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Phenotypic antimicrobial susceptibility and occurrence of selected resistance genes in gram-positive mastitis pathogens isolated from Wisconsin dairy cows

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ABSTRACT

In the United States, few intramammary antimicrobials exist that are approved for treatment of bovine mastitis; thus, ensuring judicious use of these products is a priority. The objectives of this study were to determine phenotypic susceptibility and presence of selected antimicrobial resistance genes from staphylococci, streptococci, and streptococcal-like organisms recovered from cases of clinical mastitis occurring in cows on large Wisconsin farms. *Staphylococcus aureus* (n = 35 from 19 herds), coagulase-negative staphylococci (n = 51 from 30 herds), *Streptococcus* spp. (n = 78 from 36 herds), and streptococcal-like organisms (n = 31 from 19 herds) were used in this study. All *Staphylococcus* spp. were susceptible to ceftiofur, cephalothin, and the combination of penicillin and novobiocin. Of all staphylococci, only a single *Staphylococcus epidermidis* exhibited phenotypic resistance to oxacillin. Phenotypic susceptibility to erythromycin was observed in only 8.6 and 15.7% of *Staphylococcus aureus* and coagulase-negative staphylococci, respectively. Approximately 20% of staphylococci and 13 to 22% of streptococci and streptococcal-like organisms exhibited phenotypic resistance to pirlimycin. All *Streptococcus* spp. exhibited phenotypic susceptibility to ceftiofur, cephalothin, and oxacillin. The proportion of isolates exhibiting phenotypic susceptibility to pirlimycin and sulfadimethoxine differed among *Streptococcus dysgalactiae* and *Streptococcus uberis*. All streptococcal-like organisms exhibited phenotypic susceptibility to ceftiofur, cephalothin, oxacillin, penicillin, and the combination of penicillin and novobiocin. Of all organisms tested, 36.9% did not carry any of the resistance genes (*ermC*, *blaZ*, *tetK*, or *tetM*), 35.4% carried 1 gene, and 27.7% carried multiple

genes (usually *blaZ* in combination with a *tet* gene). Eighteen (51.4%) *Staph. aureus* and 12 (48.0%) *Staphylococcus chromogenes* carried multiple resistance genes. Six (12.2%) *Strep. dysgalactiae* and no *Strep. uberis* carried multiple resistance genes. Results indicate that most gram-positive mastitis organisms were susceptible to most antimicrobials used for intramammary administration, but some resistance to drugs used for systemic treatment of mastitis was noted. The presence of selected resistance genes was not proportional to the occurrence of phenotypic resistance.

Key words: treatment, mastitis, dairy, resistance

INTRODUCTION

Mastitis continues to be a common disease of dairy cows and one of the most significant factors limiting dairy farm profitability. Recent studies have indicated that most cases of clinical mastitis occurring in dairy cows in developed dairy regions are caused by environmental pathogens (Bradley et al., 2007; Oliveira et al., 2013). Among gram-positive organisms, environmental streptococci and CNS are the most prevalent pathogens recovered from clinical cases of mastitis occurring on dairy farms (McDougall et al., 2007; Pinzón-Sanchez and Ruegg, 2011; Oliveira et al., 2013).

The use of antimicrobials to prevent or treat mastitis is one of the main reasons for administration of antimicrobials to dairy cows (Pol and Ruegg, 2007b; USDA, 2007; Saini et al., 2012). Use of antimicrobials in food-producing animals is under increasing scrutiny due to concern about potential development of antimicrobial resistance. Bacterial resistance to specific antimicrobial classes may occur intrinsically due to a lack of binding sites or other pharmacological characteristics; this can cause clinical problems, but is not considered to be a major public health issue (Neu, 1992). Acquired antimicrobial resistance has the potential for transmission to humans and is of great concern to public health authorities (Neu, 1992).

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Antimicrobial resistance of mastitis pathogens is most commonly measured using phenotypic susceptibility tests that assess the ability of a range of concentrations of selected antimicrobials to inhibit bacterial growth (Walker, 2006). In veterinary medicine, the most commonly used phenotypic methods include disk diffusion, agar dilution, and broth microdilution (Walker, 2006).

Genotypic methods are used for detection of selected antimicrobial resistance genes, and hundreds of such genes have been characterized for gram-negative and gram-positive pathogens (Woodford and Sundsfjord, 2005). These methods assess the genotype of the organism, whereas phenotypic susceptibility tests assess the phenotype (or expression of the genotype) under laboratory conditions. As compared with phenotypic methods, resistance of a microorganism to a specific antimicrobial may occur via mechanisms associated with different resistance genes, whereas phenotypic methods simply detect results of gene expression (Cockerill, 1999).

Although the overall prevalence of resistance to most antimicrobials used in intramammary (IMM) compounds is reported to be low (Rajala-Schultz et al., 2004; Pol and Ruegg, 2007a; Bengtsson et al., 2009; Oliveira et al., 2012), monitoring the development of antimicrobial resistance is important to ensure the continued health of animals and humans. In addition, carriage of antimicrobial resistance genes by mastitis pathogens may be a potential source of gene transmission, indicating the importance of monitoring for selected resistance genes (Walther and Perreten, 2007; Vanderhaeghen et al., 2010). The objectives of the current study were to determine phenotypic susceptibility and presence of selected antimicrobial resistance genes from staphylococci, streptococci, and streptococcal-like organisms (SLO) recovered from cases of clinical mastitis occurring in cows on Wisconsin herds.

MATERIALS AND METHODS

Enrollment of Farms and Selection of Isolates

Recruitment of herds, enrollment of cows, and collection of data has been described previously (Oliveira et al., 2013; Oliveira and Ruegg, 2014). In brief, dairy herds were required to have a minimum of 200 lactating and dry cows (90th percentile for herd size in WI), participate in monthly DHI testing, record animal health data into computerized records, use a milking routine that included routine fore-stripping of quarters for detection of mastitis, and administer antimicrobials to treat affected cows. Data was collected between March and November 2010.

Most farms identified clinical mastitis by observation of abnormal milk or occurrence of other local or systemic symptoms. Trained milking technicians were responsible for case detection and collection of consecutive duplicate quarter milk samples from clinically affected quarters before administration of treatment. After collection of the milk sample, cows were treated according to individual farm protocol without influence by study personnel (Oliveira and Ruegg, 2014). All milk samples were frozen (median days frozen was 27 d; Oliveira and Ruegg, 2014) and mailed to University of Wisconsin-Madison Milk Quality Laboratory. All *Staphylococcus* spp., *Streptococcus* spp. and SLO identified in the original study were eligible for analysis of antimicrobial susceptibility, but only 1 isolate from each animal was used. (Oliveira et al., 2013). After confirmation of the organism, *Staphylococcus aureus* (n = 35 from 19 herds), CNS (n = 51 from 30 herds), *Streptococcus* spp. (n = 78 from 36 herds), and SLO (n = 31 from 19 herds) isolated from cases of bovine clinical mastitis were used in the current study.

Microbiological Procedures

Upon arrival at the University of Wisconsin Milk Quality Laboratory, milk samples were thawed at room temperature, 100 μ L of milk from each duplicate sample was inoculated onto each half of a blood agar plate, and 10 μ L were plated onto a quarter of a MacConkey agar. Plates were incubated at 37°C for 24 to 48 h. Microbiologic procedures were conducted according to National Mastitis Council guidelines (National Mastitis Council, 1999). *Staphylococcus aureus* was differentiated from other staphylococci by means of mannitol and tube coagulase reactions. Suspected *Streptococcus* spp. were identified as gram-positive, catalase-negative cocci by the Christie, Atkins, Munch-Petersen test and esculin reaction. After identification at the genus level, all isolates were frozen in 20% glycerol solution and stored at -80°C until further analyses. For future analysis, frozen isolates were thawed, plated, and passed twice on blood agar plate.

Phenotypic identification of staphylococci and SLO at the species level was initially performed using API Staph and API 20 Strep System, respectively (BioMérieux Inc., Durham, NC). Final identification of staphylococci was performed using partial sequencing of the *rpoB* gene.

