

Activity of Pirlimycin against Pathogens from Cows with Mastitis and Recommendations for Disk Diffusion Tests

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Pirlimycin is an analog of clindamycin that will be recommended for therapy of bovine mastitis. It has good activity against staphylococci and streptococci, the major pathogens for bovine mastitis. Five hundred and thirty bacterial isolates recovered from cows with mastitis were studied to confirm the spectrum of activity and to develop recommendations for susceptibility testing. Pirlimycin is not active against isolates of *Enterobacteriaceae*, it varies in its activity against enterococci, and it is active against veterinary isolates of streptococci (MIC for 50% of strains tested, ≤ 0.03 to $0.06 \mu\text{g/ml}$) and staphylococci (MIC for 50% of strains tested, 0.25 to $1.0 \mu\text{g/ml}$). On the basis of levels of drug attained in the milk with recommended dosing schedules, we chose MIC breakpoints of $\leq 2 \mu\text{g/ml}$ for susceptibility and $\geq 4 \mu\text{g/ml}$ for resistance. We also recommended a disk diffusion test using a disk containing $2 \mu\text{g/ml}$ and breakpoints of ≤ 12 mm for resistance and ≥ 13 mm for susceptibility.

Bovine mastitis is an economically important disease to the dairy industry, with losses estimated at more than 2 billion dollars a year in the United States (3). At least 135 different species of microorganisms have been implicated in bovine mastitis, but the majority of these infections are caused by staphylococci and streptococci (12). Antimicrobial therapy for both lactational and dry cows is important in reducing the level of mastitis in dairy herds (3).

Antimicrobial susceptibility testing of the causal pathogens is considered an important factor in selecting the most appropriate therapeutic agent for therapy of mastitis, but no standard criteria for testing the organisms that cause bovine mastitis exist. Current criteria used for categorizing organisms as resistant or susceptible are usually based on the standard recommendations developed by the National Committee for Clinical Laboratory Standards for organisms and antimicrobial agents used for therapy of human diseases (5-7), e.g., regression statistics, population distribution, and clinical response, methods which require knowledge of the levels of the agent in tissue following usual therapy. With rare exceptions, the National Committee for Clinical Laboratory Standards breakpoints are based upon concentrations of the antimicrobial agent that are achieved in the blood of the average patient who receives the usual dosage by either an intravenous or an oral route. The exceptions are the rare antimicrobial agents for which significant levels of drug accumulate in the urine but not in blood or tissue. In the therapy of bovine mastitis, the drugs are infused directly into the udder, making concentrations in milk the major parameter for determining susceptibility breakpoints. Thus, current National Committee for Clinical Laboratory Standards guidelines, which are based on achievable levels of drug on human pathogens, may not be appropriate for determining the antimicrobial susceptibilities of bovine mastitis pathogens.

Pirlimycin is a new lincosamide antibiotic under development as a mastitis therapeutic agent (the Upjohn Company, Kalamazoo, Mich.). This compound is a clindamycin analog,

and it has potent activity against many gram-positive cocci, including staphylococci and streptococci (2, 13). The use of pirlimycin would be facilitated if antimicrobial susceptibility tests could be performed on the isolates from mastitis specimens with breakpoints developed specifically for mastitis therapy. The purposes of this study were to determine the spectrum of activity of pirlimycin against bacteria isolated from cows with mastitis and to develop breakpoints for determining categories of susceptibility by disk diffusion tests with pirlimycin when tested against mastitis pathogens.

MATERIALS AND METHODS

Bacteria. Except for the human isolates, the strains used in this study were relatively recent isolates (from 1986 to 1991) from bovine intramammary infections and had been stored on 3-mm-diameter glass beads at -70°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) in the laboratories of the Upjohn Company. Some of the staphylococci tested were human isolates that were added because of their resistance, e.g., to methicillin, and had been stored in blood at -70°C or colder temperatures. The strains were subcultured onto appropriate media and sent to the Institutes for Microbiology Research, where they were subcultured at least two times prior to being tested. The following species (numbers of strains) were included in the study: *Enterobacter aerogenes* (1), *Escherichia coli* (59), *Klebsiella oxytoca* (19), *Klebsiella pneumoniae* (32), *Enterococcus faecalis* (25), *Streptococcus agalactiae* (72), *Streptococcus dysgalactiae* (40), *Streptococcus equi* (10), *Streptococcus uberis* (52), *Staphylococcus aureus* (51 bovine and 27 human isolates), *Staphylococcus epidermidis* (56 bovine and 26 human isolates), *Staphylococcus chromogenes* (22), *Staphylococcus hyicus* (29), and *Staphylococcus xyloso* (18). The numbers of organisms tested have no correlation with the frequency at which they cause mastitis and obviously do not include every species that has been implicated in mastitis.

