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Antimicrobial resistance of bacteria isolated from dairy cow milk samples submitted for bacterial culture: 8,905 samples (1994–2001)

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Objective—To determine whether antimicrobial resistance patterns of major mastitis pathogens isolated from milk samples from dairy cows have changed over time.

Design—Retrospective study.

Sample Population—8,905 bacterial isolates obtained from milk samples submitted to the Wisconsin Veterinary Diagnostic Laboratory between January 1994 and June 2001.

Procedure—Antimicrobial susceptibility was determined by means of the Kirby-Bauer disk diffusion method. Logistic regression was used to determine whether percentages of isolates resistant to various antimicrobials changed over time.

Results—For the gram-positive mastitis pathogens, percentages of isolates resistant to various β-lactam antimicrobials did not increase over the course of the study. Percentage of *Staphylococcus aureus* isolates resistant to penicillin decreased from 49 to 30%; percentage of *Streptococcus* isolates resistant to penicillin decreased from 6 to 1%. Percentage of isolates resistant to erythromycin increased for *S aureus*, *Escherichia coli*, *Enterobacter* spp, *Enterococcus* spp, and *Pasteurella* spp. Percentage of isolates resistant to lincomycin increased for *S aureus* and *Staphylococcus* spp. Percentage of coagulase-negative *Staphylococcus* isolates resistant to pirlimycin increased from 6 to 19%. For several pathogens, percentages of isolates resistant to sulfisoxazole and to trimethoprim-sulfamethoxazole decreased. No pathogens had a significant increase in the percentage of isolates resistant to novobiocin-penicillin.

Conclusions and Clinical Relevance—Results did not indicate a trend toward increased antimicrobial resistance among mastitis pathogens isolated from milk samples from dairy cows between 1994 and 2001. Reduced resistance to β-lactam antimicrobials was identified for several gram-positive mastitis pathogens. (*J Am Vet Med Assoc* 2003;222:1582–1589)

Milk samples from cows with mastitis are frequently submitted for bacterial culture. Organisms that are obtained may be submitted for antimicrobial susceptibility testing to help guide selection of antimicrobial treatment.[1] However, cows with mastitis are usually treated before laboratory results are available, and antimicrobials are often chosen on the basis of historical data. In some of these instances, mastitis treatment may fail or be less effective because of a lack of knowledge of the susceptibility patterns of the causative organisms.[2] Results of antimicrobial susceptibility testing have been useful in predicting likelihood of a bacteriologic cure in cows with *Staphylococcus aureus* mastitis.[3,4] However, results of in vitro susceptibility tests performed with the Kirby-Bauer disk diffusion method do not always correlate with clinical outcomes, especially in cows with clinical mastitis caused by environmental mastitis pathogens.[5] Results of susceptibility testing have been used to monitor for development of antimicrobial resistance in mastitis pathogens.[6] Development of antimicrobial resistance in mastitis pathogens may affect treatment efficacy, disease control, and productivity of dairy cattle.[7] In some instances, dairy cows that develop mastitis caused by resistant bacteria may become chronically infected and be sent to slaughter prematurely.[8]

The development of antimicrobial resistance in bacteria that cause disease in animals is of increasing concern to the public.[9] Use of antimicrobials in food-producing animals is increasingly scrutinized because of the possibility that it may cause development of resistance in bacteria that infect humans.[10] Antimicrobials are used on dairy farms mostly for the treatment of mastitis.[11] Antimicrobial resistance initially develops through chromosomal mutations, but plasmids containing resistant genes can move between bacteria, and there is concern that resistant bacteria could spread to humans.[12]

Monitoring antimicrobial resistance patterns of mastitis pathogens may be important when selecting antimicrobials for treatment of affected cows.[13] Antimicrobial resistance has been identified in isolates of *S aureus*, coagulase-negative *Staphylococcus* spp, *Streptococcus* spp, and gram-negative bacteria obtained from cows with mastitis,[6,14,15] and increases and decreases in percentages of various organisms resistant to particular antimicrobials have been reported.[6,14,15]

Veterinarians have an important role in guiding the selection of antimicrobials used to prevent and treat mastitis. The development of antimicrobial resistance in mastitis pathogens has important implications for animal and human health. The purpose of the study reported here was to determine whether antimicrobial resistance patterns of major mastitis pathogens isolated from milk samples from dairy cows have changed over time.

Criteria for Selection of Cases

Records of all milk samples submitted to the Wisconsin Veterinary Diagnostic Laboratory between January 1994 and June 2001 were reviewed. Data for isolates submitted for antimicrobial susceptibility testing were included in the study.