DNA Extraction

Bacterial DNA was extracted using a heat lysis-based method. Four colonies from a pure 24-h culture

of bacteria were suspended in 200 μL of sterile water in a microcentrifuge tube. The samples were heated at 100°C for 10 min in a heat block, centrifuged at 13,000 $\times g$ for 2 min at room temperature to pellet cellular debris, then frozen at -20°C until further analyses.

Molecular Identification of *Staphylococcus* spp.

For confirmation of identification of staphylococcal species, partial *rpoB* gene sequencing was performed as described previously by Drancourt and Raoult (2002). The PCR products were purified by electroelution (Sambrook and Russell, 2001) after electrophoresis at 70 V for 1 h on a 0.7% agarose gel. The nucleotide sequences were assembled and compared with the nucleotide sequences at the GenBank database using the BLAST network service (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Strains with homology of 97% or greater were characterized as the same species (Drancourt and Raoult, 2002).

Determination of Phenotypic Antimicrobial Susceptibility

Antimicrobial susceptibility was performed using a custom-designed broth microdilution panel that included the use of quality control organisms (Sensititer; Trek Diagnostic Systems Inc., Westlake, OH). The custom panel was used because previous research indicated that isolates were frequently inhibited at the least or greatest concentrations, which reduced the ability to determine the true MIC (Pol and Ruegg, 2007a; Aparao et al., 2009b; Oliveira et al., 2012). The custom panel included all 10 of the antimicrobials included in the mastitis panel of the most commonly used commercially available broth dilution testing system (Sensititer; Trek Diagnostic Systems Inc.) and added testing for enrofloxacin and florfenicol. As compared with the commercially available mastitis panel, the custom panel included an additional 3 to 7 dilutions (depending on the antimicrobial). This method was performed in accordance with guidelines established by the Clinical Laboratory Standards Institute (CLSI, 2008). Each of the 96 wells in the custom-designed extended dilution panel for MIC determination contained serial dilutions of the following 12 antimicrobial agents as described: ampicillin (0.015–2.0 $\mu\text{g}/\text{mL}$), ceftiofur (0.015–4.0 $\mu\text{g}/\text{mL}$), cephalothin (0.03–16 $\mu\text{g}/\text{mL}$), enrofloxacin (0.06–1.0 $\mu\text{g}/\text{mL}$), erythromycin (0.015–2.0 $\mu\text{g}/\text{mL}$), florfenicol (0.5–16.0 $\mu\text{g}/\text{mL}$), oxacillin (0.12–4.0 $\mu\text{g}/\text{mL}$), penicillin (0.015–4.0 $\mu\text{g}/\text{mL}$), the combination of penicillin and novobiocin (0.03–4.0 $\mu\text{g}/\text{mL}$), pirlimycin (0.06–8.0 $\mu\text{g}/\text{mL}$), sulfadimethoxine (16–1024 $\mu\text{g}/\text{mL}$), and tetracycline (0.06–32.0 $\mu\text{g}/\text{mL}$). Two positive con-

trol wells were also included in each panel. Isolates were subcultured twice on blood agar plates, and bacterial suspensions were prepared and standardized to a 0.5 McFarland standard per the manufacturer's instructions. Aliquots (40 μL for *Staphylococcus* spp. and 80 μL for *Streptococcus* spp.) of this suspension were dispensed into each well and the plates were incubated aerobically at 36°C for 18 to 24 h. Quality control was performed in accordance with the guidelines specified by the Clinical Laboratory Standards Institute (CLSI, 2008) using *Staph.aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 as control strains. All susceptibility results from the control strains complied with the quality control ranges.

Identification of Antimicrobial Resistance Genes

Four known antimicrobial resistance genes were tested in the current study based on antimicrobial usage in enrolled herds and expected prevalence of phenotypic resistance. Detection of *ermC* was performed as described previously (Sutcliffe et al., 1996; Aparao, 2008) using conventional PCR and included the use of a positive control. All isolates were tested for the presence of *ermC* by using primers 5'-TCAAAACATA-ATATAGATAAAA-3' and 5'-GCTAATATTGTTTA-AATCGTCAAT-3' (Sutcliffe et al., 1996).

Real-time PCR was performed to detect the presence of *blaZ*, *tetK*, and *tetM* genes. All isolates were tested for the presence of *blaZ* by using primers 5'-ACGTC-TAAAAGAAGACTAGGAGATAAAGTAACAA-3' and 5'-CGAAAGCAGCAGGTGTTGAA-3'. All isolates were tested for the presence of *tetK* by using primers 5'-TGTTATGGGCGGATTATCTTTTACT-3' and 5'-AGCAAACCTCATTCCAGAAGCAACT-3'. All isolates were tested for the presence of *tetM* by using primers 5'-AGTGGAGCGATTACAGAATTAGGAA-3' and 5'-TCTGACGTTCTAAAAGCGTATTATCC-3'. Amplification reactions were performed in a total volume of 25 μL containing 12.5 μL of SYBR Green PCR Master Mix (Applied Biosystems Inc., Carlsbad, CA), 0.5 μM of each primer; 6.5 μL of H_2O , and 5 μL of template. The real-time PCR detection system used was ABI 7500 Fast (Applied Biosystems Inc.). The thermal cycling profile consisted of 4 stages: stage 1 = 50°C for 2 min; stage 2 = 95°C for 15 min; stage 3 = 40 cycles at 95°C for 15 s, followed by 60°C for 1 min; and stage 4 = 95°C for 15 s. The negative control was a reaction mixture containing all reagents but no DNA template. Data analysis was performed on the ABI 7500 instrument software (Applied Biosystems Inc.) using the dissociation melt curve analysis. The positive controls for *blaZ*, *tetM*, and *tetK* were designed by Integrated DNA Technologies (Integrated DNA Technologies Inc.,

Coralville, IA) by supplying them with the PCR amplicon target sequences and having them implement it into a selectable vector. Additionally, sequencing of the PCR products and comparison of the sequence results to the sequences at the GenBank database revealed 100% identity to similar genes in the database suggesting that the genes were correctly identified by the set of primers used.

Definitions and Classifications

Aerococcus spp., *Enterococcus* spp., and *Lactococcus* spp. were grouped and classified as SLO. Antimicrobial resistance was defined as the ability of a bacterial pathogen to survive exposure to a defined concentration of an antimicrobial agent (CLSI, 2008, 2013). The interpretative criteria for ceftiofur, the combination of penicillin and novobiocin, and pirlimycin were based on bovine mastitis (CLSI, 2008, 2013). The interpretative criteria for ampicillin, cephalothin (the class representative for cephalosporins), erythromycin, oxacillin, penicillin, sulfadimethoxine, and tetracycline were based on human data (CLSI, 2008, 2013). The interpretative criterion for enrofloxacin and florfenicol were based on cattle respiratory disease (CLSI, 2008, 2013).

Statistical Analysis

Minimum inhibitory concentration data for each antimicrobial were summarized using the 50th (MIC₅₀), and 90th (MIC₉₀) percentiles, as well as the proportion of isolates susceptible, intermediate (when applicable), or resistant. Statistical analyses were performed using SAS 9.3 (SAS Institute, 2011).

RESULTS

Characteristics of Herds

Characteristics of the herds (n = 52), cases, and mastitis treatments have been previously described (Oliveira et al., 2013; Oliveira and Ruegg, 2014). In brief, herds contained between 170 to 2,728 milking cows with an average daily milk yield of 33.5 kg/cow (range = 21.0–40.8 kg /cow) and bulk tank SCC of 219,000 cells/mL (range = 87,000–432,000 cells/mL). All herds used antimicrobial treatments for dry cow therapy. Almost all cases were treated using 1 of 5 IMM antimicrobials that are available in the United States (Oliveira and Ruegg, 2014). Of cows that received only IMM therapy, the distribution of use of IMM products was ceftiofur (75%), cephalosporin (14%), amoxicillin (5%), hetacillin (4%), and pirlimycin (3%; Oliveira and Ruegg, 2014).

