Research Note

Antimicrobial Properties of the Chelating Agent EDTA on Streptococcal Bovine Mastitis Isolates

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ABSTRACT

To determine the efficacy of the chelating agent EDTA on microbial growth, separate cultures of two streptococcal bovine mastitis isolates, *Streptococcus agalactiae* and *Streptococcus uberis*, were exposed to known concentrations of EDTA. Bacterial cultures of $10^8$ CFU/ml were exposed to concentrations of EDTA ranging from 30 to 100 mM in an in-vitro-milk environment. Multiple replications of cultures exposed to EDTA were plated during a two-hour time course. A concentration of 100 mM EDTA resulted in a 90% reduction of *S. agalactiae* and a 99% reduction of *S. uberis*. Under these experimental conditions, EDTA treatments in cultures of both isolates exhibited from 1 to 2 log reductions suggesting that EDTA is a potentially effective antimicrobial against streptococcal isolates implicated in causing bovine mastitis.

Bovine mastitis is an inflammation of mammary glands resulting from injury, bruising, or more commonly from bacterial infection. Its symptoms may include redness, swelling, decreased appetite, and decreased milk production. Mastitis is the most costly and most common disease in the dairy industry (1, 5). A variety of bacteria have been implicated in causing bovine mastitis, but the majority of cases involve one of five species, three of which are *Streptococcus* species (8). Current antibiotic therapies to prevent/treat streptococcal bovine mastitis, such as penicillin G, erythromycin, and pirlimycin have several disadvantages among which are their effects upon host body defenses, development of antibiotic-resistant bacterial strains, and the potential for antibiotic carryover in dairy products consumed by the public (3, 4, 15).

A chelating agent is a chemical compound that coordinates with a metal to form a chelate, which is a different compound whose molecules contain a closed ring of atoms, one of which is a metal atom. Chelating agents have shown various degrees of antimicrobial effectiveness, either by themselves or in combination with other antimicrobial agents (2, 13). Their biological activity has been attributed to their ability to bind free metal ions essential for bacterial metabolism and membrane stabilization (9, 14). As a chelating agent, EDTA has been demonstrated to exhibit antimicrobial properties against both Gram-positive and Gram-negative organisms (6, 7, 11). EDTA has shown limited, if any, toxicity in biological systems, although as with any antimicrobial, the concentration administered should be minimized as much as possible (14). Although EDTA may not work effectively against all bacterial strains, bacteria have not developed resistance to EDTA. Currently in the literature, there are no reports measuring the antimicrobial properties of EDTA against *Streptococcus* in an in-vitro-milk environment. The objective of this study was to measure the in-vitro-antimicrobial activity of EDTA in milk against two *Streptococcus* sp. mastitis isolates as a preliminary step in evaluating EDTA containing solutions as novel intramammary mastitis treatments.

MATERIALS AND METHODS

Isolation and identification of *Streptococcus* spp. Although no bacteria have developed any resistance to any chelating agents, it has been reported that different bacterial species have different antimicrobial susceptibilities to EDTA. Therefore, we chose to monitor two different *Streptococcus* species that are significant mastitis pathogens rather than numerous isolates within a species. *S. uberis* SMO 1015 and *S. agalactiae* SMO 886 were from routine submissions of bovine milk from dairies in Northern California archived in the mastitis collection of the Dairy Food Safety Laboratory (DFSL; Department of Population Health and Reproduction, University of California, Davis, Calif.). Pure cultures of each were established by selecting colonies from serial dilutions on 5% sheep blood agar plates, sub-culturing in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) and freezing on beads (Microbank, Pro-Lab Diagnostics, Austin, Tex.) at −70°C. To re-activate the bacteria, one bead was added to 10 ml TSB and incubated at 37°C for 24 h. These cultures were streaked for re-isolation onto 5% sheep blood agar, which were then incubated at 37°C for 24 h before being stored at 4°C. The identification of both isolates was confirmed using the API 20 Strep system (BioMérieux-Vitek Inc., Hazelwood, Mo.).
EDTA. The chelating agent used was disodium ethylenediaminetetraacetic acid (EDTA) (EM Science, Gibbstown, N.J.). Sterile solutions at pH 8.0 were prepared by dissolving EDTA in sterile distilled deionized water (ddH2O) and filtering through 0.45-μm filters (Nalge Nunc Intl., Rochester, N.Y.) to attain concentrations of 300, 400, 500, 700, 800, 900, and 1000 mM.

