Characterization of coagulase-negative staphylococcus species from cows' milk and environment based on *bap*, *icaA*, and *mecA* genes and phenotypic susceptibility to antimicrobials and teat dips

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ABSTRACT

The aim of this study was to investigate whether the main coagulase-negative staphylococcus (CNS) species involved in bovine intramammary infections (IMI) possess specific characteristics that promote colonization of the udder. Virulence markers associated with biofilm formation, antimicrobial resistance, and biocide tolerance were compared between typically contagious CNS species (*Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, and *Staphylococcus simulans*) and those rarely causing IMI (*Staphylococcus sciuri*, *Staphylococcus equorum*, and others) to find possible associations with pathogenicity. Coagulase-negative staphylococcus isolates ($n = 366$) belonging to 22 different species were analyzed by PCR for the presence of the biofilm-associated genes *bap* and *icaA*, and the methicillin resistance gene *mecA*. A selection of 82 isolates was additionally tested for their susceptibility to 5 antibiotics and 2 commercial teat dip products. Minimum inhibitory concentrations of antimicrobials were determined by Etest (AB bioMérieux, Marcy l'Etoile, France), and a microdilution method was optimized to determine minimum biocidal concentrations of teat dips. The *bap*, *icaA*, and *mecA* genes were detected significantly more in isolates from CNS species typically living in the cows' environment than in isolates from IMI-causing species. Antimicrobial resistance was mainly against erythromycin (23%) or oxacillin (16%), and was detected more often in the environmental species. The isolates least susceptible to the teat dips belonged to the IMI-causing species *Staph. chromogenes* and *Staph. simulans*. We concluded that carriage of biofilm genes and antimicrobial resistance were not associated with the ability to colonize

the mammary gland because free-living CNS species constituted a more significant reservoir of biofilm and resistance determinants than did IMI-causing species. In contrast, increased tolerance to biocides may favor the establishment of bovine IMI by some CNS species.

Key words: antimicrobial, biofilm, coagulase-negative staphylococcus, intramammary infection

INTRODUCTION

In many countries CNS are the most commonly isolated microorganisms from IMI in dairy cattle (Pitkälä et al., 2004; Tenhagen et al., 2006; Piepers et al., 2007). Although CNS IMI are rarely clinical, certain CNS species induce a stronger inflammatory reaction (Supré et al., 2011), or seem better able to persist in the udder than others (Thorberg et al., 2009; Piessens et al., 2011). Two CNS species, *Staphylococcus chromogenes* and *Staphylococcus epidermidis*, have been proposed as udder-adapted species because they cause IMI but are rare in the cows' environment (Piessens et al., 2011). Two other species, *Staphylococcus haemolyticus* and *Staphylococcus simulans*, regularly occur in the cows' environment but also commonly cause IMI, acting as so-called environmental opportunistic pathogens (Piessens et al., 2011). In contrast, a variety of other CNS species, such as *Staphylococcus equorum* and *Staphylococcus sciuri*, prevail in the barn environment but rarely cause IMI (so-called environmental CNS; Matos et al., 1991; Piessens et al., 2011).

It can be hypothesized that the main CNS species involved in bovine IMI possess specific traits that promote the colonization of the udder tissue. Biofilm production is such a trait and is assumed to promote the establishment of (persistent) IMI in cattle (Cucarella et al., 2004; Fox et al., 2005; Melchior et al., 2006), but its role in the pathogenesis of CNS mastitis is unclear. Two staphylococcal surface components have been identified that contribute significantly to biofilm formation. The

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polysaccharide intercellular adhesin (**PIA**) is a matrix molecule synthesized by the proteins encoded by the *ica*ADBC gene cluster, which is widely distributed among CNS from nosocomial infections (Cramton et al., 1999; de Silva et al., 2002) and in food-processing environments (Møretrø et al., 2003). Carriage of the *ica* operon in bovine CNS isolates has rarely been determined. The biofilm associated protein Bap is a surface protein first identified in strong biofilm-forming *Staphylococcus aureus* strains from chronic bovine mastitis and is strongly associated with the persistence of infection (Cucarella et al., 2001, 2004). Homologs of the *bap* gene and production of the encoded protein have also been demonstrated in CNS isolates from chronic mastitis cases (Tormo et al., 2005).

Mastitis is the number one reason for antimicrobial drug use in dairy cattle, and selective pressure significantly contributes to the dissemination of resistant pathogens (Catry et al., 2003). Of particular interest is the emergence of methicillin-resistant staphylococci in food-producing animals (Zhang et al., 2009; Huber et al., 2011), which are resistant to all classes of β -lactam antibiotics because of the expression of the *mecA* gene. The reduced susceptibility of opportunistic pathogens to these commonly used antimicrobials might promote their persistence in the dairy herd and long-term colonization of udder tissue.

Another preventive and widely used practice in dairy farming is postmilking teat disinfection. As is true for antimicrobials, frequent exposure to subinhibitory concentrations of teat dip components could give rise to bacterial populations with increased tolerance for these biocides (Smith et al., 2008). Biocide resistance has been found in various CNS species isolated from bulk milk and quarter milk samples, and individual biocide-resistant CNS strains can disseminate and persist within herds (Bjorland et al., 2005, 2006).

The aim of the present study was to compare specific characteristics between the CNS species typically causing IMI on the one hand and the so-called environmental species (only causing IMI sporadically) on the other hand. The investigated markers were (1) the presence of the *bap* and *icaA* loci involved in biofilm formation, (2) the presence of the *mecA* gene encoding methicillin resistance, (3) the phenotypic susceptibility to various antimicrobials, and (4) the phenotypic susceptibility to teat dip products as determined by an optimized microdilution assay.

MATERIALS AND METHODS

Bacterial Isolates

A total of 366 CNS isolates obtained during a previously described longitudinal study in 6 herds in Flan-

ders (Belgium) were included in this study (Table 1; Piessens et al., 2011). The isolates originate from dairy cows' milk ($n = 77$), teat apices ($n = 7$), and the dairy farm environment ($n = 282$), and belong to 22 different CNS species that were identified by amplified fragment length polymorphism (**AFLP**) analysis (Piessens et al., 2010, 2011). On the basis of a strict IMI definition taking into account consecutive culturing results of monthly quarter milk samples and AFLP typing of CNS isolates, only 4 CNS species were found to cause IMI in the studied herds: *Staph. chromogenes*, *Staph. epidermidis*, *Staph. simulans*, and *Staph. haemolyticus* (Piessens et al., 2011). This species group encompasses both udder-adapted and opportunistic CNS species, which are further referred to as "IMI-causing species" in the current paper, although isolates of these species cultured from the environment or teat apices are also included (Table 1). The other 18 CNS species primarily originate from the dairy farm environment (slatted floor alleyways, air in the free-stalls, used sawdust bedding, and fresh sawdust; Table 1; Piessens et al., 2011). These species are only sporadically isolated from milk samples and are referred to as "environmental species" throughout this work. Of this species group, *Staph. equorum* and *Staph. sciuri* were the most frequently isolated (Piessens et al., 2011).