Antimicrobial agents. The microorganisms were tested for susceptibilities to pirlimycin and clindamycin. Both anti-

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otics were provided (by the Upjohn Company) as powders appropriate for susceptibility testing.

Susceptibility methods. The MICs were determined and disk diffusion tests were performed according to the recommendations of the National Committee for Clinical Laboratory Standards (5, 6). Disks with various concentrations of pirlimycin ranging from 0.25 to 16 µg per disk were tested; only a limited number of isolates were tested with the 0.25-, 0.5-, and 1.0-µg disks. Commercially acquired clindamycin disks (2 µg) were also tested. Staphylococci were tested for β-lactamase production by the nitrocefin test (Cefinase; BBL Microbiology Systems) and for methicillin resistance by the oxacillin screen test (11). Prior to being tested for β-lactamase production, the isolates had been induced with oxacillin by growing the strain on a blood agar plate with an oxacillin disk and testing growth near the disk or the edge of the zone of inhibition (10).

Assay of pirlimycin in milk. The concentrations of pirlimycin in milk were determined in two studies, and the data were supplied by the Upjohn Company. In those studies, two 50-mg doses of pirlimycin were administered at 0 and 24 h into each mammary gland quarter. Samples of milk were assayed for pirlimycin at 0, 12, 24, 36, 48, 60, and 72 h. The assay method used was a modified agar diffusion bioassay (1) in which the indicator organism (*Micrococcus luteus* ATCC 9341) was sensitized with a highly basic (pH 8.5) medium. Milk samples were centrifuged at 3,000 × g at 4°C for 15 min to separate the fat from the milk. The final pH of the decanted sample was adjusted to 8.5 with 1 N NaOH. A 100-µl aliquot of the sample was added to wells cut in agar plates (Difco antibiotic medium 11; pH 8.5) seeded with the indicator organism. Zones of inhibition were measured and compared with a standard curve obtained with known concentrations of pirlimycin. The assay was sensitive to 0.02 µg of pirlimycin per ml.

Breakpoint determination. MICs and zone diameters for 2-, 4-, 8-, and 16-µg disks for each isolate were plotted as scattergrams (only data for 2-µg disks are shown). The MIC breakpoint selected was based on the concentration of pirlimycin in milk over 2 days following daily doses of 50 mg per dose per mammary gland quarter. A combination of regression analysis (excluding off-scale values for MICs and 6-mm-diameter zones) and error rate bounding was used to determine the disk diffusion breakpoints (4-7).

RESULTS

Although several disk masses were used in the study (data not shown), we chose the 2-µg disk because it yielded desirable zone diameters that generally were 15 to 30 mm for susceptible strains (generally the more reproducible range) and a regression line with a slope that permitted adequate discrimination between susceptible and resistant strains. This is the same concentration that was selected for clindamycin, of which pirlimycin is an analog, but that was not a major selection criterion (2).

The concentrations of pirlimycin in milk when given at 2 doses of 50 mg per mammary gland quarter 24 h apart are shown in Table 1. The levels at 12 and 36 h were 8.7 and 7.52 µg/ml, and for most of the 48 h the amount of drug appears to exceed 2 µg/ml (8a); this concentration was chosen as the susceptible MIC breakpoint. Thus, organisms for which MICs were ≤2 µg/ml would be categorized as susceptible.

The scattergram and linear regression line for the pirlimy-

TABLE 1. Pirlimycin residues in milk^a

Day and time (h)	Pirlimycin concn (µg/ml)		
	Mean	Range	SD
1			
0 + 12	8.73	7.5-10.05	1.07
0 + 24	0.96	0.57-1.45	0.3
2			
0 + 12	7.52	5.5-9.45	1.57
0 + 24	0.82	0.2-2.17	0.71
0 + 36	0.14	0.08-0.19	0.04
0 + 48	0.08	0.05-0.13	0.03

^a Six cows were used in this experiment.