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Procedures

Standard microbiologic methods were used for bacterial culture of milk samples submitted to the laboratory. Briefly, 0.01 mL of milk was streaked on a portion of a blood agar plate, an eosin methylene blue agar plate, and a TKT plate. Plates were incubated at 35 to 37°C overnight in a CO_2 incubator and examined for growth after 24 and 48 hours. Bacteria were identified on the basis of colony morphology and gram staining characteristics. For gram-positive cocci, catalase tests were performed to distinguish catalase-negative *Streptococci* spp from catalase-positive *Staphylococci* spp. *Streptococci* spp were identified by means of the CAMP test and growth on bile esculin agar. *Staphylococci* spp were identified by use of a coagulase test. Gram-positive bacilli were speciated by use of the catalase test and biochemical reactions as needed. Gram-negative bacilli were speciated by use of the oxidase, motility, indole and ornithin inoculate, and Simmons citrate tests. When antimicrobial susceptibility testing was requested, 1 or 2 colonies of identified pathogens were selected. Antimicrobial susceptibility testing was not performed on *Streptococcus agalactiae*, *Actinomyces pyogenes*, or *Corynebacterium bovis* isolates, according to laboratory protocol. Antimicrobial susceptibility was determined by use of the Kirby-Bauer disk diffusion method, using Mueller-Hinton agar. All inoculations were standardized.[a] Isolates were classified as susceptible, of intermediate susceptibility, or resistant on the basis of NCCLS standards.[16]

Statistical analyses—For purposes of statistical analyses, isolates classified as being of intermediate susceptibility were reclassified as resistant. A χ^2 test[b] was used to determine whether the percentage of isolates that were resistant was associated with year. Logistic regression[c] was performed to determine the probability of antimicrobial resistance by year. The logistic regression model for the percentage of resistant isolates by year included resistance as a response variable (yes vs no) and year as a continuous variable (0 to 7). For all analyses, values of $P < 0.05$ were considered significant.

Results

During the study period, antimicrobials used in susceptibility testing varied on the basis of organism, year, and laboratory protocol (**Table 1**). Susceptibility testing was performed on a total of 8,930 isolates, but results for only 8,905 isolates were included in the present study, because records for 25 isolates were incomplete. All told, antimicrobial susceptibility testing was performed on 2,132 *S aureus* isolates, 1,808 coagulase-negative *Staphylococcus* spp isolates, 1,220 *Streptococcus* spp isolates, 1,939 *Escherichia coli* isolates, 126 *Pseudomonas* spp isolates, 607 *Klebsiella* spp isolates, 143 *Pasteurella* spp isolates, 405 *Enterococcus* spp isolates, 260 *Enterobacter* spp isolates, and 265 isolates representing other bacterial species (**Table 2**). In

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Table 1—Antimicrobials used for susceptibility testing of 8,905 bacterial isolates obtained from dairy cow milk samples submitted for bacterial culture between January 1994 and June 2001

Antimicrobial	Years used	Pathogens tested
Amikacin	1994–2001	*Pseudomonas* spp
Ampicillin or amoxicillin	1994–2001	All
Cephalothin	1994–2001	All
Cloxacillin	1994–2001	*Staphylococcus* spp
Erythromycin	1994–2001	All
Gentamicin	1994–2001	*Pseudomonas* spp
Lincomycin	1994–2001	All
Novobiocin	1994–1998	*Streptococcus* spp, *Staphylococcus* spp
Novobiocin-penicillin	1998–2001	*Streptococcus* spp, *Staphylococcus* spp
Penicillin	1994–2001	All
Pirlimycin	1994–2001	*Streptococcus* spp, *Staphylococcus* spp
Sulfisoxazole	1994–2001	All
Trimethoprim-sulfamethoxazole	1994–2001	All
Tetracycline	1994–2001	All

Table 2—Results of antimicrobial susceptibility testing of bacterial pathogens isolated from milk samples from dairy cows

	Staphylococcus aureus (n = 2,132)		***Staphylococcus*** spp (n = 1,808)		***Streptococcus*** spp (n = 1,220)		***Escherichia coli*** (n = 1,939)		***Pseudomonas*** spp (n = 126)		***Klebsiella*** spp (n = 607)		***Pasteurella*** spp (n = 143)		***Enterococcus*** spp (n = 405)		***Enterobacter*** spp (n = 260)	
Antimicrobial	**No. tested**	**R (%)**	**No. tested**	**R (%)**	**No. tested**	**R (%)**	**No. tested**	**R (%)**	**No. tested**	**R (%)**	**No. tested**	**R (%)**	**No. tested**	**R (%)**	**No. tested**	**R (%)**	**No. tested**	**R (%)**
Amikacin	NT	NT	NT	NT	NT	NT	NT	NT	71	1.4	NT	NT	NT	NT	NT	NT	NT	NT
Ampicillin	2,130	34.9	1,807	30.1	1,220	1.8	1,938	21.9	126	97.6	607	89.1	143	0.7	405	11.1	260	83.1
Cephalothin	2,131	0.1	1,808	0.6	1,219	3.0	1,938	27.9	126	98.4	606	12.1	143	1.4	405	62.7	260	81.9
Cloxacillin	2,132	1.8	1,807	6.8	1,072	42.3	1,813	99.4	119	99.2	546	99.1	125	52.0	368	83.4	239	99.2
Erythromycin	2,132	6.7	1,804	14.9	1,218	16.2	1,935	99.2	126	96.0	605	99.0	143	22.4	405	38.0	260	97.7
Gentamicin	NT	NT	NT	NT	NT	NT	NT	NT	118	7.6	NT	NT	NT	NT	NT	NT	NT	NT
Lincomycin	2,116	3.2	1,791	12.3	1,167	26.4	1,848	99.8	124	96.8	582	99.1	141	95.0	390	62.1	254	98.8
Penicillin	2,130	35.4	1,806	32.6	1,220	5.1	1,926	99.6	124	99.2	604	99.5	143	1.4	402	45.8	259	98.8
Pirlimycin	2,037	4.8	1,720	13.6	1,169	20.9	NT	NT	NT	NT	NT	NT	NT	NT	369	62.9	NT	NT
Sulfisoxazole	2,124	4.5	1,793	7.6	1,026	55.6	1,930	16.3	125	79.2	605	11.7	133	33.1	337	87.5	259	7.3
TMS	2,129	0.9	1,803	3.4	1,211	14.8	1,932	3.8	126	88.9	604	3.6	142	9.9	403	56.1	260	3.1
Tetracycline	2,124	8.6	1,796	22.6	1,215	54.0	1,926	37.4	126	80.2	604	30.0	143	3.5	402	49.8	258	67.1
Novobiocin	1,073	3.1	736	21.6	144	38.9	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Novobiocin-penicillin	1,035	0.2	1,034	1.1	837	0.2	NT	NT	NT	NT	NT	NT	NT	NT	229	5.7	NT	NT

