Prevalence of Mastitis Pathogens and Their Resistance Against Antimicrobial Agents in Dairy Cows in Brandenburg, Germany

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ABSTRACT

The primary objective of this study was to determine management practices concerning mastitis in Brandenburg, Germany, the prevalence of mastitis pathogens in dairy cows, and their resistance to selected antimicrobial agents. A further objective was to study the potential effect of parity and stage of lactation on the resistance of *Staphylococcus aureus* isolates against ampicillin. Milk samples for microbiological culture were collected from 4 groups of clinically healthy cows (first lactation, >1 lactation, >50 d in milk, and >250 d in milk; 8 cows/group) in 80 dairy herds. Resistance of gram-positive pathogens against 6 antimicrobial agents was tested using the broth microdilution method. Mastitis pathogens were isolated from 26.4% of the milk samples. Coagulase-negative staphylococci (CNS, 9.1% of quarters) and *Corynebacterium bovis* (7.3%) were the pathogens most frequently isolated. Among the major pathogens, *Staph. aureus* (5.7%) and *Streptococcus uberis* (1.0%) had the highest prevalence. *Streptococcus agalactiae* was isolated in samples from 29% of the herds. Although the prevalence of most pathogens was higher in older cows, the prevalence of CNS was higher in primiparous cows. Results of the mastitis control questionnaire showed that cows with clinical mastitis were transferred to a sick cow pen in 70% of the herds. Cephalosporins were the drug of first choice for treatment of clinical mastitis cases followed by fixed combinations of antimicrobial agents, β -lactamase-resistant penicillins, and penicillin. Most farmers treated cows 3 to 4 times per case. Cloxacillin, alone or in combination, and penicillin were most often used for dry-cow therapy. Antimicrobial resistance of the pathogens was within the range of other reports. Resistance of *Staph. aureus* to ampicillin increased significantly during the first lactation. Further research is required to determine the factors that lead to the selection of *Staph. aureus* strains that are resistant to ampicillin during the first lactation.

Key words: dairy cow, mastitis, antimicrobial resistance

INTRODUCTION

Mastitis is one of the major causes of antibiotic use in dairy cows (Mitchell et al., 1998; DANMAP, 2003). Over 135 different microorganisms have been isolated from bovine intramammary infections (IMI), but the majority of infections are caused by staphylococci, streptococci, and gram-negative bacteria (Bradley, 2002). The contribution of various strains of bacteria to mastitis has shifted over time. Measures to control mastitis such as improved milking hygiene have reduced the prevalence of contagious major pathogens such as *Streptococcus agalactiae* (Hillerton et al., 1995; Myllys et al., 1998; Makovec and Ruegg, 2003a; Pitkala et al., 2004). Somatic cell counts have decreased in many countries (Myllys et al., 1998). However, other bacteria such as environmental streptococci have become more important, and the reduction in subclinical mastitis has not been accompanied by a reduction in clinical mastitis (Myllys et al., 1998).

Resistance of mastitis pathogens to antimicrobial agents is a well-documented challenge in dairy cows (Owens et al., 1997; Lotthammer and Klarmann, 1999; Trolldenier, 1999; Erskine et al., 2002; Makovec and Ruegg, 2003b; Pitkala et al., 2004). The World Health Organization (WHO) has stated that any use of antimicrobial agents is associated with the risk of inducing resistance to antimicrobial agents among bacteria (WHO, 1997). This has called for more investigations into the use of antimicrobial agents in food-producing animals and the determination of potential factors that influence the level of resistance in mastitis pathogens (Lotthammer and Klarmann, 1999; Osteras et al., 1999; Trolldenier, 1999; Aarestrup, 2005). Regional differences in resistance patterns of pathogens exist in Germany and worldwide (Salmon et al., 1998; Lotthammer and Klarmann, 1999; de Oliveira et al., 2000). However, a relationship between the resistance patterns of mastitis pathogens and the intensity of food animal husbandry in the respective regions could not be estab-

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lished (Lotthammer and Klarmann, 1999; Schröter, 2003).

Differences in resistance patterns between reports may be caused to some extent by the variation in methods used for the determination of resistance against antimicrobial agents. Early reports on resistance, but also some recent ones (Erskine et al., 2002; Makovec and Ruegg, 2003b) were based on the disk diffusion method, which has been shown not to correlate well with the minimum inhibitory concentrations (MIC) determined by dilution methods (Kibsey et al., 1994; Kelly et al., 1999). Presently, dilution methods are recommended by expert groups as the methods of choice (Erskine et al., 2004; Luhofer et al., 2004).

