Elimination kinetics of chlorhexidine in milk following intramammary infusion to stop lactation in mastitic mammary gland quarters of cows

John R. Middleton, DVM, PhD, DACVIM; Vincent R. Hebert, PhD; Lawrence K. Fox, PhD; Elizabeth Tomaszewska, MS; Jeff Lakritz, DVM, PhD, DACVIM

Objective—To evaluate the elimination kinetics of chlorhexidine in milk when used as an intramammary infusion to stop lactation in cows.

Design—Prospective study.

Animals—6 cows.

Procedure—The study was performed in 2 phases. Three cows were studied in each phase. All cows were treated with chlorhexidine suspension by infusion into a mastitic mammary gland quarter after 2 milkings 24 hours apart. Foremilk samples (100 mL) were collected from treated and untreated (controls) mammary gland quarters of each cow. Chlorhexidine was extracted from raw milk, and residue concentrations were quantified by use of high-performance liquid chromatography. Foremilk samples from days 2, 5, and 8 were analyzed in phase I, and samples from time 0 and days 3, 7, 14, 21, 28, 35, and 42 were analyzed in phase II.

Results—In phases I and II, there was no quantifiable transference of chlorhexidine to milk in untreated mammary gland quarters. Measurable chlorhexidine residues were found in milk from treated mammary gland quarters of 2 cows throughout the 42-day sample period in phase II. Estimated mean elimination half-life for chlorhexidine in milk was 11.5 days.

Conclusions and Clinical Relevance—On the basis of the long elimination half-life of chlorhexidine in milk from treated mammary gland quarters, the lack of human dietary exposure data to suggest a food tolerance for chlorhexidine in food products, and the Food and Drug Administration's published zero tolerance for chlorhexidine in uncooked edible calf tissues, we do not recommend extralabel use of chlorhexidine suspension as a treatment to stop lactation in mastitic mammary gland quarters of cows. (*J Am Vet Med Assoc* 2003; 222:1746–1749)

RUMINANTS

Chlorhexidine suspension[a] is prescribed as a treatment for cessation of lactation in chronically infected mammary gland quarters of cows. Although 2 studies[1,2] have assessed the usefulness of chlorhexidine for the cessation of lactation in chronically infected mammary gland quarters, there is a paucity of milk residue data for chlorhexidine when used in this manner. Boddie and Nickerson[1] reported that following 2 intramammary infusions of chlorhexidine diacetate 24 hours apart, antimicrobial activity could be detected in milk from the treated mammary gland quarter for as long as 35 days after infusion by use of a commercial test.[b] However, antimicrobial activity was not detected in milk from the untreated quarters following infusion. The test detects microbial inhibitors in milk, but it does not specifically detect chlorhexidine. Results of studies indicate that antibacterial activity can be detected in milk following an intramammary infusion of endotoxin to induce sterile mastitis.[3] Therefore, a positive test result cannot be considered clear evidence of residual chlorhexidine concentrations in milk. Additionally, the commercial test used is marketed for detection of microbial inhibitors in bulk tank milk, not individual cow milk. The AMDUCA states that a veterinarian prescribing extralabel use of a drug or compound must provide residue avoidance information to the producer. Appropriate residue avoidance data are not available for chlorhexidine when used as an intramammary infusion. In a previous study,[4] we reported a method for the detection of chlorhexidine residues in raw milk by use of **high-performance liquid chromatography (HPLC)**. The purpose of the study reported here was to determine the elimination kinetics of chlorhexidine in milk when used as an intramammary infusion to stop lactation in mastitic mammary gland quarters of cows. The method used to determine the elimination kinetics of chlorhexidine in milk was determined in the previous study.

Materials and Methods

This study was performed in 2 phases, each involving 3 adult lactating Holstein-Friesian cull cows with at least 1 mammary gland quarter with and without mastitis. Initially, a pilot study (phase I) was performed in 3 cows that were treated with an intramammary infusion of chlorhexidine and followed for 8 days. On the basis of results of the pilot study, a second study (phase II) was performed on 3 cows that followed the dissipation of chlorhexidine during a 42-day study period. These studies were conducted in accordance with the Washington State University and the University of Missouri Institutional Animal Care and Use Committee guidelines.

From the Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, MO 65211 (Middleton, Lakritz); the Food and Environmental Quality Laboratory, Washington State University, Richland, WA 99352 (Hebert, Tomaszewska); and the Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA 99164 (Fox).

Presented in part as a poster at the 41st Annual Meeting of the National Mastitis Council, Orlando, Fla, February 2002.

The authors thank Dorothy Newkirk, Scott Silk, and John Swain for technical assistance.

Address correspondence to Dr. Middleton.