Identification of *Staphylococcus* spp.

Based on *rpoB* gene sequencing, of 86 *Staphylococcus* spp., the most frequently identified species were *Staph. aureus* (40.7%) and *Staphylococcus chromogenes* (29.1%), followed by *Staphylococcus haemolyticus* (8.1%; Table 1). Compared with identification using the *rpoB* gene, API Staph correctly identified 58 of 86 (67.4%) isolates. Of *Staph. aureus* (n = 35) identified using the *rpoB* gene, only 80% were in agreement using API Staph (7 *Staph. aureus* were misidentified; Table 1). Of 25 *Staph. chromogenes* isolates identified using the *rpoB* gene, 12 gave different bacterial identification using API Staph (4 as *Staphylococcus epidermidis*, 3 as *Staph. aureus*, 3 as *Staphylococcus simulans*, 1 as *Staphylococcus auricularis*, and 1 as *Staphylococcus lugdunensis*). Of 3 *Staphylococcus equorum* identified using the *rpoB* gene, 2 were identified using API Staph as *Staphylococcus sciuri* and 1 as *Staphylococcus xylosum*. Of 7 *Staph. haemolyticus* isolates identified using the *rpoB* gene, 1 was identified using API Staph as *Aerococcus viridians*, 1 as *Staph. aureus*, 1 as *Staphylococcus capitis*, 1 as *Staph. chromogenes*, 1 as *Staphylococcus saprophyticus*, and 1 as *Staph. simulans*.

Phenotypic Identification of *Streptococci* spp. and SLO

Isolates were identified as *Streptococcus* spp. (n = 78; 71.6%), *Lactococcus lactis* (n = 23; 21.1%), *Aerococcus viridians* (n = 5; 4.6%), and *Enterococcus* spp. (n = 3; 2.7%; Table 2). Of *Streptococcus* spp. isolated from 36 herds, the most frequently isolated species were *Streptococcus dysgalactiae* (62.8%) and *Streptococcus uberis* (28.2%; Table 2). *Lactococcus lactis*, *Aerococcus viridians*, and *Enterococcus* spp. were isolated from 14, 5, and 3 herds, respectively.

Antimicrobial Susceptibility Testing

***Staphylococcus* spp.** All staphylococci were inhibited below the breakpoint defined for susceptibility of ceftiofur, cephalosporin, and the combination of penicillin and novobiocin (Table 3). Except for 1 isolate (identified as *Staph. epidermidis*), all isolates were inhibited by the range of concentrations tested for oxacillin. The oxacillin-resistant *Staph. epidermidis* was also phenotypically resistant to ampicillin, penicillin, and tetracycline. About 80% of *Staph. aureus* and >90% of CNS demonstrated in vitro susceptibility to ampicillin and penicillin (Table 3). Of *Staph. aureus*, only 8.6% demonstrated in vitro susceptibility to erythromycin, and 20% were not inhibited at the greatest concentration of erythromycin that was tested (Table 3). Likewise, only

Table 1. Identification of staphylococci (n = 86) isolated from clinical mastitis cases (n = 86) occurring in cows on 37 large Wisconsin dairy herds using API Staph (BioMérieux Inc., Durham, NC) compared with *rpoB* gene identification

Species	<i>rpoB</i> gene identification		API Staph			
			Correctly identified		Misidentified	
	No.	%	No.	%	No.	%
<i>Staphylococcus aureus</i>	35	40.7	28	80.0	7	20.0
<i>Staphylococcus chromogenes</i>	25	29.1	13	52.0	12	48.0
Other staphylococci	26	30.2	17	65.4	9	34.6
<i>Staphylococcus auricularis</i>	1	1.2	1		0	
<i>Staphylococcus cohnii</i>	1	1.2	1		0	
<i>Staphylococcus epidermidis</i>	4	4.7	4		0	
<i>Staphylococcus equorum</i>	3	3.5	0		3	
<i>Staphylococcus haemolyticus</i>	7	8.1	1		6	
<i>Staphylococcus hyicus</i>	4	4.7	4		0	
<i>Staphylococcus sciuri</i>	1	1.2	1		0	
<i>Staphylococcus simulans</i>	4	4.7	4		0	
<i>Staphylococcus xylosum</i>	1	1.2	1		0	
Total	86	100.0	58	67.4	28	32.6

16% of CNS were sensitive to erythromycin and 7.8% were not inhibited at the greatest concentration tested (Table 3). However, of *Staph. aureus* and CNS, 71.5 and 76.5%, respectively, were classified as having intermediate resistance to erythromycin. No mastitis-specific interpretive criteria were available for florfenicol, but 14.3 and 2.6% of *Staph. aureus* and CNS, respectively, were not inhibited by the greatest concentration of this drug. In contrast, using the interpretive criteria developed for bovine respiratory disease, almost all *Staphylococci* spp. would have been considered susceptible to enrofloxacin (Table 3). About 75% of *Staphylococci* spp. were sensi-

tive to pirlimycin, but 20% of *Staph. aureus* and 10% of CNS were not inhibited at the greatest concentration of pirlimycin that was tested. A greater proportion of *Staph. aureus* (97%) were sensitive to sulfadimethoxine as compared with CNS (79%; $P = 0.01$), and 14% of CNS were not inhibited at the greatest concentration of sulfadimethoxine (Table 3). Around 70 to 80% of *Staph. aureus* and CNS demonstrated in vitro susceptibility to tetracycline

For *Staph. aureus*, the MIC₅₀ and MIC₉₀ of ceftiofur, cephalothin, enrofloxacin, oxacillin, the combination of penicillin and novobiocin, and sulfadimethoxine were either identical or within 1 or 2 dilutions. The difference in the MIC₅₀ and MIC₉₀ was 5 to 6 dilutions for ampicillin, penicillin, and tetracycline. The MIC₉₀ could not be determined for erythromycin, florfenicol, or pirlimycin because >10% of *Staph. aureus* were not inhibited at the greatest concentration tested (Table 3). For CNS, the MIC₅₀ and MIC₉₀ for ampicillin, ceftiofur, cephalothin, enrofloxacin, erythromycin, florfenicol, oxacillin, and the combination of penicillin and novobiocin were either identical or within 1 or 2 dilutions. The differences between the MIC₅₀ and MIC₉₀ were 3, 4, and 7 dilutions for penicillin, pirlimycin, and tetracycline, respectively. The MIC₉₀ could not be determined for sulfadimethoxine because 13.7% of CNS were not inhibited at the greatest concentration tested (Table 3).

***Streptococcus* spp. and SLO.** Almost all (90–100%) *Streptococcus* spp. and SLO were inhibited by the range of concentrations tested for ampicillin, ceftiofur, cephalothin, erythromycin, oxacillin, penicillin, and the combination of penicillin and novobiocin (Table 3). Few *Streptococcus* spp. and SLO were sensitive to enrofloxacin, but using the bovine respiratory

Table 2. Identification of streptococci (n = 78) and streptococcal-like organisms (n = 31) isolated from clinical mastitis cases (n = 109) occurring in cows on 40 large Wisconsin dairy herds using API 20 Strep (BioMérieux Inc., Durham, NC)

Species	API 20 Strep	
	No.	%
<i>Streptococcus</i> spp.	78	71.6
<i>Streptococcus dysgalactiae</i>	49	62.8 ¹
<i>Streptococcus uberis</i>	22	28.2
Other streptococci		
<i>Streptococcus mitis</i>	2	2.6
<i>Streptococcus bovis</i>	3	3.8
<i>Streptococcus intermedius</i>	1	1.3
<i>Streptococcus suis</i>	1	1.3
Streptococcal-like organisms	31	28.4
<i>Aerococcus viridians</i>	5	16.1 ²
<i>Enterococcus durans</i>	1	3.2
<i>Enterococcus faecium</i>	2	6.5
<i>Lactococcus lactis</i>	23	74.2
Total	109	100.0

¹Denominator is total streptococci.²Denominator is total streptococcal-like organisms.

