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Genetic characterization of antimicrobial resistance in coagulase-negative staphylococci from bovine mastitis milk

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ABSTRACT

Coagulase-negative staphylococci (CNS; n = 417) were isolated from bovine milk and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Nineteen different species were identified, and *Staphylococcus xylosus*, *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, and *Staphylococcus sciuri* were the most prevalent species. Resistance to oxacillin (47.0% of the isolates), fusidic acid (33.8%), tiamulin (31.9%), penicillin (23.3%), tetracycline (15.8%), streptomycin (9.6%), erythromycin (7.0%), sulfonamides (5%), trimethoprim (4.3%), clindamycin (3.4%), kanamycin (2.4%), and gentamicin (2.4%) was detected. Resistance to oxacillin was attributed to the *mecA* gene in 9.7% of the oxacillin-resistant isolates. The remaining oxacillin-resistant CNS did not contain the *mecC* gene or *mecA1* promoter mutations. The *mecA* gene was detected in *Staphylococcus fleurettii*, *Staphylococcus epidermidis*, *Staph. haemolyticus*, and *Staph. xylosus*. Resistance to tetracycline was attributed to the presence of *tet*(K) and *tet*(L), penicillin resistance to *blaZ*, streptomycin resistance to *str* and *ant(6)-Ia*, and erythromycin resistance to *erm*(C), *erm*(B), and *msr*. Resistance to tiamulin and fusidic acid could not be attributed to an acquired resistance gene. In total, 15.1% of the CNS isolates were multidrug resistant (i.e., resistant to 2 or more antimicrobials). The remaining CNS isolates were susceptible to antimicrobials commonly used in mastitis treatment. Methicillin-resistant CNS isolates were diverse, as determined by *mecA* gene sequence analysis, staphylococcal cassette chromosome *mec* typing, and pulsed-field gel electrophoresis. Arginine catabolic mobile element types 1 and 3 were detected in both methicillin-resistant and methicillin-susceptible *Staph. epidermidis* and were associated with sequence types ST59 and ST111. Because this study revealed the presence of multidrug-resistant CNS in a heterogeneous CNS population, we recommend antibiogram analysis of CNS in persistent infections before treatment with antimicrobials.

Key words: methicillin-resistance, coagulase-negative staphylococci, genotyping, antibiotic resistance

INTRODUCTION

Coagulase-negative staphylococci are the microorganisms most commonly isolated from bovine milk in many countries, and they are an important cause of mastitis (Pyörälä and Taponen, 2009; Rajala-Schultz et al., 2009; Piessens et al., 2011; De Vliegher et al., 2012). The CNS are opportunistic pathogens that are usually diagnosed as a group without species identification. They cause subclinical IMI that result in an increase in SCC and reduced milk quality, leading to economic losses (Pyörälä and Taponen, 2009). Because simple subclinical CNS infections can be self-limiting, they are usually not treated with antibiotics. However, CNS often appear with other major pathogens such as *Staphylococcus aureus*, *Streptococcus* spp., or coliform bacteria. In these cases and in persistent CNS infections, the cows undergo antimicrobial treatment. Currently, β-lactam antimicrobials (including penicillin and cephalosporins), aminoglycosides (gentamicin and neomycin), and macrolides (spiramycin) are commonly used to treat mastitis in Switzerland (Büttner et al., 2011). Resistance to these antibiotics has been increasingly reported in CNS associated with bovine mastitis (Walther and Perreten, 2007; Sawant et al., 2009; Sampimon et al., 2011). The CNS may also harbor antimicrobial resistance elements and pathogenicity islands, such as the staphylococcal cassette chromosome (**SCC*mec***) element (Wielders et al., 2001; Barbier et al., 2010; Tsubakishita et al., 2010) and the arginine catabolic mobile element (**ACME**; Diep et al., 2006, 2008; Miragaia et al., 2009) that can be transferred to *Staph. aureus*. Arginine catabolic mobile elements are genomic islands in *Staph. epidermidis* that are associated with host colonization, fitness, and pathogenicity. Mobility of ACME is associated with recombinase genes present on the SCC*mec* elements (Goering et al., 2007; Diep et al., 2008). The SCC*mec* elements contain the *mec* genes—*mecA* or *mecC* ($mecA_{LGA251}$)—which

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encode alternative penicillin-binding proteins (**PBP 2a**) and confer resistance to all β-lactam antimicrobials (García-Álvarez et al., 2011; Ito et al., 2012). In *Staph. sciuri*, the *mecA* gene homolog *mecA1* is a native gene that is not part of the *mec* gene complex (Couto et al., 1996, 2000; Wu et al., 1998, 2001; Tsubakishita et al., 2010). Most *Staph. sciuri* isolates are susceptible to β-lactam antimicrobials. However, alterations in the promoter regions of *mecA1* upregulate *mecA1* expression and confer methicillin resistance (Wu et al., 2001, 2005; Couto et al., 2003). Methicillin-resistant staphylococci are often also resistant to other classes of drugs such as aminoglycosides and macrolides (Woodford 2005). Nevertheless, little is known about the molecular mechanisms of antimicrobial resistance (Lüthje and Schwarz, 2006) or the genetic background of multidrug-resistant CNS strains in bovine milk.

We identified different CNS species in milk from cows with clinical and subclinical bovine mastitis, characterized their antimicrobial resistance mechanisms, and determined whether specific methicillin-resistant and multidrug-resistant CNS clones are common in dairy cows.

MATERIALS AND METHODS

Origin of Milk Samples

Coagulase-negative staphylococci (n = 417) were isolated from milk (n = 370) obtained from cows diagnosed with clinical (n = 115) and subclinical (n = 255) mastitis and control samples (n = 47) in Switzerland. Control samples were collected from cows that had suffered from mastitis previously and had been treated; the control milk samples contained <150,000 cells/mL. The 417 isolates came from 363 different cows and from 2 different mammary quarters of 7 cows. The 363 cows originated from 195 different farms ($\mathbf{n_f}$) in the cantons of Berne (n_f = 91), Jura (n_f = 56), Fribourg (n_f = 26), Vaud (n_f = 8), Lucerne (n_f = 5), Valais (n_f = 4), Solothurn (n_f = 3), Aargau (n_f = 1), and Thurgau (n_f = 1). In 47 cases, 2 different CNS strains were found in the same milk sample.

