, adopted within the last 10 yr, has been an effective control measure. Commercial teat dips generally contain chlorides, iodides, or bromides. They are used widely to inactivate microorganisms in the distal part of the teat canal and on the teat end, thus lowering bacterial numbers available for invasion and entry into the teat canal (6, 8). On August 9, 1977, because of possible health hazards of teat dip ingredients, the Food and Drug Administration (FDA) (5) proposed that teat dips be classified as new animal drugs. The proposal characterized currently marketed teat dips as inefficient and variable in action, irritating to the animal’s skin, and presenting the possibility of contaminating dairy products with harmful residues. As new animal drugs, teat dips would be required to undergo extensive testing and approval by the FDA prior to their release for marketing.

Our interest in more effective and safer teat dip ingredients led us to test 62 branched and straight chain alkyl amines (10 to 18 carbons chain length) for their ability to inhibit growth of five microorganisms that cause about 95% of bovine mastitis (4). These primary, secondary, and tertiary amines were tested in an in vitro broth tube culture assay. Compounds with 12 to 13 carbon atoms were most active against both gram-positive (S. agalactiae, S. uberis, S. aureus) and gram-negative (E. coli, K. pneumoniae) bacteria.

The Teat Dip Committee of the National Mastitis Council has proposed protocols for three types of controlled trials which may be useful in the evaluation of a teat dip (9). Protocol A measures efficacy in reducing...
bacterial numbers on teat surfaces, Protocol B measures efficacy of a teat dip in reducing infections during experimental exposure to mastitis pathogens, and Protocol C measures efficacy of a teat dip in reducing naturally occurring new intramammary infections. The objective of the present study was to screen the most active C13 amines under Protocol A conditions as a first step in the formulation of a teat dip based upon these amines. A C13 primary, secondary, and tertiary amine and a C12 quaternary amine were tested for their ability to reduce experimentally applied populations of bacteria on the bovine teat surface.

**EXPERIMENTAL PROCEDURES**

**Test Compounds**

Four C13 compounds were selected for use in the in vivo Protocol A assay based upon the results of the in vitro study (4): a C13 primary (tridecanamine hydrochloride-compound 1), secondary methyl (N-methyltridecanamine-compound 2), secondary ethyl (N-ethyltridecanamine-compound 3), tertiary diethyl amine (N, N-dimethyltridecanamine-compound 4). In addition, a frequently used quaternary ammonium disinfectant (N, N, N-trimethyldodecanammonium chloride-compound 5) was included to provide comparison. Stock solutions of the selected compounds were prepared at a concentration of 150 mg/ml with absolute ethanol as the solvent. Immediately before use, dilutions were made with Butterfield’s phosphate diluent (BPD) (11) to yield test solutions containing 100, 500, 1,000, 3,000, 7,000, or 10,000 ~g/ml.

The primary, secondary, and tertiary amines were synthesized in our laboratory as in (4). The quaternary amine, N, N, N-trimethyldodecanammonium chloride, was purchased from Eastman Organic Chemicals, Rochester, NY 14650.

**Preparation of the Challenge Suspension**

Two representative species of mastitic pathogens were used in the in vivo study: S. agalactiae, ATCC #27956, and E. coli, Cornell 48-1, gram-positive and gram-negative, respectively. Stock cultures were maintained in refrigerated trypticase soy agar (TSA) deeps. The starter culture was prepared by adding one loop of stock culture to 10 ml sterile trypticase soy broth (TSB). After incubation for 8 h at 37 °C to initiate growth, 1 ml of the starter culture was transferred to a 300 ml Erlenmeyer flask containing 200 ml sterile TSB. Incubation for 18 h yielded a concentration of about 8.5 × 10^8 organisms/ml for E. coli and about 4.5 × 10^8 organisms/ml for S. agalactiae. Dilutions used homogenized whole milk such that the challenge pathogen suspension contained about 10^8 organisms/ml.

**Test Animals and Experimental Design**

Lactating Holstein dairy cows fed a ration of grain, corn silage, and alfalfa hay were housed in individual stanchions. As lactations ended, animals were replaced so that at least 12 were always available. All experimental procedures were prior to the first milking of the day between 0830 and 1230 h.

The 48 teats were assigned randomly into two control groups of nine and three experimental groups of ten. In this manner, three concentrations of a given compound were tested against one organism per trial. One control group, B (nine teats), was used to determine the initial number of pathogens at the time of testing. Another control group, A (nine teats), served to determine the number of pathogens removed and/or killed by the vehicle in the teat dip testing procedure.

