Relationship between results of in vitro susceptibility tests and outcomes following treatment with pirlimycin hydrochloride in cows with subclinical mastitis associated with gram-positive pathogens

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Objective—To determine the association between results of in vitro antimicrobial susceptibility tests and outcomes in cows that received intramammary treatment with pirlimycin hydrochloride for subclinical mastitis associated with gram-positive pathogens.

Design—Case-control study.

Animals—132 dairy cows (178 mammary glands with subclinical mastitis caused by 194 pathogen isolates).

Procedures—Cows with positive results for a California mastitis test (CMT) were assigned to receive 50 mg of pirlimycin via intramammary administration into each CMT-positive mammary gland every 24 hours for 2 consecutive days or no treatment. Duplicate milk samples were collected before treatment and approximately 21 days later. Target pathogens included coagulase-negative *Staphylococcus* spp (n = 118 isolates), *Streptococcus* spp (28), *Staphylococcus aureus* (7), and other gram-positive cocci (30). Antimicrobial susceptibilities were determined via broth microdilution.

Results—Overall treatment success rate was 66% (128/194) for both groups. In vitro resistance to pirlimycin ranged from 0% (0/7 isolates of *S aureus*) to 50% (13/26 isolates of other gram-positive cocci). For the treated group, 62 of 94 (66%) target pathogens were classified as treatment successes and 32 (34%) were classified as failures. Similarly for the control group, 66 of 100 (66%) target pathogens were classified as treatment successes, whereas 34 (34%) were classified as failures.

Conclusions and Clinical Relevance—Many target pathogens from cows with subclinical mastitis were eliminated without treatment, and treatment with pirlimycin did not improve the treatment success rate. Results of in vitro antimicrobial susceptibility tests were not useful as predictors of treatment success following intramammary treatment with pirlimycin. (*J Am Vet Med Assoc* 2009;234:1437–1446)

Mastitis is characterized by inflammation of the mammary gland and is almost always caused by a bacterial infection. Approximately 60% to 70% of new infections that develop during lactation do not cause obvious clinical signs and are often unrecognized.[1] These subclinical infections are a serious concern for dairy farmers because of decreased milk production, reduced milk quality, and increased transmission of pathogens that cause mastitis.

Antimicrobials are routinely used for treatment of dairy cattle affected with clinical and subclinical infections.[2] Intramammary administration is used for approximately two-thirds of antimicrobials used for treatment of cows with mastitis.[3] Intramammary treatments concentrate the drug at the site of infection and have limited systemic absorption of the drug, thus reducing the probability of unwanted adverse effects.[4] Intramam-

ABBREVIATIONS

ATCC	American Type Culture Collection
CMT	California mastitis test
MIC	Minimum inhibitory concentration
MIC_{50}	Minimum inhibitory concentration at which growth of 50% of the isolates of a bacterial species is inhibited
MIC_{90}	Minimum inhibitory concentration at which growth of 90% of the isolates of a bacterial species is inhibited
SCC	Somatic cell count

mary administration of antimicrobials during lactation is often used for treatment of cows with mastitis caused by gram-positive organisms; most antimicrobials that are administered via the intramammary route have limited efficacy against gram-negative pathogens.[5] Milk from treated cows must be discarded, and the cost-effectiveness of treatment of most cows with subclinical mastitis has not been conclusively determined. Therefore, practitioners generally reserve treatments during lactation for cows that have clinical signs of mastitis.

The selection of antimicrobials is often based on interpretation of results (susceptible, intermediate, or resistant) of in vitro antimicrobial susceptibility tests.[6] The objective of antimicrobial susceptibility testing is to perform a standardized test that will consistently predict the expected therapeutic outcome. Results of in vitro susceptibility tests (performed by use of determination of MIC or Kirby-Bauer disk diffusion–zone inhibition methods) do not consistently predict outcomes for antimicrobial treatment of cows with clinical signs of mastitis.[7–10]

Pirlimycin hydrochloride is an antimicrobial that is only formulated for intramammary administration and is commonly used for treatment of cows with mastitis.[3,11] Pirlimycin is approved for treatment of cows with subclinical or clinical mastitis caused by *Staphylococcus* spp and *Streptococcus* spp.[12,13] It is 1 of only 3 antimicrobials that have break points specifically validated for mastitis in cows. In another study,[10] investigators compared clinical and microbiologic outcomes of cows with mild to moderate signs of clinical mastitis that were treated with intramammary administration of pirlimycin; the findings revealed that clinical outcomes (number of days of abnormal milk and rate of bacteriologic cure) were not associated with results of susceptibility tests. The objective of the study reported here was to determine the relationship between results of antimicrobial susceptibility tests and outcomes of cows with subclinical mastitis that were treated with pirlimycin.

Materials and Methods

Animals—Multiparous and primiparous cows were eligible for enrollment. Specific eligibility criteria for cows included an SCC ≥ 250,000 cells/mL obtained on the test days of the preceding 2 months, 4 functional mammary glands, no episodes of clinical mastitis during the preceding 30-day period, no administration of antimicrobial treatment during the preceding 60-day period, and ≤ 180 days of lactation at the time of enrollment. The study and treatments of cattle were approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Five herds (farms A through E, respectively) located in Wisconsin that were willing to participate in the study and adhere to protocol requirements were recruited through practicing veterinarians and extension agents. Participating herds were required to have a unique identification for each adult cow, known calving dates, monthly Dairy Herd Improvement Association tests that included collection and recording of individual cow SCCs, adherence to label instructions and withdrawal times for intramammary administration of pirlimycin hydrochloride,[a] ≥ 30 cows with an individual SCC ≥ 250,000 cells/mL, no cows with *Streptococcus agalactiae* infections, and no current outbreak of *Mycoplasma bovis*. All owners of participating commercial dairy farms signed informed consent statements.

Screening, sample collection, and treatment administration—Screening of eligible cows for subclinical mastitis and collection of milk samples were performed by the authors. After farm personnel prepared each cow for milking but before the milking units were attached to the udder, the authors collected a pretreatment milk sample from each mammary gland to perform a CMT[b] to screen each mammary gland for subclinical mastitis. The CMT results were classified as negative (no visible reaction) or positive (any visible reaction including trace reactions). All cows with ≥ 1 mammary gland with positive CMT results were enrolled. Duplicate milk samples were then collected aseptically via recommended sampling procedures[14] from all mammary glands with positive CMT results; these samples were used for microbiologic examination. An additional milk sample was collected from the aforementioned mammary glands for determination of SCC.