All 366 isolates were analyzed for the presence of the *bap*, *icaA*, and *mecA* genes. On the basis of the *bap* and *icaA* PCR results, a selection of 40 strains was further tested for phenotypic biofilm formation. On the basis of distinct AFLP fingerprints, a subset of 82 isolates was selected for phenotypic susceptibility testing against 5 antimicrobials and 2 biocides (32 *Staph. haemolyticus*, 10 *Staph. chromogenes*, 10 *Staph. simulans*, 10 *Staph. epidermidis*, 10 *Staph. equorum*, and 10 *Staph. sciuri*).

bap, *icaA*, and *mecA* PCR Assays

Deoxyribonucleic acid extraction of all isolates was done as described previously (Piessens et al., 2010). Genomic DNA was analyzed by specific PCR assays for detection of the biofilm-associated gene *bap* with primers *sasp-6m* and *sasp-7c* (Tormo et al., 2005), the intracellular adhesion protein gene *icaA* with primers *ica4f* and *ica2r* (Møretrø et al., 2003), and the methicillin resistance gene *mecA* with primers *mecA1* and *mecA2* (Murakami et al., 1991). The PCR assay was performed in a 25- μ L reaction mixture containing 0.25 μ L of each primer (100 μ M; Eurogentec, San Diego, CA), 1 $\times$ PCR buffer II (Applied Biosystems, Foster City, CA), 1.5 mM $MgCl_2$ (Applied Biosystems), 0.1 mM each deoxynucleotide 5'-triphosphate, 1 U of AmpliTaq Polymerase (Applied Biosystems), and 1 μ L of bacterial DNA. Thermal cycling conditions were 5 min

Table 1. Polymerase chain reaction results for detection of the *bap*, *icaA*, and *mecA* genes in 366 bovine isolates belonging to 22 CNS species

CNS species group	Isolates (no.)	Origin (no.)		No. of PCR-positive isolates from milk/environment (% of [sub]total)		
		Milk	Environment	<i>bap</i>	<i>icaA</i>	<i>mecA</i>
IMI-causing ¹						
<i>Staph. chromogenes</i>	28	17	11			
<i>Staph. epidermidis</i>	24	12	12		0/1	7/1
<i>Staph. haemolyticus</i>	131	20	111 ²			
<i>Staph. simulans</i>	37	5	32			
Subtotal	220	54	166	0/0 (0.0)	0/1 (0.5)	7/1 (3.6)
Environmental						
<i>Staph. equorum</i>	34	5	29	5/28		
<i>Staph. sciuri</i>	31	1	30		1/8	1/28
<i>Staph. xylosus</i>	9	0	9	0/8	0/3	
<i>Staph. devriesei</i>	8	2	6			
<i>Staph. hominis</i>	7	3	4			
<i>Staph. saprophyticus</i>	7	3	4		0/1	
<i>Staph. warneri</i>	7	4	3			
<i>Staph. fleurettii</i>	6	0	6			0/6
<i>Staph. arlettae</i>	6	0	6			
<i>Staph. cohnii</i>	5	1	4		0/1	
<i>Staph. auricularis</i>	5	3	2			
<i>Staph. succinus</i>	5	0	5			
<i>Staph. capitis</i>	4	0	4		0/4	
<i>Staph. gallinarum</i>	4	0	4			
<i>Staph. vitulinus/pulvereri</i>	3	0	3			
<i>Staph. caseolyticus</i>	2	1	1			
<i>Staph. lentus</i>	2	0	2			
<i>Staph. nepalensis</i>	1	0	1			
Subtotal	146	23	123	5/36 (28.1)	1/17 (12.3)	1/34 (24.0)
Overall	366	77	289	5/36 (11.2)	1/18 (5.2)	8/35 (11.7)

¹An IMI was defined as the isolation of the same CNS strain, based on amplified fragment length polymorphism fingerprinting, at least twice in 3 consecutive monthly milk samples from the same quarter.

²Including 7 isolates originating from teat apices.

of denaturation at 95°C; 30 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 58°C (*bap* and *icaA*) or 52°C (*mecA*), and 30 s of elongation at 72°C; and a final elongation step at 72°C for 8 min. Fragments were analyzed by electrophoresis on 2% (wt/vol) agarose gels. In each PCR run, a negative (water) and positive control were coanalyzed. The strains used as positive controls were *Staphylococcus xylosus* CCM 2738^T (*bap*), *Staphylococcus capitis* ssp. *capitis* LMG 13353^T (*icaA*), and *Staph. aureus* MRSA ST 398 strain MB 4393 (*mecA*). The expected amplicon sizes were 971, 568, and 533 bp, respectively.

Phenotypic Biofilm Assay

A selection of 40 strains was further tested by a phenotypic biofilm assay. This selection included all isolates PCR-positive for *bap* or *icaA* or both ($n = 30$) of the species *Staph. epidermidis* (1 isolate), *Staph. equorum* (6 isolates), *Staph. sciuri* (9 isolates), *Staph. xylosus* (8 isolates), *Staphylococcus saprophyticus* (1 isolate), *Staphylococcus cohnii* (1 isolate), and *Staph. capitis* (4 isolates). Ten *bap* and *icaA* PCR-negative isolates were tested as well.

The assay used to test phenotypic biofilm production was based on the assay described by Christensen et al. (1985) but with some modifications. All strains were tested in triplicate. As positive and negative controls, the biofilm-producer *Staph. epidermidis* strain ATCC 35984 and the nonbiofilm-producer *Staph. epidermidis* strain ATCC 12228 were used. Isolates were grown at 37°C for 18 h in tryptone soy broth (TSB; Oxoid Ltd., Basingstoke, UK) with 2% glucose. Of this stationary culture, 100 µL was diluted in 10 mL of TSB with 2% glucose, and 100 µL of this dilution was pipetted into a flat-bottomed polystyrene 96-well microtiter plate. To prevent edge effects, all outer wells of the plates were filled with 100 µL of Ringer's solution and not used for test samples. Plates were incubated at 37°C for 24 h and thereafter growth of the strains was checked by visual assessment of turbidity in the wells. The latter was done to prevent slow-growing strains from being classified as nonbiofilm formers. Subsequently, wells were washed 3 times with Ringer's solution. Adherent cells were fixed with methanol (99%). After 15 min, the ethanol was removed and the plate was briefly air-dried. Next, 100 µL of crystal violet (0.1%) was added and the plate was incubated for 20 min. Afterward, the

staining solution was carefully removed, the plate was washed 2 times with Milli Q water (Millipore, Billerica, MA) and 150 μ L of acetic acid (33%) was added. Absorbance was measured with a microtiter plate reader at 590 nm. In each plate, a blank (TSB + 2% glucose), a positive control, and a negative control were included, each in triplicate. An isolate was considered a biofilm producer if the mean absorbance value was higher than the mean absorbance value + 3 $\times$ SD of the negative control strain.

MIC Determination by Etest

Minimum inhibitory concentrations of 5 antimicrobial agents representing different antibiotic classes were determined for a selection of 82 CNS isolates (6 different species) by an Etest (AB bioMérieux, Marcy l'Etoile, France) according to the manufacturer's guidelines. The antimicrobials were cephalothin, enrofloxacin, erythromycin, gentamicin, and oxacillin, representing the cephalosporins, fluoroquinolones, macrolides, aminoglycosides, and penicillins, respectively. *Staphylococcus aureus* ATCC 29213, *Staph. aureus* ATCC 43300, and *Enterococcus faecalis* ATCC 29213 were used for quality control, as recommended by the manufacturer. The MIC results were evaluated based on the Clinical and Laboratory Standards Institute document M31-A3 (CLSI, 2007), with resistance breakpoints of 32 μ g/mL for cephalothin, 4 μ g/mL for enrofloxacin, 8 μ g/mL for erythromycin, 8 μ g/mL for gentamicin, and 0.5 μ g/mL for oxacillin. Isolates with an MIC in the intermediate range were also categorized as resistant, and half-log MIC values were rounded up to the next upper log value of standard 2-fold dilution series (Schwarz et al., 2010).