Resistance of *Staphylococcus aureus* to penicillin or ampicillin has been extensively studied (Erskine et al., 2004). Although the MIC of penicillin against *Staph. aureus* did not differ between strains isolated from heifers and cows in one study (Watts et al., 1995), a recent report has demonstrated numerically higher proportions of penicillin-resistant CNS in older cows compared with isolates from primiparous cows (Rajala-Schultz et al., 2004). Treatment with penicillin at dry off has been proposed to exert selection pressure toward penicillin-resistant *Staph. aureus* strains (Osteras et al., 1999).

Resistance to antimicrobial agents in mastitis pathogens has 2 relevant aspects: The first is a reduction in cure rates after treatment of clinical mastitis cases (Owens et al., 1997; Sol et al., 2000). The second issue is the potential impact of transmission of resistant bacteria to humans via the food chain (Ungemach, 1999). This is not likely to occur with milk from clinical cases of mastitis, because this milk is banned from human consumption. However, clinical cases may turn into subclinical cases or latent infections. Resistant bacteria from these infections are present in the bulk tank milk and may therefore be transmitted to humans via raw milk products.

In eastern German provinces, herd sizes are bigger than in most other parts of the European Union. Limited literature exists on the contribution of different mastitis pathogens to the mastitis problem of these dairy herds and on their management practices concerning mastitis problems.

Therefore, the objectives of this study were to determine management practices concerning mastitis, and the prevalence of mastitis pathogens in clinically healthy quarters of dairy cows in Brandenburg, Germany. A further objective was to determine the susceptibility of these bacteria to 6 antimicrobial agents that are or have been commonly used in dairy cows. Finally, we investigated whether the prevalence of ampicillin resistance of *Staph. aureus* was influenced by parity or stage of lactation.

MATERIALS AND METHODS

The study was conducted on 80 dairy farms selected from the respondents to a mail survey on dairy cow management practices with particular respect to mastitis. A standardized questionnaire had been sent to 845 farms that were members of the Brandenburg Dairy Herd Improvement Association (Landeskontrollverband Brandenburg, Waldsiedersdorf, Germany). The German province of Brandenburg is located around the German capital (Berlin). Two hundred farms (23.7%) responded to the questionnaire and indicated willingness for further cooperation. A convenience sample comprising 96 of the respondents was chosen to participate in the study. The selection of herds was carried out to achieve representative distribution across the province concerning size and location of the farms. Of the selected farms, 16 were removed from the analysis for reasons of poor data quality or limited data access.

All farms were visited once between July 2001 and October 2002 by the same investigator. Management practices, housing conditions, and milking routines were evaluated and documented on a standardized data capture form. Milking routines were recorded by observation of routine milking over one milking period. Management of cows with mastitis was recorded as observed during the visit. For events that could not be observed during the visit (e.g., dry-cow routines, treatment protocols), management was recorded as indicated by the farm manager in a questionnaire covering 20 items.

Aseptic quarter foremilk samples were collected from 4 groups of animals: 1) Primiparous cows at the beginning of the lactation (≤ 50 DIM); 2) primiparous cows at the end of lactation (≥ 250 DIM); 3) older cows ($\geq$ second lactation) at the beginning of the lactation (≤ 50 DIM); and 4) older cows ($\geq$ second lactation) at the end of lactation (≥ 250 DIM).

Eight clinically healthy cows of each of the 4 groups were sampled at milking time before cluster attachment on each farm. Cows were selected in order of their appearance in the milking parlor during the visit. Cows with blind quarters were included, but they only contributed 3 samples per cow (Table 1). Samples were cooled and shipped to the laboratory at the same day.

Microbiological Analyses

Milk samples (0.01 mL) were plated on blood agar (blood agar base no. 2, Oxoid, Wesel, Germany, with 5% sheep blood) that contained 0.1% esculin. Preliminary identification was by colony morphology, hemolysis, and Gram staining. Samples were regarded as positive for environmental pathogens if a minimum of 1,000 cfu of the respective pathogen was determined. For *Strep. agalactiae* and *Staph. aureus*, a minimum of 100 cfu

Table 1. Prevalence of mastitis pathogens in samples from 4 different groups of cows (n = 2,560)