After collection of pretreatment milk samples and routine milking were completed, each enrolled cow was randomly assigned to the treatment or control group. For cows assigned to the treatment group, all CMT-positive mammary glands received an intramammary infusion of 50 mg of pirlimycin administered by the authors. A second infusion was administered by farm personnel approximately 24 hours later. As per the product label, milk was discarded for a period of 36 hours after the second infusion. No treatments were administered to any CMT-positive mammary glands of cows assigned to the control group. The authors returned to each farm approximately 21 days after enrollment for collection of posttreatment milk samples from all enrolled cows; all milk samples collected at approximately 21 days after enrollment were obtained by use of the same collection protocol used for the pretreatment milk samples.

Bacterial isolation—Somatic cell counts were determined by use of flow cytometry at a commercial Dairy Herd Improvement Association laboratory.[c] Milk samples used for microbiologic examination were immediately cooled to 4°C and transported to the Milk Quality Research laboratory located at the University of Wisconsin, Madison. At the laboratory, samples were processed immediately or stored at –20°C for ≤ 1 week. Except for the inoculum volume, laboratory procedures were performed in accordance with NMC guidelines.[14] In brief, 0.1 mL from each of the duplicate milk samples was inoculated onto half of a blood agar plate. Plates were incubated at 37°C for 18 to 24 hours and then evaluated for growth of bacterial colonies. An infection was defined as the isolation of ≥ 3 identical colonies from both duplicate samples. Samples were considered contaminated when ≥ 3 dissimilar colonies were recovered from the same sample.

After the initial inoculation of blood agar plates, the remaining milk samples were incubated at 37°C for 18 hours. When no growth (0 colonies) was detected on the initial set of plates, 0.01-mL aliquots from the corresponding incubated duplicate milk samples were inoculated onto half of a blood agar plate. Plates were incubated at 37°C for 18 to 24 hours and then evaluated for growth of bacterial colonies. The same aforementioned definitions of infection and contamination were used for microbiologic results of the incubated milk samples, with the exception that *Bacillus* spp were excluded from analysis.

Bacterial identification—All gram-positive, catalase-positive cocci were classified as *Staphylococcus* spp

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and further identified by use of a commercial *Staphylococcus* identification system.[d] All β-hemolytic staphylococci that were mannitol- and coagulase-positive isolates were classified as *Staphylococcus aureus*. Remaining staphylococci were broadly grouped together as coagulase-negative *Staphylococcus* spp. The gram-positive, catalase-negative cocci were initially classified as *Streptococcus* spp and characterized on the basis of their growth on esculin medium and results of CAMP tests. Further speciation of probable streptococci was performed by use of a commercial *Streptococcus* identification system.[e] Gram-positive cocci that were not identified by use of the aforementioned identification systems were classified as other gram-positive cocci pathogens. After identification, all gram-positive cocci pathogens isolated were stored in 20% glycerol at –80°C until required for further characterization.

Antimicrobial susceptibility and MIC determination—Antimicrobial susceptibility and in vitro MIC of gram-positive cocci pathogens were determined by use of a commercial broth microdilution method that included the use of quality-control organisms.[f] This method adhered to the guidelines established by the Clinical Laboratory Standards Institute.[15] Each half of a 96-well plate of a standard mastitis panel[g] used for antimicrobial susceptibility testing and MIC determination against pathogens that cause mastitis contained serial dilutions (1:2) of 10 antimicrobial agents: ampicillin (0.12 to 8.00 µg/mL), ceftiofur (0.5 to 4.0 µg/mL), cephalothin (2 to 16 µg/mL), erythromycin (0.25 to 4.00 µg/mL), oxacillin (2.0 to 4.0 µg/mL), penicillin (0.015 to 8.000 µg/mL), penicillin-novobiocin (1.0 to 8.0 µg/mL), pirlimycin (0.5 to 4.0 µg/mL), sulfadimethoxine (32 to 256 µg/mL), and tetracycline (1.0 to 8.0 µg/mL). Each panel included 3 positive control wells (wells that did not contain antimicrobials).

Isolates went through 2 cycles of inoculation and growth on blood agar plates, and final bacterial suspensions were prepared and standardized to a 0.5 McFarland standard by use of a nephlometer[f] as per manufacturer's instructions. Aliquots (50 µL) of this suspension were dispensed into each well, and plates were incubated aerobically at 36°C for 18 to 24 hours. In vitro MIC values of the antimicrobial agents for the various pathogens were obtained by use of an electronic detector[f] and transferred to a database for analysis. Quality-control tests were performed with ATCC strains *S aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212.[15]

Definitions—An intramammary infection was defined as the isolation of ≥ 3 identical colonies from both duplicate milk samples from a mammary gland. Target pathogens were defined as gram-positive cocci isolated from pretreatment samples. On the basis of results for the culture method, pathogens isolated from the initial aliquots of the milk samples (milk was not incubated) were termed recovery by use of initial culture, whereas pathogens recovered from the aliquots of incubated milk samples were termed recovery by use of milk incubation.

Outcome for each isolate of target pathogens was classified as a treatment failure (failure) or a treatment success (success) for the purposes of statistical analysis.

A failure was defined as isolation of the same bacterial species from both duplicates of the pretreatment milk sample and at least one of the duplicate posttreatment milk samples from the same mammary gland. A success was defined as no bacterial isolates cultured from the posttreatment milk sample or isolation of a different bacterial species in the posttreatment milk sample, compared with the bacterial species isolated in the pretreatment milk sample from the same mammary gland.