cin MICs and zone diameters obtained with the 2-µg disk are shown in Fig. 1. On the basis of the linear regression analysis and error rate bounding, the zone diameter breakpoints selected were ≤12 mm for resistance and ≥13 mm for susceptibility. With these breakpoints, one isolate (*S. agalactiae*) would be classified as a very major error (or falsely susceptible; i.e., resistant by MIC and susceptible by disk diffusion) (10). This represents 0.19% of the total population and 0.56% of the resistant population (MICs of ≥4 µg/ml). Twenty-one (3.9% of the total population) of the isolates were classified as major errors (falsely resistant; i.e., susceptible by MIC and resistant by disk diffusion); these isolates were 19 *E. faecalis* isolates and one isolate each of *S. agalactiae* and *S. uberis*. If the enterococci were re-

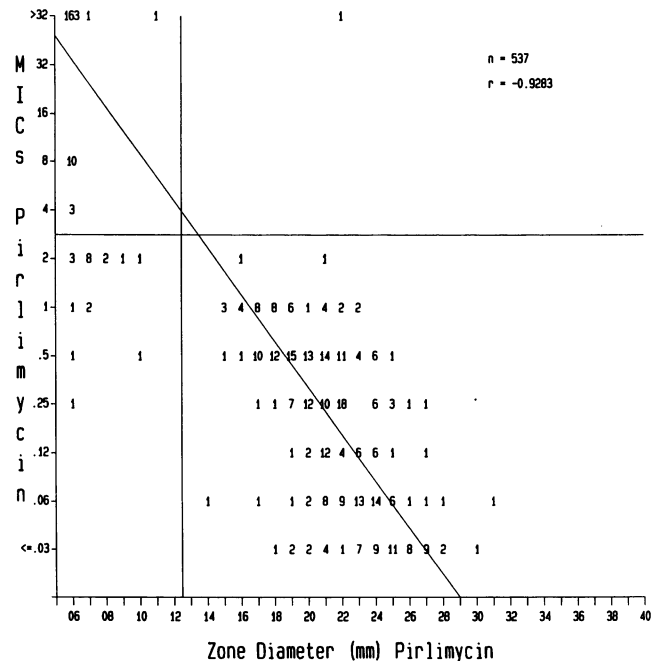


FIG. 1. Scattergram for pirlimycin MICs and zone diameters (2 µg/ml) obtained in tests with gram-negative and -positive mastitis pathogens and some human strains of staphylococci (n = 537). The horizontal line represents the MIC breakpoint (≤2 µg/ml is susceptible), and the vertical line represents the disk diffusion breakpoint (≥13 mm is susceptible). See the text for species tested. For the regression calculations, the extreme values (≥32 and ≤0.03 µg/ml and 6 mm) were excluded.

TABLE 2. MICs of pirlimycin and clindamycin for selected bovine mastitis pathogens and some human isolates

Species and/or isolates (no. of isolates)	MIC ($\mu\text{g/ml}$) ^a					
	Pirlimycin			Clindamycin		
	Range	50%	90%	Range	50%	90%
<i>Enterobacteriaceae</i>						
<i>E. aerogenes</i> (1)	>32			>32		
<i>E. coli</i> (59)	>32			>32		
<i>K. oxytoca</i> (19)	>32			>32		
<i>K. pneumoniae</i> (32)	>32			>32		
Enterococci						
<i>E. faecalis</i> (25)	≤ 0.03 –8	2	2	≤ 0.015 –32	8	16
Streptococci						
<i>S. agalactiae</i> (72)	≤ 0.03 –>32	0.06	0.5	≤ 0.015 –>32	0.03	4
<i>S. dysgalactiae</i> (40)	≤ 0.03 –>32	0.06	1	≤ 0.015 –>32	0.03	16
<i>S. equi</i> (10)	≤ 0.03 –>32	≤ 0.03	8	≤ 0.015 –>32	≤ 0.015	16
<i>S. uberis</i> (52)	≤ 0.03 –>32	0.06	>32	≤ 0.015 –>32	0.03	>32
Staphylococci						
<i>S. aureus</i> ^b						
All (78)	0.06–>32	1	>32	≤ 0.015 –>32	0.25	>32
Vet (51)	0.06–>32	0.5	1	0.03–>32	0.12	0.25
Human (27)	0.5–>32	>32	>32	≤ 0.015 –>32	>32	>32
<i>S. chromogenes</i> (22)						
<i>S. epidermidis</i> ^b	0.25–1	0.5	0.5	0.06–2	0.12	0.5
All (82)	0.12–>32	0.25	>32	≤ 0.015 –>32	0.12	>32
Vet (56)	0.12–>32	0.25	0.5	≤ 0.015 –>32	0.12	0.12
Human (26)	0.12–>32	>32	>32	≤ 0.015 –>32	>32	>32
<i>S. hyicus</i> (29)						
	0.25–1	0.5	1	0.03–16	0.12	0.25
<i>S. xylosus</i> (18)						
	0.5–2	0.5	1	0.12–>32	0.5	2

^a 50% and 90%, MICs for 50 and 90% of isolates tested, respectively.