Phase I—Milk samples were obtained from all 4 mammary quarters of each cow for bacteriologic culture. *Staphylococcus aureus* was identified on bacteriologic culture of milk from the treated mammary gland quarters of 2 cows. *Klebsiella* sp was identified on bacteriologic culture of milk from the treated mammary gland quarter of 1 cow. The infected mammary gland quarter of each cow received an intramammary infusion of 28 mL of chlorhexidine suspension[a] (1 g chlorhexidine hydrochloride in 28 mL proprietary base) after 2 milkings 24 hours apart.[1,2] The treated mammary gland quarter was not milked for the rest of that lactation period, except to collect samples for the study. The remaining untreated mammary gland quarters were milked twice daily. One cow was treated in the right rear quarter, 1 was treated in the right front quarter, and 1 was treated in the left front quarter. Individual foremilk samples (100 mL) were collected from all 4 mammary gland quarters of each cow at time 0 (immediately before first infusion of chlorhexidine suspension); day 1 (immediately before second infusion [24 hours after first infusion]); day 2 (24 hours after second infusion); and days 3, 4, 5, 6, 7, and 8, respectively. Samples were stored in 100-mL bottles at –20°C until analyzed. Milk samples from days 2, 5, and 8 from all 4 mammary gland quarters (1 treated and 3 untreated controls) were thawed at 22°C, and chlorhexidine residues were quantified by use of HPLC. Samples from days 3, 4, 6, and 7 were not analyzed.

Phase II—Milk samples were obtained from mammary gland quarters selected for study for bacteriologic culture. *S aureus* was identified on bacteriologic culture of milk from mastitic mammary gland quarters from all 3 cows. No bacterial growth was identified on bacteriologic culture of milk from clinically normal mammary gland quarters selected as controls from all 3 cows. Treatments were administered as described for phase I. Two cows were treated in the left front mammary gland quarter, and 1 cow was treated in the right rear mammary gland quarter. The right rear mammary gland quarter on 2 cows was untreated and used as a control, and the left front mammary gland quarter on 1 cow was untreated and used as a control. Foremilk samples (100 mL) were collected from each treated and untreated control mammary gland quarter at time 0 (immediately before first infusion), on day 2 (24 hours after second infusion), and on days 3, 7, 14, 21, 28, 35, and 42, respectively. Milk samples were stored in 100-mL bottles at –20°C until analyzed. Milk samples collected at time 0; day 3 (48 hours after second infusion); and days 7, 14, 21, 28, 35, and 42 were analyzed to quantify chlorhexidine residues as in phase I. Samples from day 2 were not analyzed.

Residue determination—Each analytic sample set consisted of a minimum of 6 treated and untreated mammary gland quarter foremilk samples, 2 **quality control (QC)** whole milk samples containing a known concentration of chlorhexidine, 1 QC whole milk sample without chlorhexidine to be used as a control, and 1 reagent blank containing chlorhexidine. For each sample, 10 g of milk were weighed into a 250-mL centrifuge bottle. The QC and reagent blank samples contained a known volume of a solution of 1 mg chlorhexidine[c]/mL of acetate buffer (10mM; pH 3.6). Reagent blanks containing chlorhexidine were prepared for a range of anticipated residue concentrations. Chlorhexidine extraction from milk and methods for quantification of chlorhexidine residues by use of HPLC have been described.[4] The method's **limit of detection (LOD)** and **limit of quantification (LOQ)** are 0.1 and 0.4 ppm, respectively.[4]

Statistical analyses—Means ± SD were calculated for chlorhexidine concentrations in milk from treated mammary gland quarters at each data collection point in phases I and II of the study. A noncompartmental analysis was performed by use of computer software[d] to determine the **maximum concentration (Cmax)**, **minimum concentration (Cmin)**, **elimination half life ($T_{1/2}$ elim)**, **area under the curve (AUC)**, and percentage AUC that was extrapolated for chlorhexidine in milk from treated mammary gland quarters of cows in phase II of the study. Area under the curve was calculated from time 0 to infinity. The formula for extrapolation to infinity was the following:

$$AUC = Cp\ last/\beta$$

where Cp last is chlorhexidine concentration in the last sample of milk, and β is the elimination rate constant.

Results

For each analytic run, a multipoint linearity assay was performed with calibration standards ranging from 1 through 100 mg/mL. The detector response was found to be linear; linear regression indicated the coefficient of determination (r^2) was ≥ 0.995 for all standard curves during the study. Mean ± SD recovery rate from QC whole milk samples containing chlorhexidine in phase I was 82 ± 10%. Mean ± SD recovery rate from QC whole milk samples containing chlorhexidine in phase II was 83 ± 9%.