Target pathogen PCR assays—Isolates of target pathogens from the pretreatment and posttreatment milk samples classified as failures and that were identified by use of the aforementioned identification criteria as *Staphylococcus* spp or *Streptococcus* spp were further confirmed as being identical isolates by use of published PCR-based methods.[16,17] In brief, genomic DNA was extracted by use of a commercially available kit.[h] Primer sets[17] were used for the amplification of *S aureus*, *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus intermedius*, *Staphylococcus simulans*, *Streptococcus dysgalactiae*, and *Streptococcus uberis*. A different series of primer sets[16] was used for the amplification of *Staphylococcus hyicus*, *Staphylococcus warneri*, *Staphylococcus lentus*, and *Staphylococcus sciuri*. All primers were synthesized by the University of Wisconsin Biotechnology Center.[i] A typical PCR reaction mixture (volume, 25 µL) contained 5X reaction buffer,[j] 2.5mM $MgCl_2$,[k] 200µM of each deoxynucleoside triphosphate,[k] 5mM forward primers, 5mM reverse primers, 1.25 units of DNA polymerase,[l] and nuclease-free water. All PCR assays were performed on a DNA thermal cycler.[m]

Cycling conditions for the amplification reaction of *S aureus*, *S chromogenes*, *S epidermidis*, *S intermedius*, *S simulans*, *S dysgalactiae*, and *S uberis* were an initial denaturation at 94°C for 1 minute; followed by 30 cycles consisting of denaturation at 93°C for 1 minute, annealing of primer at 55°C for 1 minute, and extension at 72°C for 1 minute; and a final elongation at 72°C for 5 minutes. Cycling conditions for amplification of *S hyicus*, *S warneri*, *S lentus*, and *S sciuri* were an initial denaturation at 94°C for 2 minutes; followed by 25 cycles consisting of denaturation at 94°C for 1 minute, annealing of primers at 47°C for 7 minutes, and extension at 72°C for 2 minutes; and a final elongation at 72°C for 7 minutes. One negative control reaction consisting of the PCR mixture without any bacterial DNA was also included in each series of amplifications.

Amplification products were analyzed by use of electrophoresis in a 1.4% agarose gel at 105 V for 75 minutes with a Tris–boric acid–EDTA (45mM Tris-HCL, 45mM boric acid, and 1mM EDTA) buffer. A 100-base pair ladder[n] was loaded into every tenth well of each gel. Following electrophoresis, gels were stained in an ethidium bromide solution (0.05 mg/L) and examined under UV fluorescence. Detection of a band at the expected product size was interpreted as a positive result.

Statistical analysis—Data for statistical analysis were only included from variables that included at least 5 instances of the outcome of interest. This resulted in the data from one of the farms (farm E) and the data from all *S aureus* isolates being excluded from all of the models.

The association between the results of in vitro susceptibility tests of pirlimycin and treatment outcomes for isolates of target pathogens from cows in the treatment group was evaluated by use of logistic regression.[o] For this analysis, only data from cows that received treatment with pirlimycin were included. The full logistic regression model was as follows: treatment outcome (failure or success) = pirlimycin susceptibility (susceptible or resistant) + pathogen type (coagulase-negative *Staphylococcus* spp, other gram-positive cocci, or *Streptococcus* spp) + farm of origin (A, B, C, or D) + parity (1 or > 1) + culture method (initial culture or milk incubated) + number of days of lactation + $\log_{10}$ pretreatment SCC.

The relationship between MIC of pirlimycin and treatment outcome for isolates of target pathogens from cows in the treatment group was examined by use of survival analysis.[p] Only data from cows that received treatment with pirlimycin were included in this analysis. Survival analysis of isolates based on treatment outcome (success or failure) was performed by use of Kaplan-Meier survival curves, with time defined as pirlimycin concentration in the wells of the mastitis-panel plate and event defined as inhibition of bacterial growth. Isolates that were not inhibited at the greatest concentration were right censored. Log-rank and Wilcoxon tests were used to test the null hypothesis of no difference in the survival functions between strata (success or failure).

The association between group (treatment or control) and treatment outcome was examined by use of logistic regression.[o] The effects of farm, parity, pathogen type, culture method, number of days of lactation, and $\log_{10}$ pretreatment SCC were included in the model. The full logistic regression model was as follows: treatment outcome (failure or success) = group (treatment or control) + farm (A, B, C, or D) + parity (1 or > 1) + pathogen type (coagulase-negative *Staphylococcus* spp, other gram-positive cocci, or *Streptococcus* spp) + culture method (initial culture or milk incubated) + number of days of lactation + $\log_{10}$ pretreatment SCC.

The effects of variables that could be associated with recovery of pathogens on the basis of culture method (initial culture or milk incubated) were evaluated by use of logistic regression.[o] The full logistic regression model was as follows: recovery required milk incubation (yes or no) = farm (A, B, C, or D) + parity (1 or > 1) + pathogen type (coagulase-negative *Staphylococcus* spp, other gram-positive cocci, or *Streptococcus* spp) + group (treatment or control) + pirlimycin susceptibility (susceptible or resistant) + treatment outcome (failure or success) + number of days of lactation + $\log_{10}$ pretreatment SCC.

The effects of selected variables on bacterial resistance to pirlimycin were examined by use of logistic regression.[o] The full logistic regression model was as follows: pirlimycin resistant (yes or no) = farm (A, B, C, or D) + parity (1 or > 1) + pathogen type (coagulase-negative *Staphylococcus* spp, other gram-positive cocci, or *Streptococcus* spp) + number of days of lactation + $\log_{10}$ pretreatment SCC. Analysis of variance[q] was used to test for differences in $\log_{10}$ SCCs for pretreatment and posttreatment milk samples between cows in the treatment and control groups. Significance was set at a value of $P < 0.05$ for all models.

Results

Enrollment of cows and selection of mammary glands—For the 5 herds that met eligibility criteria, 262 cows and 1,048 mammary glands were eligible for enrollment (**Table 1**). After CMT screening, 220 cows and 451 mammary glands (2.1 mammary glands/cow) were enrolled. Of the enrolled cows, 16 were excluded (missing samples [n = 5], development of clinical mastitis within 3 weeks after enrollment [4], premature end of lactation [2], culling [1], death [1], and other reasons [3]). Consequently, data from 204 cows and 421 mammary glands were retained in the study.

Pathogens isolated—Results were obtained for the various pathogens.