**In Vivo Germicidal Assay on Teat Skin Surface**

Teats of the test animals initially were scrubbed thoroughly with Septisol (75 hexachlorophene solution) to remove debris, rinsed with water, and dried with sterile 5.1 cm gauze pads. Immediately prior to testing, the teat surface was scrubbed with absolute ethanol to remove foreign bacteria and allowed to air dry. Both control and experimental teats were then dipped for 5 s into a suspension of the test organism to a depth that displaced a volume of 10 ml and were allowed to drain for 15 min. The control teats (B) then were dipped in 75 ml sterile BPD (30 1-s dips with a 5-s
To determine the initial number of pathogens on the teat surface, drainage periods between each 10 dips were exposed for 5 s (depth to displace 10 ml) to either the experimental test compound (E) or to the BPD alone (control group A).

After 15 min, to allow interaction between the test compound and pathogen, the experimental (E) and type A control teats were dipped in 75 ml sterile BPD as described above to remove the remaining pathogens. As a final step, all teats were scrubbed with absolute ethanol to remove any remaining pathogens. Recovery solutions were capped and stored at 5 C until processed in the laboratory, usually within 1 h.

In the laboratory, recovery samples were shaken to distribute the pathogens evenly, and 1 ml each was transferred to triplicate petri dishes. In dilution bottles containing 99 ml sterile BPD, dilutions of 1:100 were prepared and transferred in 1-ml quantities to triplicate petri dishes. Approximately 15 ml sterile melted TSA at about 43 C were added to the plates, swirled for even distribution of the organisms, and allowed to cool. When solidified, plates were overlaid with an additional 15 ml TSA to provide uniform counting conditions. After incubation for 24 h at 37 C, the number of colonies/plate was counted and used to calculate the number of colony forming units (CFU)/ml. Results were expressed as log reductions from the initial bacterial populations in a manner similar to that used by Philpot et al. (10). The following equation was used to calculate log reduction:

\[
\text{log reduction} = \log \frac{\text{CFU initially present (control B)}}{\text{CFU surviving after treatment (group E)}}
\]

**Statistical Methods**

The data were log reductions (LR) and examined as a factorial arrangement in a completely random design with four types of compounds vs. seven concentrations by the General Linear Models (GLM) procedure of the Statistical Analysis System (SAS) program (1). Observations for the two test organisms, S. agalactiae and E. coli, were analyzed separately.

<table>
<thead>
<tr>
<th>Compound number</th>
<th>Bacterial species</th>
<th>Formula</th>
<th>Concentration in µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Teidocainehydrochloride</td>
<td>CH₃(CH₂)₁₄NH₂·HCl</td>
<td>X (\times) 10,000</td>
</tr>
<tr>
<td>2</td>
<td>N-methyldodecanamine</td>
<td>CH₃(CH₂)₁₃NHCH₃</td>
<td>X (\times) 7,000</td>
</tr>
<tr>
<td>3</td>
<td>N-n-dimethyldodecanamine</td>
<td>CH₃(CH₂)₁₃N(CH₃)₂</td>
<td>X (\times) 5,000</td>
</tr>
<tr>
<td>4</td>
<td>N,N-n′-dimethyldodecaneammonium chloride</td>
<td>CH₃(CH₂)₁₃N(CH₃)₂Cl</td>
<td>X (\times) 3,000</td>
</tr>
<tr>
<td>5</td>
<td>N,N′-dimethyldodecaneammonium chloride</td>
<td>CH₃(CH₂)₁₃N(CH₃)₂Cl</td>
<td>X (\times) 1,000</td>
</tr>
</tbody>
</table>

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RESULTS AND DISCUSSION

Standardization of
In Vivo Assay

Initial trials indicated that the use of distilled water as a recovery vehicle was inefficient because of the destruction of organisms by the sudden change in osmotic pressure. The substitution of Butterfield's phosphate diluent (BPD) at physiologic pH 7.2 provided a much more efficient vehicle. Trial times were set at 15 min, as longer periods between application of compound and recovery caused a loss of organisms due to dehydration on the teat surface. Use of scrubbing techniques to recover organisms also proved ineffective in our hands because of the variability in the size and texture of the teat surface scrubbed, pressure and vigor used between successive teats, and loss of organisms trapped within the cotton fibers of the swab. Dipping teats 10 times for 1-s intervals in BPD removed approximately 90% of the organisms and an additional 20 dips recovered the remaining 10%. The method adopted consisted of 30 dips of 1 s each with drainage between each 10.

Approximately .1 ml of the milk inoculum containing either $2 \times 10^6$ total colony forming units of $E. coli$ or approximately $6 \times 10^5$ total colony forming units of $S. agalactiae$ remained on the teat surface following the 5-s inoculation dip. This represents the approximate number of bacteria at the beginning of each trial. Use of Butterfield's phosphate diluent (BPD) alone demonstrated an average log reduction of .87.
for *S. agalactiae* in 77 observations and an average of .84 for *E. coli* in 69 observations. This represents the reduction caused by the physical application of the vehicle to suspend the test compounds. A trial used 3 to 6% solutions of absolute ethanol in BPD. Recovery demonstrated average log reductions of .98 and .82, respectively, against *S. agalactiae* and *E. coli*. An average log reduction of .63 was obtained when the test pathogens were exposed to BPD alone within the same trial, thus discounting the ethanol used as a carrier solvent for the test compounds as significantly affecting experimental results.