MBC Determination of Teat Dips by a Microdilution Test

Optimization of a Teat Dip Susceptibility Test. Susceptibility tests were developed for 2 commercial ready-to-use teat dip products: one containing 0.15% (wt/vol) of available iodine and 2% (vol/vol) emollient (pH 4.8 to 5.0; further referred to as "iodine dip"), and one containing 0.42% (wt/vol) chlorhexidine and 10.5% (vol/vol) emollient (pH 7.0; further referred to as "chlorhexidine dip"). A microdilution plate assay was optimized for determination of the minimum biocidal concentrations (MBC) of both products for 2 contact times, and recommendations described in the European standard suspension test (EN 1656; CEN, 2000) were followed for dilution or neutralization of the biocides. The neutralizer consisted of 3% polysorbate 80 (vol/vol), 0.3% lecithin (wt/vol), 0.5% sodium thiosulfate

(wt/vol), 0.1% L-histidine (wt/vol), 0.1% peptone (wt/vol), and 0.3% sodium chloride (wt/vol; Merck KGaA, Darmstadt, Germany), and was prepared according to the EN 1656 standard. In preliminary tests, the toxicity and efficiency of the neutralizer were tested for both products with the *Staph. aureus* control strain ATCC 6538 and 6 CNS test strains (one each of *Staph. chromogenes*, *Staph. epidermidis*, *Staph. haemolyticus*, *Staph. simulans*, *Staph. sciuri*, and *Staph. equorum*) using tryptone soy agar (TSA; Oxoid Ltd.) as the recovery medium for the determination of viable bacteria (EN 1656; CEN, 2000). The iodine product was effectively neutralized for all 7 strains, but neutralization of the chlorhexidine teat dip was insufficient, leading to carryover of the product after exposure. Alternatively, neutralizer efficiency was tested using Dey-Engley (D/E) neutralizing agar (BD Biosciences, Franklin Lakes, NJ) as the recovery medium. The components in the D/E neutralizing agar effectively quenched the residual activity of both products and a successful recovery of viable bacteria was obtained. In addition, the absence of lethal effects of the experimental conditions was verified with the 7 test strains for both products according to the EN 1656 standard (CEN, 2000). In the final microdilution plate assay, the D/E neutralizing broth (BD Biosciences) was used as the recovery medium both to avoid carryover and to facilitate reading of the MBC (bacterial growth induces a color change of the medium from purple to yellow).

MBC Determination. Isolates were grown on TSA at 37°C for 24 h and were transferred at least 2 times before testing. Bacterial suspensions were made in diluent (EN 1656; CEN, 2000) and adjusted to approximately 3.10^8 cfu/mL (McFarland standard 2). For each tested isolate, appropriate dilutions were spread on TSA for determination of bacterial counts in the inoculum. 2-fold dilutions of both products were freshly made in sterile distilled water and distributed into microtiter plates (160 μ L/well), which were inoculated within 2 h after preparation. Subsequently, bacterial suspensions of the 10^{-2} dilution ($\sim 3.10^6$ cfu/mL) were diluted 2-fold in the designated interfering substance (10% skim milk; Oxoid Ltd.), and 40 μ L of this mixture was used to inoculate the challenge plates. Each isolate was challenged in duplicate against 5 concentrations of the iodine dip (1,200 to 75 ppm) and 6 concentrations of the chlorhexidine dip (3,360 to 105 ppm) at 30°C for 2 contact times (5 and 30 min). One column of the challenge plates was filled with water as a control for test conditions and neutralizer toxicity. After the contact time, 20- μ L quantities of the test suspensions were transferred to plates containing 180 μ L of neutralizer per well. After 5 min of neutralization at room temperature, 20- μ L quantities were transferred

to plates containing D/E neutralizing broth (200 μ L/well) for recovery of viable bacteria. Recovery plates were read after 48 h of incubation at 37°C, and the MBC was defined as the lowest concentration for which no growth was visible (no color change). In this assay, the MBC represented the biocide concentration that resulted in a 2- to 3-log reduction in viable counts for the respective contact time. A deviation of one dilution step between duplicates was accepted and the MBC values of repeated tests were averaged. Repeatability of the microdilution assay was tested for 4 CNS strains (one each of *Staph. chromogenes*, *Staph. epidermidis*, *Staph. simulans*, and *Staph. haemolyticus*) by repeating the test on another day starting from a fresh culture. The MBC values among repeats differed no more than one dilution, indicating repeatability of the protocol.

Determination of Bactericidal Activity of Teat Dips by EN 1656

For a selection of CNS strains, the bactericidal activity of the undiluted iodine dip and that of the chlorhexidine dip were determined according to the EN 1656 standard (CEN, 2000) except that D/E neutralizing agar was used for the recovery and counting of viable bacteria.

Sequence Analysis of *mecA* Amplicons

A selection of *mecA* amplicons ($n = 10$) was sequenced by a commercial sequencing facility (Macrogen, Seoul, Korea). The same primers as for the PCR were used for the sequencing reaction, and sequence comparisons were done using the Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.gov/BLAST/>).

Statistical Analysis

All statistical analyses were done with Statistica software version 9 (StatSoft, Tulsa, OK). The associations between groups (IMI-causing vs. environmental CNS species) and PCR results (positive vs. negative) were analyzed using 2×2 tables and chi-squared or Fisher exact tests as appropriate. To compare antimicrobial and biocide susceptibility between both species groups, survival of both groups was plotted using a Kaplan-Meier survival analysis, with the MIC or MBC values defined as the time to event (Sampimon et al., 2011). When isolates were killed at the lowest test concentration or were still growing at the highest test concentration, observations were coded as left or right censored, respectively. The log-rank test was used to search for

significant differences in survival between IMI-causing and environmental species. Significance was set at $P < 0.05$.

RESULTS

Presence of *bap*, *icaA*, and *mecA* Genes

In total, *bap* was found in 11.2% (41/366) of CNS isolates and *icaA* in 5.2% (19/366; Table 1). Environmental CNS species were significantly more *bap*-positive (28.1%) or *icaA*-positive (12.3%) than isolates belonging to the IMI-causing species (0.0 and 0.5%, respectively; $P < 0.001$). The *bap*-positive isolates belonged to 2 CNS species, whereas the *icaA*-positive isolates were distributed over 6 CNS species. The presence of *bap* was common among *Staph. equorum* isolates (97.1%, 33/34). Of the *Staph. xylosus* isolates, 5/9 were positive for *bap* solely, and 3/9 were positive for both *bap* and *icaA*. The *icaA* gene was found in 29.0% (9/31) of the *Staph. sciuri* isolates. Furthermore, *icaA* was found in 1/24 *Staph. epidermidis*, 4/4 *Staph. capitis*, 1/5 *Staph. cohnii*, and 1/7 *Staph. saprophyticus* isolates.