ISOLATE DISTRIBUTION

Pretreatment milk samples obtained from the 421 mammary glands (213 from cows in the treated group and 208 from cows in the control group) were evalu-

Table 1—Description of the subjects in a study conducted to evaluate results of in vitro antimicrobial susceptibility tests and cows with subclinical mastitis that received intramammary treatment with pirlimycin hydrochloride.

Farm	Cows/herd	Milk production (kg)*	BTSCC (× 1,000 cells/mL)	Eligible† Cows	Enrolled‡ Cows	Enrolled‡ Glands	Retained§ Cows	Retained§ Glands	Used for analysis of treatment outcomes‖ Cows	Used for analysis of treatment outcomes‖ Glands	Target pathogens¶
A	1,447	40.1	177	64	55	110	52	104	31	38	39
B	356	35.9	286	30	27	58	22	52	15	23	29
C	1,422	38.6	185	74	63	132	60	126	41	58	61
D	685	37.2	194	76	63	127	58	115	39	51	54
E	520	35.3	221	18	12	24	12	24	6	8	11
Total	4,430	38.2#	212.6#	262	220	451	204	421	132	178	194

*To convert to pounds of milk, multiply value by 2.2. †Cows with SCC > 250,000 cells/mL on Dairy Herd Improvement Association test days for the preceding 2 months, 4 functional mammary glands, and no episodes of clinical mastitis in the preceding 30 days. ‡Cows with ≥ 1 mammary gland with positive CMT results. §Cows and mammary glands retained in the study after application of exclusion criteria. ‖Cows and mammary glands from which gram-positive cocci were isolated. ¶Total number of isolates of gram-positive cocci cultured from milk samples obtained from mammary glands of cows retained in the study on that farm. #Represents mean value for the 5 farms.
BTSCC = Bulk tank SCC.

ated for bacterial growth. No pathogens were isolated from 136 (32.3%) milk samples, and 27 (6.4%) milk samples were contaminated (**Table 2**). The distribution of pathogens recovered from pretreatment milk samples was coagulase-negative *Staphylococcus* spp (118 [28.0%] isolates), *Bacillus* spp (34 [8.1%]), gram-negative pathogens (33 [7.8%]), other gram-positive cocci (30 [7.1%]), *Streptococcus* spp (28 [6.7%]), yeast (8 [1.9%]), and *S aureus* (7 [1.7%]).

Posttreatment milk samples obtained from the 421 mammary glands were evaluated for bacterial growth. No pathogens were isolated from 138 (32.8%) milk samples, and 35 (8.3%) milk samples were contaminated. The distribution of pathogens recovered from posttreatment milk samples was coagulase-negative *Staphylococcus* spp (125 [29.7%] isolates), other gram-positive cocci (38 [9.0%]), gram-negative pathogens (26 [6.2%]), *Bacillus* spp (19 [4.5%]), *Streptococcus* spp (18 [4.3%]), *S aureus* (14 [3.3%]), and yeast (8 [1.9%]).

Overall, 194 isolates of target pathogens were obtained from milk samples collected from 178 mammary glands of 132 cows with subclinical mastitis (Table 1). Of these, 94 (48.5%) isolates were obtained from milk samples collected from 92 mammary glands of 68 cows in the treatment group, and 100 (51.5%) isolates were obtained from milk samples collected from 86 mammary glands of 64 cows in the control group.

Of the 94 isolates of target pathogens obtained from mammary glands of cows in the treatment group, 62 (66%) were classified as successes and 32 (34%) were classified as failures. Similarly, of the 100 isolates of target pathogens obtained from mammary glands of cows in the control group, 66 (66%) were classified as successes, whereas 34 (34%) were classified as failures. Treatment with pirlimycin did not significantly ($P = 1.0$) improve the rate of treatment success for isolates of target pathogens associated with subclinical mastitis (**Table 3**).

PATHOGEN SPECIES AND OUTCOME

Pathogen type was not significantly ($\chi^2 = 1.61$; $P = 0.44$) associated with outcome. For isolates of coagulase-negative *Staphylococcus* spp recovered from mammary glands of cows in the treatment group, 42 of 59 (71%) isolates were classified as successes and 17 (29%) were classified as failures, whereas 47 of 71 (66%) obtained from cows in the control group were classified as successes and 24 (34%) were classified as failures (Table 3). For isolates of *Streptococcus* spp recovered from mammary glands of cows in the treatment group, 11 of 19 (58%) were classified as successes and 8 (42%) were classified as failures, whereas 7 of 12 (58%) obtained from cows in the control group were classified as successes and 5 (42%) were classified as failures.

Table 2—Summary of pathogens isolated from pretreatment milk samples obtained from mammary glands of cows retained in the study.

Culture result	Total		Treatment group*		Control group†	
	No.	%	No.	%	No.	%
No growth	136	32.3	66	31.0	70	33.7
Contaminated‡	27	6.4	14	6.6	13	6.3
CNS	118	28.0	59	27.7	59	28.4
Bacillus spp	34	8.1	14	6.6	20	9.6
Gram-negative organisms	33	7.8	21	9.9	12	5.8
Streptococcus spp	28	6.7	18	8.5	10	4.8
Yeast	8	1.9	2	0.9	6	2.9
Staphylococcus aureus	7	1.7	3	1.4	4	1.9
Other gram-positive cocci	30	7.1	16	7.5	14	6.7
Total	**421**	**100**	**213**	**100.1§**	**208**	**100.1§**

*Administered 2 doses of pirlimycin (50 mg/dose) via intramammary infusion, with a 24-hour interval between doses. †No intramammary treatments were administered. ‡Culture yielded ≥ 3 dissimilar colonies. §Column does not total to 100% because of rounding.
CNS = Coagulase-negative *Staphylococcus* spp.

Table 3—Summary of treatment successes for isolates of target pathogens (n = 194) based on treatment group.