Table 3. Distribution of MIC for *Staphylococcus aureus* (n = 35), CNS (n = 51), *Streptococcus* spp. (n = 78) and streptococcal-like organisms (SLO; n = 31) isolated from clinical mastitis cases (n = 195) occurring in cows on 37 large Wisconsin dairy herds

Antimicrobial	BP ² (ug/mL)	Etiology	No.	S ³ (%)	Percentage of isolates at each indicated MIC ⁴ (ug / mL)															NI ⁵
					0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	
Ampicillin	≤0.25	<i>Staph. aureus</i>	35	77	<u>57.1</u>	5.7	8.6	5.7	0.0	14.3	8.6	0.0	— ⁶	—	—	—	—	—	0.0	
	≤0.25	CNS ⁷	51	92	27.5	<u>35.3</u>	7.8	21.6	0.0	5.9	0.0	2.0	—	—	—	—	—	—	0.0	
	≤0.25	<i>Strep. spp.</i>	78	97	<u>55.1</u>	5.1	20.5	15.4	1.3	2.6	0.0	0.0	—	—	—	—	—	—	0.0	
	≤8.00 ⁸	SLO	31	97	3.2	6.5	19.4	<u>58.1</u>	9.7	0.0	0.0	3.2	—	—	—	—	—	—	0.0	
	≤2.00	<i>Staph. aureus</i>	35	100	2.9	0.0	0.0	<u>17.1</u>	<u>31.4</u>	37.1	11.4	0.0	0.0	—	—	—	—	—	0.0	
Cephalothin	≤8.00	CNS	51	100	0.0	0.0	0.0	9.8	31.4	39.2	17.7	2.0	0.0	—	—	—	—	—	0.0	
		<i>Strep. spp.</i>	78	100	<u>52.6</u>	27.0	12.8	5.1	2.6	0.0	0.0	0.0	0.0	—	—	—	—	—	0.0	
		SLO	31	100	0.0	32.3	<u>45.2</u>	6.5	12.9	0.0	3.2	0.0	0.0	—	—	—	—	—	0.0	
		<i>Staph. aureus</i>	35	100	—	14.3	20.0	<u>37.1</u>	14.3	5.7	5.7	0.0	0.0	2.9	0.0	—	—	—	0.0	
		CNS	51	100	—	2.0	25.5	<u>47.1</u>	15.7	7.8	2.0	0.0	0.0	0.0	—	—	—	—	0.0	
Enrofloxacin	<0.25 ⁹	<i>Strep. spp.</i>	78	100	—	0.0	29.5	<u>20.5</u>	15.4	14.1	5.1	11.5	3.9	0.0	0.0	—	—	—	0.0	
		SLO	31	100	—	0.0	3.2	0.0	3.2	6.5	0.0	<u>58.1</u>	22.6	6.5	0.0	—	—	—	0.0	
		<i>Staph. aureus</i>	35	97	—	—	5.7	<u>42.9</u>	48.57	2.9	0.0	—	—	—	—	—	—	—	0.0	
		CNS	51	98	—	—	9.8	<u>62.8</u>	25.5	2.0	0.0	—	—	—	—	—	—	—	0.0	
		<i>Strep. spp.</i>	78	9	—	—	2.6	1.3	5.1	50.0	33.0	—	—	—	—	—	—	—	7.7	
Erythromycin	≤0.50	SLO	31	10	—	—	3.2	3.2	3.2	22.6	64.5	—	—	—	—	—	—	—	3.2	
		<i>Staph. aureus</i>	35	9	2.9	0.0	0.0	0.0	0.0	5.7	<u>68.6</u>	2.9	—	—	—	—	—	—	20.0	
		CNS	51	16	0.0	0.0	0.0	0.0	0.0	15.7	60.8	15.7	—	—	—	—	—	—	7.8	
		<i>Strep. spp.</i>	78	95	2.6	0.0	16.7	<u>64.1</u>	11.5	0.0	0.0	3.9	—	—	—	—	—	—	1.3	
		SLO	31	90	0.0	0.0	3.2	<u>54.8</u>	32.2	9.7	0.0	0.0	—	—	—	—	—	—	0.0	
Florfenicol	<2.0 ⁹	<i>Staph. aureus</i>	35	37	—	—	—	—	—	0.0	2.9	34.3	<u>48.6</u>	0.0	0.0	—	—	—	14.3	
		CNS	51	65	—	—	—	—	—	0.0	5.9	<u>58.8</u>	23.5	2.0	0.0	—	—	—	2.6	
		<i>Strep. spp.</i>	78	97	—	—	—	—	—	5.1	<u>44.9</u>	47.4	2.6	0.0	0.0	—	—	—	0.0	
		SLO	31	61	—	—	—	—	—	0.0	3.2	<u>58.1</u>	35.5	0.0	3.2	—	—	—	0.0	
		<i>Staph. aureus</i>	35	100	—	—	—	<u>100.0</u>	0.0	0.0	0.0	0.0	0.0	—	—	—	—	—	0.0	
Oxacillin	≤2.00	<i>Staph. aureus</i>	51	98	—	—	—	<u>88.2</u>	7.8	2.0	0.0	0.0	0.0	—	—	—	—	—	0.0	
		CNS	78	100	—	—	—	<u>77.0</u>	3.9	15.4	3.9	0.0	0.0	—	—	—	—	—	0.0	
		<i>Strep. spp.</i>	31	100	—	—	—	12.9	6.5	<u>45.1</u>	16.1	19.4	0.0	—	—	—	—	—	0.0	
		SLO	35	80	<u>71.4</u>	0.0	5.7	2.9	5.7	8.6	0.0	0.0	5.7	—	—	—	—	—	0.0	
		<i>Staph. aureus</i>	35	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.0	
Penicillin	≤0.12	CNS	51	90	<u>62.8</u>	0.0	17.7	9.8	2.0	0.0	0.0	3.9	0.0	—	—	—	—	—	3.9	
		<i>Strep. spp.</i>	78	91	48.7	6.4	26.9	9.0	9.0	0.0	0.0	0.0	0.0	—	—	—	—	—	0.0	
		SLO	31	100	3.2	3.2	16.1	19.4	54.8	0.0	0.0	3.2	0.0	—	—	—	—	—	0.0	
		<i>Staph. aureus</i>	35	100	—	<u>94.3</u>	2.9	0.0	2.9	0.0	0.0	0.0	0.0	—	—	—	—	—	0.0	
		CNS	51	100	—	<u>100.0</u>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—	—	—	—	—	0.0	
Penicillin-novobiocin	≤0.12	CNS	78	99	—	<u>64.1</u>	23.1	9.0	2.6	1.3	0.0	0.0	0.0	—	—	—	—	—	0.0	
		<i>Strep. spp.</i>	31	100	—	9.7	35.5	<u>48.4</u>	6.5	0.0	0.0	0.0	0.0	—	—	—	—	—	0.0	
		SLO	31	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.0	

Continued

Table 3 (Continued). Distribution of MIC for *Staphylococcus aureus* (n = 35), CNS (n = 51), *Streptococcus* spp. (n = 78) and streptococcal-like organisms (SLO¹; n = 31) isolated from clinical mastitis cases (n = 195) occurring in cows on 37 large Wisconsin dairy herds