Phase I—Mean ± SD chlorhexidine concentration was not determined at time 0 in phase I, because phase I was performed as an exploratory pilot study to determine if chlorhexidine could be detected in milk following treatment. Mean ± SD chlorhexidine concentration in milk from treated mammary gland quarters on day 2 (24 hours after second infusion) was 27.8 ± 25.8 ppm (**Fig 1**). Chlorhexidine concentrations were uniformly less than the LOQ in milk from all control mammary gland quarters on day 2. Mean ± SD chlorhexidine concentration in milk from treated mammary gland quarters declined to 13.9 ± 3.6 ppm on day 5 and 5.2 ± 2.8 ppm on day 8 (7 days after second infusion). Chlorhexidine concentrations remained less than the LOQ in milk from all but 1 control mammary gland quarter on day 5 and were consistently below the LOQ in milk from all control mammary gland quarters on day 8. On the basis of data for chlorhexidine in milk from treated mammary gland quarters in phase I, the study period was extended to 42 days in phase II. Because mean chlorhexidine con-

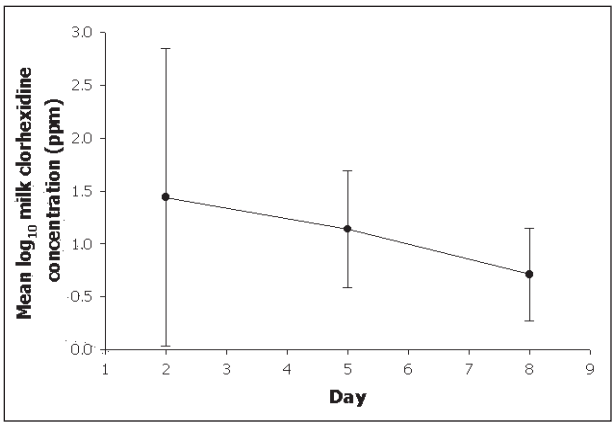

Figure 1—Mean ± SD elimination of chlorhexidine from milk of treated mammary gland quarters in 3 cows in phase I of the study.

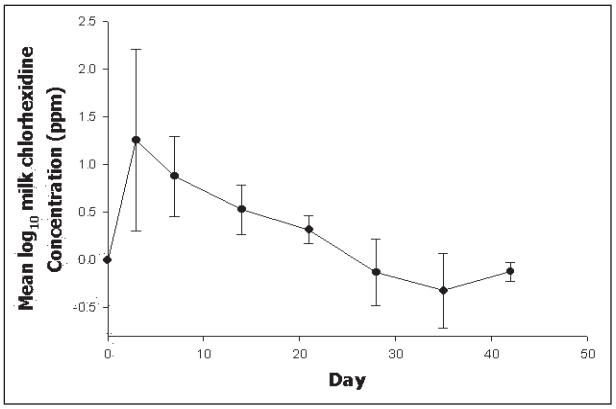

Figure 2—Mean ± SD elimination of chlorhexidine from milk of treated mammary gland quarters in 3 cows in phase II of the study.

centrations in milk from untreated mammary gland quarters were consistently less than the LOQ in phase I, a single control mammary gland quarter was studied in phase II.

Phase II—Chlorhexidine concentrations at time 0 (before first infusion) were less than the LOQ in milk from all mammary gland quarters. Mean ± SD chlorhexidine concentration in milk from treated mammary gland quarters on day 3 (48 hours after second infusion) was 18.1 ± 9.0 ppm (**Fig 2**). Chlorhexidine concentration in milk from treated mammary gland quarters declined during the 42-day study period in 2 cows, whereas 1 cow had a second peak, indicating an increase in chlorhexidine concentration in milk from the treated mammary gland quarter on day 21. Chlorhexidine concentrations in milk from treated mammary gland quarters on day 42 were greater than the LOQ for 2 cows (0.7 and 1.6 ppm, respectively) and less than the LOQ for 1 cow. Chlorhexidine concentration in milk from the untreated control mammary gland quarter of each cow was consistently less than the LOQ throughout phase II.

Pharmacokinetics—Pharmacokinetic analyses were performed on data from phase II of the study only. A noncompartmental model[d] was used to determine the elimination kinetics of chlorhexidine in milk from treated mammary gland quarters. Estimated mean ± SD $T_{1/2}$ elim of chlorhexidine residues in milk from treated mammary gland quarters was 11.5 ± 5.9 days. Mean ± SD AUC was 144.2 ± 55.0 $\mu g \bullet d \bullet mL^{-1}$ with a calculated mean ± SD Cmax of 18.1 ± 9.0 ppm and mean ± SD Cmin of 0.5 ± 0.4 ppm. Mean ± SD percentage AUC that was extrapolated was 5 ± 4%.