Pathogen type	Treatment group*			Control group†		
	No. of successes‡	No. of isolates	%	No. of successes‡	No. of isolates	%
CNS	42	59	72	47	71	66
Streptococcus spp	11	19	58	7	12	58
S aureus	1	3	33	1	4	25
Other gram-positive cocci§	8	13	62	11	13	85
Total	**62**	**94**	**66‖**	**66**	**100**	**66‖**

‡A success was defined as no bacterial isolates cultured from the posttreatment milk sample or isolation of a different bacterial species in the posttreatment milk sample, compared with the bacterial species isolated from the pretreatment milk sample. §Other gram-positive cocci include *Aerococcus* spp, *Corynebacterium* spp, *Kokuria* spp, *Enterococcus* spp, and *Micrococcus* spp. ‖Represents the weighted mean value for the 4 categories of pathogen types.
See Table 2 for remainder of key.

For isolates of other gram-positive cocci recovered from mammary glands of cows in the treatment group, 8 of 13 (62%) were classified as successes and 5 (38%) were classified as failures, whereas 11 of 13 (85%) obtained from cows in the control group were classified as successes and 2 (15%) were classified as failures. For isolates of *S aureus* recovered from mammary glands of cows in the treatment group, 1 of 3 (33%) was classified as a success and 2 (67%) were classified as failures, whereas 1 of 4 (25%) isolated from mammary glands of cows in the control group was classified as a success and 3 (75%) were classified as failures.

CONFIRMATION OF TREATMENT FAILURES

On the basis of results of the aforementioned identification criteria, 54 isolates of target pathogens initially classified as failures were coagulase-negative *Staphylococcus* spp (n = 41) or *Streptococcus* spp (13). The PCR-based amplification of the 16S-23S rRNA intergenic spacer region was used to identify the species of staphylococci and streptococci that were isolated from the pretreatment and posttreatment milk samples, which confirmed that 35 (85%) isolates of coagulase-negative *Staphylococcus* spp were failures and 9 (69%) isolates of *Streptococcus* spp were failures.

SCC—Data for SCC were available for milk samples obtained from 212 mammary glands of cows in the treatment group and 208 mammary glands of cows in the control group. There was no significant (P = 0.69) difference in the mean ± SD SCCs from pretreatment milk samples between those obtained from cows in the treatment group ($\log_{10}$ SCC, 5.4 ± 0.94 cells/mL) versus those obtained from cows in the control group ($\log_{10}$ SCC, 5.4 ± 0.98 cells/mL). There was no significant (P = 0.06) difference in SCCs of posttreatment milk samples between those obtained from cows in the treatment group ($\log_{10}$ SCC, 5.5 ± 0.89 cells/mL) versus those obtained from cows in the control group ($\log_{10}$ SCC, 5.7 ± 0.76 cells/mL).

Antimicrobial susceptibility and outcome for isolates from cows in the treatment group—For the 94 isolates of target pathogens cultured from milk samples of cows in the treatment group, there was no significant (χ^2 = 0.37; P = 0.54) association between the results of antimicrobial susceptibility tests and treatment outcomes. Of these isolates, 68 (72%) and 26 (28%), respectively, were classified as susceptible and resistant to pirlimycin. Of the 68 isolates of target pathogens in which in vitro susceptibility to pirlimycin was detected, 46 (68%) were classified as successes and 22 (32%) were classified as failures. Similarly, of the 26 isolates of target pathogens in which in vitro resistance to pirlimycin was detected, 16 (62%) were classified as successes and 10 (38%) were classified as failures.

In vitro MIC and outcome for isolates from cows in the treatment group—For the 32 isolates of target pathogens classified as failures, 16 (50%) were inhibited by a pirlimycin concentration of 0.5 µg/mL, 5 (16%) were inhibited by a pirlimycin concentration of 1.0 µg/mL, 1 (3%) was inhibited by a pirlimycin concentration of 2.0 µg/mL, and 2 (6%) were inhibited by a pirlimycin

concentration of 4.0 µg/mL. The highest pirlimycin concentration tested did not inhibit the growth of 8 (25%) isolates, and these were right censored in the analysis.

Similarly, for the 62 isolates of target pathogen classified as successes, 44 (71%) were inhibited by a pirlimycin concentration of 0.5 µg/mL, 1 (2%) was inhibited by a pirlimycin concentration of 1.0 µg/mL, 1 (2%) was inhibited by a pirlimycin concentration of 2.0 µg/mL, and 5 (8%) were inhibited by a pirlimycin concentration of 4.0 µg/mL. The highest pirlimycin concentration tested did not inhibit the growth of 11 (18%) isolates, and these were right censored in the analysis.

Overall antimicrobial susceptibility to pirlimycin—Overall, a significant association was detected between pathogen type and in vitro susceptibility to pirlimycin (**Table 4**). The other gram-positive cocci isolates were more likely to be resistant to pirlimycin, whereas coagulase-negative *Staphylococcus* spp isolates were less likely to be resistant to pirlimycin, compared with resistance to pirlimycin for *Streptococcus* spp isolates. Most isolates of coagulase-negative *Staphylococcus* spp (106/130 [81.5%]), *Streptococcus* spp (22/31 [71%]), and *S aureus* (7/7 [100%]) were classified as susceptible to pirlimycin (**Table 5**). A lesser proportion of other gram-positive cocci isolates (13/26 [50%]) were classified as susceptible to pirlimycin.

Analysis of the MIC distribution revealed that a pirlimycin MIC of 0.5 µg/mL (lowest concentration tested) inhibited the growth of most isolates of coagulase-negative *Staphylococcus* spp (93/130 [72%]), *Streptococcus* spp (16/31 [52%]), *S aureus* (4/7 [57%]), and other gram-positive cocci (9/26 [35%]; Table 5). The pirlimycin MIC_{50} for isolates of coagulase-negative *Staphylococcus* spp, *Streptococcus* spp, and *S aureus* was 0.5 µg/mL, and the pirlimycin MIC_{50} for isolates of other gram-positive cocci was 2.0 µg/mL. The pirlimycin MIC_{90} for *S aureus* isolates was 2.0 µg/mL. Because > 10% of the isolates of coagulase-negative *Staphylococcus* spp (20/130 [15.4%]), *Streptococcus* spp (5/31 [16%]), and other gram-positive cocci (9/26 [35%]) were not inhibited by the highest concentration of pirlimycin that was tested (4.0 µg/mL), the MIC_{90} of those isolates could not be determined.