Antimicrobial	BP ² (ug/mL)	Etiology	No.	S ³ (%)	Percentage of isolates at each indicated MIC ⁴ (µg / mL)																	
					0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	NI ⁵
Pirlmycin	≤2.00	<i>Staph. aureus</i>	35	77	—	—	0.0	0.0	8.6	57.1	5.7	5.7	2.9	0.0	—	—	—	—	—	20.0		
		CNS	51	75	—	—	2.0	5.9	<u>43.1</u>	15.7	5.9	3.9	11.8	—	—	—	—	—	—	9.8		
		<i>Strep.</i> spp.	78	81	—	—	3.9	34.6	<u>12.8</u>	16.7	9.0	3.9	0.0	6.4	—	—	—	—	—	12.8		
		SLO	31	71	—	—	3.2	6.5	32.3	<u>19.4</u>	3.2	6.5	0.0	—	—	—	—	—	—	22.6		
Sulfadimethoxine	≤256.0	<i>Staph. aureus</i>	35	97	—	—	—	—	—	—	—	—	—	—	<u>77.1</u>	17.1	2.9	0.0	0.0	2.9	0.0	0.0
		CNS	51	79	—	—	—	—	—	—	—	—	—	—	45.1	<u>9.8</u>	5.9	17.7	0.0	3.9	3.9	13.7
		<i>Strep.</i> spp.	78	54	—	—	—	—	—	—	—	—	—	—	5.1	11.5	7.7	29.5	10.3	0.0	2.6	33.3
		SLO	31	42	—	—	—	—	—	—	—	—	—	—	32.3	0.0	0.0	9.7	0.0	0.0	0.0	58.1
Tetracycline	≤4.00	<i>Staph. aureus</i>	35	83	—	—	5.7	<u>65.7</u>	2.9	0.0	2.9	0.0	5.7	8.6	2.9	5.7	—	—	—	—	—	0.0
		CNS	51	74	—	—	9.8	39.2	<u>5.9</u>	3.9	5.9	3.9	3.9	0.0	7.8	17.7	—	—	—	—	—	1.0
		<i>Strep.</i> spp.	78	51	—	—	3.9	2.6	5.1	2.6	<u>34.6</u>	2.6	2.6	1.3	9.0	33.3	—	—	—	—	—	2.6
		SLO	31	71	—	—	0.0	12.9	25.8	<u>19.4</u>	6.5	6.5	0.0	0.0	9.7	12.9	—	—	—	—	—	6.5

¹*Lactococcus lactis*, *Aerococcus viridians*, and *Enterococcus* spp.

²BP = breakpoint, MIC at which an isolate is considered susceptible according to CLSI (2008) guideline .

³S = percent of susceptible isolates according to CLSI (2008).

⁴Dash indicates values not tested for the indicated antimicrobial; MIC₅₀ value is underlined; MIC₉₀ value is in bold; an underlined and bold value is both the MIC₅₀ and MIC₉₀.

⁵Isolates that were not inhibited at the highest concentration of the antimicrobial tested.

⁶*Staphylococcus chromogenes*.

⁷*Staphylococcus chromogenes*, *Staphylococcus auricularis*, *Staphylococcus colmii*, *Staphylococcus epidermidis*, *Staphylococcus equorum*, *Staphylococcus haemolyticus*, *Staphylococcus hyicus*, *Staphylococcus sciuri*, *Staphylococcus simulans*, and *Staphylococcus xylosum*.

⁸Breakpoint for enterococci.

⁹No breakpoints for mastitis organisms exist, breakpoint is for bovine respiratory disease.

Table 4. Distribution of resistance genes and combinations of resistance genes in *Staphylococcus aureus* (n = 35), CNS¹ (n = 51), *Streptococcus* spp. (n = 78), and streptococcal-like organisms² (SLO, n = 31) isolated from clinical mastitis samples occurring in cows on 37 large Wisconsin dairy herds

Etiology	No.	No. (%) of genes detected	Single gene				Contains multiple genes					
			<i>ermC</i>	<i>blaZ</i>	<i>tetK</i>	<i>tetM</i>	<i>ermC</i> and <i>tetK</i>	<i>blaZ</i> and <i>tetK</i>	<i>blaZ</i> and <i>tetM</i>	<i>tetK</i> and <i>tetM</i>	<i>ermC</i> , <i>blaZ</i> , and <i>tetK</i>	<i>blaZ</i> , <i>tetK</i> , and <i>tetM</i>
<i>Staph. aureus</i>	35	8 (23)	0	5	3	1	2	5	3	0	1	7
CNS	51	16 (31)	1	4	7	1	0	7	6	1	2	6
<i>Strep.</i> spp.	78	37 (47)	0	9	6	17	0	3	2	2	0	2
SLO	31	11 (36)	0	1	7	7	0	3	0	2	0	0
Total	195	72 (37)	1	19	23	26	2	18	11	5	3	15

¹*Staphylococcus chromogenes*, *Staphylococcus auricularis*, *Staphylococcus cohnii*, *Staphylococcus epidermidis*, *Staphylococcus equorum*, *Staphylococcus haemolyticus*, *Staphylococcus hyicus*, *Staphylococcus sciuri*, *Staphylococcus simulans*, and *Staphylococcus xylosum*.

²*Lactococcus lactis*, *Aerococcus viridians*, and *Enterococcus* spp.

disease breakpoints most would have been characterized as having intermediate sensitivity. Whereas most *Streptococcus* spp. were sensitive to florfenicol, only 61% of SLO were sensitive to this compound. Overall, 81% of *Streptococcus* spp. were sensitive to pirlimycin as compared with 71% of SLO (Table 3). However, the distribution of sensitivity to pirlimycin among species was 83.7% (*Strep. dysgalactiae*), 68.2% *Strep. uberis*, 100% (other *Streptococcus* spp.), and 71% (SLO). A large proportion of *Streptococcus* spp. and SLO were resistant to sulfadimethoxine (Table 3). The greatest concentration of sulfadimethoxine that was tested failed to inhibit 33.3 and 58.1% of *Streptococcus* spp. and SLO, respectively (Table 3). A greater proportion of *Strep. dysgalactiae* (81.6%) were sensitive to sulfadimethoxine as compared with *Strep. uberis* (31.8%; $P < 0.001$). Approximately 50% of the *Streptococcus* spp. and 71% of SLO demonstrated in vitro susceptibility to tetracycline (Table 3). Differences in the proportion of isolates sensitive to tetracycline were not observed for *Strep. dysgalactiae* or *Strep. uberis*.

For *Streptococcus* spp. and SLO, the MIC₅₀ and MIC₉₀ for ceftiofur, enrofloxacin, erythromycin, florfenicol, oxacillin, and the combination of penicillin and novobiocin were either the same or within 1 to 2 dilutions. For both cephalothin and penicillin, the differences in the MIC₅₀ and MIC₉₀ were 3 to 4 dilutions for *Streptococcus* spp. and 0 to 1 dilution for SLO. The MIC₉₀ of pirlimycin could not be determined for either *Streptococcus* spp. or SLO because >10% of the isolates were not inhibited at the greatest concentration tested (Table 3). Neither the MIC₅₀ nor MIC₉₀ of sulfadimethoxine for SLO could be determined because 58.1% of organisms were not inhibited by the greatest concentration tested. The differences between the MIC₅₀ and MIC₉₀ of tetracycline were 5 and 6 dilutions for *Streptococcus* spp. and SLO, respectively.

Detection of Antimicrobial Resistance Genes

***Staphylococcus* spp.** Of 86 *Staphylococcus* spp., *blaZ* was detected (either alone or in combination with other genes) most frequently (n = 46 isolates), followed by *tetK* (n = 41), *tetM* (n = 26), and *ermC* (n = 6; Table 4). Of *Staphylococcus* spp., 62 (72.1%) carried at least 1 of the selected resistance genes. Eight (22.8%) *Staph. aureus* and 16 CNS (31.4%) carried no resistance genes (Table 4). Eighteen (51.4%) *Staph. aureus* carried multiple resistance genes. Of these isolates, 5 carried *blaZ* and *tetK* and 7 carried *blaZ*, *tetK*, and *tetM* (Table 4). Twelve (48.0%) *Staph. chromogenes* carried multiple resistance genes. Of these isolates, 4 carried *blaZ*, *tetK*, and *tetM*. A similar proportion (42.3%) of the other CNS species carried multiple resistance genes. Of these isolates, 4 carried *blaZ* and *tetM* and 3 carried *blaZ*, *tetK*, and *tetM* (Table 4).