**Effectiveness of the Amines in Reducing Bacterial Numbers on the Teat Surface**

The comparative effectiveness of the primary, secondary, tertiary, and quaternary amine compounds was determined against *S. agalactiae* and *E. coli* (Table 1). The five compounds were compared at concentrations of 100, 500, 1,000, 3,000, 7,000, and 10,000 ppm. Activity was greater against the gram-positive *S. agalactiae* than against the gram-negative *E. coli*. Graphic representations of the data are plotted along with the actual treatment means by compound for both organisms in Figures 1 to 5.

Of the various amines tested, the tertiary amine was most active, producing a log reduction of 4 (reduction of bacterial number from $10^6$ to $10^2$) at a concentration of 3,000 ppm in the teat dip. The relative order of effectiveness for the amines was: dimethyl tertiary > methyl secondary > ethyl secondary > primary = quaternary.

**Activity against Gram-Positive and Gram-Negative Bacteria**

The results in Table 1 and Figures 1 to 5 show greater activity of the amines against *S. agalactiae* than against *E. coli*. Differences among amines in the concentrations necessary to obtain maximum antibacterial effects are to be expected between gram-negative (*E. coli*) and gram-positive (*S. agalactiae*) bacteria. Although there appear to be no previous studies comparing amines as topical agents against gram-positive and gram-negative bacteria, Philpot et al. (10) demonstrated results similar to these with a quaternary ammonium compound. Their study reported log reductions of 5.09 and 2.12 for *S. agalactiae* and *E. coli*, respectively, from a .18% solution of quaternary ammonium compound (unspecified). They found greater activity against gram-positive organisms than against gram-negative species for iodophor and hypochlorite disinfectants as well. The differences in susceptibility between gram-positive and gram-negative organisms generally are attributed to differences in the structure of the cell wall. While both classes have an inner murein layer, gram-positive bacteria possess only a single outer layer composed mainly of teichoic acids. Gram-negative organisms possess three outer layers consisting of a phospholipid bilayer sandwiched between an inner layer of...
lipoprotein and an outer layer of lipopolysaccharide (7). The more complex cell wall of gram-negative organisms have been postulated to account for their superior resistance to heat, pressure, and many antibacterial agents.

Structure-Activity Relationships

Although the patterns of activity are the same for the amines tested against a particular organism, the relative magnitude of activity varied with chemical structure at a given concentration. The relative order of effectiveness suggested from the results is: dimethyl tertiary > methyl secondary > ethyl secondary > primary = quaternary for both S. agalactiae and E. coli. At a concentration of 3,000 µg/ml, the dimethyl substituted amine demonstrated an 85.2 and 92.1% greater log reduction against E. coli than the monoethyl and monomethyl substituted amines, respectively. Against S. agalactiae, the same concentration of the dimethyl substituted amine demonstrated 95.9% greater activity than the monoethyl substitution.

All of the substituted amines showed higher antibacterial activity at lower concentrations than the quaternary ammonium compound, N, N, N-trimethyldodecane ammonium chloride (DAC). In the trials with E. coli, a concentration 10,000 µg/ml of the DAC was necessary for a log reduction in the same range as demonstrated by the monomethyl and monoethyl substituted amines at 3,000 µg/ml. The dimethyl substituted amines produced 80.0 and 97.5% higher activity at 3,000 and 10,000 µg/ml, respectively, than DAC at 10,000 µg/ml.

In trials against S. agalactiae, a 10,000 µg/ml concentration of DAC was necessary to obtain results equal to those of any of the substituted amines at a concentration of 1,000 µg/ml. Concentrations of 3,000 and 10,000 µg/ml of N, N-dimethyltridecanamine demonstrated 87.4 and 95.6% greater log reductions, respectively, than the DAC at 10,000 µg/ml.

CONCLUSIONS

This study has demonstrated the effectiveness of the primary, secondary, and tertiary C13 amines in reducing the number of S. agalactiae and E. coli on teat surfaces. The N, N-dimethyltridecanamine was the most effective compound against the two organisms with the two secondary amines demonstrating less but similar activity. The three substituted amines demonstrated greater inhibitory activity and were active at much lower concentrations than dodecytrimethylammonium chloride, a quaternary ammonium disinfectant. The results suggest that these amines may be useful as potent, effective antibacterial agents for incorporation into teat dips.

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REFERENCES