Isolation and Identification of CNS

Milk samples were centrifuged at 590 × *g* for 10 min at room temperature. The milk pellets were cultivated on tryptone soy agar containing 5% defibrinated sheep blood (Becton, Dickinson and Co., Franklin Lakes, NJ) and incubated at 37°C for 18 to 24 h. Staphylococci were selected based on colony morphology, gram-positive staining of cocci, and catalase production and were subcultured on tryptone soy agar containing 5% defibrinated sheep blood.

The isolates were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (**MALDI-TOF MS**) analysis using the ethanol–formic acid extraction method for better resolution (Microflex LT, Bruker Daltonics GmbH, Bremen, Germany; Application Note MT-80, Bruker Daltonics GmbH). Species identification was considered valid when the matching score with reference spectra of the MALDI Biotyper v3.0 database (Bruker Daltonics GmbH) was ≥2, according to the criteria proposed by the manufacturer. Isolates whose measured spectra had score <2.0 were further identified by DNA sequencing of the 16S rDNA (Kuhnert et al., 1996). The CNS strains were stored at −80°C in trypticase soy medium containing 30% glycerin (Becton, Dickinson and Co.).

DNA Extraction and Amplification

To obtain total DNA, cells were incubated in 100 μL of Tris-EDTA buffer containing 0.1 mg/mL lysostaphin for 15 min at 37°C; then, 450 μL of lysis buffer (0.1 *M* Tris-HCl, pH 8.5, 0.05% Tween 20, 0.24 mg/mL proteinase K) was added and incubated at 60°C for 45 min. The DNA was then denatured at 95°C for 15 min. The PCR was performed with HOT FIREPol DNA Polymerase (Solis BioDyne, Tartu, Estonia) using the primers and conditions listed in Table 1.

Antimicrobial Resistance Tests

The CNS isolates were tested for antimicrobial susceptibility with the broth microdilution technique (Clinical and Laboratory Standards Institute, 2009) using Sensititre susceptibility plates (NLEUST plates; Trek Diagnostics Systems, East Grinstead, UK) that contained the following 19 antimicrobials: chloramphenicol, ciprofloxacin, clindamycin, dalfopristin-quinupristin, erythromycin, fusidic acid, gentamicin, kanamycin, linezolid, mupirocin, oxacillin, penicillin, rifampicin, streptomycin, sulfamethoxazole, tetracycline, tiamulin, trimethoprim, and vancomycin. The resistance breakpoints were those proposed for CNS in the guidelines of the European Committee on Antimicrobial Susceptibility Testing (**EUCAST**, www.eucast.org; Table 2), except for streptomycin and kanamycin, for which breakpoints came from the French Society for Microbiology (www.sfm-microbiologie.org). The production of β-lactamase was tested on nitrocefin dry slides (Becton, Dickinson and Co.) using colonies grown on Mueller Hinton agar for 18 h at 37°C with 0.05 μg/mL penicillin to induce β-lactamase production (Schnellmann et al., 2006). The

Table 1. Primers and primer sequences used in the study

Target gene	Primer name (F = forward; R = reverse) and sequence		Size of PCR fragment (bp)	Annealing emperature (°C)
mecA, *mecA1*, *mecA2*	mecAuniv-F	5′-AAAAGATAAATCTTGGGGTG	525	51
	mecAuniv-R	5′-CCTTGTTTCATYTTGAGTTC		
mecA	mecA-1	5′-AAAATCGATGGTAAAGGTTGGC	533	54
	mecA-2	5′-AGTTCTGCAGTACCGGATTTGC		
mecA1	mecA1-sc-F	5′-ATTAATCATCGCCATCGTGA	663	52
	mecA1-sc-R	5′-TTTGTATCTTGATTCATATTTTGAACA		
mecC	mecC-F	5′-CAGCCAGATTCATTTGTACC	486	54
	mecC-R	5′-AACATCGTACGATGGGGTAC		
mecA1 promoter	mecAscK1-F	5′-CATATATATATTTATACGCTCATC	335	50
	mecAsc-R	5′-TTCAATGGCATCAATTGTTTC		
mecA[1] (full-length gene)	mecA-F7	5′-GATAACACCTGCTACAC	2,194	51
	mecA-R7	5′-AAGGGAGAAGTAACAGC		

[1]Primers annealing external to *mecA* for amplification and sequencing of the full-length gene.

antimicrobial resistance genes were detected by using a custom-made microarray (AMR+ve-2 array tubes, Alere Technologies GmbH, Jena, Germany; Perreten et al., 2005). The microarray results were analyzed using the IconoClust program (Alere Technologies GmbH), and the data were interpreted visually.

Characterization of the mec Genes and SCCmec Elements

All isolates displaying a MIC for oxacillin above the resistance breakpoint (MIC >0.25 μg/mL), which suggests the presence of an alternative penicillin-binding protein (based on Clinical and Laboratory Standards Institute and EUCAST), were additionally tested by PCR for the *mecA*, *mecA1*, and *mecC* genes (García-Álvarez et al., 2011; Ito et al., 2012) using the primers listed in Table 1. The complete nucleotide sequences of the *mecA* genes were obtained by PCR amplification with the mecA-F7 and mecA-R7 primers (Table 1). Sequencing was performed on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA). The SCC*mec* types were determined by the Kondo method (Kondo et al., 2007).

Table 2. Distribution of antimicrobial resistance phenotypes in CNS

		Resistance phenotype							
		Total (n_{total} = 417)		Clinical mastitis (n_{total} = 115)		Subclinical mastitis (n_{total} = 255)		Control milk (n_{total} = 47)	
Antimicrobial substance	Breakpoint (μg/mL)	No.	%	No.	%	No.	%	No.	%
Oxacillin	R >0.25	196	47.0	65[a]	56.5	112[a]	43.9	19	40.4
Fusidic acid	R >1.0	141	33.8	44	38.3	81	31.8	16	34.0
Tiamulin	R >2.0	133	31.9	34	29.6	85	33.3	14	29.8
Penicillin	R >0.125	97	23.3	26	22.6	63	24.7	8	17.0
Tetracycline	R >2.0	66	15.8	18	15.7	37	14.5	11	23.4
Streptomycin	R >16	40	9.6	9	7.8	29	11.4	2	4.3
Erythromycin	R >2.0	29	7.0	7	6.1	18	7.1	4	8.5
Sulfamethoxazole	R >128	21	5.0	13[b]	11.3	8	3.1	0[b]	0.0
Trimethoprim	R >4.0	18	4.3	6	5.2	11	4.3	1	2.1
Clindamycin	R >0.5	14	3.4	3	2.6	10	3.9	1	2.1
Chloramphenicol	R >8.0	13	3.1	4	3.5	8	3.1	1	2.1
Gentamicin	R >1.0	10	2.4	5	4.4	5	2.0	0	0.0
Kanamycin	R >16.0	10	2.4	5	4.4	5	2.0	0	0.0
Quinupristin-dalfopristin	R >2.0	0	0.0	0	0.0	0	0.0	0	0.0
Rifampicin	R >0.5	0	0.0	0	0.0	0	0.0	0	0.0
Ciprofloxacin	R >1.0	0	0.0	0	0.0	0	0.0	0	0.0
Mupirocin	R >256	0	0.0	0	0.0	0	0.0	0	0.0

[a]Denotes a significant difference ($P = 0.03$) in the number of oxacillin-resistant isolates from clinical and subclinical mastitis cases, as determined by Fisher's exact test.