Species	n	%	Primiparous		≥ Second lactation	
			≤50 DIM (%)	≥250 DIM (%)	≤50 DIM (%)	≥250 DIM (%)
Negative	7,296	73.6	80.2 ^{ac}	72.1 ^{bc}	76.3 ^{ad}	65.9 ^{bd}
CNS	903	9.1	10.4 ^c	10.7 ^c	7.8 ^d	7.6 ^d
<i>Corynebacterium bovis</i>	722	7.3	2.8 ^{ac}	8.1 ^{bc}	6.0 ^{ad}	12.3 ^{bd}
<i>Staphylococcus aureus</i>	569	5.7	3.8 ^a	6.3 ^{bc}	4.7 ^a	8.2 ^{bd}
<i>Streptococcus uberis</i>	98	1.0	0.6	0.4 ^c	1.0 ^a	1.9 ^{bd}
<i>Streptococcus</i> spp. ¹	78	0.8	0.8	0.6	0.8	1.0
<i>Streptococcus agalactiae</i>	70	0.7	0.3 ^c	0.5	1.1 ^d	0.9
<i>Streptococcus dysgalactiae</i>	49	0.5	0.3	0.3	0.8	0.6
Coliforms	28	0.3	0.2	0.2	0.3	0.4
<i>Enterococcus faecalis</i>	23	0.2	0.1	0.4	0.2	0.2
<i>Enterococcus faecium</i>	19	0.2	0.2	0.2	0.2	0.2
Others ²	55	0.6	0.4	0.3	0.7	0.8
Analyzed samples	9,910	100	2,506	2,455	2,474	2,475
Contaminated/broken (n)	124		20	56	36	12
Total samples (n)	10,034		2,526	2,511	2,510	2,487
Dry quarters (n)	206		34	49	50	73

^{a,b}Significant difference between stages of lactation ($P < 0.05$).

^{c,d}Significant difference between parity classes ($P < 0.05$).

¹Includes all *Streptococcus* spp. that are not explicitly mentioned.

²Includes *Arcanobacterium pyogenes*, yeasts, *Prototheca* spp., and *Pseudomonas* spp.

was required. *Staphylococcus aureus* was differentiated from CNS using a commercial test kit (Slidex Staph Plus, BioMerieux, Nürtingen, Germany). Streptococci were differentiated by the Christie, Atkins, Munch-Petersen phenomenon and esculin hydrolysis. *Streptococcus dysgalactiae* and *Strep. agalactiae* were further identified using Lancefield grouping (streptococcal grouping kit, Oxoid) with *Strep. dysgalactiae* being positive for C and *Strep. agalactiae* for B. *Enterococcus* spp. and *Strep. uberis* were further identified with biochemical methods. The isolates were stored at -80°C for further investigation.

Determination of the MIC

The MIC of the bacteria was determined using the microbroth dilution method in accordance with instructions M31-A2 of the Clinical Laboratory Standards Institute (CLSI, 2002). The evaluation was undertaken visually with the help of a semiautomatic readout device (SensiTouch, MCS Diagnostics, Swalmen, The Netherlands). To validate the results obtained, the bacterial counts of the bacterial suspension and the purity of the inoculum were determined. Reference strains *Staph. aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; Braunschweig, Germany) were examined in parallel. The MIC level was defined as the minimum concentration of an antimicrobial agent that inhibited visible growth.

Minimum inhibitory concentrations of 6 antimicrobial agents (ampicillin, oxacillin, amoxicillin/clavula-

nate, gentamicin, cefquinome, and streptomycin) were determined for the gram-positive pathogens *Staph. aureus* (n = 199), *Strep. uberis* (n = 69), *Strep. dysgalactiae* (n = 30), and *Enterococci* (n = 27) using commercial microtiter plates. *Streptococcus agalactiae* was not tested because it was assumed to be generally susceptible to penicillin.

Statistical Analyses

The association of IMI with group was tested using logistic regression with group (1 to 4), quarter (1 to 4), and herd as categorical covariates and the presence of the respective pathogen as binary outcome. Logistic regression was run twice for each pathogen with 2 reference groups for the covariate group; i.e., group 1 and group 4. This was done to be able to better identify the effects of parity and stage of lactation. Only pathogens that were isolated from a minimum of 50 quarters were selected as outcome variables. Separate models for the different pathogens were calculated with quarters that carried a pathogen different from the outcome pathogen being coded as not infected with the pathogen in question. Blind quarters and samples that could not be analyzed were coded as missing values.

To control for possible interactions between the quarters of individual cows, the same analysis was performed on the cow level. Cows were coded as positive for a respective pathogen, if at least one quarter was infected with the pathogen, otherwise negative.

In addition to the analysis by pathogen, a ratio between major contagious and environmental pathogens

was calculated to summarize the contribution of environmental and contagious pathogens to the infection pattern of the 4 groups. Contagious pathogens in this calculation were *Staph. aureus* and *Strep. agalactiae*. Environmental pathogens were all *Streptococcus* spp. (except *Strep. agalactiae*), all enterococci, and coliforms.