The *mecA* was detected in 11.7% (43/366) of CNS isolates, and *mecA*-positive isolates were distributed over 3 species (Table 1). Environmental CNS were significantly more *mecA*-positive (24.0%) than the IMI-causing species (3.6%; $P < 0.001$). The *mecA* was detected in 33.3% (8/24) of *Staph. epidermidis* isolates, and was common in *Staph. sciuri* and *Staphylococcus fleurettii* [93.5% (29/31) and 100% (6/6), respectively]. In 22.6% (7/31) of the *Staph. sciuri* isolates, *mecA* was found concomitantly with *icaA*.

For all *Staph. chromogenes* isolates (28/28), a *mecA* amplicon with a slightly higher position than the control was detected on gel. Therefore, the *mecA* amplicons of a selection of PCR-positive *Staph. chromogenes* ($n = 4$), *Staph. epidermidis* ($n = 2$), *Staph. fleurettii* ($n = 2$), and *Staph. sciuri* ($n = 2$) were sequenced. For the *Staph. chromogenes* amplicons, no homology with *mecA* was found in a BLAST search, indicating nonspecific amplification. The sequences obtained from the other species were confirmed to be *mecA* (2 *Staph. epidermidis*, 2 *Staph. fleuretti*, and 1 *Staph. sciuri*) or a *mecA* homolog (1 *Staph. sciuri*).

Phenotypic Biofilm Formation

Nine out of the 30 *bap*- or *icaA*-positive CNS strains, or both, produced biofilm in the phenotypic assay (Table 2). The biofilm producers were *Staph. epidermidis* (1), *Staph. sciuri* (3), and *Staph. xylosus* (5). None of the 10 *bap*- or *icaA*-negative strains produced biofilm.

Table 2. Phenotypic biofilm formation of *bap* and/or *icaA* PCR-positive strains and selected *bap* and *icaA* PCR-negative strains

Species	Strain no.	Source	PCR result		Biofilm formation
			<i>bap</i>	<i>icaA</i>	
<i>Staph. epidermidis</i>	MB 4756	Bedding stock	–	+	+
<i>Staph. epidermidis</i>	MB 4649	Air stall	–	–	–
<i>Staph. epidermidis</i>	MB 4692	Milk	–	–	–
<i>Staph. equorum</i>	MB 4618	Bedding stall	+	–	–
<i>Staph. equorum</i>	MB 4663	Air stall	+	–	–
<i>Staph. equorum</i>	MB 4707	Overshoe	+	–	–
<i>Staph. equorum</i>	MB 4715	Milk	+	–	–
<i>Staph. equorum</i>	MB 4769	Bedding stock	+	–	–
<i>Staph. equorum</i>	MB 4841	Milk	+	–	–
<i>Staph. equorum</i>	MB 4732	Overshoe	–	–	–
<i>Staph. sciuri</i>	MB 4746	Milk	–	+	+
<i>Staph. sciuri</i>	MB 4770	Overshoe	–	+	–
<i>Staph. sciuri</i>	MB 4808	Overshoe	–	+	–
<i>Staph. sciuri</i>	MB 4855	Bedding stock	–	+	+
<i>Staph. sciuri</i>	MB 5143	Overshoe	–	+	–
<i>Staph. sciuri</i>	MB 5172	Bedding stall	–	+	–
<i>Staph. sciuri</i>	MB 5174	Bedding stall	–	+	–
<i>Staph. sciuri</i>	MB 5179	Bedding stall	–	+	–
<i>Staph. sciuri</i>	MB 5202	Bedding stock	–	+	+
<i>Staph. sciuri</i>	MB 4684	Overshoe	–	–	–
<i>Staph. sciuri</i>	MB 4704	Bedding stock	–	–	–
<i>Staph. xylosus</i>	MB 5127	Air stall	+	–	–
<i>Staph. xylosus</i>	MB 5175	Bedding stock	+	–	+
<i>Staph. xylosus</i>	MB 5181	Bedding stock	+	–	–
<i>Staph. xylosus</i>	MB 5211	Bedding stock	+	–	+
<i>Staph. xylosus</i>	MB 5217	Bedding stock	+	–	+
<i>Staph. xylosus</i>	MB 5132	Air stall	+	+	+
<i>Staph. xylosus</i>	MB 5146	Overshoe	+	+	–
<i>Staph. xylosus</i>	MB 5209	Bedding stock	+	+	+
<i>Staph. xylosus</i>	MB 5124	Overshoe	–	–	–
<i>Staph. saprophyticus</i>	MB 5123	Air stall	–	+	–
<i>Staph. saprophyticus</i>	MB 5148	Milk	–	–	–
<i>Staph. saprophyticus</i>	MB 5164	Bedding stock	–	–	–
<i>Staph. cohnii</i>	MB 5170	Bedding stock	–	+	–
<i>Staph. cohnii</i>	MB 5135	Bedding stall	–	–	–
<i>Staph. cohnii</i>	MB 5193	Milk	–	–	–
<i>Staph. capitis</i>	MB 4862	Air stall	–	+	–
<i>Staph. capitis</i>	MB 5191	Air stall	–	+	–
<i>Staph. capitis</i>	MB 5192	Bedding stock	–	+	–
<i>Staph. capitis</i>	MB 5197	Air stall	–	+	–

Antimicrobial Susceptibility

All 82 CNS isolates were susceptible to cephalothin and gentamicin according to Etest, and only one isolate had intermediate resistance to enrofloxacin (Table 3). Nineteen isolates (23.2%) were classified as (intermediate) resistant to erythromycin, and 13 (15.9%) to oxacillin. No significant difference was found in survival between IMI-causing and environmental CNS species against increasing erythromycin concentrations (log-rank test; $P > 0.05$), but the environmental CNS species survived significantly higher oxacillin concentrations than the IMI-causing species (Figure 1; log-rank test; $P < 0.001$). A significant difference was also observed between *mecA*-positive and *mecA*-negative CNS isolates in survival against increasing oxacillin concentrations (Figure 2; log-rank test; $P < 0.001$).

Species distribution and resistance patterns of the 82 isolates are given in Table 4. Fifty-one isolates (62.2%) were susceptible to all 5 tested antimicrobials. The species with the highest proportion of pansusceptible isolates were *Staph. haemolyticus* (90.6%) and *Staph. simulans* (90.0%). Twenty-nine isolates showed resistance to a single compound, either erythromycin (22.0%) or oxacillin (13.4%), and 1 *Staph. sciuri* isolate was resistant to both. Another *Staph. sciuri* isolate was resistant to oxacillin and intermediate resistant to enrofloxacin. Erythromycin-resistant isolates were found among all 6 CNS species, with the highest proportions in *Staph. equorum* (90.0%) and *Staph. epidermidis* (30.0%). The oxacillin-resistant isolates belonged to 3 species: 8 *Staph. sciuri*, 3 *Staph. epidermidis*, and 2 *Staph. chromogenes*, but carriage of *mecA* was confirmed in only 10 (7 *Staph. sciuri* and 3 *Staph. epidermidis*) out of 13

Figure 1. Percentage survival of 82 CNS isolates with increasing concentrations of oxacillin. Dotted line: CNS belonging to IMI-causing species ($n = 62$); solid line: CNS belonging to environmental species ($n = 20$); +: left censored (log-rank test: $P < 0.001$).

by PCR. One *Staph. sciuri* isolate and 2 *Staph. chromogenes* isolates with oxacillin MIC of 0.5 to 1.0 µg/mL were *mecA*-negative.