R = Resistant. NT = Not tested. TMS = Trimethoprim-sulfamethoxazole.

Table 3—Results of logistic regression analysis to determine, for bacterial pathogens isolated from milk samples from dairy cows, whether the percentage of isolates resistant to various β-lactam antimicrobials changed with year

Pathogen	Ampicillin		Cephalothin		Cloxacillin		Penicillin	
	OR	*P* value	OR	*P* value	OR	*P* value	OR	*P* value
Staphylococcus aureus	0.92	< 0.001	0.92	0.778	0.79	0.002	0.91	< 0.001
Staphylococcus spp	1.02	0.336	1.02	0.880	1.00	0.942	1.02	0.421
Streptococcus spp	0.87	0.154	0.86	0.048	0.98	0.602	0.83	0.003
Escherichia coli	1.03	0.274	1.02	0.416	1.26	0.110	1.14	0.395
Klebsiella spp	1.09	0.110	1.03	0.545	0.99	0.949	0.51	0.137
Enterobacter spp	1.26	0.004	1.05	0.529	1.22	0.578	1.48	0.184
Enterococcus spp	1.01	0.897	1.04	0.330	1.26	< 0.001	0.85	< 0.001
Pasteurella spp	0.74	0.515	0.55	0.207	1.59	< 0.001	0.55	0.207
Pseudomonas spp	1.04	0.872	1.20	0.598	0.99	0.836	0.81	0.636

OR = Odds ratio.

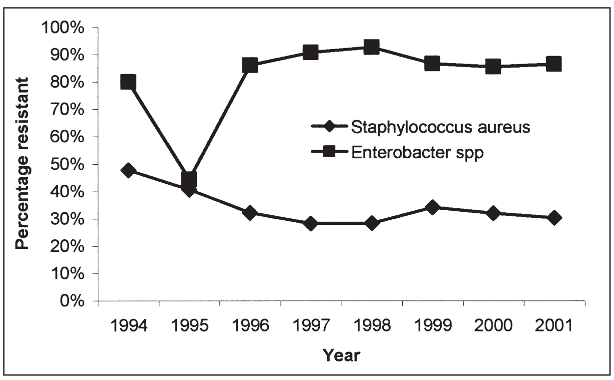

Figure 1—Percentages of *Staphylococcus aureus* (n = 2,132) and *Enterobacter* spp (260) isolates obtained from dairy cow milk samples submitted for bacterial culture between January 1994 and June 2001 that were found to be resistant to ampicillin.

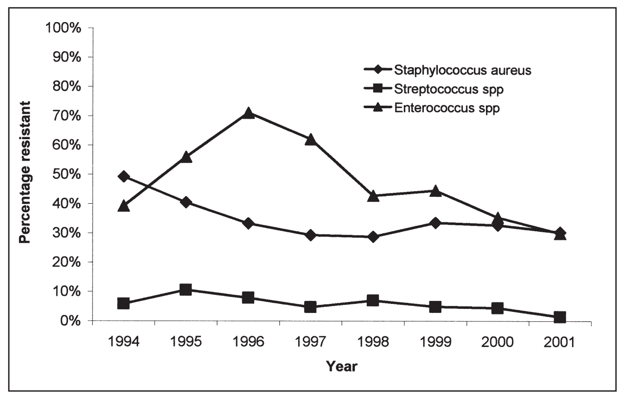

Figure 3—Percentages of *S aureus* (n = 2,132), *Streptococcus* spp (1,220), and *Enterococcus* spp (405) isolates obtained from dairy cow milk samples submitted for bacterial culture between January 1994 and June 2001 that were found to be resistant to penicillin.