The association of group with *Staph. aureus* resistance to ampicillin was tested using binary logistic regression with resistance to the antimicrobial agent as the binary outcome and group, herd, and quarter as covariates. The analysis was carried out for 2 different breakpoints (0.25 and 0.5 mg/L) to account for different breakpoints set in different countries. This analysis was not performed for the other bacteria and for other antimicrobial agents against *Staph. aureus*, because the number of isolates was limited and variability was low.

All logistic regressions were carried out using SPSS software (SPSS 12.0, SPSS Inc., Munich, Germany).

RESULTS

Average herd size in the remaining herds was 300 cows with 56% of the herds between 100 and 299 cows and 2 herds with more than 900 cows. The mean test-day SCC of the herds in the month of the visit was 372,000 cells/mL, with a range from 158,000 to 754,000 cells/mL. Cows produced 22.1 kg of milk/d (including dry cows) on average.

Management Practices

Cows with clinical cases of mastitis were transferred into a sick cow pen in 56 of the 80 farms. Another 8 farms housed these animals together with other cows producing nonsaleable milk (i.e., fresh cows). Of the 16 farms (20%) that did not separate the sick and treated cows from the producing herd, 2 had their cows in the herd without marking them. They relied on the accuracy of the person in the milking parlor. One herd used the software provided by the milking parlor to block sick cows from the bulk tank. The other 13 herds marked the cows with color bands around the legs.

Results of the questionnaire on mastitis management indicated that in 35% of the farms cephalosporins (cefoperazone and cefquinome) were used as the first choice of treatment for clinical mastitis. Oxacillin or cloxacillin was used on 17% and penicillin was the first choice on 13% of the farms. Oxytetracycline was used on 6% of the farms and fixed commercial combinations of antimicrobial agents were used on 27% of farms. The fixed combinations were variable, but in the majority of cases they contained 1 antimicrobial agent directed against gram-positive and 1 against gram-negative bacteria.

Eighteen farms did not give information on the type of product they used. Information on type of treatment (intramammary or systemic) was not requested.

Three or more treatments per case of clinical mastitis were carried out by 47% of the farmers that responded to this question (77 of 80). Information on the interval between treatments was not requested. Three farmers (4%) administered a minimum of 2 treatments. The other farmers treated "until the secretion looked normal again" (47%) or SCC dropped according to California Mastitis Test results (3%).

Nineteen farmers did not report the maximum number of treatments for one case of mastitis. Six farmers performed a therapy "until there was success". Of the farmers with more detailed answers (55 of 80), most (45%) gave a maximum of 3 to 4 treatments. Twelve farmers (22%) treated more and longer, 16 (29%) less often.

Twelve farmers (15%) changed the antimicrobial agent during the treatment of mastitis cases. Seventeen (22%) did not do so and most of the farmers changed the antimicrobial agent from time to time (63%). Two farmers did not answer this question.

Blanket dry-cow therapy was performed on 67 of the 79 farms that provided an answer to this question. Another 12 farmers used dry-cow therapy selectively for cows with high SCC (10 farmers, threshold not specified) or a case of clinical mastitis during lactation (3 herds). Twelve farmers did not provide information about the medicinal products they currently used for dry cows. Cloxacillin alone (46%) or in combination (21%) was the antimicrobial agent most often used at dry-off. Penicillin was used on 31% of the farms and neomycin on 24%. Other antimicrobial agents used for this purpose were framycetin (21% in combination with penicillin), streptomycin and nafcillin (6% in combination with penicillin), cefoperazone, ampicillin, and erythromycin (1% each).

Microbiological Cultures

A total of 9,910 milk samples collected from 2,529 cows were included in the study. For technical reasons (contamination or broken samples), 124 samples (1.2%) of 31 cows could not be included. Two hundred and six quarters (2.0%) were blind.

Of the samples included in the study, 2,614 (26.4%) samples contained mastitis pathogens (Table 1). *Corynebacterium bovis* and CNS were the predominant findings, accounting for 62.2% of the positive samples. Among the major pathogens isolated, *Staph. aureus* was the predominant contagious agent (21.8%) and *Strep. uberis* (3.7% of the positive samples) was the major environmental pathogen.