[b]Denotes a significant difference ($P = 0.02$) in the number of sulfamethoxazole-resistant isolates from clinical mastitis cases and control milk, as determined by Fisher's exact test.

Analysis of the mecA1 Promoter Region in Staph. sciuri

Staphylococcus sciuri isolates carrying a *mecA1* homolog (n = 37) were analyzed for a point mutation (Wu et al., 2001) in the promoter region by restriction analysis of PCR products amplified with primers mecAscK1-F and mecAsc-R (Table 1). The 335-bp PCR product was tested for *Psi*I cleavage using the manufacturer's suggested conditions (New England BioLabs, Beverly, MA); *Psi*I recognizes the mutated promoter sequence TATAAT but not the wild-type sequence TATATT.

Genotyping of Methicillin-Resistant CNS

Methicillin-resistant, *mecA*-positive CNS isolates and multidrug-resistant, *mecA*-negative *Staph. epidermidis* isolates were genotyped by pulsed-field gel electrophoresis (**PFGE**). Analysis of *Sma*I-digested chromosomal DNA was performed as described previously (Schnellmann et al., 2006). Digested DNA was separated by gel electrophoresis in a contour-clamped homogeneous electric field DRIII device (Bio-Rad Laboratories Inc., Richmond, CA) with a ramped pulse time of 5 to 40 s at 6 V/cm for 21 h at 12°C. The lambda ladder PFG marker (New England BioLabs) was used as a size reference. The digital PFGE pattern images were analyzed with the BioNumerics software (Applied Maths, Kortrijk, Belgium), and the PFGE profiles were defined by the DNA banding patterns and criteria of Tenover et al. (1995).

Multilocus Sequence Typing

All *Staph. epidermidis* isolates (n = 15) were examined by multilocus sequence typing (**MLST**), which is based on the sequencing of internal fragments of 7 housekeeping genes (Thomas et al., 2007). Allele and sequence type (**ST**) numbers were assigned according to the *Staph. epidermidis* MLST database (http://sepidermidis.mlst.net/).

Detection of ica and ACME

The *Staph. epidermidis* isolates (n = 15) were tested by PCR for the biofilm operon *ica* (Gu et al., 2005) and ACME. The presence and type of ACME was determined using the primer pairs AIPS.27 and AIPS.28 for *arcA* and AIPS.45 and AIPS.46 for *opp3* gene clusters (Diep et al., 2008).

Statistical Analysis

Antimicrobial resistance phenotypes (Table 2) were compared using the Fisher exact test. This test is useful when the sample size is small (zero in some cells), and the test evaluates the hypothesis that the 2 column percentages in a 2 × 2 table are equal. Statistical analysis was performed with the statistical software NCSS 2007 (www.ncss.com). The overall level of statistical significance was set to $P < 0.05$.

RESULTS

Prevalence and Identification of CNS

In total, 97.8% of the CNS isolates (n = 408) were clearly identified at the species level by MALDI-TOF MS analysis. The most frequent CNS species were *Staph. xylosus*, *Staph. chromogenes*, *Staph. sciuri*, and *Staph. haemolyticus* (Table 3). The remaining 2.2% were identified by 16S rDNA analysis as *Staph. chromogenes* (n = 1), *Staph. saprophyticus* (n = 1), or novel *Staphylococcus* species (n = 7; Table 3). Neither clinical nor subclinical mastitis could be correlated with the presence of individual bacterial species. Similar species were identified in control milk samples with low SCC. Of the 417 CNS, 268 isolates were the only species present in the milk from which they originated, and 149 isolates were present together with at least one other species. *Staphylococcus xylosus* (n = 92), *Staph. chromogenes* (n = 56), *Staph. haemolyticus* (n = 22), and *Staph. sciuri* (n = 21) were the predominant species among the 268 isolates that had only a single CNS species present (Table 4). Among the mixed cultures, 18 CNS isolates were co-purified with *Staph. aureus* (12.1%), 83 isolates were present with *Streptococcus* spp. (55.7%), and 48 isolates (32.2%) were coincident in milk with at least one other bacterium (e.g., *Trueperella pyogenes*, *Escherichia coli*, *Corynebacterium bovis*, or a mix of more than 3 different bacteria; Table 4). In the milk samples, none of the CNS was found more frequently as a single agent than together with other bacteria, with the exception of *Staph. chromogenes*, which was not often found together with *Staph. aureus* or streptococci (Table 4).

Analysis of Antimicrobial Resistance Phenotypes and Genotypes

Oxacillin resistance, which is the indicator of *mec* gene-mediated methicillin resistance, was the most frequent resistance phenotype (47.0% of isolates), followed by resistance to fusidic acid (34.1%), tiamulin (31.9%), penicillin (23.3%), tetracycline (15.8%), streptomycin (9.6%), and erythromycin (7.0%; Table 2). Resistance to 2 or more antibiotics was observed in 15.1% of the CNS isolates. Multidrug-resistant isolates were found in milk from clinical (n = 21) and subclinical (n = 34)

Table 3. Prevalence of CNS and distribution of different CNS strains in clinical and subclinical mastitis milk and control milk