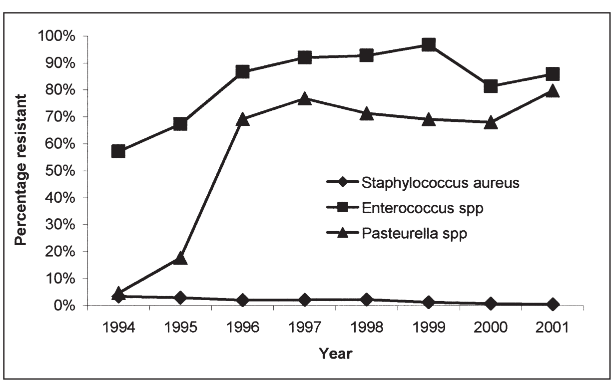

Figure 2—Percentages of *S aureus* (n = 2,132), *Enterococcus* spp (405), and *Pasteurella* spp (143) isolates obtained from dairy cow milk samples submitted for bacterial culture between January 1994 and June 2001 that were found to be resistant to cloxacillin.

general, high percentages of the gram-negative bacteria were resistant to β-lactam and macrolide antimicrobials.

For all of the gram-positive pathogens, the percentage of isolates resistant to β-lactam antimicrobials did not increase significantly over time (**Table 3**). The percentage of *S aureus* isolates resistant to ampicillin decreased significantly ($P < 0.001$), from 48% in 1994 to 30% in 2001 (**Fig 1**), but the decrease was not linear (goodness-of-fit *P* value = 0.007). The percentage of *Enterobacter* isolates resistant to ampicillin increased significantly ($P = 0.004$), from 80% in 1994 to 87% in 2001, but the increase was not linear (goodness-of-fit *P* value = 0.005). The percentage of *Streptococcus* isolates resistant to cephalothin decreased significantly ($P = 0.048$), from 4% in 1994 to 1% in 2001, in a linear fashion (goodness-of-fit *P* value = 0.517); no other organisms had a significant change in the percentage of isolates resistant to cephalothin. The percentage of *S aureus* isolates resistant to cloxacillin decreased significantly ($P = 0.002$) in a linear fashion (goodness-of-fit *P* value = 0.949), from 3% in 1994 to 0.4% in 2001 (**Fig 2**). The percentage of *Enterococcus* isolates resistant to cloxacillin increased significantly ($P < 0.001$), from 57 to 86% (goodness-of-fit *P* value = 0.001), and the percentage of *Pasteurella* isolates resistant to cloxacillin increased significantly ($P < 0.001$), from 5 to 80% (goodness-of-fit *P* value = 0.016); for both organisms, the increases were not linear. The percentage of *S aureus* isolates resistant to penicillin decreased significantly ($P < 0.001$), from 49 to 30% (goodness-of-fit *P* value = 0.009; **Fig 3**); the percentage of *Streptococcus* isolates resistant to penicillin decreased significantly ($P = 0.003$), from 6 to 1% (goodness-of-fit *P* value = 0.501); and the percentage of *Enterococcus* isolates resistant to penicillin decreased significantly ($P < 0.001$), from 39 to 30% (goodness-of-fit *P* value = 0.022). Decreases in percentages of *S aureus* and *Enterococcus* isolates resistant to penicillin were not linear.

Table 4—Results of logistic regression analysis to determine, for bacterial pathogens isolated from milk samples from dairy cows, whether the percentage of isolates resistant to selected macrolide and lincosamide antimicrobials changed with year

	Erythromycin		Lincomycin		Pirlimycin	
Pathogen	**OR**	***P* value**	**OR**	***P* value**	**OR**	***P* value**
Staphylococcus aureus	1.20	< 0.001	1.18	0.003	1.01	0.909
Staphylococcus spp	1.02	0.481	1.22	< 0.001	1.23	< 0.001
Streptococcus spp	1.00	0.984	1.00	0.994	0.93	0.053
Escherichia coli	1.35	0.014	0.99	0.395	NT	NT
Klebsiella spp	1.14	0.461	1.12	0.561	NT	NT
Enterobacter spp	1.73	0.020	1.47	0.193	NT	NT
Enterococcus spp	1.11	0.027	1.04	0.353	0.97	0.473
Pasteurella spp	1.63	< 0.001	1.01	0.928	NT	NT
Pseudomonas spp	1.10	0.640	0.80	0.319	NT	NT

OR = Odds ratio. NT = Not tested.

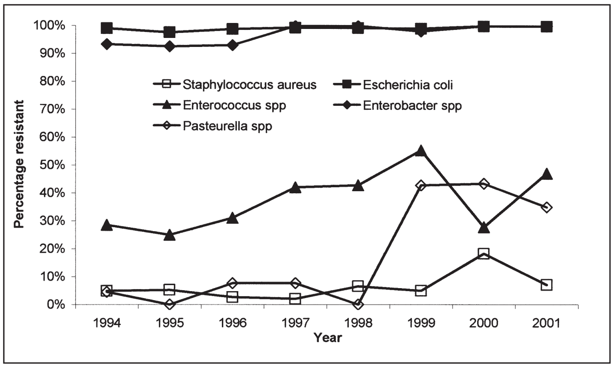

Figure 4—Percentages of *S aureus* (n = 2,132), *Escherichia coli* (1,939), *Enterococcus* spp (405), *Enterobacter* spp (260), and *Pasteurella* spp (143) isolates obtained from dairy cow milk samples submitted for bacterial culture between January 1994 and June 2001 that were found to be resistant to erythromycin.