Effect of group, herd, and quarter was tested for CNS, *C. bovis*, *Staph. aureus*, *Strep. uberis*, *Strep. agalactiae*, other streptococci, and negative samples. More samples were negative in primiparous than in older cows ($P < 0.001$) and in early lactation than in late lactation ($P < 0.001$). The prevalence of CNS was lower in older than in primiparous cows ($P < 0.001$), but did not differ between early and late lactation. On the other hand, the prevalence of *C. bovis* was higher in late lactation ($P < 0.001$) and in multiparous cows ($P < 0.001$). The prevalence of *Staph. aureus* increased during lactation ($P < 0.001$). In late lactation, it was higher in older cows than in primiparous cows ($P < 0.05$). *Streptococcus agalactiae* was more prevalent in older than in primiparous cows in early ($P = 0.001$) and late lactation ($P = 0.05$). However, there was no significant effect of stage of lactation on the prevalence. In older cows, the prevalence of *Strep. uberis* increased during lactation ($P = 0.01$) and was higher in late lactation than in primiparous cows in late lactation ($P < 0.001$).

A second analysis, carried out at the cow level to control for possible interactions between the quarters, delivered the same significant differences between the groups with one exception. The difference in prevalence of *Strep. uberis* between early- and late-lactation multiparous cows was not significant on the cow level.

The overall ratio between contagious major pathogens (*Staph. aureus* and *Strep. agalactiae*) and environmental major pathogens (nonagalactiae streptococci, coliforms) was between 1.73 and 2.13 in groups 1, 3, and 4, respectively. In contrast, late-lactation primiparous cows (group 2) showed a ratio of 3.25 indicating a substantial increase in contagious pathogens (4.1 to 6.6%) compared with environmental pathogens (2.2 to 2.1%) during the first lactation. In older cows, the increase in contagious pathogens was within the same range (5.7 to 8.9%), but there was also an increase in environmental pathogens (3.3 to 4.2%).

There was great variation between farms concerning the prevalence of mastitis pathogens. Only CNS were identified in samples from all farms (Table 2).

Antibiotic Resistance

A total of 325 isolates of 5 species from 300 cows of 75 herds were tested against 6 antimicrobial agents (Table 3). Twenty-five cows contributed 2 isolates. In 19 cases, these were from different species; in 6 cases, they were from the same species. The maximum number of isolates per farm was 10; of these, no more than 8 were from the same species.

For the analysis of effects on the risk of ampicillin resistance in *Staph. aureus* isolates, 4 isolates were dropped because 4 cows had contributed 2 isolates. The

risk differed significantly between groups ($P = 0.036$). Based on a breakpoint of 0.25 mg/L, the proportion of *Staph. aureus* isolates that were resistant to ampicillin was lower in early- (31%) than in late-lactation primiparous cows (61%) and in early- and late-lactation multiparous cows (47 and 48%, respectively). The risk of resistance to ampicillin was 8.8 times higher in late lactation primiparous cows than in early lactation primiparous cows [odds ratio (OR) = 8.8, 95% CI = 1.8–41.7]. In early- (OR = 1.5, CI = 0.3–6.5) and late-lactation multiparous cows (OR = 3.1, CI = 0.8–11.4), the risk was not significantly increased compared with early-lactation primiparous cows. Quarter and herd were not significantly associated with resistance of *Staph. aureus* to ampicillin. Using the breakpoint 0.5 mg/L, OR were similar, but the overall proportion of isolates classified as resistant was lower (data not shown).

DISCUSSION

Prevalence of Pathogens

Minor pathogens were the predominant group of bacteria isolated from milk samples in this study. This is in agreement with other German and European reports (Sobiraj et al., 1997; Pitkala et al., 2004). *Staphylococcus* spp., other than *Staph. aureus*, also had the highest prevalence in reports from the United States (Wilson et al., 1997; Makovec and Ruegg, 2003a).

The analysis of factors associated with IMI on the quarter level is always confronted with the complex interrelationship of quarters and the effect of one pathogen on the risk of infection with another. To cope with the first obstacle we chose to perform the analysis on both the quarter and cow levels. The close-to-perfect agreement of the differences between groups indicates that for the purpose of this study, the interaction between quarters of individual cows was of minor importance.

A possible interaction between pathogens was ignored in this study. This seemed reasonable, because we did not study the risk of new infections in cows over time but in different groups of cows. Because about 75% of the quarters were negative, the possible effect of the interaction was probably small.