Biocide Susceptibility

The MBC of 1 *Staph. haemolyticus* isolate could not be determined because no recovery of the unexposed control was obtained, indicating possible toxicity of the neutralizer or the experimental conditions for this strain. The MBC of the 81 CNS isolates for 2 contact times is given in Table 5 (iodine dip) and Table

Figure 2. Percentage survival of 82 CNS isolates with increasing concentrations of oxacillin. Dotted line: *mecA*-positive CNS isolates ($n = 13$); solid line: *mecA*-negative CNS isolates ($n = 69$); +: censored (log-rank test: $P < 0.001$).

Table 3. Minimum inhibitory concentrations (µg/mL) of 5 antimicrobial agents as determined by Etest¹ for 82 CNS isolates from milk ($n = 33$), teat apices ($n = 7$), and the dairy farm environment ($n = 42$)

Antimicrobial agent	No. of isolates with MIC										No. (%) of (intermediate) resistant isolates
	<0.125	0.125	0.25	0.5	1	2	4	8	64	>256	
Cephalothin	58	7	13	4							0
Enrofloxacin	35	26	15	5	1 ²						1
Erythromycin	27	19	15	2		3 ²	3 ²	2 ³	1 ³	10 ³	19 (23.2)
Gentamicin	80	2									0
Oxacillin	41	9	19	5 ³	4 ³	1 ³	3 ³				13 (15.9)

¹AB bioMérieux (Marcy l'Etoile, France).

²Intermediate resistant.

³Resistant.

Table 4. Species distribution and resistance patterns of 82 bovine CNS isolates tested for their susceptibility to enrofloxacin (EF), erythromycin (EM), and oxacillin (OX) by Etest¹

CNS species group	Isolates (no.)	Origin (no.)		Phenotypic resistance profile ² [no. (%)]				No. (%) pansusceptible
		Milk	Environment	EM	OX	OX-EM	OX-EF	
IMI-causing ³								
<i>Staph. chromogenes</i>	10	7	3	2 (20.0)	2 (20.0)			6 (60.0)
<i>Staph. epidermidis</i>	10	6	4	3 (30.0) ⁴	3 (30.0) ⁵			4 (40.0)
<i>Staph. haemolyticus</i>	32	12	20 ⁶	3 (9.4)				29 (90.6)
<i>Staph. simulans</i>	10	4	6	1 (10.0)				9 (90.0)
Subtotal	62	29	33	9 (14.5)	5 (8.1)			48 (77.4)
Environmental								
<i>Staph. equorum</i>	10	3	7	9 (90.0)				1 (10.0)
<i>Staph. sciuri</i>	10	1	9		6 (60.0) ⁷	1 (10.0) ⁵	1 (10.0) ⁵	2 (20.0)
Subtotal	20	4	16	9 (45.0)	6 (30.0)	1 (5.0)	1 (5.0)	3 (15.0)
Total	82	33	49	18 (22.0)	11 (13.4)	1 (1.2)	1 (1.2)	51 (62.2)

¹AB bioMérieux (Marcy l'Etoile, France).²Number (%) of intermediate resistant or resistant isolates per species or group.³An IMI was defined as the isolation of the same CNS, based on amplified fragment length polymorphism fingerprinting, at least twice in 3 consecutive monthly milk samples from the same quarter.⁴One out of 3 of isolates was *mecA* positive.⁵*mecA* positive isolate(s).⁶Including 7 isolates originating from teat apices.⁷Five out of 6 isolates were *mecA* positive.

6 (chlorhexidine dip). No significant differences were found between the IMI-causing and environmental species in survival against increasing biocide concentration (data not shown). The isolates most tolerant to the iodine dip belonged to the species *Staph. simulans* (Table 5). The isolates most tolerant to the chlorhexidine dip were *Staph. chromogenes* and *Staph. simulans* (Table 6).

In addition, the bactericidal activity of the individual undiluted products against the 2 least susceptible isolates in the microdilution assay was determined according to the EN 1656 standard (CEN, 2000), except that D/E neutralizing agar was used for recovery and

counting of viable bacteria. After 30 min of exposure to the respective undiluted products, more than a 5-log reduction in bacterial counts was obtained in all tests, indicating adequate bactericidal activity of both products against all tested CNS strains at user concentrations.

DISCUSSION

Biofilm Genes

This study was conducted to compare characteristics between CNS species causing IMI (*Staph. chromogenes*, *Staph. epidermidis*, *Staph. simulans*, and *Staph. haemo-*

Table 5. In vitro susceptibility of 81 CNS isolates exposed for 5 and 30 min to 2-fold dilutions of the iodine teat dip (range 75 to 1,200 ppm)

MBC ¹ of iodine (ppm) after exposure for		Isolates (no.)	IMI-causing species ² [no. (%)]				Environmental species [no. (%)]	
5 min	30 min		<i>Staph. chromogenes</i> (n = 10)	<i>Staph. epidermidis</i> (n = 10)	<i>Staph. haemolyticus</i> (n = 31)	<i>Staph. simulans</i> (n = 10)	<i>Staph. equorum</i> (n = 10)	<i>Staph. sciuri</i> (n = 10)
75	75	44	1 (10.0)	6 (60.0)	29 (93.5)	—	7 (70.0)	—
112.5	75	4	—	1 (10.0)	2 (6.5)	—	1 (10.0)	—
150	75	3	—	2 (20.0)	—	1 (10.0)	—	—
150	125.5	1	—	—	—	—	—	1 (10.0)
150	150	28	9 (90.0)	1 (10.0)	—	7 (70.0)	2 (20.0)	9 (90.0)
300	150	1	—	—	—	1 (10.0) ³	—	—
450	225	1	—	—	—	1 (10.0) ³	—	—

¹MBC = minimum biocidal concentration.²An IMI was defined as the isolation of the same CNS, based on amplified fragment length polymorphism fingerprinting, at least twice in 3 consecutive monthly milk samples from the same quarter.³Bactericidal activity of the respective teat dip was tested against the 2 least susceptible isolates (EN 1656; CEN, 2000); 30 min of exposure to the undiluted product resulted in >5-log reduction in viable bacteria.

Table 6. In vitro susceptibility of 81 CNS isolates exposed for 5 and 30 min to 2-fold dilutions of the chlorhexidine teat dip (range 105 to 3,360 ppm)

MBC ¹ of chlorhexidine (ppm) after exposure for		Isolates (no.)	IMI-causing species ² [no. (%)]				Environmental species [no. (%)]	
5 min	30 min		<i>Staph.</i> <i>chromogenes</i> (n = 10)	<i>Staph.</i> <i>epidermidis</i> (n = 10)	<i>Staph.</i> <i>haemolyticus</i> (n = 31)	<i>Staph.</i> <i>simulans</i> (n = 10)	<i>Staph.</i> <i>equorum</i> (n = 10)	<i>Staph.</i> <i>sciuri</i> (n = 10)
105	105	6	1 (10.0)	—	1 (3.2)	—	4 (40.0)	—
157.5	105	3	—	—	2 (6.5)	—	1 (10.0)	—
210	105	7	—	1 (10.0)	1 (3.2)	—	4 (40.0)	1 (10.0)
210	210	32	2 (20.0)	2 (20.0)	21 (67.7)	2 (20.0)	1 (10.0)	4 (40.0)
315	210	9	—	4 (40.0)	2 (6.5)	1 (10.0)	—	2 (20.0)
315	315	1	—	—	1 (3.2)	—	—	—
420	210	20	5 (50.0)	3 (30.0)	3 (9.7)	6 (60.0)	—	3 (30.0)
420	315	1	1 (10.0)	—	—	—	—	—
420	420	2	1 (10.0) ³	—	—	1 (10.0) ³	—	—

¹MBC = minimum biocidal concentration.