For several bacterial species, the percentage of isolates resistant to macrolide antimicrobials increased over time **(Table 4)**. Percentage of isolates resistant to erythromycin increased significantly for *S aureus*, *E coli*, *Enterobacter* spp, *Enterococcus* spp, and *Pasteurella* spp isolates **(Fig 4)**. The percentage of *S aureus* isolates resistant to erythromycin increased significantly ($P < 0.001$), from 5 to 7% (goodness-of-fit P value < 0.001), and the percentage of *Enterococcus* isolates resistant to erythromycin increased significantly ($P = 0.027$), from 29 to 47% (goodness-of-fit P value = 0.017), but the increases were not linear. The percentage of *E coli* isolates resistant to erythromycin increased significantly ($P = 0.014$), from 99 to 100% (goodness-of-fit P value = 0.418); the percentage of *Enterobacter* isolates resistant to erythromycin increased significantly ($P = 0.02$), from 93 to 100% (goodness-of-fit P value = 0.766), and the percentage of *Pasteurella* isolates resistant to erythromycin increased significantly ($P < 0.001$), from 5 to 35% (goodness-of-fit P value = 0.212). The percentage of *S aureus* isolates resistant to lincomycin increased significantly ($P < 0.001$), from 4 to 7% (goodness-of-fit P value < 0.001); and the percentage of *Staphylococcus* isolates resistant to lincomycin increased significantly ($P < 0.001$), from 8 to 18% (goodness-of-fit P value = 0.010), but increases were not linear. The percentage of *Staphylococcus* isolates resistant to pirlimycin increased significantly ($P < 0.001$), from 6 to 19%, but the increase was not linear (goodness-of-fit P value = 0.039).

The percentage of *Staphylococcus* isolates resistant to tetracycline increased significantly ($P = 0.01$), from 18 to 25% (goodness-of-fit P value = 0.821; **Table 5**). The percentage of *Pseudomonas* isolates resistant to tetracycline increased significantly ($P = 0.005$), from 62 to 90%, but the increase was not linear (goodness-of-fit P value = 0.035). Percentages of *S aureus*, *Staphylococcus* spp, *Streptococcus* spp, *E coli*, *Klebsiella* spp, *Enterobacter* spp, and *Pasteurella* spp isolates resistant to sulfisoxazole decreased significantly, whereas the percentage of *Pseudomonas* isolates resistant to sulfisoxazole increased significantly **(Fig 5 and 6)**. The percentage of *S aureus* isolates resistant to sulfisoxazole decreased significantly ($P = 0.001$), from 7 to 4% (goodness-of-fit P value = 0.213). The percentage of *Staphylococcus* isolates resistant to sulfisoxazole decreased significantly ($P = 0.001$), from 16 to 5% (goodness-of-fit P value = 0.291), and the percentage of *Streptococcus* isolates resistant to sulfisoxazole decreased significantly ($P < 0.001$), from 65 to 44% (goodness-of-fit P value = 0.270). The percentage of *E coli* isolates resistant to sulfisoxazole decreased significantly ($P < 0.001$), from 26 to 14% (goodness-of-fit P value = 0.069); the percentage of *Klebsiella* isolates resistant to sulfisoxazole decreased significantly ($P < 0.001$) from 27 to 4% (goodness-of-fit P value = 0.732); the percentage of *Enterobacter* isolates resistant to sulfisoxazole decreased significantly ($P < 0.001$), from 33 to 0% (goodness-of-fit P value = 0.721), and the percentage of *Pasteurella* isolates resistant to sulfisoxazole decreased significantly ($P = 0.001$), from 57 to 20% (goodness-of-fit P value = 0.829). The percentage of *Pseudomonas* isolates resistant to sulfisoxazole increased significantly ($P = 0.037$), from 69 to 90% (goodness-of-fit P value = 0.973).

The percentage of *S aureus* isolates resistant to trimethoprim-sulfamethoxazole decreased significantly ($P < 0.001$), from 2 to 0% (goodness-of-fit P value = 0.913; **Fig 7**); the percentage of *Staphylococcus* isolates resistant to trimethoprim-sulfamethoxazole decreased significantly ($P < 0.001$), from 5 to 1% (goodness-of-fit P value = 0.052); the percentage of *Streptococcus* isolates resistant to trimethoprim-sulfamethoxazole decreased significantly ($P < 0.001$), from 22 to 7%

Table 5—Results of logistic regression analysis to determine, for bacterial pathogens isolated from milk samples from dairy cows, whether the percentage of isolates resistant to tetracycline and various sulfonamides changed with year

	Tetracycline		Sulfisoxazole		Trimethoprim-sulfamethoxazole	
Pathogen	**OR**	***P* value**	**OR**	***P* value**	**OR**	***P* value**
Staphylococcus aureus	1.04	0.290	0.86	0.001	0.64	0.001
Staphylococcus spp	1.07	0.010	0.81	< 0.001	0.80	< 0.001
Streptococcus spp	1.04	0.197	0.86	< 0.001	0.86	< 0.001
Escherichia coli	1.02	0.285	0.91	< 0.001	1.02	0.739
Klebsiella spp	1.05	0.212	0.78	< 0.001	0.72	0.002
Enterobacter spp	1.06	0.382	0.46	< 0.001	0.72	0.382
Enterococcus spp	0.97	0.532	0.88	0.087	0.90	0.017
Pasteurella spp	1.30	0.244	0.78	0.001	0.69	0.006
Pseudomonas spp	1.39	0.005	1.73	0.037	1.16	0.263

OR = Odds ratio.