**Evaluation of EDTA as an antimicrobial.** Colonies of either *S. uberis* SMO 1015 or *S. agalactiae* SMO 886 on 5% sheep blood agar were each inoculated into 40 ml of TSB and incubated at 37°C for 24 h. Two milliliters of each culture were again inoculated into 40 ml TSB. The initial optical density at 580 nm was measured and the culture incubated at 37°C until balanced growth, as determined by a tenfold increase of the initial optical density, was attained (5 to 6 h). Based on corresponding growth curves comparing the optical density to CFU/ml, the typical culture reached approximately 10⁸ CFU/ml. Bacterial cells were harvested by three cycles of centrifugation at 2,500 rpm for 15 min followed by resuspension in 15 ml of 10 mM sodium phosphate buffer, pH 7.4. The bacterial suspension was adjusted to 1 to 2 x 10⁸ CFU/ml in the same buffer via comparison of the optical density at 620 nm of suspended bacteria with previously determined optical density versus CFU/ml growth curve data.

Ten microliters of the suspensions of either *S. uberis* SMO 1015 or *S. agalactiae* SMO 886 in buffer and ten microliters of pre-determined concentrations of EDTA in ddH2O were added to 80 μl ultra-high temperature (UHT) milk (Real Fresh Milk, distributed by Advanced Food Products LLC, Visalia, Calif.). The total volume of the incubation mixture was 100 μl, to attain a final bacterial concentration of 1 to 2 x 10⁵ CFU/ml and final EDTA concentrations ranging from 30 to 100 mM. Control incubation mixtures used in the determination of the log reduction of each variable were prepared by substituting 10 μl sterile ddH2O without EDTA along with the 10 μl of each bacterial concentration to 80 μl UHT milk. These mixtures were incubated at 39°C in a shaking water bath. Samples of 10 μl were taken at timed intervals of 0, 15, 30, 60, and 120 min and serially diluted into 990 μl of 10 mM sodium phosphate buffer. Samples of 50 μl were plated on sheep blood agar plates (Spiral Biotech, Norwood, Mass.;) in duplicate onto 5% sheep blood agar and incubated at 37°C for 48 h.

Bacterial concentrations from colony counts were determined using a ProToCOL plate counter (Synoptics Ltd., Frederick, Md.) and reported as CFU/ml. Log reduction was calculated by the formula: log (Control CFU/ml / Test CFU/ml). The maximum log reduction number that can be determined by this method is 4.00 (99.99% reduction).

**Statistical analysis.** Means of bacterial log reduction values were calculated from six experimental replications. Statistical data analysis (Analysis of Variance, ANOVA) was performed on log-transformed data using the General Linear Models procedure of SAS (12). The analysis was conducted to determine the significance of the effects of the EDTA concentrations and the exposure time. The assumptions of these models were checked using a Wilk-Shapiro test of normality and a Levene test of homoscedasticity. Then post hoc comparisons were calculated among combinations of the EDTA treatments for each combination of bacterial strain and time point. These comparisons were accomplished using Fisher’s protected least significant differences (PLSD). Significant differences were determined at *P* < 0.05, unless otherwise noted.

## RESULTS

EDTA was effective in reducing microbial populations of both *S. agalactiae* SMO 886 and *S. uberis* SMO 1015 in an in-vitro-milk environment. Table 1 shows the average log reductions of both *S. agalactiae* SMO 886 and *S. uberis* SMO 1015 during a two-hour incubation. As expected, log reductions generally increased for both isolates as EDTA concentration increased. After 120 min, numbers of *S. agalactiae* SMO 886 were reduced by only 0.23 logs in 30 mM EDTA, but in 100 mM EDTA, cultures were reduced by 0.97 logs. For cultures of *S. uberis* SMO 1015 after 120 min, 30 mM EDTA reduced populations by 0.69 logs, while 100 mM EDTA increased the reduction to 2.05 logs.

*S. uberis* SMO 1015 appeared more susceptible to EDTA than did *S. agalactiae* SMO 886. For example, after 120 min the *S. uberis* isolate was reduced by 1.40 logs in 60 mM EDTA while the *S. agalactiae* isolate was reduced by only 0.52 logs by the same EDTA concentration. The same pattern existed at earlier time points, where after 15 min in 60 mM EDTA, cultures of the *S. agalactiae* were reduced by only 0.03 logs, compared to 0.39 log reduction of the *S. uberis* under the same conditions.

