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Effects of Antibiotic Treatment of Nonlactating Dairy Cows on Antibiotic Resistance Patterns of Bovine Mastitis Pathogens

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Antibiotic resistance patterns of the major groups of bovine mastitis pathogens (*Streptococcus agalactiae*, other streptococci, *Staphylococcus aureus*, and *Staphylococcus epidermidis*) were examined by determining the minimum inhibitory concentration (MIC) of 13 different antibiotics against bacterial isolates from dairy cattle. The bacterial strains were obtained from milk samples from each cow in 21 New York state dairy herd surveys. In 12 herd surveys (high antibiotic-use group), all 365 cows received antibiotic infusions into the udder at the cessation of each lactation cycle. The 324 animals in the other nine herd surveys (low antibiotic-use group) did not routinely receive antibiotics during the nonlactation period. The MICs from the two groups were compared by calculating for each bacterial group the average MIC, the antibiotic concentration necessary to inhibit 90% of the isolates, and the antibiotic concentration necessary to inhibit 50% of the isolates. Increased resistance to all 13 antibiotics was observed with *Streptococcus agalactiae* isolates from the high antibiotic use herds. However, there was relatively little difference between the two groups in the resistance patterns of the other bacterial species examined. The most important finding of the study was the identification of a multiple beta-lactam resistance phenotype in *Streptococcus agalactiae*.

Bovine mastitis is the greatest economic and animal health problem facing the dairy farmer. In herds where minimal control measures are practiced, accumulated losses may be as high as 10 to 15% of the potential herd production (1). Approximately 90% of all bovine udder infections in the United States are caused by *Streptococcus agalactiae* (group B), non-group B streptococci (including *Streptococcus faecalis*, *Streptococcus uberis*, and *Streptococcus dysgalactiae*), *Staphylococcus aureus*, and *Staphylococcus epidermidis*. Coliforms, *Corynebacterium* spp., and *Pasteurella* spp. (1, 2) are occasionally responsible for clinical infections.

Antibiotic treatment has become the primary method for controlling mastitis. There are numerous clinical and economic disadvantages inherent in the treatment of mastitis in lactating cows, including the rapid dilution of the antimicrobial agent, the loss of saleable milk, and the possible presence of antibiotic residues in milk reaching the consumer (4, 11). For these reasons, infusion of antibiotics into the udders of nonlactating animals (referred to as dry treatment or dry cow therapy) has become an increasingly popular means of mastitis control. This form of treatment can be utilized for animals suffering from clinical mastitis during the previous lactation period or can be used prophylactically, since most new infections occur during the first three weeks after the cessation of lactation (8). In addition to avoiding the disadvantages of lactation therapy described above, dry cow therapy may be more cost effective than diagnosing individual cases, and treatment of subclinical infections, which could serve as foci for new herd infections, is not overlooked.

Dry treatment preparations usually consist of beta-lactam antibiotics utilized alone or in combination with agents such as streptomycin, furaltadone, or novobiocin (1). When insoluble salts of these antibiotics are formulated with an adsorbing agent such as aluminum monostearate in an oil base, antimicrobial activity in the dry udder can be retained for 2 to 4 weeks (13). In comparison, after intramammary administration of antibiotics to lactating cows, antimicrobial activity is retained for less than 96 h (13). The persistent nature of dry treatment preparations could thus provide a strong selective pressure for the acquisition and maintenance of drug resistance genes. In clinical mastitis, antibiotic

treatment failures are often attributed to multiple antibiotic resistance, particularly among staphylococci and gram-negative pathogens (3, 5). Presently, it is difficult to isolate mastitis pathogens that are completely sensitive to all commonly used antibiotics. Because of the important role of antibiotics in the control of mastitis, we felt that it would be advisable to examine the antibiotic resistance of bovine udder pathogens from dairy herds. The major variable, in terms of the antibiotic selection pressure in these herds, was the extent of dry cow therapy employed by the dairy farmer. The results of our initial study are presented in this paper.