²An IMI was defined as the isolation of the same CNS, based on amplified fragment length polymorphism fingerprinting, at least twice in 3 consecutive monthly milk samples from the same quarter.

³Bactericidal activity of the respective teat dip was tested against the 2 least susceptible isolates (EN 1656; CEN, 2000); 30 min of exposure to the undiluted product resulted in >5-log reduction in viable bacteria.

lyticus) and those that are primarily free living in the environment (*Staph. equorum*, *Staph. sciuri*, and other CNS species). The biofilm mode of growth has been associated with persistence of IMI because it protects bacteria against the lethal effects of the host defense system and antimicrobial treatment (Melchior et al., 2006). Both *Bap* and the product of the *ica* operon (PIA) promote adhesion, cell-to-cell interactions, and bacterial accumulation of staphylococci, and the presence of *Bap* has especially been associated with persistence of IMI (Cucarella et al., 2004; Tormo et al., 2005). Therefore, we investigated whether CNS species causing bovine IMI possibly harbor these genes more often than other CNS species primarily colonizing extramammary niches. However, no association was found between the presence of these loci and an increased ability to establish IMI. On the contrary, carriage of *bap* and *icaA* genes, as well as in vitro biofilm production, was detected almost exclusively in CNS from the barn environment. Strong biofilm-forming CNS have been recovered from food-processing environments as well (Møretrø et al., 2003). Biofilm formation might promote CNS survival in the environment, where bacteria have to cope with changing conditions (e.g., humidity and ambient temperature), mechanical shear, or disinfection.

The failure to detect *bap* or *icaA* genes does not necessarily imply the inability to form a biofilm because the biofilm-forming process of CNS can be mediated by factors other than *Bap* or PIA (Cucarella et al., 2004; Fredheim et al., 2009). Furthermore, the presence of *bap* or *ica* loci showing low similarity with the currently

available sequences cannot be ruled out. Nonetheless, the primers used to amplify the *bap* gene have been designed from the *Staph. aureus bap* gene, from which the sequence has been determined to be more than 97% similar to the *bap* homologs in *Staph. epidermidis*, *Staph. simulans*, *Staph. chromogenes*, and *Staphylococcus hyicus* mastitis isolates, and 74% similar to the *bap* gene of *Staph. xylosus* (Tormo et al., 2005). The *icaA* primers used have been designed from conserved regions in *icaA* genes of *Staph. aureus*, *Staph. epidermidis*, and *Staphylococcus caprae* (Møretrø et al., 2003). Whereas the *icaA* genes of clinical *Staph. haemolyticus* and *Staph. epidermidis* strains have been described as closely related (Fredheim et al., 2009), the *icaA* genes of 38 strains belonging to 9 food-related CNS species were less conserved, with sequence similarities of 55 to 82% and considerable sequence diversity between strains within species (Møretrø et al., 2003). Given that these genes were detected in a variety of species in this study, it is concluded that *bap*- and *ica*-mediated biofilm formation likely does not play a role in the pathogenesis of IMI-causing CNS species. These results are in accordance with a recent study by Simojoki et al. (2012), in which no association was found between biofilm production and the persistence of IMI caused by CNS.

Antimicrobial Resistance

Although the importance of CNS as a main cause of mastitis has not been univocally established, their tendency toward increased antimicrobial resistance is

a matter of concern (Gentilini et al., 2002; Botrel et al., 2010). Because of their commensal lifestyle, CNS could serve as a reservoir of transferable resistance determinants for other mastitis pathogens. In the present study, the *mecA* gene was found in only 3 out of 22 analyzed CNS species. Carriage of *mecA* in the IMI-causing CNS species was limited, but was found in one-third of the *Staph. epidermidis* isolates. The occurrence of *mecA*-positive *Staph. epidermidis* from bovine milk samples has been reported previously (Sawant et al., 2009; Fessler et al., 2010; Sampimon et al., 2011), and clonal dissemination of multi-drug-resistant *Staph. epidermidis* strains carrying *mecA* within herds has been observed (Sawant et al., 2009; Fessler et al., 2010). The occurrence of methicillin-resistant *Staph. epidermidis* in cattle might require more attention, and some researchers even recommend culling of animals infected with methicillin-resistant CNS (Gentilini et al., 2002).

Among the CNS species prevailing in the environment, *mecA* was found commonly in isolates of the species *Staph. sciuri* and *Staph. fleurettii*. In a recent Swiss study, *Staph. sciuri* and *Staph. fleurettii* constituted the largest proportion of methicillin-resistant CNS isolated from bulk tank milk samples (Huber et al., 2011). Although these CNS species are rarely associated with IMI, both *Staph. fleurettii* and *Staph. sciuri* frequently occur in the dairy farm environment (Huber et al., 2011; PiesSENS et al., 2011), thus representing a considerable reservoir of the *mecA* gene. However, it is known that *Staph. sciuri* and *Staph. fleurettii* inherently harbor a *mecA* homolog that is closely related to the *mecA* gene of MRSA, but is not located on a mobile genetic element and does not necessarily confer phenotypic methicillin resistance (Tsubakishita et al., 2010). Interpretation of the results should be done prudently because the carriage of a nonfunctional *mecA* homolog might be of minor importance.

A selection of 82 CNS isolates belonging to 6 CNS species was tested phenotypically for their susceptibility to antimicrobial agents belonging to 5 different antibiotic classes. The MIC values for enrofloxacin, gentamicin, and cephalothin were well below the recommended breakpoints (CLSI, 2007). The observed effectiveness of these antimicrobials against CNS is in accordance with other studies (Trinidad et al., 1990; Gentilini et al., 2002; Sampimon et al., 2011). However, erythromycin-resistant isolates were found in each of the 6 CNS species tested, with the highest proportion being *Staph. equorum*. This is consistent with a previous study in which erythromycin resistance was significantly more common in *Staph. equorum* than in other CNS species (Sampimon et al., 2011). Oxacillin-resistant isolates were found among *Staph. chromogenes*, *Staph. epider-*

midis, and *Staph. sciuri* isolates. It has been proposed that CNS from animals with oxacillin MIC of 0.5 to 2.0 µg/mL should be confirmed by *mecA*-PCR before they are reported as methicillin resistant (Fessler et al., 2010). Consequently, the borderline resistance observed in 2 *Staph. chromogenes* and 1 *Staph. sciuri* *mecA*-negative isolates cannot be considered methicillin resistance and is likely mediated by another mechanism (e.g., hyperexpression of β-lactamases; Chambers, 1997). By contrast, the *mecA*-positive *Staph. sciuri* and *Staph. epidermidis* isolates were significantly less susceptible to oxacillin than the *mecA*-negative CNS, although oxacillin MIC of the *mecA*-positive isolates were relatively low (0.5 to 4 µg/mL). Furthermore, no cross-resistance to cephalothin was observed. Lack of cross-resistance to cephalothin has been described before in heteroresistant human *Staph. epidermidis* strains (Thornsberry and McDougal, 1983). These findings emphasize the difficulties of correct assessment of methicillin resistance in CNS species by phenotypic methods (Chambers, 1997; Fessler et al., 2010).