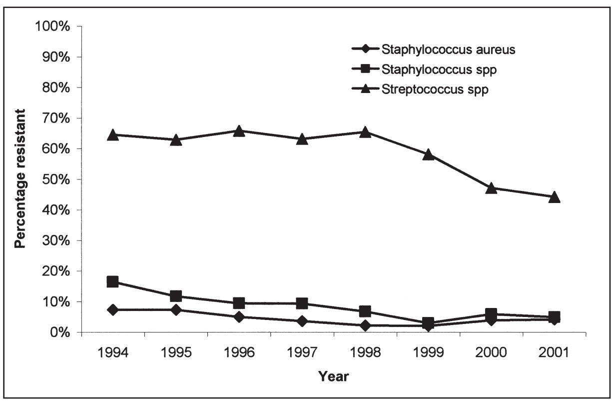

Figure 5—Percentages of *S aureus* (n = 2,132), *Staphylococcus* spp (1,808), and *Streptococcus* spp (1,220) isolates obtained from dairy cow milk samples submitted for bacterial culture between January 1994 and June 2001 that were found to be resistant to sulfisoxazole.

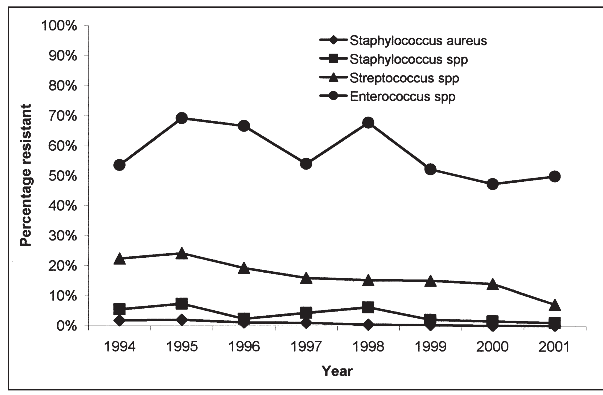

Figure 7—Percentages of *S aureus* (n = 2,132), *Staphylococcus* spp (1,808), *Streptococcus* spp (1,220), and *Enterococcus* spp (405) isolates obtained from dairy cow milk samples submitted for bacterial culture between January 1994 and June 2001 that were found to be resistant to trimethoprim-sulfamethoxazole.

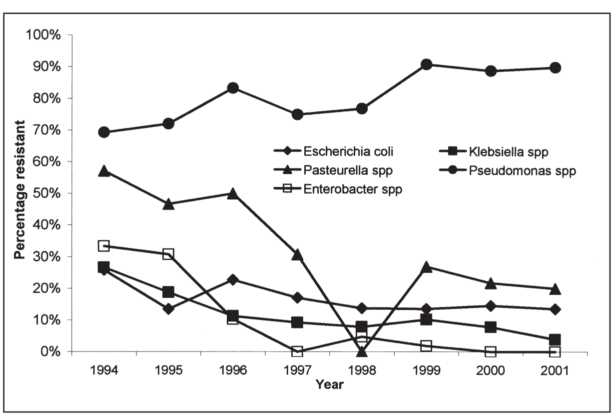

Figure 6—Percentages of *E coli* (n = 1,939), *Klebsiella* spp (607), *Pasteurella* spp (143), *Pseudomonas* spp (126), and *Enterobacter* spp (260) isolates obtained from dairy cow milk samples submitted for bacterial culture between January 1994 and June 2001 that were found to be resistant to sulfisoxazole.

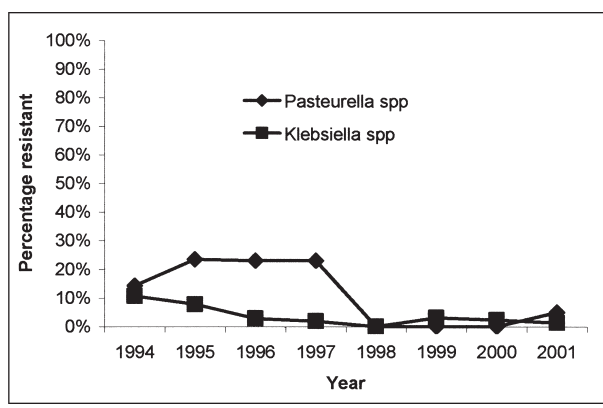

Figure 8—Percentages of *Pasteurella* spp (n = 143) and *Klebsiella* spp (607) isolates obtained from dairy cow milk samples submitted for bacterial culture between January 1994 and June 2001 that were found to be resistant to trimethoprim-sulfamethoxazole.