***Streptococcus* spp. and SLO.** Of 78 *Streptococcus* spp., 41 (52.6%) carried at least 1 of the selected resistance genes. We detected *tetM* (either alone or in combination with other genes) most frequently (n = 23 isolates), followed by *blaZ* and *tetK*, which were detected in 16 and 13 isolates, respectively (Table 4). No *Streptococcus* spp. carried the *ermC* gene. None of the selected resistance genes were found in 21 of 49 (42.8%) *Strep. dysgalactiae* or 16 of 22 (72.7%) *Strep. uberis*. At least one of the selected resistance genes was identified in all of the 7 other species of streptococci. Six (12.2%) *Strep. dysgalactiae* carried multiple resistance genes. Of these isolates, 2 carried *blaZ* and *tetM*, 2 carried *tetK* and *tetM*, 1 carried *blaZ* and *tetK*, and 1 carried *blaZ*, *tetK* and *tetM*. No *Strep. uberis* carried multiple genes.

Of 31 SLO, 20 (64.5%) carried at least 1 of the selected resistance genes (Table 4). We detected *tetK* (either alone or in combination with other genes) most frequently (n = 12), followed by *tetM* (n = 9) and *blaZ*

Table 5. Comparison of phenotypic and genotypic antimicrobial resistance for selected genes in *Staphylococcus aureus* (n = 35), CNS¹ (n = 51), *Streptococcus* spp. (n = 78), and streptococcal-like organisms² (SLO; n = 31) isolated from clinical mastitis samples occurring in cows on 37 large Wisconsin dairy herds

Organism	Susceptibility test category	Erythromycin		Pirlimycin		Penicillin		Tetracycline		
		No.	No. (%) of <i>ermC</i> detected	No.	No. (%) of <i>ermC</i> detected	No.	No. (%) of <i>blaZ</i> detected	No.	No. (%) of <i>tetK</i> detected	No. (%) of <i>tetM</i> detected
<i>Staph. aureus</i> (n = 35)	Susceptible	3	0 (0)	27	1 (4)	28	14 (50)	29	13 (45)	9 (31)
	Intermediate	25	1 (4)	— ³	—	—	—	3	3 (100)	1 (33)
	Resistant ⁴	7	2 (29)	8	2 (25)	7	7 (100)	3	2 (67)	1 (33)
CNS (n = 51)	Susceptible	7	1 (14)	38	2 (5)	46	20 (43)	37	15 (41)	12 (32)
	Intermediate	40	1 (3)	—	—	—	—	0	—	—
	Resistant	4	1 (25)	13	1 (8)	5	5 (100)	14	8 (57)	3 (21)
<i>Strep.</i> spp. (n = 78)	Susceptible	74	0 (0)	63	0 (0)	71	15 (21)	40	7 (18)	5 (13)
	Intermediate	0	—	—	—	—	—	2	1 (50)	0 (0)
	Resistant	4	0 (0)	15	0 (0)	7	2 (29)	36	4 (14)	18 (50)
SLO (n = 31)	Susceptible	28	0 (0)	22	0 (0)	31	4 (13)	22	9 (41)	5 (23)
	Intermediate	3	0 (0)	—	—	—	—	0	—	—
	Resistant	0	—	9	0 (0)	0	—	9	3 (33)	4 (44)

¹*Staphylococcus chromogenes*, *Staphylococcus auricularis*, *Staphylococcus cohnii*, *Staphylococcus epidermidis*, *Staphylococcus equorum*, *Staphylococcus haemolyticus*, *Staphylococcus hyicus*, *Staphylococcus sciuri*, *Staphylococcus simulans*, and *Staphylococcus xylosus*.

²*Lactococcus lactis*, *Aerococcus viridians*, and *Enterococcus* spp.

³Organisms are not classified in this category.

⁴Includes organisms that were not inhibited at the greatest concentration tested.

(n = 4). No SLO carried the *ermC* gene. Seven SLO carried *tetK* or *tetM*. Of 5 SLO that carried multiple resistance genes, 3 carried *blaZ* and *tetK* and 2 carried *tetK* and *tetM* (Table 4).

Comparison of Phenotypic Resistance and Occurrence of Resistance Genes. The proportion of isolates with phenotypic resistance did not correspond with the proportion of isolates identified with the selected resistance genes (Table 5). No *Staph. aureus* that demonstrated phenotypic susceptibility to erythromycin carried the *ermC* gene; however, *ermC* was identified in 1 of 7 phenotypically susceptible CNS (a *Staph. chromogenes*) (Table 5). We also detected *ermC* in 2 of 7 *Staph. aureus* and 1 of 4 CNS that were phenotypically resistant to erythromycin; all of these isolates were not inhibited by the greatest concentration of erythromycin that was tested. Likewise, *ermC* was detected in 25% of *Staph. aureus* and 8% of CNS that were phenotypically resistant to pirlimycin (Table 5). Two CNS and 1 *Staph. aureus* that demonstrated in vitro susceptibility to pirlimycin carried the *ermC* gene (Table 5). In contrast, all *Staphylococcus* spp. that demonstrated phenotypic resistance to penicillin (n = 12) carried *blaZ*. About 40 to 50% of staphylococci that were phenotypically susceptible to penicillin carried the *blaZ* gene (Table 5). Only 3 *Staph. aureus* demonstrated phenotypic resistance to tetracycline; *tetK* and *tetM* were identified in 2 and 1 of the resistant isolates, respectively (Table 5). Of 14 CNS that were resistant to tetracycline, *tetK* and *tetM* were identified in 8 (57%) and 3 (21%), respectively. These same genes were found

in about 30 to 45% of staphylococci that exhibited phenotypic susceptibility to tetracycline (Table 5).

The gene *ermC* was not identified in any streptococci or SLO. Of 71 streptococci that were phenotypically susceptible to penicillin, *blaZ* was detected in 15 (21%) (Table 5). Of 7 streptococci that were phenotypically resistant to penicillin, *blaZ* was found in 2 (29%; Table 5). We identified *blaZ* in 7 of 44 (16%) and 1 of 21 (4.8%) *Strep. dysgalactiae* and *Strep. uberis* that exhibited phenotypic susceptibility to penicillin, respectively. Based on the use of the enterococci breakpoints, all SLO were considered to be susceptible to penicillin and *blaZ* was recovered from 13% of this group of organisms. Overall, the selected tetracycline resistance genes were identified in 13 to 18% of phenotypically susceptible streptococci, in contrast to 14 to 50% of phenotypically resistant streptococci (Table 5). Of *Strep. dysgalactiae* (n = 22) that were phenotypically resistant to tetracycline, 3 and 12 isolates carried *tetK* and *tetM*, respectively. Of *Strep. uberis* (n = 10) that demonstrated phenotypic resistance to tetracycline, *tetM* was identified in 3 organisms and no *tetK* was identified. The proportion of SLO that carried either *tetK* or *tetM* was about the same (23–44%) for both phenotypically susceptible and resistant organisms (Table 5).

DISCUSSION

The dairy farms enrolled in our study were reasonably representative of larger Wisconsin dairy herds that contribute the majority of milk used in processing. The

mastitis pathogens (Oliveira et al., 2013) and treatments (Oliveira and Ruegg, 2014) used on these dairy farms were typical of many larger dairy farms in the upper Midwestern region of the United States. Whereas *Staph. aureus* was recovered from clinical cases on 20 farms, it was sporadically recovered and accounted for <4% of clinical cases that were enrolled in the overall study (Oliveira et al., 2013). Of CNS, *Staph. chromogenes* and *Staph. haemolyticus* were most frequently identified. These species have been commonly reported in varying proportions in previous studies that also used genotypic identification, and *Staph. chromogenes* is typically the most commonly identified species (Taponen et al., 2006; Capurro et al., 2009; Sampimon et al., 2009; Park et al., 2011).