Discussion

Although residue avoidance data are available, the method for detecting milk residues was not specific for chlorhexidine; therefore, false-positive results may occur by use of that technique.[1] By using recently published methodology[4] for quantifying chlorhexidine residues in raw milk, we were able to determine the elimination kinetics for chlorhexidine when used as an intramammary infusion to stop lactation in mastitic mammary gland quarters. Phase I was performed as a feasibility study to determine whether chlorhexidine could be detected in raw milk following intramammary infusion. The results of Phase I indicated that our detection method was adequate and that the data collection period needed to be > 7 days after infusion. Hence, a second study of longer duration was performed (Phase II).

Chlorhexidine concentrations in milk from untreated mammary gland quarters in phase II were consistently less than the LOQ. Two cows in phase II had quantifiable chlorhexidine concentrations in milk from the treated mammary gland quarter on day 42. These data in combination with estimated $T_{1/2}$ elim of 11.5 days indicate that chlorhexidine will persist in milk from treated mammary gland quarters for a prolonged period after treatment. When chlorhexidine suspension is used as a treatment to stop lactation in a mastitic mammary gland quarter, the objective is to not milk the treated quarter for the rest of the lactation period in which it is treated. However, milking of the nontreated mammary gland quarters is continued. Chlorhexidine concentrations greater than the LOD were found in milk from a few untreated mammary gland quarters in phase I, although the concentrations were less than the LOQ. Therefore, in an inflamed udder, there may be some transference of chlorhexidine to milk in untreated mammary gland quarters. Accidental milking of a treated mammary gland quarter may cause a chlorhexidine residue in bulk tank milk for 42 days or longer. Because blood samples were not collected in our studies, we do not know whether chlorhexidine is distributed into plasma and other tissues. Similarly, we did not perform analysis for the principal chlorhexidine metabolite, *p*-chloroaniline, in milk or plasma. Actual residue time of chlorhexidine in milk of cows that have no milk removed from the treated mammary gland quarter may be longer than reported here, because in order to conduct these studies, we had to withdraw 100 mL of milk from treated quarters once per week for 6 weeks. Therefore, we were removing a small amount of chlorhexidine from the mammary gland with each sampling.

Information published by the Environmental Protection Agency states that chlorhexidine diacetate is of low to moderate toxicity, highly toxic when applied to the eye, and causes liver effects in animal studies.[5] Although the chlorhexidine residues detected in milk following treatment for cessation of lactation are well below any probable population-adjusted dose, to our knowledge, there are no human dietary exposure data for tolerance levels of chlorhexidine in food products.

The FDA has established a tolerance of zero for chlorhexidine residues in uncooked edible tissues of calves.[6] The detection limit of the HPLC procedure described here was 0.1 ppm. Therefore, although the concentrations of chlorhexidine in milk from untreated mammary gland quarters may be less than the LOD, trace concentrations of chlorhexidine may be found in milk from untreated mammary gland quarters. Although the HPLC technique described here is sensitive, as with any assay, it is not possible to guarantee a zero residue in milk from treated or untreated mammary gland quarters.

On the basis of our findings, FDA regulations, and the information from the EPA, we cannot recommend extralabel use of chlorhexidine as a treatment to stop lactation in mastitic mammary gland quarters of dairy cows.

[a]Nolvasan suspension, Fort Dodge Animal Health, Fort Dodge, Iowa.
[b]Delvotest, DSM Food Specialties, Menomonee Falls, Wis.
[c]Chlorhexidine diacetate, Sigma Chemical Co, St Louis, Mo.
[d]TopFit version 2.0, Gustav Fischer, New York, NY.

References

1. Boddie RL, Nickerson SC. Permanent drying off of chronic mastitic quarters. *Louisiana Cattlemen* 1993;Nov:10–11.
2. Middleton JR, Fox LK. Therapeutic cessation of lactation of *Staphylococcus aureus* infected mammary quarters. *J Dairy Sci* 2001; 84:1976–1978.
3. Tyler JW, Cullor JS, Erskine RJ, et al. Milk antimicrobial drug residue assay results in cattle with experimental, endotoxin-induced mastitis. *J Am Vet Med Assoc* 1992;201:1378–1384.
4. Hebert VR, Middleton JR, Tomaszewska E, et al. Methodology for quantifying residues of chlorhexidine in raw dairy milk. *J Agric Food Chem* 2003;51:567–570.
5. United States Environmental Protection Agency. R.E.D. Facts. Reregistration Eligibility Decision, EPA-738-F-96-025. 1996; 1–5.
6. United States Food and Drug Administration. *Tolerances for residues of new animal drugs in food: chlorhexidine*. Title 21 Code of Federal Regulations. Washington, DC: Office of the Federal Register National Archives and Records Administration, 2002;556: 120.