A significant association was detected between in vitro susceptibility and the culture method (Table 4). Isolates recovered by use of initial culture were less likely (odds ratio, 0.35) to be resistant to pirlimycin, compared with the likelihood of resistance for isolates recovered by use of milk incubation. Parity, farm, group, number of days of lactation, and $\log_{10}$ pretreatment SCC were not significantly associated with pirlimycin resistance.

Of 130 isolates of coagulase-negative *Staphylococcus* spp, 65 (50%) were recovered by use of initial culture and 65 (50%) were recovered by use of milk incubation. Fewer susceptible isolates (47/65 [72%]) were obtained when recovery was via milk incubation, compared with the number of susceptible isolates (59/65 [91%]) obtained when recovery was via initial culture.

Association between culture method and pathogen type—Isolates of 80 of 194 (41.2%) target pathogens were recovered by use of milk incuba-

Table 4—Risk factors associated with pirlimycin resistance of isolates of target pathogens cultured from milk samples obtained from cows with subclinical mastitis.

Variable	Category	No. of isolates	Resistant isolates No.	Resistant isolates %	Odds ratio (95% CI)
Categorical					
Parity ($\chi^2 = 1.37$; $P = 0.24$)	1	66	18	27.2	ND
	> 1	110	25	22.7	ND
Farm ($\chi^2 = 5.42$; $P = 0.14$)	A	38	14	36.8	ND
	B	27	8	29.6	ND
	C	58	11	18.9	ND
	D	53	10	18.8	ND
Pathogen type	CNS	121	21	17.3	0.31 (0.11, 0.89)
($\chi^2 = 14.02$; $P < 0.001$)	Other gram-positive cocci	25	13	52.0	2.16 (0.65, 7.19)
	Streptococcus spp	30	9	30.0	Ref
Group ($\chi^2 = 0.39$; $P = 0.52$)	Treatment*	88	25	28.4	ND
	Control†	88	18	20.4	ND
Milk incubation‡	No	105	21	20.0	0.35 (0.15, 0.83)
($\chi^2 = 5.66$; $P = 0.01$)	Yes	71	22	30.9	Ref
Quantitative					
No. of days of lactation ($\chi^2 = 2.51$; $P = 0.11$)	—	—	—	—	—
Log$_{10}$ pretreatment SCC ($\chi^2 = 0.01$; $P = 0.90$)	—	—	—	—	—

‡Incubation of the milk samples at 37°C for 18 hours was required for pathogens to be recovered.
CI = Confidence interval. ND = Not determined. Ref = Referent group.
See Table 2 for remainder of key.

Table 5—Pirlimycin susceptibility profiles and MIC distributions for isolates of target pathogens cultured from milk samples obtained from cows with subclinical mastitis.

Pathogen type	No. of isolates	Susceptibility to pirlimycin Susceptible (%)	Susceptibility to pirlimycin Resistant (%)	Isolates inhibited at each indicated MIC (%) 0.5 µg/mL	1.0 µg/mL	2.0 µg/mL*	4.0 µg/mL	NI	MIC$_{50}$ (µg/mL)	MIC$_{90}$ (µg/mL)	Ratio of break point concentration to MIC$_{50}$
CNS	130	81.5	18.5	71.5	7.7	2.3	3.1	15.4	0.5	ND	4.0
Streptococcus spp	31	71.0	29.0	51.6	9.7	9.7	13.0	16.0	0.5	ND	4.0
Other gram-positive cocci	26	50.0	50.0	34.6	7.7	7.7	15.4	34.6	2.0	ND	1.0
S aureus	7	100	0	57.1	28.6	14.3	NA	NA	0.5	2.0	4.0

*Break point concentration (ie, isolates with an MIC ≤ 2 µg/mL were classified as susceptible to pirlimycin).
NI = Not inhibited at the highest concentration tested. ND = Not determined because $> 10\%$ of isolates were not inhibited by the highest concentration tested. NA = Not applicable.
See Table 2 for remainder of key.

tion, whereas 114 (58.8%) were recovered by use of initial culture. A significant ($\chi^2 = 14.30$; $P = 0.002$) association was detected between culture method (initial culture or milk incubated) and the type of pathogen isolated. The distribution of isolates of target pathogens via the culture method was as follows: coagulase-negative *Staphylococcus* spp (initial culture [n = 65]; milk incubated [65]), *Streptococcus* spp (initial culture [23]; milk incubated [8]), other gram-positive cocci (initial culture [19]; milk incubated [7]), and *S aureus* (initial culture [7]; milk incubated [0]).

Association between culture method and outcome—Culture method was significantly ($\chi^2 = 6.40$; $P = 0.01$) associated with treatment outcome. Of the 114 isolates recovered via initial culture, 47 (41.2%) were classified as failures and 67 (58.8%) were clas-

sified as successes. Of the 80 isolates recovered via milk incubation, 19 (24%) were classified as failures and 61 (76%) were classified as successes. Isolates of target pathogens recovered by use of milk incubation were 2.2 times as likely to be classified as successes as those recovered by use of initial culture.

Association between culture method and antimicrobial susceptibility—Results of in vitro susceptibility tests were significantly ($\chi^2 = 4.22$; $P = 0.03$) associated with culture method. Of 80 isolates of target pathogens recovered by use of milk incubation, 25 (31%) were classified as resistant to pirlimycin and 55 (69%) were classified as susceptible. Of 114 isolates of target pathogens recovered by use of initial culture, 21 (18.4%) were classified as resistant to pirlimycin and 93 (81.6%) were classified as susceptible.

Discussion

Pirlimycin is a lincosamide antimicrobial that is an analogue of clindamycin. Its activity is based on binding to the 50S ribosomal subunit of bacterial ribosomes, which subsequently interferes with protein synthesis and suppresses bacterial growth.[18] In vitro and clinical activity of pirlimycin against most gram-positive pathogens that cause mastitis in cows (including *S aureus, Staphylococcus* spp, *Streptococcus* spp, and some *Enterococcus* spp) have been reported.[13,19,20] One study[20] revealed that many staphylococci produce β-lactamase (ranging from 21.6% of *S aureus* isolates to 84% of *S epidermidis* isolates). Pirlimycin is an important alternative for the treatment of cows with mastitis caused by pathogens that are capable of β-lactamase production; the production of β-lactamase does not influence the antimicrobial mechanism of pirlimycin.[13] One in vitro study[19] has revealed that pirlimycin has a postantimicrobial effect against *S aureus*, which results in increased susceptibility of *S aureus* organisms to phagocytosis after the organisms are exposed to subinhibitory concentrations of pirlimycin.