	Total		Clinical mastitis milk		Subclinical mastitis milk		Control milk[1]	
CNS	No.	%	No.	%	No.	%	No.	%
Total strains	417	100.0	115	100.0	255	100.0	47	100.0
Staphylococcus xylosus	150	36.0	43	37.4	95	37.2	12	25.5
Staphylococcus chromogenes	70	16.8	20	17.4	40	15.7	10	21.3
Staphylococcus sciuri	37	8.9	10	8.7	25	9.8	2	4.3
Staphylococcus haemolyticus	35	8.4	8	6.9	23	9.0	4	8.5
Staphylococcus devriesei	18	4.3	7	6.0	11	4.3	0	0.0
Staphylococcus warneri	17	4.1	4	3.5	6	2.3	7	14.8
Staphylococcus simulans	16	3.8	1	0.9	13	5.1	2	4.3
Staphylococcus epidermidis	15	3.6	4	3.5	10	3.9	1	2.1
Staphylococcus fleurettii	12	2.9	6	5.2	4	1.6	2	4.3
Staphylococcus succinus	9	2.2	0	0.0	7	2.8	2	4.3
Staphylococcus vitulinus	9	2.2	5	4.4	4	1.6	0	0.0
Staphylococcus hyicus	6	1.4	2	1.7	4	1.6	0	0.0
Staphylococcus equorum	6	1.4	1	0.9	2	0.8	3	6.3
Staphylococcus saprophyticus	2	0.5	1	0.9	1	0.4	0	0.0
Staphylococcus auricularis	2	0.5	1	0.9	1	0.4	0	0.0
Staphylococcus capitis	2	0.5	0	0.0	2	0.8	0	0.0
Staphylococcus cohnii	2	0.5	0	0.0	0	0.0	2	4.3
Staphylococcus hominis	1	0.2	0	0.0	1	0.4	0	0.0
Staphylococcus lentus	1	0.2	0	0.0	1	0.4	0	0.0
Staphylococcus spp.	7	1.6	2	1.7	5	1.9	0	0.0

[1]Milk taken from cows after mastitis treatment.

mastitis cases and in control milk (n = 8). Oxacillin resistance was significantly more frequent in clinical mastitis isolates (56.5%) than in subclinical mastitis isolates (43.9%), whereas sulfamethoxazole resistance was significantly more frequent in clinical mastitis isolates (11.3%) than in control milk. No significant

Table 4. Distribution of CNS as single agent and associated with other pathogens in the milk samples

	Proportion of CNS (n = 417)								
				Occurring in a mixed culture (n = 149)					
	Total (n = 417)	Occurring as a single agent (n = 268)		With *Staph. aureus* (n = 18)		With *Streptococcus* spp. (n = 83)		With other bacteria[1] (n = 48)	
CNS	No.	No.	%	No.	%	No.	%	No.	%
Staphylococcus xylosus	150	92	34.3	7	38.9	34	41.0	17	35.4
Staphylococcus chromogenes	70	56	20.9	1	5.6	5	6.0	8	16.7
Staphylococcus sciuri	37	21	7.8	1	5.6	10	12.0	5	10.4
Staphylococcus haemolyticus	35	22	8.2	0	0.0	9	10.8	4	8.3
Staphylococcus devriesei	18	13	4.9	0	0.0	3	3.6	2	4.2
Staphylococcus warneri	17	11	4.1	1	5.6	3	3.6	2	4.2
Staphylococcus simulans	16	14	5.2	0	0.0	2	2.4	0	0.0
Staphylococcus epidermidis	15	10	3.7	2	11.1	2	2.4	1	2.1
Staphylococcus fleurettii	12	4	1.5	1	5.6	4	4.8	3	6.3
Staphylococcus succinus	9	5	1.9	2	11.1	2	2.4	0	0.0
Staphylococcus vitulinus	9	2	0.7	3	16.7	0	0.0	4	8.3
Staphylococcus hyicus	6	2	0.7	0	0.0	3	3.6	1	2.1
Staphylococcus equorum	6	5	1.9	0	0.0	1	1.2	0	0.0
Staphylococcus saprophyticus	2	0	0.0	0	0.0	2	2.4	0	0.0
Staphylococcus auricularis	2	1	0.4	0	0.0	1	1.2	0	0.0
Staphylococcus capitis	2	2	0.7	0	0.0	0	0.0	0	0.0
Staphylococcus cohnii	2	2	0.7	0	0.0	0	0.0	0	0.0
Staphylococcus hominis	1	0	0.0	0	0.0	1	1.2	0	0.0
Staphylococcus lentus	1	1	0.4	0	0.0	0	0.0	0	0.0
Staphylococcus spp.	7	5	1.9	0	0.0	1	1.2	1	2.1

[1]Indicates at least one species other than *Staphylococcus aureus* and *Streptococcus* spp. (e.g., *Trueperella pyogenes*, *Escherichia coli*, *Corynebacterium bovis*) or a mix of more than 3 different bacteria.

difference in resistance between isolates was observed for the other antimicrobials tested (Table 2).

Oxacillin resistance was attributed to the *mecA* gene present in 9.7% (n = 19) of the oxacillin-resistant isolates (n = 196). The *mecA* gene was detected in *Staph. fleurettii* (11/12), *Staph. epidermidis* (6/15), *Staph. haemolyticus* (1/37), and *Staph. xylosus* (1/155) isolates. The *mecA* or *mecC* gene was not detected in the other 177 oxacillin-resistant isolates (90.3%; Table 5). These isolates exhibited an oxacillin MIC of 0.5 or 1.0 μg/mL, which is just above the clinical resistance breakpoint. Twenty of these 177 oxacillin-resistant *mecA*- and *mecC*-negative isolates contained a *blaZ* gene expressing a β-lactamase. The remaining isolates were also negative for *blaZ* (Table 5). Among them, all *Staph. sciuri* isolates (n = 37) contained the *mecA1* gene, and exhibited low-level resistance to oxacillin (MIC between 0.5 and 1.0 μg/mL). The low oxacillin resistance was due to the absence of the T→A mutation in the −10 promoter sequence (Wu et al., 2001), as demonstrated by *Psi*I restriction analysis.

Resistance to other antimicrobials correlated with the presence of the associated resistance genes (Table 5): the β-lactamase gene *blaZ*; the tetracycline efflux genes *tet*(L) and *tet*(K); the streptomycin adenyltransferase and nucleotidyltransferase genes *ant(6)-Ia* and *str*; the chloramphenicol acetyltransferase genes cat_{pC221} and cat_{pC223}; the gentamicin acetyltransferase gene *aac(6')-Ie*; the kanamycin-neomycin phosphotransferase genes *aph(2')-Ia* and *aph(3')-III*; the macrolide and lincosamide 23S rRNA methylase genes *erm*(B) and *erm*(C); the macrolide efflux gene *msr*; the lincosamide nucleotidyltransferase gene *lnu*(A); and the trimethoprim-resistant dihydrofolate reductase genes *dfr*(A), *dfr*(D), *dfr*(G), and *dfr*(K). In a few strains, resistance to erythromycin, clindamycin, streptomycin, gentamicin, chloramphenicol, and trimethoprim could not be explained by the presence of any of the tested genes, suggesting new antimicrobial resistance mechanisms in CNS (Table 5).