^b All, vet, human, all isolates, isolates from animals, and isolates from humans, respectively.

moved, there would be one very major error (false susceptibility) and two major errors (false resistance).

The activities of pirlimycin and clindamycin against these selected mastitis and human isolates, as determined by MICs, are shown in Table 2. Pirlimycin, like clindamycin, had little activity against the gram-negative isolates, as indicated by MICs of >32 $\mu\text{g/ml}$ and zone diameters of 6 mm (no zone). Pirlimycin and clindamycin were, on the other hand, active against the gram-positive cocci tested in this study, although less so for *E. faecalis*. Although some isolates of *S. aureus*, *S. epidermidis*, and the other coagulase-negative staphylococcal species were resistant (MIC, ≥ 4 $\mu\text{g/ml}$), MICs for the susceptible isolates generally were ≤ 0.5 $\mu\text{g/ml}$. There were also some resistant isolates of streptococci, but the MICs for susceptible strains were generally lower than those for the staphylococcal isolates, e.g., ≤ 0.03 $\mu\text{g/ml}$. The *E. faecalis* isolates were less susceptible to these drugs than were staphylococci and streptococci. The MICs of pirlimycin for *E. faecalis* were mostly 1 and 2 $\mu\text{g/ml}$, although MICs for 6 of the 22 isolates were ≤ 0.5 $\mu\text{g/ml}$. For most of these enterococcal isolates, pirlimycin MICs were two to four times lower than the MICs of clindamycin. The zone sizes also reflect the lesser activities of both agents for *E. faecalis*; those isolates for which MICs were ≥ 0.5 $\mu\text{g/ml}$ had pirlimycin and clindamycin zone sizes of 6 to 10 mm, while those for which MICs were <0.5 $\mu\text{g/ml}$ had zone diameters indicating susceptibility (>12 mm).

The zone diameters for the 2- μg pirlimycin disk for the species tested in this study ranged from 6 (no zone) to 31 mm, as can be seen in Fig. 2. The zones correlated well with

the MICs, with a linear regression coefficient of -0.93 . The MICs and zone diameters for pirlimycin closely paralleled those obtained for clindamycin (2- μg disk).

A comparison of pirlimycin MICs and clindamycin zone diameters is shown in the scattergram in Fig. 2. These data show that one could use clindamycin zones and the same breakpoint recommended for pirlimycin (≥ 13 mm equals susceptibility) to predict for pirlimycin susceptibility. The five strains that were very major errors (with numbers of isolates in parentheses) were *S. uberis* (1), *S. epidermidis* (2), *S. aureus* (1), and *S. agalactiae* (1).

The β -lactamase and methicillin susceptibility results for the staphylococcal species are shown in Table 3. All of the species had significant percentages of strains that produced β -lactamase, ranging from 27.6% for *S. hyicus* to 84% for *S. epidermidis*. The susceptibilities to methicillin of these staphylococci varied and were largely influenced by the human isolates. All isolates of *S. chromogenes* and *S. xylosus* and 93.1% of *S. hyicus* isolates were susceptible to methicillin. The overall methicillin resistance rates were 6.9% for *S. hyicus*, 29.1% for *S. epidermidis*, and 30.8% for *S. aureus*, with most of the methicillin-resistant *S. epidermidis* and *S. aureus* isolates being human isolates. Only 3.9% of the mastitis *S. aureus* and 7.4% of the mastitis *S. epidermidis* isolates were resistant to methicillin. All strains but one (95.8%) of *S. aureus* that were resistant to methicillin were also resistant to pirlimycin, and 73.9% of the methicillin-resistant *S. epidermidis* isolates were also resistant to pirlimycin. The two methicillin-resistant strains of *S. hyicus* were susceptible to pirlimycin.