Pirlimycin is formulated as an aqueous gel for use as an intramammary infusion. It is one of the most commonly used intramammary antimicrobials on dairy farms in Wisconsin.[3] Pirlimycin is one of the few intramammary compounds approved for administration at 24-hour intervals and has a relatively short milk withholding period (36 hours after the last infusion). These characteristics are desirable for most farmers. Pirlimycin is 1 of only 3 antimicrobials that have in vitro susceptibility break points specific for mastitis in cows. Pirlimycin was selected as the test compound for the study reported here because of its spectrum of activity, label indication for treatment of subclinical mastitis, and availability of in vitro susceptibility break points specific for mastitis in cows.

Subclinical mastitis is defined on the basis of the inflammatory response to infection, which is typically measured by use of the SCC. The SCC threshold used in the study reported here was selected on the basis of the results of studies[21,22] that indicated a threshold of approximately 200,000 to 250,000 cells/mL is optimal to reduce diagnostic error in field conditions. The study reported here was not designed to specifically determine treatment efficacy, but it did include a negative control group.

Results of other studies[8,23,24] have revealed that mammary glands of cows with mastitis caused by minor pathogens (coagulase-negative *Staphylococcus* spp, *Corynebacterium* spp, and other bacteria that induce a minimal inflammatory response) have relatively high rates of recovery without any treatment, and treatment does not appreciably enhance the probability of cure. The rate of success without treatment in the study reported here was similar to that of another report[8] and was not influenced by intramammary treatment. However, a study[11] conducted by the manufacturer of pirlimycin reported greater cure rates after pirlimycin was used for the treatment of cows with subclinical mastitis caused by coagulase-negative *Staphylococcus* spp and *Streptococcus* spp, compared with the cure rate in cows that did not receive treatment.

When the study reported here was conducted, the label dosage for pirlimycin was 1 tube (50 mg)/affected mammary gland administered every 24 hours for 2 doses. Subsequently, the label was amended to allow treatment every 24 hours for up to 8 consecutive doses. The longer duration of intramammary treatment improves efficacy of treatment for cows with subclinical mastitis caused by invasive pathogens, such as *S aureus* and some *Streptococcus* spp.[25,26]

Coagulase-negative *Staphylococcus* spp were the most common isolates obtained from mammary glands with subclinical mastitis in the study reported here. In most cases of mastitis caused by coagulase-negative *Staphylococcus* spp, the superficial tissue in the mammary gland is the site of infection. The outcomes of cows with mastitis caused by coagulase-negative *Staphylococcus* spp are not consistently improved by the use of extended treatment protocols (> 2 doses) for pirlimycin treatment, compared with outcomes for treatment with 2 doses of pirlimycin.[25,26] These pathogen-specific differences in response to treatment emphasize the need for practitioners to use microbiologic analysis of milk samples when developing appropriate treatment protocols.

The definition of cure or success varies among studies and can influence the detected outcome. Factors that influence cure or success include the frequency of sampling, case definition, pathogen type, and laboratory methods. In the study reported here, treatment success was defined on the basis of duplicate posttreatment milk samples obtained on a single day. This sampling schedule may likely have resulted in an overestimation of the actual rate of treatment success; pathogens that shed at less than the detection limit (< 30 colony-forming units/mL) of the isolation methods used in this study would have been potentially misclassified as treatment successes. This limitation did not influence the overall study conclusion because the same sampling schedule was used for both groups.

Most researchers use identification of coagulase-negative *Staphylococcus* spp at the genus level to define treatment cure or success of mastitis caused by these pathogens.[27] This can result in overestimation of treatment failures and underestimation of cures or successes. In the present study, identification of pathogens at the species level was used to define treatment outcomes and was confirmed by use of published PCR-based methods. The PCR assay confirmed the validity of the reported rate of success. It is unlikely that the isolates of coagulase-negative *Staphylococcus* spp recovered from milk samples obtained from mammary glands with subclinical mastitis were transient infections or contaminants from the teat skin or streak canal; the selection criteria included an increased SCC at the cow level with confirmation by use of positive CMT results at the mammary gland level.

The susceptibility test results for pirlimycin detected in the study reported here were similar to those in previous reports.[3,10,28] Because pirlimycin has validated break points for mastitis in cows, it is reasonable to expect an association between results of antimicrobial susceptibility tests and treatment outcomes. However, in the study reported here, classification of a pathogen as resistant or susceptible to pirlimycin was not associated with treatment outcomes of mammary glands treated with pirlimycin. In addition, the in vitro MIC values were not associated with treatment outcomes.

Specific break points for pirlimycin for mastitis in cows were developed by use of disk diffusion tests.[13] The diameter of the growth inhibition zones from the disk diffusion assay was used to calculate MIC values. Organisms with MIC values ≤ 2 μg/mL were classified as susceptible, and organisms with MIC ≥ 4 μg/mL were classified as resistant. That study[13] included the administration of 50 mg of pirlimycin/mammary gland 2 times at 24-hour intervals. The concentration of pirlimycin in milk was 8.7 μg/mL at 12 hours and 7.52 μg/mL at 36 hours after the initial dose (12 hours after the second dose). These concentrations exceeded the break point used to categorize susceptibility (≤ 2 μg/mL). However, the concentrations of pirlimycin were less than the break point at 24 (0.96 μg/mL), 48 (0.82 μg/mL), 56 (0.14 μg/mL), and 78 (0.08 μg/mL) hours after the initial dose. Ideally, additional data derived from studies that use smaller intervals between time points for sample collection would be needed to better understand the relationship between concentration of pirlimycin in milk and MIC of pirlimycin for pathogens that cause mastitis.