Resistance to fusidic acid was not due to the known fusidic acid resistance genes *fus*(B), and *fus*(C), suggesting the appearance of new resistance genes or mutations in the elongation factor G *fus*(A) (Farrell et al., 2011). Similarly, no known tiamulin resistance genes (*vga* or *lsa*) were detected in the tiamulin-resistant strains. Resistance to sulfonamides was not further characterized.

Multiple combinations of these genes were found in 4.8% of the CNS isolates. The most frequent resistance genes detected in combination were those conferring resistance to oxacillin, tetracycline, penicillin, streptomycin, gentamicin, kanamycin, erythromycin, and clindamycin (Table 6). The presence of several genes in one isolate was linked to the presence of *mecA* in *Staph. epidermidis* (n = 6), *Staph. sciuri* (n = 3), *Staph. haemolyticus* (n = 1), and *Staph. fleurettii* (n = 1). The other *mecA* positive isolates [*Staph. fleurettii* (n = 10) and *Staph. xylosus* (n = 1)] contained only the *mecA* gene. The CNS isolates lacking *mecA*, but containing several other resistance genes were classified as *Staph. chromogenes* (n = 3), *Staph. epidermidis* (n = 3), *Staph. haemolyticus* (n = 1), and *Staph. warneri* (n = 2; Table 6). All CNS isolates containing several resistance genes were found in milk samples from cows presenting with subclinical or clinical mastitis (Table 6). Although one *Staph. xylosus* isolate contained a *mecA* gene, multidrug resistance was never observed in *Staph. xylosus*, the most frequently detected CNS in our study.

Genotyping of Methicillin-Resistant CNS

The CNS strains containing the *mecA* gene (n = 19) were further analyzed for *mecA* sequences, clonality, and SCC*mec* and ACME types. Two different *mecA* genes with slight sequence differences were detected in *Staph. epidermidis* isolates (Figure 1). Four *mecA* genes that slightly differed from each other were found in *Staph. fleurettii* (Figure 1). The *mecA* genes detected in *Staph. haemolyticus* and *Staph. xylosus* also differed slightly from each other and from those in *Staph. epidermidis* and *Staph. fleurettii* (Figure 1).

Analysis of methicillin-resistant *Staph. epidermidis* (n = 6) and *Staph. fleurettii* (n = 11) by PFGE showed that strains of the same species were not clonally related, except for 4 *Staph. fleurettii* from 4 different farms that showed 2 similar PFGE patterns (Figure 1). Methicillin-resistant *Staph. epidermidis* belonged to ST59 (n = 2), ST55, ST89, and the new strains ST452 and ST454, whereas *Staph. epidermidis* strains lacking the *mecA* gene (methicillin-sensitive *Staph. epidermidis*, **MSSE**) and displaying a multidrug-resistance profile belonged to ST111 (n = 4), ST184 (n = 1), ST293 (n = 1), and to the new strain ST453 (n = 1); MSSE also showed a different PFGE profile (data not shown).

Several different SCC*mec* elements were detected among the methicillin-resistant CNS isolates (Figure 1). *Staphylococcus epidermidis* strains contained SCC*mec* IV (n = 4), SCC*mec* V (n = 1), and one nontypeable SCC*mec* related to types IV and VI. *Staphylococcus haemolyticus* (n = 1) and *Staph. xylosus* (n = 1) both contained a nontypeable SCC*mec*. The *mecA* gene of *Staph. fleurettii* strains (n = 11) were associated with the class A *mec* gene complex and could not be assigned to a known SCC*mec* element (Figure 1). Two methicillin-resistant *Staph. epidermidis* strains, ST59-SCC*mec* IV and ST454-SCC*mec* V, contained a type 2 and type 3 ACME, respectively; ACME type 1 was

Table 5. Distribution of antimicrobial resistance and antimicrobial resistance genes in CNS from bovine milk