Besides the minor pathogens, *Staph. aureus* was still the pathogen with the highest prevalence in clinically healthy animals. This is in accordance with data from other countries (Poelarends et al., 2001; Giannechini et al., 2002). In our study, the prevalence of *Staph. aureus* was higher in late than in early lactation. In late lactation, multiparous cows carried more *Staph. aureus* infections than primiparous cows, whereas the difference in early lactation was not significant. This may be due to the widespread use of dry-cow therapy

Table 2. Prevalence of mastitis pathogens in quarter foremilk samples from 80 dairy herds in Brandenburg, Germany

Species	Prevalence (%)				Herds negative (%)
	Mean	Median	Minimum	Maximum	
<i>Staphylococcus aureus</i>	5.7	2.75	0	31.7	10.0
<i>Streptococcus agalactiae</i>	0.7	0	0	8.9	71.3
Nonagalactiae streptococci	2.7	2.3	0	13.7	10.0
<i>Streptococcus uberis</i>	1.0	0.8	0	8.9	45.0
<i>Streptococcus dysgalactiae</i>	0.5	0	0	4.8	63.8
<i>Enterococcus</i> spp.	0.4	0	0	2.4	63.8
Coliforms	0.3	0	0	4.8	78.8
CNS	9.1	8.0	2.3	27.3	0
<i>Corynebacterium bovis</i>	7.3	6.4	0	22.5	10.0

in the herds studied that is known to reduce the prevalence of *Staph. aureus* effectively. The higher susceptibility of older cows to IMI with *Staph. aureus* in the course of lactation is in accordance with a report on a higher susceptibility of quarters that had been infected with *Staph. aureus* previously (Zadoks et al., 2001), which is more likely in older cows. However, it is not clear whether these are new infections or flare-ups of old infections that were not fully eliminated.

Streptococcus agalactiae was still found in about 30% of the herds. This has also been reported for other European countries (Poelarends et al., 2001). Data from the United States suggest a somewhat higher prevalence of this species. In contrast to 2.7% of our bacteriologically

positive samples, *Strep. agalactiae* was found in 13.1% of the positive samples in New York and Pennsylvania (Wilson et al., 1997) and in 7.7% of the positive samples in Wisconsin (Makovec and Ruegg, 2003a). However, these papers report data from years ago and the samples in these 2 studies were from diagnostic laboratories and likely included more samples from problem herds and problem cows. Makovec and Ruegg (2003a) described a constant decline of the prevalence of *Strep. agalactiae* in their diagnostic material with a prevalence of 3% in the last year of their investigation, which is similar to our results.

As expected, *Strep. uberis* had the highest prevalence among the environmental pathogens (Morin et al.,

Table 3. Minimum inhibitory concentrations of 6 antimicrobial agents against *Staphylococcus aureus* (n = 199), enterococci (n = 27), *Streptococcus uberis* (n = 69), and *Streptococcus dysgalactiae* (n = 30) isolated from clinically healthy udder quarters of dairy cows

Antimicrobial agent ¹	Species	MIC, ¹ mg/L															
		0.008	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
Ampicillin	<i>Enterococcus</i> spp.			4	1	5	2	3	9	3	0	0	0	0			
	<i>Staph. aureus</i>			19	46	44	22	9	32	17	7	1	0	2			
	<i>Strep. dysgalactiae</i>			27	1	0	1	0	1	0	0	0	0	0			
	<i>Strep. uberis</i>			33	15	14	5	2	0	0	0	0	0	0			
Oxacillin + 2 % NaCl	<i>Enterococcus</i> spp.				2	3	0	0	5	3	0	3	6	1	2	2	
	<i>Staph. aureus</i>				5	34	81	66	9	3	1	0	0	0	0	0	
	<i>Strep. dysgalactiae</i>				24	4	0	0	1	0	0	0	1	0	0	0	
	<i>Strep. uberis</i>				19	13	2	4	23	6	2	0	0	0	0	0	
Amoxicillin/ clavulanic acid	<i>Enterococcus</i> spp.					8	5	3	11								
	<i>Staph. aureus</i>					97	38	42	22								
	<i>Strep. dysgalactiae</i>					29	0	0	1								
	<i>Strep. uberis</i>					54	15	0	0								
Cefquinome	<i>Enterococcus</i> spp.	4	0	1	1	3	3	1	0	1	5	6	1				
	<i>Staph. aureus</i>	0	0	0	0	2	33	80	52	3	0	0	0				
	<i>Strep. dysgalactiae</i>	25	2	0	0	1	0	0	0	0	1	0	0				
	<i>Strep. uberis</i>	24	6	4	7	18	2	0	0	0	0	0	0				
Gentamicin	<i>Enterococcus</i> spp.					0	0	1	2	5	2	3	10	3	1		
	<i>Staph. aureus</i>					55	92	33	9	6	1	2	0	0	1		
	<i>Strep. dysgalactiae</i>					3	1	2	16	6	1	0	1	0	0	0	
	<i>Strep. uberis</i>					2	5	2	2	6	11	14	18	8	1		
Streptomycin	<i>Enterococcus</i> spp.							0	0	0	0	1	2	2	3	8	11
	<i>Staph. aureus</i>							1	15	16	68	72	17	2	4	2	2
	<i>Strep. dysgalactiae</i>							1	0	1	9	10	3	0	0	1	5
	<i>Strep. uberis</i>							0	0	0	2	0	4	9	10	19	25