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MATERIALS AND METHODS

Bacterial isolates. Mastitis surveys were performed on dairy herds classified as either high antibiotic use or low antibiotic use by the extent of dry cow therapy employed on the farm. Twelve herd surveys (365 cows) in which all cows were routinely given dry treatment formed the high-use group, and nine herd surveys (324 cows) where dry treatment was not employed or utilized only for clinical treatment formed the low-use group. About 80% of the cows sampled from high-use herds received benzalthine cephapirin (Bristol Laboratories, Syracuse, N.Y.), 500 mg per udder quarter, upon entry into the dry period of the lactation cycle. The other dry treatment products consisted of several different beta-lactams, some of which were in combination with aminoglycosides or novobiocin. To obtain bacterial isolates, a composite milk sample from all four quarters of each cow in every herd was collected aseptically after a cleansing of the teat ends with 70% alcohol. The milk samples were cultured on Todd-Hewitt agar plates (Difco Laboratories, Detroit, Mich.) supplemented with 5% sheep blood. Suspected streptococcal and staphylococcal isolates were subcultured and identified as *Streptococcus agalactiae*, other streptococci, *Staphylococcus aureus*, or *Staphylococcus epidermidis* by the procedure of Carter (2). The identity of *Streptococcus agalactiae* isolates as Lancefield group B was confirmed serologically. Since every animal in the herds used in the study was cultured, the vast majority of isolates collected (>95%) were from subclinical infections rather than acute mastitis cases (acute cases may have received therapy during the lactation period when the sample was obtained). Furthermore, information obtained from the herd managers revealed that at the time of sampling, less than 5% of the lactating cows in the herds were receiving antibiotic treatment for clinical mastitis. Thus, the predominant antibiotic selective pressure on the bacterial strains isolated from high-use herds was the dry treatment of the cows before the present lactation cycle. The strains collected in this study represent the pool of subclinical infections which could serve as the source for future clinical mastitis cases in the respective herds of origin. To avoid obtaining multiple isolates of the same strain from an individual animal, we only analyzed one strain of a given species per milk sample. In addition, we carefully examined the resistance patterns obtained within each herd for repeating identical resistance phenotypes to verify that the results were not affected by repeated isolation of a dominant clone within the herd.

Antimicrobial agents. The following antibiotics were utilized for susceptibility testing (all antimicrobial agents were obtained from Sigma Chemical Co., St. Louis, Mo., except where noted.): penicillin G, ampicillin, methicillin, cephalosporin C, chloramphenicol, novobiocin, kanamycin, erythromycin, lincomycin, gentamicin, tetracycline, streptomycin (Calbiochem-Boehring, La Jolla, Calif.), and cephalothin (Eli Lilly & Co., Indianapolis, Ind.).

MIC determinations. Minimum inhibitory concentrations (MICs) for the 13 antibiotics were determined for each isolate by a standardized agar dilution technique (12), except that the basic medium employed was Todd-Hewitt agar supplemented with 5% sheep blood when streptococcal isolates were tested. Antibiotic plates were spot inoculated by a Steers replicator with standardized overnight Todd-Hewitt broth cultures diluted to approximately 5×10^6 CFU/ml. The MIC was defined as the lowest concentration of an antibiotic at which no growth was visible after incubation of the agar plates for 24 h at 37°C.

Statistical analysis. To determine whether routine dry treatment was associated with increased antibiotic resistance levels in mastitis-causing, gram-positive cocci, average MIC data were compiled and statistically analyzed. Average MICs were calculated by totaling individual MICs and dividing by the number of strains tested. When determining the average MIC, values which exceeded the MIC endpoint were incorporated into the calculations as having that endpoint. Within the four pathogen divisions, high and low antibiotic-use average MICs for each antibiotic were compared by two-sample Student *t* tests. Student *t* test values were computed to test the null hypothesis ($MIC_{ave}1 - MIC_{ave}2 \neq 0$) were calculated, where MIC_{ave} 1 is the average MIC of sample 1 and $MIC_{ave}2$ is the average MIC of sample 2. In addition, the concentrations of antibiotics necessary to inhibit growth of 90% (MIC_{90}) and 50% (MIC_{50}) of the isolates in a given category were calculated from the MIC data.