When the data was analyzed statistically, it was found that there were no significant differences in the log reduction of either of the isolates between 50 and 70 mM concentrations of EDTA at 120 minutes exposure times, but there was significantly more log reduction when concentrations greater than 90 mM were applied. Thus, 50 and 90

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### Table 1. Average log reductions of *S. agalactiae* SMO 886 and *S. uberis* SMO 1015 in EDTA at 15, 30, 60, and 120 min

<table>
<thead>
<tr>
<th>EDTA (mM)</th>
<th><em>S. agalactiae</em> SMO 886</th>
<th><em>S. uberis</em> SMO 1015</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>-0.01 D</td>
<td>0.04 C</td>
</tr>
<tr>
<td>40</td>
<td>-0.06 D</td>
<td>0.09 C</td>
</tr>
<tr>
<td>50</td>
<td>0.02 CD</td>
<td>0.33 B</td>
</tr>
<tr>
<td>60</td>
<td>0.03 BC</td>
<td>0.39 B</td>
</tr>
<tr>
<td>70</td>
<td>0.03 BC</td>
<td>0.36 B</td>
</tr>
<tr>
<td>80</td>
<td>0.09 A</td>
<td>0.45 AB</td>
</tr>
<tr>
<td>90</td>
<td>0.07 AB</td>
<td>0.60 A</td>
</tr>
<tr>
<td>100</td>
<td>0.14 A</td>
<td>0.49 AB</td>
</tr>
</tbody>
</table>

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* a *n* = 6 for each condition at each time point. Means within columns having the same letter are not significantly different (*P* > 0.05). Letters are compared with each separate time point and bacterial species.
mM concentrations were two concentrations defining significant log reductions for both isolates.

**DISCUSSION**

Overall, 100 mM EDTA exhibited a 90 to 99% reduction at 120 minutes in the number of viable bacteria of both *Streptococcus* isolates from mastitic dairy cows. Increasing the concentration of EDTA and measuring the log reductions at time points longer than two hours may show even greater log reductions. However, increasing the concentrations from 50 mM to 70 or 80 mM in general did not significantly increase bacterial log reductions. Only when 90 mM was used, a significant log reduction of both bacterial species was observed as compared to that in 50 mM concentrations. Therefore 50 mM and 90 mM EDTA may be two significant concentrations to examine further in vivo experiments. These results may be significant because even though a concentration of 50 mM resulted in half the log reductions noticed in 90 to 100 mM, the results were obtained with half the concentration of EDTA which may be better tolerated by the host tissues. In preliminary in vivo experiments, low concentrations of EDTA-containing solutions caused limited inflammation of the mammary gland mucosa lining (data not shown).

While chelating agents, including EDTA, have demonstrated antimicrobial properties against various Gram-negative bacteria, the picture has not been as clear against Gram-positive bacteria, particularly *Streptococcus* spp. (2, 6, 7). Although the release of lipopolysaccharide (LPS) and the destabilization of the LPS layer of the membrane have been suggested as possible mechanisms involved in the antimicrobial effect of EDTA in Gram-negative bacteria (10), the mode of action for EDTA against Gram-positive bacteria remains undefined because of differences between the Gram-positive and Gram-negative cell membranes. However, our results demonstrate that even though there was a difference in the extent of antimicrobial action both *Streptococcal* species where still significantly reduced by exposure to EDTA in the milk environment during the conditions studied. These results are encouraging given that *Streptococcal* mastitis can be difficult to treat with conventional antibiotics as well as the occurrence of antibiotic resistant strains. Given the fact that fewer antimicrobial drugs may be available to treat food animals in the future, alternative treatments such as EDTA may become even more important to insure the safety of our milk supply.

In summary, EDTA was found to effectively reduce the microbial populations of two *Streptococcus* isolates from mastitic dairy cows. Further testing including in vivo studies is needed before the potential of EDTA as a novel alternative to antibiotic treatment for *Streptococcal* bovine mastitis can be recommended. However, solutions containing EDTA may also find application as teat dips and/or a milk equipment disinfection strategy in attempts to prevent the spread of such contagious mastitis pathogens.

**ACKNOWLEDGMENTS**

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**REFERENCES**