¹MIC₅₀ are displayed in italics; MIC₉₀ in bold; bold and italic entries indicate that MIC₅₀ and MIC₉₀ were identical.

2001; Poelarends et al., 2001). *Strep. uberis* was more prevalent at the end of lactation in older than in primiparous cows, whereas it did not differ significantly between the age groups in early lactation (Todhunter et al., 1995; Zadoks et al., 2001). In contrast to the prevalence of *Staph. aureus*, the prevalence of *Strep. uberis* in primiparous cows did not increase during lactation. This explains the increase of the ratio between contagious and environmental pathogens in primiparous cows at the end of lactation (Zadoks et al., 2001).

Antibiotic Resistance

Data on the amount of antimicrobial agents used in dairy cows and on treatment procedures applied by practitioners and farmers are limited (Grave et al., 1999; DANMAP, 2003) or anecdotal when problems occur that are associated with treatment protocols (Schukken et al., 2000). Although the overall use of antimicrobial agents is lower in dairy cows than it is in the pork or poultry industries (DANMAP, 2003), routine treatments like dry-off therapy are widely recommended and implemented on almost all large farms in Germany. Cephalosporins, penicillins, and synthetic penicillins that are resistant to β -lactamase account for most of the treatments in the study farms. This is in line with reports from Denmark (DANMAP, 2003).

Comparing the classification of bacteria as susceptible or resistant requires reference to the breakpoints used. For several antimicrobial agents, no generally accepted veterinary breakpoints are available (Kietzmann et al., 2004). Overall, the proportion of isolates that were resistant to the antimicrobial agents tested was within the range of other reports from Germany using the same breakpoints (Trolldenier and Wagner, 2001; Schröter, 2003; Wallmann et al., 2003, 2004). Reports based on the agar gel-diffusion method are difficult to compare with those performed with dilution methods because there is only limited agreement between the results of the 2 methods (Kibsey et al., 1994; Kelly et al., 1999; Schwarz et al., 2003).

Enterococci. *Enterococcus* spp. are frequently used as indicator bacteria for the development of antimicrobial resistance (DANMAP, 2003). Limited information is available from random samples of bacteria in dairy cows, and even less on mastitis pathogens isolated from milk samples (Rossitto et al., 2002; Pitkala et al., 2004). The susceptibility data of the examined *enterococci* showed no resistance to ampicillin (breakpoint 8 mg/L). This result and the MIC₉₀ of 1 mg/L correspond with data from other reports (Watts et al., 1995; Rossitto et al., 2002; Schlegelova et al., 2002). The MIC₉₀ of oxacillin and cefquinome for enterococci were high in our study (64 and 8 mg/L, respectively). Resistance to oxa-

cillin and cephalosporins is common among enterococci (Watts et al., 1995; Myllys et al., 1998; Rossitto et al., 2002; Schlegelova et al., 2002). The MIC₉₀ of streptomycin was lower than in other reports (Schlegelova et al., 2002; DANMAP, 2003).

Staphylococcus aureus. In our study, the MIC₉₀ of ampicillin for *Staph. aureus* isolates was higher than in other studies (Salmon et al., 1998; Schröter, 2003; Wallmann et al., 2004) but in agreement with an international study (de Oliveira et al., 2000). Some authors reported a reduction in the proportion of *Staph. aureus* isolates that were resistant to β -lactams over the last decade when the disk diffusion method was used (Erskine et al., 2002; Makovec and Ruegg, 2003b). Salmon et al. (1998) reported striking differences in the efficacy of penicillin and cephalosporins against *Staph. aureus* isolates isolated from heifers in New Zealand and Denmark.

The MIC₉₀ of oxacillin for *Staph. aureus* was in the same range as in most other studies (de Oliveira et al., 2000; Gentilini et al., 2000; Yoshimura et al., 2002; Schröter, 2003). The MIC₉₀ of streptomycin for *Staph. aureus* was lower than in a recent report from the Czech Republic (Schlegelova et al., 2002), but slightly higher than the 2 mg/L reported from a German survey (Schröter, 2003). For gentamicin, the MIC₉₀ of 0.5 mg/L underlines the results of other recent studies (Yoshimura et al., 2002; Schröter, 2003; Wallmann et al., 2004).