RESULTS

Resistance profiles of streptococci. Penicillin G was the most effective beta-lactam antibiotic against *Streptococcus agalactiae* isolates from both groups and cephalosporin C was the least effective (Table 1). Elevated levels of resistance to the beta-lactams was encountered in a number of strains isolated from high-use herds. Some of these strains exhibited an MIC of 5 μg/ml for penicillin G, with cross-resistance to the other beta-lactams. Elevated resistances to the other antibiotics were also more prevalent in the isolates from high-use herds, with increased levels of tetracycline and aminoglycoside resistance especially common. Two-sample Student *t*

TABLE 1. Comparative susceptibility of *Streptococcus agalactiae* isolates from high and low antibiotic-use dairy herds[a]

Antimicrobial agent	Average MIC (μg/ml) for the following isolates:		Range for the following isolates:		ΔMIC[b]	MIC_{90} for the following isolates:		MIC_{50} for the following isolates:	
	High use	Low use	High use	Low use		High use	Low use	High use	Low use
Penicillin G	1.33	0.22	≤0.1–5	≤0.1–0.5	+	5	0.5	0.5	≤0.1
Ampicillin	3.22	0.27	≤0.1–20	≤0.1–0.5	+	20	0.5	0.5	≤0.1
Methicillin	8.07	1.25	0.5–50	≤0.1–5	+	50	5	5	1
Cephalosporin C	72.35	56.92	20–100	20–100	+	100	100	50	50
Cephalothin	2.07	0.23	≤0.1–0.5	≤0.1–0.5	+	5	0.5	0.5	≤0.1
Tetracycline	18.94	7.00	≤2–128	≤2–64	+	32	16	16	≤2
Streptomycin	251.35	88.46	100–>500	50–200	+	500	100	200	100
Kanamycin	257.35	117.30	50–100	50–200	+	500	200	200	100
Gentamicin	160.29	43.26	≤25–>500	≤1–500	+	>500	50	50	≤25
Erythromycin	92.03	39.38	≤1–>500	≤1–50	−	500	≤1	≤1	≤1
Lincomycin	62.58	4.76	≤1–>500	≤1–50	+	100	≤1	≤1	≤1
Novobiocin	45.29	11.46	4–128	≤2–64	+	128	8	16	8
Chloramphenicol	22.05	7.69	≤5–100	≤5–40	+	50	≤5	10	≤5

[a] Data based on 68 isolates from high-use herds and 26 from low-use herds.

[b] +, For the computed Student *t* test value, the null hypothesis of (average MIC of high-use isolates) − (average MIC of low-use isolates) ≠ 0 must be accepted when $P \leq 0.05$; −, the null hypothesis must be rejected when $P \leq 0.05$.

tests comparing the average MICs for the two groups revealed a statistically significant difference for every antibiotic except erythromycin. However, average MICs for erythromycin were numerically different, and the MIC_{90} showed a dramatic variation. A biomodal distribution profile, with susceptible strains displaying an MIC of <1 and resistant strains with an MIC of >500, probably resulted in large standard deviations in average MICs for erythromycin, accounting for the negative Student *t* test values.

The average MICs of 10 of the 12 antibiotics tested against the other streptococci (Table 2) were not statistically different in the two groups of isolates. In general, the MIC_{90}s and MIC_{50}s also showed smaller differences than those observed with the *Streptococcus agalactiae* isolates, and in the case of cephalosporin C, the two groups had the same MIC_{90}, even though the average MICs were significantly different. Thus, the differences in average resistance levels to cephalosporin C probably have little clinical significance in these organisms. Penicillin G was the most effective agent against these isolates in vitro.