Overall, a higher proportion of the environmental (85.0%) than the IMI-causing CNS (22.6%) showed antimicrobial resistance to 1 or more compounds, notably to oxacillin and erythromycin. Analogous with these findings, others have found higher proportions of resistant isolates among CNS species rarely colonizing the udder compared with CNS species commonly causing IMI (Persson Waller et al., 2011; Sampimon et al., 2011). These results indicate that commensal and environmental CNS might be more significant in the development and spread of resistance in dairy farms, which goes against the general assumption that antimicrobial resistance is associated with virulence. This could be explained by the fact that the environment, in contrast to the udder, harbors a mixed and large microbial population, providing a better climate for horizontal transfer than the relatively sterile environment of the udder. The determinants for oxacillin and erythromycin resistance in staphylococci are often located on mobile genetic elements favoring their distribution across staphylococcal species (Lüthje and Schwarz, 2006; Zhang et al., 2009). Long-term residency in a polybacterial environment (e.g., bovine skin or environment) may have favored the acquisition of resistance genes carried on mobile genetic elements, especially in an environment where antimicrobials are intensively used. Ecological information on potential changes in susceptibility of extramammary CNS is virtually nonexistent. Continued surveillance of resistance in mastitis pathogens and commensals is important to ensure the future efficacy of antimicrobials for use in dairy cattle.

Biocide Susceptibility

No standard method is available for testing of the susceptibility of microorganisms to biocides, and several researchers have been making use of end-point microdilution or plating methods (Bjorland et al., 2005; Smith et al., 2008). However, biocides have no specific targets in the bacterial cell and exert considerable residual activity after exposure. Therefore, neutralization is recommended after exposure for standardized determination of the MBC. A microdilution method including a neutralization step was optimized to test isolates against 5 iodine and 6 chlorhexidine concentrations in microtiter plates for 2 contact times. The initial problem with neutralizing chlorhexidine was reported before (Kampf et al., 2005; Mehrgan et al., 2006), but was overcome by using a commercial recovery broth containing neutralizing components. In the assay, both the iodine and chlorhexidine teat dips were effective against all CNS strains if used at the ready-to-use concentrations (0.15% iodine, 0.42% chlorhexidine). However, sublethal concentrations of the tested biocides allowed some *Staph. chromogenes* and *Staph. simulans* strains to survive other CNS species. Possible explanations for these findings might be the presence of efflux pumps, which are known to be widespread among CNS from animals (Bjorland et al., 2005), or differences in the permeability of the cell wall. The ability to survive at higher biocide concentrations could provide a selective advantage for these CNS strains, especially when sublethal concentrations would be used in practice. Although the biological relevance of our findings remains uncertain, more study on biocide susceptibility of bovine CNS isolates is warranted because this feature might explain why CNS have become so successful as mastitis pathogens despite the general use of postmilking teat disinfection.

CONCLUSIONS

Biofilm genes and antimicrobial resistance were found more commonly in environmental than in IMI-causing CNS species, contradicting the assumption that these characteristics have a major role in the ability to colonize the mammary gland. Except for *Staph. epidermidis*, CNS species causing IMI did not harbor the *mecA* gene. The environmental CNS species, particularly *Staph. sciuri* and *Staph. fleurettii*, constitute a more significant *mecA*-reservoir than the CNS species involved in IMI. A few *Staph. chromogenes* and *Staph. simulans* strains showed higher tolerance to biocides, which might provide a possible colonization advantage for these species.

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REFERENCES

- Bjorland, J., T. Steinum, B. Kvitle, S. Waage, M. Sunde, and E. Heir. 2005. Widespread distribution of disinfectant resistance genes among staphylococci of bovine and caprine origin in Norway. *J. Clin. Microbiol.* 43:4363–4368.
- Bjorland, J., T. Steinum, M. Sunde, S. Waage, S. Sviland, H. Oppegard, and E. Heir. 2006. Deletion of pT181-like sequence in an *smr*-encoding mosaic plasmid harboured by a persistent bovine *Staphylococcus warneri* strain. *J. Antimicrob. Chemother.* 57:46–51.
- Botrel, M. A., M. Haenni, E. Mornigat, P. Sulpice, J. Y. Madec, and D. Calavas. 2010. Distribution and antimicrobial resistance of clinical and subclinical mastitis pathogens in dairy cows in Rhone-Alpes, France. *Foodborne Pathog. Dis.* 7:479–487.
- Catry, B., H. Laevens, L. A. Devriese, G. Opsomer, and A. Kruif. 2003. Antimicrobial resistance in livestock. *J. Vet. Pharmacol. Ther.* 26:81–93.
- CEN (European Committee for Standardization). 2000. EN 1656: Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in the veterinary area—Test method and requirements (phase 2, step 1). Eur. Comm. Stand., Brussels, Belgium.
- Chambers, H. F. 1997. Methicillin resistance in staphylococci: Molecular and biochemical basis and clinical implications. *Clin. Microbiol. Rev.* 10:781–791.
- Christensen, G. D., W. A. Simpson, J. J. Younger, L. M. Baddour, F. F. Barrett, D. M. Melton, and E. H. Beachey. 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: A quantitative model for the adherence of staphylococci to medical devices. *J. Clin. Microbiol.* 22:996–1006.
- CLSI (Clinical and Laboratory Standards Institute). 2007. Performance standard for antimicrobial disk and diffusion susceptibility tests for bacterial isolates from animals; approved standard—Third edition. CLSI document M31-A3. Clin. Lab. Stand. Inst., Wayne, PA.
- Cramton, S. E., C. Gerke, N. F. Schnell, W. W. Nichols, and F. Gotz. 1999. The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infect. Immun.* 67:5427–5433.
- Cucarella, C., C. Solano, J. Valle, B. Amorena, I. Lasa, and J. R. Penades. 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J. Bacteriol.* 183:2888–2896.
- Cucarella, C., M. A. Tormo, C. Ubeda, M. P. Trotonda, M. Monzon, C. Peris, B. Amorena, I. Lasa, and J. R. Penades. 2004. Role of biofilm-associated protein bap in the pathogenesis of bovine *Staphylococcus aureus*. *Infect. Immun.* 72:2177–2185.
- de Silva, G. D. I., M. Kantzanou, A. Justice, R. C. Massey, A. R. Wilkinson, N. P. J. Day, and S. J. Peacock. 2002. The *ica* operon and biofilm production in coagulase-negative staphylococci associated with carriage and disease in a neonatal intensive care unit. *J. Clin. Microbiol.* 40:382–388.
- Fessler, A. T., C. Billerbeck, K. Kadlec, and S. Schwarz. 2010. Identification and characterization of methicillin-resistant coagulase-negative staphylococci from bovine mastitis. *J. Antimicrob. Chemother.* 65:1576–1582.
- Fox, L. K., R. N. Zadoks, and C. T. Gaskins. 2005. Biofilm production by *Staphylococcus aureus* associated with intramammary infection. *Vet. Microbiol.* 107:295–299.