Information on the susceptibility of *Staph. aureus* from milk to amoxicillin/clavulanic acid is rare in the literature. The MIC₉₀ of 1 mg/L determined in this study is similar to other recent German studies (Schröter, 2003; Wallmann et al., 2004) but higher than published for Germany in an international study (de Oliveira et al., 2000).

Streptococcus uberis. All *Strep. uberis* isolates were susceptible to ampicillin, amoxicillin/clavulanic acid, and cefquinome according to breakpoints published by Clinical Laboratory Standards Institute (CLSI, 2002). This supports data from several other reports (Owens et al., 1997; Trolldenier, 1999; Rossitto et al., 2002; Pitkala et al., 2004). The MIC₉₀ of 2 mg/L for oxacillin was within the range of other studies. In an older German study, Trolldenier (1999) also determined a MIC₉₀ of 2 mg/L. Rossitto et al. (2002) reported a slightly lower MIC₉₀ of 1 mg/L for milk samples from California. Salmon et al. (1998) reported 16 mg/L, but they only tested 15 isolates from Denmark.

Streptococcus dysgalactiae. Minimum inhibitory concentrations of ampicillin and cefquinome were low for *Strep. dysgalactiae*. Rossitto et al. (2002) reported a MIC₉₀ of 0.06 mg/mL of ampicillin for 152 isolates of *Strep. dysgalactiae*; they did not test lower concentra-

tions. In our study, we found 0.03 mg/L to be inhibitory as well. Likewise, the MIC₉₀ of 0.125 mg/L determined for oxacillin in our study is below the tested concentration in the study reported by Rossitto et al. (2002). Trolldenier (1999) reported an MIC₉₀ of 0.016 mg/L for ampicillin and 0.09 mg/L for oxacillin.

Factors Affecting Antibiotic Resistance. The proportion of ampicillin-resistant *Staph. aureus* isolates was significantly lower in primiparous cows in early lactation than in the same age group in late lactation. To our knowledge, this difference has not been described before. Recently, Rajala-Schultz et al. (2004) reported a numerical difference in the proportion of penicillin-resistant CNS between primiparous and older cows. Drying off with antimicrobial drugs designed for lactation therapy has been proposed as a risk factor for subclinical mastitis with penicillin-resistant *Staph. aureus* strains (Osteras et al., 1999). Although a difference between age groups may have been caused by dry cow therapy selectively eliminating the strains susceptible to penicillin, this cannot explain the difference between early and late lactation in primiparous cows.

It has been proposed that strains susceptible to penicillin are also more susceptible to lactation therapy (Sol et al., 2000). This may be a selective pressure during lactation; however, treatment records of the cows that were sampled were not available.

Another possible explanation is the genetic diversity of strains found in early lactation primiparous cows and in older cows. Recently, it has been demonstrated that in herds with good milking hygiene, sporadic IMI with *Staph. aureus* are caused by a variety of strains even in the same herd, indicating an environmental origin of the strains (Sommerhäuser et al., 2003). In herds with poor milking hygiene, strains were more homogeneous. Infections present in primiparous cows at the onset of lactation are likely to be of environmental origin too, because these animals have not been confronted with the milking process. Sommerhäuser et al. (2003) have proposed that these strains might also be less adapted to the mammary gland and therefore be more easily eliminated and less easily spread within the herd. With respect to the results of our study this may be a cause of the increasing proportion of ampicillin resistant isolates not related to use of antimicrobial agents but to the ecology of the respective *Staph. aureus* strains. Further research will be needed to investigate this hypothesis.

CONCLUSIONS

Coagulase-negative staphylococci had the highest prevalence in clinically healthy cows, especially in pri-

miparous cows. *Staphylococcus aureus* was the predominant major pathogen. The prevalence of *Staph. aureus* increased with age and stage of lactation. *Streptococcus uberis* was the most frequent environmental pathogen isolated from the animals. Although its prevalence increased during lactation in older cows, it did not increase in primiparous cows.

Antimicrobial resistance determined in our study was in line with other reports. Interestingly, a lower proportion of ampicillin-resistant isolates of *Staph. aureus* was found in early lactation primiparous cows. This finding indicates the need for further investigation of the epidemiology of resistance against penicillin in *Staph. aureus* isolated from bovine mammary glands.

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