Antimicrobial substance	Phenotypic resistance (%)	Resistance genes[1]	Resistance genes in resistant isolates		Species (no.)
			No.	%	
Oxacillin[2]	47.0	*mecA*	19	9.7	*Staph. fleurettii* (11), *Staph. epidermidis* (6), *Staph. haemolyticus* (1), *Staph. xylosus* (1)
		mecA1	37	18.9	*Staph. sciuri* (37)
		No *mecA/A1/C*; *blaZ*	20	10.2	*Staph. chromogenes* (9), *Staph. xylosus* (5), *Staph. cohnii* (2), *Staph. saprophyticus* (2), *Staph. warneri* (2)
		No *mecA/A1/C*; no *blaZ*	120	61.2	*Staph. xylosus* (97), *Staph. vitulinus* (8), *Staph. succinus* (5), *Staph. chromogenes* (2), *Staph. devriesei* (1), *Staph. equorum* (1), *Staph. hyicus* (1), *Staph. lentus* (1), *Staph. simulans* (1), *Staph. warneri* (1), *Staphylococcus* spp. (2)
Penicillin[3]	23.3	*blaZ*	88	90.7	*Staph. chromogenes* (37), *Staph. devriesei* (11), *Staph. haemolyticus* (11), *Staph. xylosus* (9), *Staph. epidermidis* (9), *Staph. warneri* (3), *Staph. cohnii* (2), *Staph. saprophyticus* (2), *Staph. auricularis* (1), *Staph. capitis* (1), *Staph. fleurettii* (1), *Staph. hominis* (1)
		No β-lactamase[4]	8	8.3	*Staph. fleurettii* (8)
		Unknown	1	1.0	*Staph. xylosus* (1)
Tetracycline	15.8	*tet*(K)	62	95.4	*Staph. xylosus* (33), *Staph. warneri* (7), *Staph. epidermidis* (6), *Staph. sciuri* (4), *Staph. chromogenes* (3), *Staph. simulans* (3), *Staph. fleurettii* (2), *Staph. vitulinus* (2), *Staph. haemolyticus* (1), *Staphylococcus* sp. (1)
		tet(L)	3	4.6	*Staph. chromogenes* (3)
Streptomycin	9.6	*str*	36	90.0	*Staph. chromogenes* (12), *Staph. epidermidis* (7), *Staph. sciuri* (5), *Staph. haemolyticus* (4), *Staph. devriesei* (3), *Staph. warneri* (2), *Staph. simulans* (1), *Staph. vitulinus* (1), *Staph. fleurettii* (1)
		ant(6)-Ia	3	7.5	*Staph. epidermidis* (2), *Staph. haemolyticus* (1)
		Unknown	1	2.5	*Staph. haemolyticus* (1)
Erythromycin	7.0	*erm*(B)	2	7.4	*Staph. chromogenes* (1), *S fleurettii* (1)
		erm(C)	11	40.8	*Staph. epidermidis* (6), *Staph. haemolyticus* (4), *Staph. warneri* (1)
		msr	6	22.2	*Staph. xylosus* (3), *Staph. epidermidis* (2), *Staph. hominis* (1)
		Unknown	8	29.6	*Staph. equorum* (3), *Staph. xylosus* (1), *Staph. cohnii* (1), *Staph. fleurettii* (1), *Staph.* spp. (2)
Clindamycin	3.4	*erm*(B)	2	14.3	*Staph. chromogenes* (1), *Staph. sciuri* (1)
		erm(C)	8	57.2	*Staph. haemolyticus* (4), *Staph. epidermidis* (4)
		lnu(A)	1	7.1	*Staph. xylosus* (1)
		Unknown	3	21.4	*Staph. xylosus* (1), *Staph. fleurettii* (1), *Staph. lentus* (1)
Chloramphenicol	3.1	cat_{pC221}	7	53.8	*Staph. epidermidis* (3), *Staph. xylosus* (2), *Staph. chromogenes* (1), *Staph. sciuri* (1)
		cat_{pC223}	4	30.8	*Staph. cohnii* (1), *Staph. haemolyticus* (1), *Staph. simulans* (1), *Staph. xylosus* (1)
		Unknown	2	15.4	*Staph. haemolyticus* (1), *Staph. simulans* (1)
Kanamycin	2.4	*aac(6')-Ie–aph(2')-Ia*	7	70.0	*Staph. epidermidis* (4), *Staph. sciuri* (1), *Staph. chromogenes* (1), *Staph. fleurettii* (1)
		aph(3')-III	3	30.0	*Staph. haemolyticus* (2), *Staph. epidermidis* (1)
Gentamicin	2.4	*aac(6')-Ie–aph(2')-Ia*	7	70.0	*Staph. epidermidis* (4), *Staph. sciuri* (1), *Staph. chromogenes* (1), *Staph. fleurettii* (1)
		Unknown	3	30.0	*Staph. haemolyticus* (2), *Staph. xylosus* (1)
Trimethoprim	1.2	*dfr*(A)	1	20.0	*Staph. epidermidis* (1)
		dfr(D)	2	40.0	*Staph. sciuri* (1), *Staph. fleurettii* (1)
		dfr(G)	1	20.0	*Staph. vitulinus* (1)
		dfr(K)	1	20.0	*Staph. chromogenes* (1)

[1]Antimicrobial resistance genes and their functions: *mecA* and *mecA1* = penicillin-binding proteins; *blaZ* = β-lactamase; *tet*(K) and *tet*(L) = tetracycline efflux proteins; *str* = streptomycin nucleotidyltransferase; *ant(6)-Ia* = streptomycin adenyltransferase; *erm*(B) and *erm*(C) = macrolide, lincosamide, and streptogramin B 23S rRNA methylase; *msr* = macrolide efflux gene; *lnu*(A) = lincosamide nucleotidyltransferase; cat_{pC221} and cat_{pC223} = chloramphenicol acetyltransferases; *aac(6')-Ie–aph(2')-Ia* = gentamicin, kanamycin, and neomycin acetyltransferase; *aph(3')-III* = kanamycin and neomycin phosphotransferase; *dfr*(A), *dfr*(D), *dfr*(G),*dfr*(K) = trimethoprim-resistant dihydrofolate reductases.

[2]Oxacillin, indicator antimicrobial for the presence of an alternative penicillin-binding protein (PBP 2a) encoded by *mec* genes.

[3]Penicillin used for the prediction of a β-lactamase.

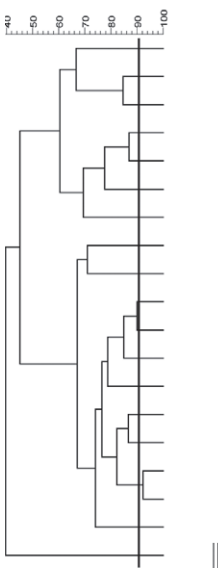
[4]Does not produce β-lactamase, but contained *mecA*.

Table 6. Occurrence of multiple antimicrobial resistance genes in CNS isolated from the milk of 20 different cows suffering from bovine mastitis