(goodness-of-fit *P* value = 0.591); and the percentage of *Enterococcus* isolates resistant to trimethoprim-sulfamethoxazole decreased significantly (*P* = 0.017), from 54 to 50% (goodness-of-fit *P* value = 0.469). The percentage of *Klebsiella* isolates resistant to trimethoprim-sulfamethoxazole decreased significantly (*P* = 0.002), from 11 to 1% (goodness-of-fit *P* value = 0.539; **Fig 8**), and the percentage of *Pasteurella* isolates resistant to trimethoprim-sulfamethoxazole decreased significantly (*P* = 0.005), from 14 to 5% (goodness-of-fit *P* value = 0.183).

The percentage of *Staphylococcus* isolates resistant to novobiocin decreased significantly (*P* = 0.007), from 29 to 0% (goodness-of-fit *P* value = 0.072), and the percentage of *Streptococcus* isolates resistant to novobiocin decreased significantly ($P < 0.001$), from 77 to 0% (goodness-of-fit P value = 0.857). No bacterial species had significant changes in the percentage of isolates resistant to the combination of novobiocin and penicillin. *Pseudomonas* isolates were tested for susceptibility to amikacin and gentamicin; no significant changes in the percentages of isolates resistant to these antimicrobials were observed.

Discussion

Results of the present study support previous suggestions that the percentage of gram-positive bacterial isolates from cows with mastitis that are resistant to β-lactam antimicrobials is decreasing. In contrast, for some organisms, an increase in the percentage of isolates resistant to macrolide antimicrobials was found. Consistent reductions in percentages of isolates resistant to sulfisoxazole and trimethoprim-sulfamethoxazole were identified for a number of bacteria. Although resistance to specific antimicrobials appeared to increase in a few bacterial species, most had no significant changes in patterns of antimicrobial resistance over time.

Data for the present study consisted of results of antimicrobial susceptibility testing of a large number of bacterial isolates obtained from milk samples submitted to a central veterinary reference laboratory over a 6.5-year period. Laboratory protocols changed very little during the study period, allowing analysis of changes in antimicrobial susceptibility patterns. However, results were limited by the nature of the available data. With the exception of differentiating *Enterococcus* isolates and identifying *S agalactiae* isolates, *Streptococcus* isolates were not speciated by the laboratory. Significant differences in resistance among streptococcal species have been reported,[17] and relative differences in isolation of streptococcal species over the years of the study could have influenced our results. In addition, isolates were tested for antimicrobial susceptibility only at the request of the submitting veterinarian. Many of the isolates were probably obtained from cows with clinical signs of mastitis, but it was not possible to determine the reason for submission of individual milk samples, and some samples may have been submitted from cows with subclinical mastitis. Selected pathogens were tested for susceptibility to some antimicrobials for only part of the time period of the study. For example, testing for susceptibility to pirlimycin began during 1994. Pirlimycin is labeled as effective against gram-positive cocci, so the laboratory only tested *Streptococcus* and *Staphylococcus* isolates for susceptibility to pirlimycin. Laboratory protocols were modified in 1998, when a disk containing a combination of novobiocin and penicillin replaced the novobiocin disk. This change was made because commercially available products for the treatment of mastitis included a combination of these antimicrobials.

Kirby-Bauer disk diffusion was used to determine antimicrobial susceptibility of isolates in the present study. This technique is the most widely used method in veterinary medicine for determining susceptibility of animal pathogens.[18] The primary disadvantage of using this method when monitoring development of resistance is that outcomes are reported on a qualitative basis (sensitive, intermediate, or resistant), and subtle changes in susceptibility may not be apparent. In addition, it is difficult to compare outcomes of antimicrobial susceptibility testing among studies because of differences in the origin of isolates, laboratory procedures, and interpretive guidelines, and percentages of isolates susceptible to particular antimicrobials may vary from 1 study to the next.[6,19,20] For example, in a study[20] of dairy cows in Argentina with subclinical or clinical mastitis, a large percentage of *S aureus* isolates were reported to be resistant to penicillin,[20] but in another study,[19] the overall level of resistance of *S aureus* to antimicrobials was generally low.

In general, β-lactam antimicrobials, macrolides, and lincosamides are considered to be more effective against gram-positive bacteria than gram-negative bacteria. Tetracyclines and sulfonamides are regarded as broad-spectrum antimicrobials with greater activity against gram-negative pathogens. The overall levels of resistance found in our study for specific mastitis pathogens and various antimicrobials were comparable to levels of resistance that others have reported,[6,20] although we found gram-positive bacteria to be resistant to β-lactam antimicrobials less often than previously reported.

When evaluating changes in resistance by year, a significant (ie, < 0.05) goodness-of-fit P value for the logistic regression model suggested that a linear model did not accurately fit the data. Significant goodness-of-fit P values were identified for a few pathogens, indicating that factors other than year were influencing changes in antimicrobial resistance. The nature of our data did not allow us to identify these factors, because additional information was not available. Factors influencing resistance patterns included the reason for collection of milk samples (ie, subclinical vs clinical mastitis), past antimicrobial use on farms submitting milk samples for susceptibility testing, and the number of isolates tested in any given year. Our model identified changes in the percentage of bacterial isolates resistant to tested antimicrobials by year, but in some instances, other factors would need to be considered to create a model that completely describes the data.