Staphylococci. Methicillin and cephalothin were the most effective beta-lactam antibiotics against high and low antibiotic-use groups of

TABLE 2. Comparative susceptibilities of isolates of streptococci other than *Streptococcus agalactiae* from high and low antibiotic-use dairy herds[a]

Antimicrobial agent	Average MIC (μg/ml) for the following isolates:		Range for the following isolates:		ΔMIC[b]	MIC_{90} for the following isolates:		MIC_{50} for the following isolates:	
	High use	Low use	High use	Low use		High use	Low use	High use	Low use
Penicillin G	1.33	0.99	≤0.1–10	≤0.1–10	−	5	5	0.5	0.5
Ampicillin	1.62	1.42	≤0.1–10	≤0.1–10	−	5	5	0.5	0.5
Methicillin	34.21	17.62	≤0.1–100	≤0.1–10	+	100	50	10	5
Cephalosporin C	75.09	58.41	20–100	20–100	+	100	100	100	50
Cephalothin	13.64	11.25	0.5–100	≤0.1–100	−	50	50	1	1
Tetracycline	48.94	24.78	≤20–256	≤20–256	−	256	32	4	≤2
Streptomycin	210.00	212.88	≤25–500	≤25–500	−	500	500	200	200
Kanamycin	184.91	154.59	≤25–500	≤25–500	−	500	500	100	100
Gentamicin	65.57	85.20	≤25–500	≤25–500	−	100	500	≤25	≤25
Erythromycin	20.25	23.29	≤1–500	≤1–500	−	≤1	10	≤1	≤1
Lincomycin	24.72	36.31	≤1–500	≤1–500	−	10	50	≤1	≤1
Novobiocin	40.23	26.53	≤2–256	≤2–256	−	128	64	16	8
Chloramphenicol	10.19	11.22	≤5–50	≤5–50	−	20	10	10	10

[a] Data based on 53 isolates from high-use herds and 49 isolates from low-use herds.

[b] See footnote *b*, Table 1.

TABLE 3. Comparative susceptibility of *Staphylococcus aureus* isolates from high and low antibiotic-use dairy herds[a]

Antimicrobial agent	Average MIC (μg/ml) for the following isolates:		Range for the following isolates:		ΔMIC[b]	MIC_{90} for the following isolates:		MIC_{50} for the following isolates:	
	High use	Low use	High use	Low use		High use	Low use	High use	Low use
Penicillin G	48.05	60.96	≤0.1->100	≤0.1->100	−	>100	>100	1	>100
Ampicillin	46.12	63.01	0.5->100	0.5->100	−	>100	>100	1	>100
Methicillin	9.48	8.62	1–100	5–50	−	5	5	5	5
Cephalosporin C	73.96	82.90	50–100	50–100	−	100	100	50	100
Cephalothin	6.30	7.13	0.5–100	0.5–50	−	10	10	1	5
Tetracycline	54.58	51.32	4–256	4–256	−	256	256	8	16
Streptomycin	243.23	257.24	≤25–500	≤25–500	−	500	500	200	200
Kanamycin	148.96	119.54	≤25–500	≤25–500	−	200	200	100	100
Gentamicin	82.29	79.93	≤25–500	≤25–500	−	200	200	25	25
Erythromycin	71.08	78.55	≤1–500	≤1–500	−	100	100	5	50
Lincomycin	26.56	42.49	≤1–500	≤1–500	−	10	5	1	5
Novobiocin	13.88	12.42	≤2–256	≤2–256	−	16	16	4	2
Chloramphenicol	20.63	41.05	10–100	10–100	−	20	100	20	40

[a] Data based on 48 isolates from high-use herds and 76 isolates from low-use herds.
[b] See footnote *b*, Table 1.