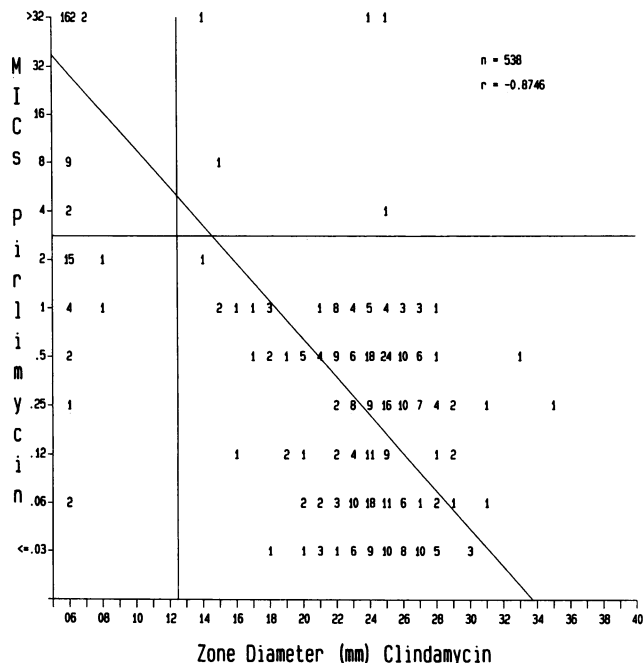


FIG. 2. Scattergram for pirlimycin MICs and clindamycin zone diameters (2 µg per disk) obtained in tests with gram-negative and -positive mastitis pathogens (the same strains used in Fig. 1). The horizontal line represents the MIC breakpoint (≤ 2 µg/ml is susceptible), and the vertical line represents the disk diffusion breakpoint (≥ 13 mm is susceptible).

DISCUSSION

The breakpoints that have been used for determining the categories of susceptibility (susceptible, moderately susceptible, intermediate, and resistant) for drugs used in the therapy of infections in animals have been those breakpoints used for therapy of infections in humans (5-7). Among the problems with using these breakpoints in animal medicine

are that dosages are different, the pharmacokinetics for these drugs are different in different host animals, and the pathogens are often different from those in humans. In almost all cases for antimicrobial agents in human microbiology, breakpoints are based on concentrations of the drug attained in the blood of average patients with the usual dosage of the drug, although it is recognized that the level of the drug in infected tissues may not be the same as in that blood and that the levels attained in different individuals may vary significantly. There are, however, a few antimicrobial agents used for human infections for which the breakpoints are based solely on levels in urine, e.g., nitrofurantoin and nalidixic acid, drugs which yield no significant levels in blood but are concentrated in the urine. Thus, these drugs are used only for urinary tract infections (5, 6).

The latter situation is somewhat analogous to that which exists in the therapy of bovine mastitis in that the antimicrobial agents are infused directly into the individual quarters of the udder, yielding concentrations of the drugs in milk that are dose dependent but that also will be removed during the milking process and that are unrelated to concentrations in blood. Therefore, we chose to use levels in milk achieved by intramammary infusion of pirlimycin into the udder to determine the disk diffusion breakpoints for susceptible and resistant categories. On the basis of the data shown in Fig. 1, we chose 2 µg/ml as the MIC breakpoint, i.e., any organism for which an MIC is ≤ 2 µg/ml is, by definition, susceptible to pirlimycin, whereas an organism for which an MIC is > 2 µg/ml is resistant to pirlimycin. The subsequent decision about a disk diffusion breakpoint had to be based on this MIC breakpoint. As shown in Fig. 2, breakpoints of ≤ 12 mm for resistance and ≥ 13 mm for susceptibility correctly predict the category of susceptibility for almost all strains. The number of very major errors (false susceptibility) is well within the rates that have been suggested as acceptable (10). Although the number of major errors (false resistance) is larger, it is also within the accepted range (10).

The role that milk might play in the activity of the antimicrobial agent has not been considered in determining this breakpoint. There are some indications that milk may adversely affect the activities of some antimicrobial agents. For example, Owens and Watts reported that the addition of milk to test agar resulted in smaller zones of inhibition when *S. aureus* was tested against novobiocin, streptomycin, gentamicin, tetracycline, and vancomycin (8). Sandholm et al. reported similar results in that MICs for *S. aureus* were increased when milk was added to the broth (9). Similarly, the effects of tissue and body fluids on antimicrobial agents are generally not considered when breakpoints for antimicrobial agents used with human pathogens are set (5-7). This is a practical decision, since it is not possible to create the exact conditions of an infective process for every infection, and even if that could be done, it would be unreasonable and unaffordable for a clinical laboratory to do so. Therefore, the goals in routine antimicrobial susceptibility testing are to perform a standardized test and to use a standard breakpoint to yield a result that will consistently predict the outcome of therapy for an infection. Experimental trials using the 50-mg dose of pirlimycin for treatment of clinical mastitis resulted in a bacteriological cure in 67% of mammary gland quarters (14). Thus, the breakpoints established in this study should correlate well with clinical efficacy and should enhance the predictability of therapy.