Agreement between identification of species of staphylococci using the *rpoB* gene and API Staph was approximately 70%, which was similar to previous research (Matthews et al., 1990; Sampimon et al., 2011). Half of the most common *Staphylococcus* spp. were misidentified using API Staph, indicating the need for a more reliable test when identification at the species level is required. Most importantly, the phenotypic method used in the current study misidentified 20% of *Staph. aureus*. The failure to properly identify cows infected with *Staph. aureus* could delay resolution of a herd problem and dairy professionals should recognize limitations of current testing methodologies. In regions where *Staph. aureus* is still a significant cause of mastitis, alternative methods of confirming the diagnosis (such as use of chromogenic media or *nuc*-PCR) should be considered (Virgin et al., 2009; Graber et al., 2013). To identify species of streptococci and SLO, biochemical and phenotypic identification was used because previous researchers have demonstrated full agreement between genotypic and phenotypic identification methods used for identification of *Strep. uberis*, *Strep. dysgalactiae*, and *Streptococcus agalactiae* (Wyder et al., 2011).

Several studies have described antimicrobial susceptibility profiles of bovine mastitis pathogens collected from Wisconsin dairy cows (Makovec and Ruegg, 2003; Pol and Ruegg, 2007a; Oliveira et al., 2012), but no studies have been conducted specifically using isolates collected from larger modern dairy farms that produce the majority of processed milk. The conventional dairy herd population from which isolates were collected in 2004 and 2005 (Pol and Ruegg, 2007a) was composed of smaller Wisconsin dairy herds that were matched with similar organic herds. Whereas neither the previous (Pol and Ruegg, 2007a) nor current studies were specifically designed to estimate prevalence of resistance, comparison of the results is worthwhile. As compared with Pol and Ruegg (2007a), no overall increasing or

decreasing trend in the proportion of resistant mastitis pathogens could be discerned. Ceftiofur and cephalosporin were the most common IMM products used in the herds enrolled in the current study (Oliveira and Ruegg, 2014), and, similar to Pol and Ruegg, (2007a), almost none of the pathogens examined in the current study were phenotypically resistant to those compounds. Likewise, both studies reported minimal in vitro resistance to oxacillin and the combination of penicillin and novobiocin. Changes in resistance to ampicillin and penicillin were not consistent. As compared with Pol and Ruegg (2007a), the proportion of resistant *Staph. aureus* was numerically greater in the current study; however, that trend did not apply to CNS (decreased resistance) or *Streptococcus* spp. (similar proportion of resistant isolates). Some indication exists that resistance of *Staph. aureus* to penicillin has declined. Makovec and Ruegg (2003) analyzed results of milk samples submitted for antimicrobial resistance testing to the Wisconsin state diagnostic laboratory from 1994 to 2001. Compared with the 20% of resistant *Staph. aureus* identified in the present study, they reported that the percentage of *Staph. aureus* isolates resistant to penicillin decreased from 49 (1994) to 30% (2001). It is possible that declining resistance to penicillin is related to changes in treatment protocols or is a result of aggressive culling of chronically affected cows in large dairy herds. However, the current study was conducted using isolates collected from Wisconsin dairy herds and it is likely that results may differ from isolates collected in larger herds of other US regions, indicating the need for broader studies throughout the United States.

Pirlimycin was the only antimicrobial that is found in commercially available IMM products used in the United States that demonstrated a considerable proportion of resistant isolates. Pol and Ruegg (2007a) reported that the proportion of pirlimycin resistant organisms was 0 (of 46 *Staph. aureus*), 18 (of 141 CNS), and 24% (of 42 *Streptococcus* spp.). In the current study, the proportion of *Staph. aureus* resistant to pirlimycin was greater than in Pol and Ruegg (2007a), but similar proportions of resistance to pirlimycin were detected in the other organisms. Similar to Pol and Ruegg, (2007a), a considerable proportion of CNS and *Streptococcus* spp. were resistant to sulfadimethoxine and tetracycline. In the United States, sulfadimethoxine is approved for treatment of dairy cattle that are affected with bacterial pneumonia or necrotic pododermatitis. Use of sulfadimethoxine for treatment of any other condition is expressly prohibited by US regulations, but it continues to be used on a small number of farms (Oliveira and Ruegg, 2014). This drug is not expected to achieve a therapeutic concentration in the udder (Baggot, 2006) and it should not be used for mastitis therapy. Whereas

the systemic administration of tetracycline is allowable under US extra-label guidelines, it has only moderate lipid solubility and the higher pH of mastitic milk is thought to reduce its antimicrobial activity (Baggot, 2006); thus, use of systemic tetracycline for mastitis therapy should be discouraged.

Many organisms tested using the extended dilution MIC panel were inhibited at concentrations well below the values included in the commercially available panel. Use of custom panel with extended dilutions highlighted that some organisms have considerable ranges between the MIC₅₀ and MIC₉₀, perhaps indicating a shift in the underlying microbial populations. Given the uncertainty about the validity of non-species- and disease-specific breakpoints, monitoring of the distribution of inhibition may be more useful for detection of selection toward more resistant organisms.

Overall, phenotypic resistance to most selected antimicrobials among *Staphylococcus* spp. was uncommon and was similar to previous research; this supports previous research indicating that resistance to antimicrobials is not widespread among gram-positive mastitis pathogens (Lüthje and Schwarz, 2006; Rajala-Schultz et al., 2004; Sawant et al., 2009; Sampimon et al., 2011; Oliveira et al., 2012). Ceftiofur was the most common intramammary antimicrobial used by herds enrolled in our study, so it is encouraging that no phenotypic resistance to this antimicrobial was detected in these mastitis pathogens.

Methicillin resistance in *Staphylococcus* spp. is a cause of both hospital- and community-acquired infections of humans and is a public health concern (Aarestrup and Schwarz, 2006). Phenotypic resistance to oxacillin is one method that is used to detect methicillin resistance of staphylococci. Detection of *mecA* and *mecC* is advised when resistance to methicillin is the subject of research interest, but was not performed on all isolates in the current study because previous studies have established that methicillin resistance is infrequent in *Staph. aureus* recovered from cases of bovine mastitis (Virgin et al., 2009; Hata et al., 2010; Vanderhaeghen et al., 2010). Only 1 isolate (identified as *Staph. epidermidis*) demonstrated phenotypic resistance to oxacillin. That isolate was determined to be positive for *mecA* by PCR testing at the Illinois State Diagnostic Laboratory (Urbana, IL). Resistance to methicillin and multidrug antimicrobial resistance have been previously described in *Staph. epidermidis* isolated from bovine mastitis (Sawant et al., 2009; Sampimon et al., 2011). Whereas this type of resistance appears to be infrequent, it is theoretically possible that humans and dairy cattle could exchange resistance genes and other researchers have recommended that animals that carry methicillin-resistant staphylococci should be culled (Gentilini et al., 2002).

About 20% of *Staph. aureus* and 8% of CNS demonstrated phenotypic resistance to erythromycin, and most isolates were classified as having intermediate resistance. Erythromycin was not reported to be used in treatments given on these dairy farms (Oliveira and Ruegg, 2014), and the approved IMM erythromycin product has not been available in the United States for many years. However, pirlimycin is used as an IMM product and it is possible that resistance to erythromycin is a result of shared genetic mechanisms.

With the exception of pirlimycin, tetracycline, and sulfadimethoxine, almost all *Streptococcus* spp. demonstrated susceptibility to most of the selected antimicrobials. According to many researchers, phenotypic resistance to tetracycline is the most common form of resistance among species of streptococci (Erskine et al., 2002; Guérin-Faubleé et al., 2002; Makovec and Ruegg, 2003; Bengtsson et al., 2009; Nam et al., 2009). This was confirmed in the present study, where almost half of the isolates demonstrated phenotypic resistance to tetracycline.

Differences among species in resistance to sulfadimethoxine were identified for streptococci. Most *Strep. dysgalactiae* demonstrated susceptibility to sulfadimethoxine in contrast to widespread resistance of *Strep. uberis*. Likewise, 68% of *Strep. uberis* and 18% of *Strep. dysgalactiae* were not inhibited at the greatest concentration of sulfadimethoxine that was tested. Pol and Ruegg (2007a) reported that 21.4% (n = 17) of *Streptococcus* spp. isolated from subclinical mastitis demonstrated resistance to sulfadimethoxine, but the isolates were not identified at the species level. The use of sulfadimethoxine for treatment of mastitis is not permitted by the US Food and Drug Administration, so this use should be actively discouraged.