- Fredheim, E. G. A., C. Klingenberg, H. Rohde, S. Frankenberger, P. Gaustad, T. Flaegstad, and J. E. Sollid. 2009. Biofilm formation by *Staphylococcus haemolyticus*. *J. Clin. Microbiol.* 47:1172–1180.
- Gentilini, E., G. Denamiel, A. Betancor, M. Rebuelto, M. R. Ferme-pin, and R. A. De Torres. 2002. Antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine mastitis in Argentina. *J. Dairy Sci.* 85:1913–1917.
- Huber, H., D. Ziegler, V. Pfluger, G. Vogel, C. Zweifel, and R. Stephan. 2011. Prevalence and characteristics of methicillin-resistant coagulase-negative staphylococci from livestock, chicken carcasses, bulk tank milk, minced meat, and contact persons. *BMC Vet. Res.* 7:6. <http://dx.doi.org/10.1186/1746-6148-7-6>.
- Kampf, G., M. Shaffer, and C. Hunte. 2005. Insufficient neutralization in testing a chlorhexidine-containing ethanol-based hand rub can result in a false positive efficacy assessment. *BMC Infect. Dis.* 5:48. <http://dx.doi.org/10.1186/1471-2334-5-48>.
- Lüthje, P., and S. Schwarz. 2006. Antimicrobial resistance of coagulase-negative staphylococci from bovine subclinical mastitis with particular reference to macrolide-lincosamide resistance phenotypes and genotypes. *J. Antimicrob. Chemother.* 57:966–969.
- Matos, J. S., D. G. White, R. J. Harmon, and B. E. Langlois. 1991. Isolation of *Staphylococcus aureus* from sites other than the lactating mammary gland. *J. Dairy Sci.* 74:1544–1549.
- Mehrgan, H., F. Elmi, M. Reza Fazeli, A. Reza Shaverdi, and N. Samadi. 2006. Evaluation of neutralizing efficacy and possible microbial cell toxicity of a universal neutralizer proposed by the CTPA. *Iran. J. Pharm. Res.* 2006:173–178.
- Melchior, M. B., H. Vaarkamp, and J. Fink-Gremmels. 2006. Biofilms: A role in recurrent mastitis infections? *Vet. J.* 171:398–407.
- Møretro, T., L. Hermansen, A. L. Holck, M. S. Sidhu, K. Rudi, and S. Langsrud. 2003. Biofilm formation and the presence of the intercellular adhesion locus *ica*, among staphylococci from food and food processing environments. *Appl. Environ. Microbiol.* 69:5648–5655.
- Murakami, K., W. Minamide, K. Wada, E. Nakamura, H. Teraoka, and S. Watanabe. 1991. Identification of methicillin-resistant strains of staphylococci by polymerase chain-reaction. *J. Clin. Microbiol.* 29:2240–2244.
- Persson Waller, K., A. Aspán, A. Nyman, Y. Persson, and U. Grönlund Andersson. 2011. CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis. *Vet. Microbiol.* 152:112–116.
- Piepers, S., L. De Meulemeester, A. de Kruif, G. Opsomer, H. W. Barkema, and S. De Vliegher. 2007. Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. *J. Dairy Res.* 74:478–483.
- Piessens, V., K. Supre, M. Heyndrickx, F. Haesebrouck, S. De Vliegher, and E. Van Coillie. 2010. Validation of amplified fragment length polymorphism genotyping for species identification of bovine associated coagulase-negative staphylococci. *J. Microbiol. Methods* 80:287–294.
- Piessens, V., E. Van Coillie, B. Verbist, K. Supré, G. Braem, A. Van Nuffel, L. De Vuyst, M. Heyndrickx, and S. De Vliegher. 2011. Distribution of coagulase-negative *Staphylococcus* species from milk and environment of dairy cows differs between herds. *J. Dairy Sci.* 94:2933–2944.
- Pitkälä, A., M. Haveri, S. Pyörälä, V. Myllys, and T. Honkanen-Buzalski. 2004. Bovine mastitis in Finland 2001—Prevalence, distribution of bacteria, and antimicrobial resistance. *J. Dairy Sci.* 87:2433–2441.
- Sampimon, O. C., T. J. G. M. Lam, D. J. Mevius, Y. H. Schukken, and R. N. Zadoks. 2011. Antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine milk samples. *Vet. Microbiol.* 150:173–179.
- Sawant, A. A., B. E. Gillespie, and S. P. Oliver. 2009. Antimicrobial susceptibility of coagulase-negative *Staphylococcus* species isolated from bovine milk. *Vet. Microbiol.* 134:73–81.
- Schwarz, S., P. Silley, S. Simjee, N. Woodford, E. van Duijkeren, A. P. Johnson, and W. Gaastra. 2010. Editorial: Assessing the antimicrobial susceptibility of bacteria obtained from animals. *J. Antimicrob. Chemother.* 65:601–604.
- Simojoki, H., P. Hyvönen, C. Plumed, S. Ferrer, S. Taponen, and S. Pyörälä. 2012. Is the biofilm formation and slime producing ability of coagulase-negative staphylococci associated with the persistence and severity of intramammary infection? *Vet. Microbiol.* 158:344–352.
- Smith, K., C. G. Gemmell, and I. S. Hunter. 2008. The association between biocide tolerance and the presence or absence of *qac* genes among hospital-acquired and community-acquired MRSA isolates. *J. Antimicrob. Chemother.* 61:78–84.
- Supré, K., F. Haesebrouck, R. N. Zadoks, M. Vanechoutte, S. Piepers, and S. De Vliegher. 2011. Some coagulase-negative *Staphylococcus* species affect udder health more than others. *J. Dairy Sci.* 94:2329–2340.
- Tenhagen, B. A., G. Koster, J. Wallmann, and W. Heuwieser. 2006. Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. *J. Dairy Sci.* 89:2542–2551.
- Thorberg, B. M., M. L. Danielsson-Tham, U. Emanuelson, and K. P. Waller. 2009. Bovine subclinical mastitis caused by different types of coagulase-negative staphylococci. *J. Dairy Sci.* 92:4962–4970.
- Thornsberry, C., and L. K. McDougal. 1983. Successful use of broth microdilution in susceptibility tests for methicillin-resistant (heteroresistant) staphylococci. *J. Clin. Microbiol.* 18:1084–1091.
- Tormo, M. A., E. Knecht, F. Gotz, M. Lasa, and J. R. Penades. 2005. *Bap*-dependent biofilm formation by pathogenic species of *Staphylococcus*: Evidence of horizontal gene transfer? *Microbiology* 151(Pt. 7):2465–2475.
- Trinidad, P., S. C. Nickerson, and D. G. Luther. 1990. Antimicrobial susceptibilities of staphylococcal species isolated from mammary glands of unbred and primigravid dairy heifers. *J. Dairy Sci.* 73:357–362.
- Tsubakishita, S., K. Kuwahara-Arai, T. Sasaki, and K. Hiramatsu. 2010. Origin and molecular evolution of the determinant of methicillin resistance in staphylococci. *Antimicrob. Agents Chemother.* 54:4352–4359.
- Zhang, Y., S. Agidi, and J. T. LeJeune. 2009. Diversity of staphylococcal cassette chromosome in coagulase-negative staphylococci from animal sources. *J. Appl. Microbiol.* 107:1375–1383.