Staphylococcus aureus (Table 3). Nearly 50% of high and low antibiotic-use isolates were resistant to 100 μg of penicillin G and ampicillin per ml, suggesting the production of a beta-lactamase. Of both high- and low-use isolates, 100% were resistant to 2 μg of tetracycline per ml. Gentamicin was the most active aminoglycoside, although the MIC_{90} was 200 μg/ml for both groups. All isolates from both groups were resistant to 5 μg of chloramphenicol per ml.

For each antibiotic, the high antibiotic-use group did not demonstrate statistically higher resistance levels when compared with the low antibiotic-use group.

Cephalothin and methicillin were the most active beta-lactam antibiotics against *Staphylococcus epidermidis* (Table 4). In the case of ampicillin, penicillin, and streptomycin, the high-use group showed an increase in resistance based on average MICs, as well as MIC_{90} and MIC_{50} levels. Kanamycin and gentamicin were the most effective aminoglycosides against both groups. Greater than 90% of the high-use isolates were inhibited by 25 μg of either antibiotic per ml, but there was no increased resistance in the high-use isolates. There were no significant differences in resistances to the other antibiotics tested.

DISCUSSION

In spite of the important role that antibiotics play in the control of bovine mastitis, there have

TABLE 4. Comparative susceptibilities of *Staphylococcus epidermidis* isolates from high and low antibiotic-use dairy herds[a]

Antimicrobial agent	Average MIC (μg/ml) for the following isolates:		Range for the following isolates:		ΔMIC[b]	MIC_{90} for the following isolates:		MIC_{50} for the following isolates:	
	High use	Low use	High use	Low use		High use	Low use	High use	Low use
Penicillin G	54.70	15.28	≤0.1->100	≤0.1->100	+	>100	50	>100	0.5
Ampicillin	54.82	13.48	≤0.1->100	≤0.1->100	+	>100	>100	>100	0.5
Methicillin	5.42	5.63	1–10	1–50	−	10	5	5	5
Cephalosporin C	71.54	79.02	10–100	20–100	−	100	100	50	100
Cephalothin	1.40	3.48	0.5–5	0.5–50	−	5	5	1	0.5
Tetracycline	51.69	59.69	≤2–256	4–256	−	256	256	8	8
Streptomycin	181.73	76.47	≤25–500	≤25–500	+	500	100	50	≤25
Kanamycin	44.23	53.92	≤25–500	≤25–500	−	≤25	100	≤25	≤25
Gentamicin	29.81	29.90	≤25–100	≤25–100	−	≤25	50	≤25	≤25
Erythromycin	24.92	22.37	≤1–500	≤1–500	−	50	10	≤1	≤1
Lincomycin	22.92	23.75	≤1–500	≤1–500	−	10	10	≤1	≤1
Novobiocin	37.39	24.12	≤2–256	≤2–256	−	128	32	8	8
Chloramphenicol	25.19	19.41	≤5–500	≤5–100	−	40	40	10	20

[a] Data based on 26 isolates from high-use herds and 51 isolates from low-use herds.
[b] See footnote *b*, Table 1.

only been a few studies of the antibiotic resistance of mastitis pathogens (3, 9). Consequently, little information is available on the prevalent resistance patterns of the bacterial strains that serve as the source of new infections. Furthermore, little is known about the effects of various herd management practices on the incidence of resistance in these microorganisms. In the present study, we attempted to catalog some of the current resistance trends in bovine udder pathogens, and we also examined the possible influences of dry cow therapy on resistance. This was done by comparing resistance patterns from the four major groups of mastitis pathogens isolated from surveys of herds where the extent of dry treatment was varied. The incidence of clinical mastitis in the lactating cows which served as the source of all bacterial isolates was low, and the vast majority of the strains came from cows which showed no clinical signs of infection (and had not received antibiotics during the lactation period sampled). Therefore, we reasoned that any antibiotic treatment received before the start of lactation would be of primary importance as a selective factor in this bacterial population, which represented the major source of impending clinical outbreaks of mastitis in the herds sampled.