The proportion of *Strep. uberis* that have been reported to be phenotypically resistant to enrofloxacin has varied among studies from 3.8 (Rossitto et al., 2002) to 22% (Roesch et al., 2006). Using the breakpoints for bovine respiratory disease, more than half of *Strep. dysgalactiae*, *Strep. uberis*, and SLO were classified as having intermediate resistance to enrofloxacin, and 3 to 7% were not inhibited at the greatest concentration of enrofloxacin that was tested. Previous researchers have also demonstrated a large proportion of phenotypic resistance of SLO to different antimicrobials (Rossitto et al., 2002; Rajala-Schultz et al., 2004; Pol and Ruegg, 2007a). In spite of US regulations prohibiting the use of enrofloxacin in lactating cows, 1 herd enrolled in the current study used enrofloxacin to treat adult cows experiencing respiratory disease and 8 herds used this drug to treat only calves (which is an allowable usage). However, due to the absence of validated breakpoints for mastitis pathogens, conclusions about trends in the

development of resistance to enrofloxacin in mastitis pathogens would be speculative.

The criteria used to select the 4 antimicrobial genes included in our study was based on the proportion of isolates that demonstrated phenotypic resistance for the tested antimicrobials, the expected prevalence of these genes among isolates (based on previous research), and the history of antimicrobial usage in the enrolled dairy herds. To date, 10 genes have been identified encoding resistance to macrolides (such as erythromycin), and *ermC* is the most commonly reported resistance gene (Eady et al., 1993; Jensen et al., 1999; Aarestrup and Schwarz, 2006). At least 38 tetracycline resistance genes have been found, and the *tetK* gene has been commonly found in species of staphylococci (Schwarz and Chaslus-Dancla, 2001). The *tetM* gene seems to be identified more commonly in species of staphylococci other than *Staph. aureus* (Schwarz and Chaslus-Dancla, 2001; Lüthje and Schwarz, 2006).

Of all isolates included in this study, 63% carried at least 1 of the 4 selected resistance genes. Similar to Sawant et al. (2009), only a few species of staphylococci (mainly *Staph. aureus* and *Staph. chromogenes*) carried the *ermC* gene. As also reported by Sawant et al. (2009), 1 *Staph. epidermidis* carried *ermC*; this isolate also carried 2 other genes and was the only isolate that exhibited phenotypic resistance to oxacillin. A study conducted in Germany (Lüthje and Schwarz, 2006) reported that *ermC* is the most prevalent *erm* gene recovered from cases of bovine subclinical mastitis caused by CNS and most of the isolates exhibited phenotypic resistance to both erythromycin and pirlimycin. In the present study, whereas phenotypic resistance to pirlimycin was not uncommon, few isolates carried the *ermC* gene. Isolates that demonstrated phenotypic susceptibility to erythromycin but resistance to pirlimycin are likely to harbor *lnuB* gene, which encodes a nucleotidyltransferase that mediates resistance to lincosamides, but not to macrolides (Lüthje and Schwarz, 2006; Schmitt-Van de Leemput and Zadoks, 2007; Apparao et al., 2009b). Apparao (2008) tested the presence of both genes (*ermC* and *lnuB*) in gram-positive pathogens isolated from mastitis before cows received IMM pirlimycin and reported that the prevalence was 8% for *ermC* and 3% for *lnuB*. The *lnuB* gene was not tested in the present study because the *ermC* gene was expected to be commonly present in *Staphylococcus* spp. and also confers resistance to more than 1 antimicrobial. Potentially, phenotypic resistance to pirlimycin and not to erythromycin of *Strep. uberis* could be related to the presence of *lnuB* gene or other unknown mechanisms (Schmitt-Van de Leemput and Zadoks, 2007).

Beta-lactams are important antimicrobials that are often used for prevention and treatment of dairy cattle

diseases (Sawant et al., 2009; Pol and Ruegg, 2007b). About 10 to 20% of *Staphylococcus* spp. demonstrated phenotypic resistance to penicillin, and the *blaZ* gene was identified in all resistant *Staph. aureus* and CNS. However, in agreement with previous research (Haveri et al., 2005), about 40 to 50% of phenotypically sensitive *Staph. aureus* and CNS also carried the *blaZ* gene. There are several possibilities as to why results of phenotypic susceptibility testing may differ from results of genotypic testing. Multiple mechanisms of resistance often exist, but researchers usually only test for a limited number of genes. For example, 21 genes are known to be associated with macrolide-lincosamide-streptogramin resistance. Another possibility could be a mutation of the primer annealing site, as hypothesized by Haveri et al. (2005). However, this is an unlikely explanation because the discrepancy was found in many isolates from different farms and for all genes assessed in the current study. Haveri et al., (2005) suggested that differences in identification of the *blaZ* gene and the phenotypic development of resistance may be attributable to use of incorrect resistance breakpoints. Those authors suggested that phenotypically susceptible isolates that carry resistance genes should be considered as potentially resistant (Haveri et al., 2005). Breakpoints are discriminatory antimicrobial concentrations used to interpret results of phenotypic susceptibility tests and define isolates as susceptible, intermediate, or resistant (CLSI, 2008). Only ceftiofur, the combination of penicillin and novobiocin, and pirlimycin have validated breakpoints for mastitis, and the application of breakpoints that were defined for other pathogens and different disease conditions for bovine mastitis pathogens has been criticized (Watts and Yancey, 1994; Schwarz et al., 2010). Several studies have demonstrated that in vitro susceptibility tests are not predictive of clinical outcomes (Hoe and Ruegg, 2005; Apparao et al., 2009a; Apparao et al., 2009b) and a clear need exists for more research to better understand the validity and interpretation of both phenotypic and genotypic tests used to determine susceptibility of mastitis pathogens.

Similar to results of the present study, the combination of genotypic and phenotypic resistance to both penicillin and tetracycline has been previously reported for *Staph. aureus* isolated from bovine IMI (Waage et al., 2002; Haveri et al., 2005). Of all isolates, 43% carried *blaZ* and a *tet* gene; however, this was more prevalent in *Staph. aureus* compared with other species. Resistance to tetracycline and macrolides is often found in the same mobile unit, Tn1545 (De Leener et al., 2004). Waage et al. (2002) reported that 99 of 107 penicillin and tetracycline resistant *Staph. aureus* isolates from bovine mastitis occurring in 18 herds belonged to the same strain. Based on these data, Aarestrup

and Schwarz (2006) hypothesized that the combined penicillin and tetracycline resistance had spread mainly via transmission of a single strain. Clustering of *Staph. aureus* strains within herds could explain the finds in the present study, but this was not tested, as all of the *Staph. aureus* that contained the combination of *blaZ* and *tet* genes came from 8 herds.

CONCLUSIONS

The presence of selected resistance genes was not proportional to the occurrence of phenotypic resistance. The majority of the organisms contained at least 1 of the 4 tested resistance genes. Whereas the presence of resistance genes did not correspond with the occurrence of phenotypic resistance, *blaZ* and *tet* genes were identified in a large proportion of the isolates. There was a clear discrepancy between results of phenotypic and genotypic testing for antimicrobial resistance, which suggest that a broader selection of genes should be tested for and further research should be directed toward validating resistance breakpoints. Most gram-positive mastitis organisms were susceptible to most antimicrobials used for IMM administration but some resistance to drugs used for systemic treatment of mastitis was noted and about 25% of *Staphylococcus* spp. were resistant to pirlimycin. All *Staph. aureus* were susceptible to oxacillin. All isolates were susceptible to both of the cephalosporins that were tested, suggesting that resistance is not emerging to these commonly used IMM compounds. For a few antimicrobials (ampicillin, florfenicol, sulfadimethoxine, and pirlimycin) differences in the proportion of susceptibility, as well MIC₅₀ and MIC₉₀, existed among species of staphylococci and streptococci. This study does not support the evidence of emerging widespread resistance among mastitis pathogens; however, the proportion of some organism resistant to tetracycline and sulfonamides indicate that usage of these drugs should be strongly discouraged.

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