The duration for which the concentration of pirlimycin in milk is greater than the pirlimycin MIC for a pathogen is an important determinant of efficacy. The concentration of free (not bound to protein or lipid) pirlimycin at the site of infection is responsible for the therapeutic effect. Milk and mammary gland tissue contain pathogens that cause mastitis, and the concentration of any antimicrobial at the actual site of an intramammary infection could impact clinical efficacy. Milking frequency may influence the concentration of any antimicrobial at the site of infection, and this issue needs further study as more herds adopt more frequent daily milking schedules. Some herds that participated in the present study had a milking schedule of 2 times/d, and other herds had a milking schedule of 3 times/d. The effect of milking frequency on treatment outcomes of mastitis was not specifically evaluated in this study and should be investigated in future studies.

The usefulness of antimicrobial susceptibility testing would be improved if the test results were good predictors of treatment outcomes. The earliest report[29] of a lack of agreement between results of antimicrobial susceptibility tests and treatment outcomes was in 1955. The ability of an antimicrobial to reach the site of infection and maintain an effective concentration is an important determinant of treatment efficacy.[30] With respect to cows with mastitis, detailed knowledge of the pharmacokinetic behavior of antimicrobials is lacking, and the concentration at the site of infection is not clearly defined.[1] The lack of pharmacokinetic data for antimicrobials used to treat mastitis is considered one of the primary reasons for the poor performance of susceptibility tests with respect to treatment outcomes after intramammary administration.

The growth patterns of bacteria in vivo in milk versus in vitro in the test media used in antimicrobial susceptibility tests are also potential issues for the performance of these assays.[1] Bacteria in test media multiply rapidly and are susceptible to antimicrobials, whereas pathogens that cause mastitis have much lower multiplication rates in milk.[1] The in vitro antimicrobial activity of drugs in milk is markedly reduced, compared with the in vitro antimicrobial activity of drugs in Mueller-Hinton medium.[6] This is particularly apparent with antimicrobials that are highly bound to lipids or proteins or when milk contains numerous leukocytes. Milk casein can also reduce in vitro antimicrobial activity; the addition of milk to test agar causes smaller zones of inhibition.[31]

Pathologic and immunologic changes in mammary glands with mastitis may play an important role in altering in vitro activity of antimicrobials, compared with antimicrobial activity in healthy mammary glands.[32] Similarly, the effects of tissue and body fluids on antimicrobial agents are not considered when break points for antimicrobials used for treatment of humans with illnesses caused by bacterial pathogens are set.[15,33] This is a practical decision because it is not possible to create the exact conditions of an infective process for every type of infection. Also, it would be unreasonable to expect a clinical laboratory to have culture protocols that are specific for every type of infection.

Treatment failure in animals with infectious diseases is a multifactorial problem that involves complex interactions among host immune responses, the pathogen, and the concentration of the antimicrobial chosen for treatment.[34] In the study reported here, failure of antimicrobials to significantly improve the elimination of intramammary infections caused by target pathogens suggests that clearance of intramammary infections ultimately depends on host immune responses. Unfortunately, relatively little is known about the relationship between cow characteristics and the prognosis for subclinical mastitis caused by these target pathogens.[25]

Recovery of coagulase-negative *Staphylococcus* spp was enhanced by incubating the milk samples prior to culturing. The pathogens isolated from incubated milk samples had several characteristics that differed from those pathogens isolated from initial culture. There was a higher rate of treatment success for isolates recovered by use of milk incubation, compared with the rate for isolates recovered via initial culture. The higher rate of success probably was evident because pathogens that required milk incubation for isolation originated in mammary glands that were shedding fewer bacterial organisms; shedding larger numbers of bacterial organisms decreases the likelihood of cure.[25,35]

Pirlimycin's spectrum of activity is only for gram-positive organisms, and pirlimycin treatment has been associated with an increase in the risk of clinical mastitis caused by gram-negative pathogens.[36] It has been suggested that pirlimycin be used with extreme caution in herds that have a higher incidence of mastitis caused by gram-negative pathogens.[37] All cows in the present study underwent rigorous udder preparation followed by careful disinfection of the teats prior to sample collection and administration of treatments. Despite this strict protocol of udder and teat disinfection, 3 cows with high milk production assigned to the treatment group from 1 herd developed severe clinical mastitis within 48 hours after receiving the first intramammary infusion of pirlimycin. Gram-negative coliform bacteria were recovered from 2 of these cows, and no pathogens were isolated from the third. One of the cows from which a coliform pathogen was isolated died within 72 hours after the first pirlimycin administration.

The study reported here suggests that results of in vitro antimicrobial susceptibility tests and MIC values cannot be used as predictors of treatment success following treatment with intramammary infusions of pirlimycin to cows with subclinical mastitis. Coagulase-

negative *Staphylococcus* spp were isolated from milk samples of most of the mammary glands with subclinical mastitis, and these isolates had a high rate of success without treatment. Administration of pirlimycin did not improve the treatment success rate for isolates of target pathogens obtained from these cows. Future research should be directed toward studying other factors that might be associated with treatment success.

a. Pirsue, Pfizer Animal Health, Kalamazoo, Mich.
b. California mastitis test kit, ImmuCell, Portland, Me.
c. Agsource CRI, Verona, Wis.
d. API Staph, bioMerieux, Durham, NC.
e. API 20 Strep, bioMerieux, Durham, NC.
f. Sensititre, Trek Diagnostics, Westlake, Ohio.
g. Sensitouch, Trek Diagnostics, Westlake, Ohio.
h. DNeasy kit, Qiagen, Valencia, Calif.
i. DNA Synthesis Facility, University of Wisconsin Biotechnology Center, Madison, Wis.
j. 5✕ Flexi Buffer, Promega, Madison, Wis.
k. Promega, Madison, Wis.
l. Taq DNA polymerase, Promega, Madison, Wis.
m. 9600 GeneAmp PCR System, Perkin-Elmer, Waltham, Mass.
n. New England Biolabs, Ipswich, Mass.
o. PROC LOGISTIC, SAS, version 9.1, SAS Institute Inc, Cary, NC.
p. PROC LIFETEST, SAS, version 9.1, SAS Institute Inc, Cary, NC.
q. PROC GLM, SAS, version 9.1, SAS Institute Inc, Cary, NC.

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RUMINANTS