The resistance profiles obtained from MIC determinations carried out with the high and low antibiotic-use herd isolates were compared by statistical analysis of the average MIC for each antibiotic and by determination of the MIC_{50} and MIC_{90}. The average MICs gave an overall indication of the level of resistance in the bacterial population sampled, whereas the MIC_{50} and MIC_{90} provided information on the distribution of MICs in the population. In most cases, there was general agreement in resistance patterns determined by the different methods. However, the comparison of erythromycin resistance in *Streptococcus agalactiae* by average MIC and MIC_{90} gave the opposite results (Table 1). This was attributed to the fact that there was an extremely large difference in the MIC of resistant and susceptible strains, resulting in a large standard deviation in the average MICs. This made the difference in the average MICs between the two groups statistically insignificant. However, the large increase in the MIC_{90} for the high-use isolates showed that the high-level erythromycin resistance phenotype was indeed more prevalent in *S. agalactiae* strains from the high-use herds. On the other hand, the smaller variation in observed MICs for cephalosporin C against *S. agalactiae* enabled us to identify an increase in average MICs among the high-use isolates which was not evident from the MIC_{90} and MIC_{50}. Thus, there were advantages to using both methods.

The largest differences in resistance profiles were observed with the *S. agalactiae* isolates (Table 1). The levels of beta-lactam resistance observed in certain strains isolated from the high-use herds have not, to our knowledge, been previously reported for human or bovine isolates of group B streptococci. We are currently investigating the mechanism and transferability of this resistance phenotype in more detail, as well as assessing the clinical significance of beta-lactam resistance in clinical failures of beta-lactams in streptococcal mastitis therapy. Although the implications of the increased resistance of these strains are not clear at present, it should be emphasized that the prevailing assumption of uniform penicillin sensitivity in bovine *S. agalactiae* isolates may now be clinically incorrect. Our observations may also be relevant to the potential for development of increased penicillin resistance in human group B streptococci. Human strains with MICs of 0.8 μg/ml have been isolated (6), and the incidence of infection and mortality in human neonatal disease caused by penicillin-resistant *S. agalactiae* is increased by the prophylactic use of penicillin (10).

In contrast to the results obtained with the group B streptococci, there was relatively little difference in the susceptibility patterns of the other streptococci and staphylococci from the two groups of herds. Multiple antibiotic resistance was quite common among both groups of isolates. Unlike *S. agalactiae*, other streptococci and staphylococci are not obligate udder pathogens in the bovine host. Therefore, they may be subject to increased antibiotic selection pressure from various environmental sources. Although New York state dairy cattle do not routinely receive antibiotics in their feed, the dairy environment may often be subjected to low-level antibiotic contamination through nutritional supplements used for other animals (e.g., calves) on the farm, inadvertent contamination of commercial feed during mixing, accidental exposure of nontreated animals to mastitis treatments used by the farmer, etc. (7). These factors could mask the selective effects of dry treatment in organisms that readily survive outside, as well as within, the bovine mammary gland. However, the slight increases in resistance that were observed in staphylococci from high-use herds (Tables 3 and 4) were seen with beta-lactams and streptomycin, which are the most frequently used antibiotics in dry treatment preparations. Thus, there may have been some small selective effect of the dry treatment on these organisms as well.

Our data indicate that the numerous advantages of routine dry treatment as a form of mastitis control should be weighed against the possible increases in drug resistance in *S. aga-*

lactiae which may be related to this type of antibiotic use. Clearly, more information is needed that is relevant to the clinical significance of the resistance observed in the present study. It may also be beneficial to test the efficacy of novel antibiotic combinations such as gentamicin with a beta-lactam in mastitis control, since gentamicin was the most effective aminoglycoside in our study. However, any new treatment formulations used against this disease should be developed with the goal of minimizing the evolution of streptococcal resistance to important antimicrobial agents such as penicillin.

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