Isolate	CNS species	Mastitis	Resistance[1,2]
mecA positive			
M1529/10	*Staph. epidermidis*	Subclinical	*erm*(C): CLI, ERY; *tet*(K): TET; *str*: STR; *blaZ*: PEN; *aac(6')-Ie–aph(2')-Ia*: GEN-KAN; *dfr*(A): TMP; *mecA*: OXA
M1186/10	*Staph. epidermidis*	Clinical	*erm*(C): CLI, ERY; *tet*(K): TET; *ant(6)-Ia*: STR; *blaZ*: PEN; *aac(6')-Ie–aph(2')-Ia*: GEN-KAN; *mecA*: OXA
M4460/09	*Staph. fleurettii*	Clinical	*erm*(B): CLI, ERY; *str*: STR; *blaZ*: PEN; *aac(6')-Ie–aph(2')-Ia*: GEN-KAN; *dfr*(D): TMP; *mecA*: OXA
M1570/10	*Staph. haemolyticus*	Clinical	*erm*(C): CLI, ERY; *tet*(K): TET; *blaZ*: PEN; *aph(3')-III*: KAN; *mecA*: OXA
M744/10	*Staph. epidermidis*	Subclinical	*str*: STR; cat_{pC221}: CHL; *aac(6')-Ie–aph(2')-Ia*: GEN-KAN; *erm*(C): CLI. ERY; *mecA*: OXA
M1383/10	*Staph. epidermidis*	Subclinical	*erm*(C): CLI, ERY; *blaZ*: PEN; *aac(6')-Ie–aph(2')-Ia*: GEN-KAN; *mecA*: OXA
M1965/10	*Staph. sciuri*	Clinical	*tet*(K): TET; *str*: STR; *aac(6')-Ie–aph(2')-Ia*: GEN-KAN; *dfr*(D): TMP; *mecA1*: OXA
M8/10	*Staph. epidermidis*	Clinical	*ant(6)-Ia*: STR; *blaZ*: PEN; cat_{pC221}: CHL; *aph(3')-III*: KAN; *erm*(C): CLI, ERY; *mecA*: OXA
M1201/10	*Staph. sciuri*	Subclinical	*tet*(K): TET; *str*: STR; cat_{pC221}: CHL; *mecA1*: OXA
M703/10	*Staph. epidermidis*	Subclinical	*blaZ*: PEN; *msr*: ERY; *mecA*: OXA
M3901/09	*Staph. sciuri*	Clinical	*tet*(K): TET; *str*: STR; *mecA1*: OXA
mecA negative			
M425/10	*Staph. chromogenes*	Subclinical	*erm*(B): CLI, ERY; *tet*(L): TET; *blaZ*: PEN; cat_{pC221}: CHL; *dfr*(K): TMP
M47/10	*Staph. chromogenes*	Subclinical	*tet*(L): TET; *str*: STR; *blaZ*: PEN; *aac(6')-Ie–aph(2')-Ia*: GEN-KAN
M1256/10	*Staph. warneri*	Subclinical	*str*: STR; *blaZ*: PEN; *erm*(C): CLI, ERY
M4233–1/09	*Staph. epidermidis*	Subclinical	*erm*(C): CLI-ERY; *tet*(K): TET; *str*: STR
M4298–1/09	*Staph. epidermidis*	Subclinical	*tet*(K): TET; *str*: STR; *blaZ*: PEN; cat_{pC221}: CHL
M46/10	*Staph. chromogenes*	Subclinical	*tet*(K): TET; *str*: STR; *blaZ*: PEN
M523/10	*Staph. epidermidis*	Clinical	*tet*(K): TET; *str*: STR; *blaZ*: PEN
M619–2/10	*Staph. haemolyticus*	Subclinical	*ant(6)-Ia*: STR; *blaZ*: PEN; *aph(3')-III*: KAN
M1094–1/10	*Staph. warneri*	Subclinical	*tet*(K): TET; *str*: STR; *blaZ*, PEN

[1]Antimicrobial resistance genes and their functions: *erm*(B) and *erm*(C) = macrolide, lincosamide, and streptogramin B 23S rRNA methylase; *tet*(K) and *tet*(L) = tetracycline efflux proteins; *str* = streptomycin nucleotidyltransferase; *blaZ* = β-lactamase; *aac(6')-Ie–aph(2')-Ia* = gentamicin, kanamycin, and neomycin acetyltransferase; *dfr*(A), *dfr*(D), *dfr*(G),*dfr*(K) = trimethoprim-resistant dihydrofolate reductases; *mecA* and *mecA1* = penicillin-binding proteins; *ant(6)-Ia* = streptomycin adenyltransferase; *aph(3')-III* = kanamycin and neomycin phosphotransferase; cat_{pC221} and cat_{pC223} = chloramphenicol acetyltransferases; *msr* = macrolide efflux gene.

[2]Antibiotics: CLI = clindamycin; TET = tetracycline; STR = streptomycin; PEN = penicillin; KAN = kanamycin; GEN = gentamicin; TMP = trimethoprim; ERY = erythromycin; OXA = oxacillin; CHL = chloramphenicol.



Strains	Species	Anamnesis	*mecA*	Identity (%) to *mecA* of *S. aureus* N315	SCC*mec* type	ST	ACME type
M1570/10	*S. haemolyticus*	clinical	*mecA*[a]	99.85	NT	-	-
M1186/10	*S. epidermidis*	clinical	*mecA*[b]	99.95	IV	89	-
M1383/10	*S. epidermidis*	subclinical	*mecA*[b]	99.95	IV, VI	452	-
M1529/10	*S. epidermidis*	subclinical	*mecA*[b]	99.95	IV	59	2
M744/10	*S. epidermidis*	subclinical	*mecA*[b]	99.95	IV	59	-
M8/10	*S. epidermidis*	clinical	*mecA*[c]	99.90	IV	55	-
M703/10	*S. epidermidis*	subclinical	*mecA*[d]	99.90	V	454	3
M143/10	*S. fleurettii*	clinical	*mecA*[e]	99.80	class A[j]	-	-
M3783/09	*S. fleurettii*	subclinical	*mecA*[f]	99.70	class A[j]	-	-
M1125-2/10	*S. fleurettii*	clinical	*mecA*[e]	99.80	class A[j]	-	-
M404-1/10	*S. fleurettii*	clinical	*mecA*[e]	99.80	class A[j]	-	-
M1191-2/10	*S. fleurettii*	subclinical	*mecA*[e]	99.80	class A[j]	-	-
M1961/10	*S. fleurettii*	subclinical	*mecA*[e]	99.80	class A[j]	-	-
M205/10	*S. fleurettii*	control milk	*mecA*[g]	99.70	class A[j]	-	-
M4276-1/09	*S. fleurettii*	subclinical	*mecA*[e]	99.80	class A[j]	-	-
M277/10	*S. fleurettii*	clinical	*mecA*[e]	99.80	class A[j]	-	-
M545/10	*S. fleurettii*	clinical	*mecA*[c]	99.80	class A[j]	-	-
M4460/09	*S. fleurettii*	clinical	*mecA*[h]	98.95	class A[j]	-	-
M1545/10	*S. xylosus*	clinical	*mecA*[i]	99.85	NT	-	-