The **odds ratio (OR)** from the logistic regression analysis can be used to determine the rate at which the prevalence of resistance is increasing or decreasing each year. Statistically significant odds ratios < 1.0 reflect a reduced odds of observing resistance during any given year, compared with the year before. Statistically significant odds ratios > 1.0 reflect an increased odds of observing resistance during any given year, compared with the year before. For example, the OR of 0.79 for resistance of *S aureus* isolates to cloxacillin can be interpreted to mean that the likelihood that *S aureus* would be resistant to cloxacillin was 0.79 that of the previous year.

In our study, *Enterococcus* spp were the only gram-positive bacteria for which resistance to any β-lactam antimicrobial (cloxacillin) increased. None of the major gram-positive mastitis pathogens had evidence of an increase in resistance to β-lactam antimicrobials during the period of this study. A decrease in resistance to penicillin was found for *S aureus*, *Streptococcus* spp, and *Enterococcus* spp isolates. In agreement with results of a previous study,[6] a high percentage (> 95%) of streptococcal isolates were found to be susceptible to ampicillin, cephalothin, and penicillin. Another study[7] reported a slight decrease in susceptibility to penicillin for *Streptococcus uberis* isolates and no change in susceptibility to penicillin for *Streptococcus dysgalactia* isolates. The percentage of *S aureus* isolates in the present study that were resistant to ampicillin and cloxacillin decreased, as did the percentage of *Streptococcus* spp isolates that were resistant to cephalothin. Our results agree with results of other studies[6,14,15] in which resistance of *S aureus* to ampicillin and penicillin also decreased.[6,14,15]

Relatively low cure rates for cows with chronic mastitis have encouraged some practitioners to reduce the use of intramammary antimicrobial treatments.[21] Our data did not allow us to identify reasons for these low cure rates, but the possibility that changes in the use of antimicrobial treatments for mastitis are contributing to this trend is an area for further research. Significant increases in percentages of *Enterobacter* and *Pasteurella* isolates resistant to ampicillin and cloxacillin, respectively, were detected in the present study. Most of these gram-negative isolates were resistant to these agents, and the increases in percentages of isolates that were resistant were not linear over time. Relatively few *Pasteurella* isolates were included in this study, and it is possible that a large number of submissions from 1 or 2 farms during a single year could have influenced results for this organism. The percentage of *Staphylococcus* isolates that were resistant to β-lactam antimicrobials was similar or slightly greater than the percentage of *S aureus* isolates that were resistant. To our knowledge, changes in resistance patterns of *Staphylococcus* spp have not been reported previously, and our study found no changes in percentage of *Staphylococcus* isolates resistant to β-lactam antimicrobials.

Chromosomal resistance to macrolide (erythromycin) and lincosamide (lincomycin and pirlimycin) antimicrobials can develop rapidly, and isolates that are resistant to macrolides should be considered to be cross-resistant to lincosomides.[17,22] According to laboratory protocols, most gram-negative pathogens in the present study were not tested for resistance to pirlimycin, but most were resistant to erythromycin and lincomycin. In our study, percentages of *S aureus* isolates resistant to erythromycin and lincomycin increased, as did percentages of *Staphylococcus* isolates resistant to lincomycin and pirlimycin.

Few changes in antimicrobial resistance to tetracycline have been reported.[6,14,15] In our study, only *Staphylococcus* and *Pseudomonas* had significant increases in percentages of isolates resistant to tetracycline. Increased susceptibility among *S dysgalactiae* with no change in susceptibility among *S aureus* and *S uberis* has been previously reported.[6] In the present study, streptococcal isolates were not speciated, preventing direct comparisons with previous research.

Sulfonamides are broad-spectrum antimicrobials with activity against gram-positive and gram-negative bacteria. Decreased therapeutic value of sulfonamides had been reported because of widespread acquired resistance, but less resistance occurs when sulfonamides are used in combination with trimethoprim.[23] In our study, bacteria were tested for resistance against sulfisoxazole and trimethoprim-sulfamethoxazole. All bacteria, except *Pseudomonas* spp, were less likely to be resistant to trimethoprim-sulfamethoxazole than to sulfisoxazole. In our study, the percentage of *S aureus* isolates resistant to sulfonamides was less than the percentage reported in 1974, but greater than percentages reported in 1988 and 1997.[14,15] The percentage of *Staphylococcus* isolates resistant to sulfonamides in our study was greater than the percentage reported in 1988 but less than the percentage reported in 1997.[14,15] Our study results were similar to results of a previous study[6] of gram-negative bacteria in that we identified consistent decreases in resistance to sulfisoxazole and trimethoprim-sulfamethoxazole for almost all isolates. Most sulfonamides were removed from use in dairy cattle during the 1990s, and it appears that bacterial resistance has decreased, possibly as a result of less exposure.

[a]Prompt Inoculation System, Becton-Dickinson Microbiology Systems, Sparks, Md.
[b]Proc FREQ, SAS version 8, SAS Institute Inc, Cary, NC.
[c]Proc LOGISTIC, SAS version 8, SAS Institute Inc, Cary, NC.

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