Pirlimycin is very active against staphylococci and streptococci, the major causes of bovine mastitis, but there are resistant strains. Therefore, routine antimicrobial suscepti-

TABLE 3. β -Lactamase production and methicillin resistance in staphylococcal species^a

Species and/or isolates	% Isolates with the following characteristic(s) ^b :				
	Able to produce β -lactamase	Meth ^s	Meth ^r	Meth ^r + pirlimycin ^s	Meth ^r + pirlimycin ^r
<i>S. aureus</i> ^c					
All	46.2	69.2	30.8	4.2	95.8
Vet	21.6	96.1	3.9	50.0	50.0
Human	92.6	18.5	81.5	0	100.0
<i>S. epidermidis</i> ^c					
All	84.0	70.9	29.1	26.1	73.9
Vet	83.6	92.6	7.4	75.0	25.0
Human	84.6	24.0	76.0	15.8	84.2
<i>S. chromogenes</i>	47.6	100	0	0	0
<i>S. hyicus</i>	27.6	93.1	6.9	100	0
<i>S. xylosus</i>	38.9	100	0	0	0

^a β -Lactamase production was determined by nitrocefin after induction with oxacillin. Methicillin resistance was determined by the oxacillin screen test (11).

^b Meth^s and Meth^r, methicillin susceptible and resistant, respectively; pirlimycin^s and pirlimycin^r, pirlimycin susceptible and resistant, respectively.

^c All, vet, human, all isolates, isolates from animals, and isolates from humans, respectively.

bility testing is indicated and recommended. The resistant strains are easily separated from the susceptible strains by the disk diffusion test, since in our test most of the resistant strains had no zones of inhibition (indicated by 6 mm) compared with the susceptible strains, whose zones were mostly >20 mm. Most of the enterococcal strains would be designated resistant by this disk test, even though they were determined by MICs to be mostly borderline resistant, but those strains of *E. faecalis* designated susceptible by MICs would be designated susceptible by this disk diffusion test. All of the gram-negative bacilli were easily and correctly distinguished by the disk diffusion test to be resistant, since they had no zones of inhibition (6 mm).

That significant numbers of the staphylococci produce β -lactamase has no bearing on the activity of pirlimycin, since β -lactamase has no activity on this lincosamide. However, it does point out that many of the staphylococci will be resistant to penicillins and some cephalosporins and that pirlimycin may serve as an alternative agent for therapy of staphylococcal mastitis. Methicillin resistance appears to be uncommon in bovine *S. aureus* and *S. epidermidis*, but if these isolates become resistant to methicillin, there will likely be implications for pirlimycin, since it is well established that methicillin-resistant staphylococci from humans are often resistant to clindamycin, an analog of pirlimycin, as well as many other classes of antimicrobial agents, even though the unique penicillin-binding protein responsible for methicillin resistance is not responsible for lincosamide resistance (11). These data confirm this relationship among the human staphylococcal strains in that most of the strains that were methicillin resistant were also pirlimycin resistant. The occurrence of methicillin resistance in *S. hyicus*, a veterinary isolate, is of interest, but it should be pointed out that the oxacillin screen test has not been used extensively with this species, if at all. Therefore, it is unknown whether this is the same kind of resistance as is known to occur with *S. aureus* and *S. epidermidis*. It would be prudent for veterinarians, however, to be concerned about the occurrence of methicillin-resistant staphylococci in animal medicine.

It is clear from these data that one could also use the disk diffusion test with the 2- μ g clindamycin disk to predict for pirlimycin susceptibility or resistance if one used the same breakpoint (≥ 13 mm equals susceptibility, ≤ 12 mm equals resistance). It is not our purpose here to recommend this procedure, but it is conceivable that there may be situations in which such an adaptation might be useful. For example, clindamycin is available for use in dogs, and a veterinary laboratory might use clindamycin as a class drug for pirlimycin.

In summary, pirlimycin is a new analog of clindamycin that is being proposed for therapy of bovine mastitis. It has marked activity against staphylococci and streptococci, the major pathogens for mastitis. A disk diffusion test using a 2- μ g disk and breakpoints of ≤ 12 mm for resistance and ≥ 13 mm for susceptibility will discriminate between susceptible

and resistant strains judged by MIC breakpoints of ≤ 2 μ g/ml for susceptibility and ≥ 4 μ g/ml for resistance.

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