Figure 1. Genetic background and properties of methicillin-resistant CNS from bovine mastitis milk from Switzerland. The phylogenetic tree was constructed from pulsed-field gel electrophoresis (PFGE) patterns of 19 methicillin-resistant CNS isolates. Cluster analysis was generated by Bionumerics 6.6 (Applied Maths, Kortijk, Belgium). The dotted line indicates the cut-off value of ≥90% determining clonality between the isolates, according to the criteria of Tenover et al. (1995). (a) *mecA* of *Staphylococcus haemolyticus* M1570/10 (EMBL accession no. HE978799); (b) *mecA* identical to *mecA* of *Staphylococcus epidermidis RP26A* (EMBL accession no. CP000029); (c) *mecA* of *Staph. epidermidis* M8/10 (EMBL accession no. HE978797); (d) *mecA* of *Staph. epidermidis* M703/10 (EMBL accession no. HE978798); (e) *mecA* identical to *mecA* of *Staphylococcus fleurettii* M143/10 (EMBL accession no. HE978795); (f) *mecA* of *Staph. fleurettii* M3783/09 (EMBL accession no. HE978796); (g) *mecA* of *Staph. fleurettii* M205/10 (EMBL accession no. HE978794); (h) *mecA* of *Staph. fleurettii* M4460/09 (EMBL accession no. HE861945); (i) *mecA* of *Staphylococcus xylosus* M1545/10 (EMBL accession no. HE978800); (j) *mecA* of *Staph. fleurettii* has been reported to be chromosomally encoded and only contains part of the class A *mec* gene complex; it is not associated with a staphylococcal cassette chromosome (SCC*mec*) element (Tsubakishita et al., 2010). NT = not typeable.

detected in 4 MSSE ST111 and in 1 MSSE ST456. None of the *Staph. epidermidis* isolates carried the biofilm-formation operon *ica* (Figure 1).

DISCUSSION

Many diverse CNS species have been identified in bovine milk, and MALDI-TOF MS is a reliable and rapid method to identify CNS species (Loonen et al., 2012). We observed that a short ethanol-formic acid extraction is necessary for accurate identification. The CNS in milk were frequently detected as single bacterial species, suggesting that these species were the infectious agents. However, the presence of these CNS species was not correlated with a clinical mastitis diagnosis (Table 3). The most frequently occurring species in this study were *Staph. xylosus*, *Staph. chromogenes*, *Staph. sciuri*, and *Staph. haemolyticus*, as reported in other studies (Piessens et al., 2011; Supré et al., 2011; Waller et al., 2011). Although *Staph. xylosus* is not known to cause mastitis, it was detected in 35.9% of the milk samples in our study and as a single species in 22.8% of those samples, emphasizing previous conclusions that *Staph. xylosus* is an underestimated pathogenic CNS in bovine mastitis (Supré et al., 2011). Additionally, two-thirds of the *Staph. xylosus* isolates were resistant to oxacillin but lacked a known *mec* gene. The absence of a *mecA* gene was also observed in other oxacillin-resistant CNS isolates (Table 5). The presence of a *blaZ* overexpressing β-lactamase may explain decreased susceptibility to oxacillin in some of the strains, as has been described in borderline oxacillin-resistant *Staph. aureus* (McDougal and Thornsberry, 1986). For the other oxacillin-resistant isolates lacking *mec* and *blaZ* genes, independent mechanisms (which may not be related to an acquired resistance gene) explain the decreased susceptibility to oxacillin with MIC in the range of 0.5 to 2.0 μg/mL. Oxacillin resistance in *Staph. sciuri* may also depend on *mecA1* gene overexpression. Alterations to the promoter region of *mecA1* are necessary for high-level *mecA1* expression and oxacillin resistance in *Staph. sciuri* (Wu et al., 2001, 2005; Couto et al., 2003). In our study, none of the *Staph. sciuri* strains contained the −10 promoter mutation that is associated with oxacillin resistance (Wu et al., 2001). However, the MIC for these isolates were between 0.5 and 1.0 μg/mL, values that are above

the CLSI and EUCAST resistance breakpoints. The clinical and therapeutic relevance of decreased susceptibility to oxacillin remains to be clarified. The oxacillin breakpoint may be set low to properly gauge resistance in CNS from bovine mastitis cases (Fessler et al., 2010), and detection of acquired *mec* genes may be necessary for correct interpretation of the antibiogram.

The *mecA* gene was detected in *Staph. epidermidis*, *Staph. fleurettii*, *Staph. haemolyticus*, and *Staph. xylosus*. Three *mecA* genes that differed from each other in only a few base pairs were found in the methicillin-resistant *Staph. fleurettii* and *Staph. epidermidis* isolates, suggesting the independent acquisition of the *mecA* gene in these species. This conclusion is supported by the observation that the different SCC*mec* elements were detected in individual *Staph. epidermidis* isolates, and that all but one *Staph. fleurettii* isolate contained a *mecA* gene associated with the class A *mec* gene complex. It has been reported that the *mecA*-containing region in *Staph. fleurettii* is not associated with a SCC*mec* element but is encoded chromosomally within a part of the class A *mec* gene complex (Tsubakishita et al., 2010). It is therefore expected that the Kondo typing method (Kondo et al., 2007) detects the chromosomal class A *mec* gene complex in *Staph. fleurettii*. Genetic diversity was confirmed by PFGE, which showed that, except for 2 pairs of *Staph. fleurettii* with similar PFGE profiles, all methicillin-resistant CNS isolates had different PFGE profiles. Despite different PFGE profiles, *Staph. epidermidis* isolates belonging to the ST111 and ST59 groups were predominant in bovine mastitis cases, suggesting that a specific clonal lineage of *Staph. epidermidis* has adapted to the udder environment (Piessens et al., 2012). Half of the *Staph. epidermidis* isolates contained an ACME operon, which may be involved in host adaptation in humans (Miragaia et al., 2009). The ACME were mainly observed in the *Staph. epidermidis* ST59 or ST111 groups, suggesting that it may also play a role in host adaptation in cows. Additionally, *Staph. epidermidis* was the predominant CNS species among those that contained multiple antimicrobial resistance genes. Multiple resistance genes were also found in *Staph. sciuri*, *Staph. chromogenes*, *Staph. haemolyticus*, and *Staph. fleurettii*, and these genes were frequently associated with the presence of the *mecA* gene. Genes conferring resistance to clinically relevant antimicrobials such as the penicillins, macrolides, lincosamides, and aminoglycosides were also detected. In total, 15.1% of the isolates studied were resistant to more than 2 antimicrobials, and some strains were virtually resistant to all antimicrobials authorized for the treatment of mastitis. The remaining CNS isolates were susceptible to antimicrobials commonly used in mastitis treatment.

Our study demonstrated that CNS species in milk from cows experiencing mastitis are generally susceptible to the antimicrobials commonly used for treatment. However, CNS have the potential to acquire resistance genes, leading to therapeutic failures. Some multidrug-resistant isolates, especially *Staph. epidermidis*, *Staph. chromogenes*, and *Staph. haemolyticus*, are present in bovine mastitis milk and may resist antimicrobial treatment. An antibiogram is therefore recommended for targeted therapy, and chronically infected cows should be culled from the herd.

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