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
polysaccharide intercellular adhesin (PIA) is a matrix molecule synthesized by the proteins encoded by the icaADBC gene cluster, which is widely distributed among CNS from nosocomial infections (Crampton et al., 1999; de Silva et al., 2002) and in food-processing environments (Moretro 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 Flanders (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 icaA1f and icaA2r (Moretro 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-μL reaction mixture containing 0.25 μL of each primer (100 μM), Eurogentec, San Diego, CA), 1× PCR buffer II (Applied Biosystems, Foster City, CA), 1.5 mM MgCl₂ (Applied Biosystems), 0.1 mM each deoxyribonucleotide 5’-triphosphate, 1 U of AmpliTaq Polymerase (Applied Biosystems), and 1 μL of bacterial DNA. Thermal cycling conditions were 5 min
of denaturation at 95°C; 30 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 58°C (\textit{bap} and \textit{icaA}) or 52°C (\textit{mec}A), 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 \textit{Staphylococcus xylosus} CCM 2738T (\textit{bap}), \textit{Staphylococcus capitis} ssp. \textit{capitis} LMG 13353T (\textit{icaA}), and \textit{Staph. aureus} MRSA ST 398 strain MB 4393 (\textit{mec}A). 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 \textit{bap} or \textit{icaA} or both (\textit{n} = 30) of the species \textit{Staph. epidermidis} (1 isolate), \textit{Staph. equorum} (6 isolates), \textit{Staph. sciuri} (9 isolates), \textit{Staph. xylosus} (8 isolates), \textit{Staphylococcus saprophyticus} (1 isolate) \textit{Staphylococcus cohnii} (1 isolate), and \textit{Staph. capitis} (4 isolates). Ten \textit{bap} and \textit{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 \textit{Staph. epidermidis} strain ATCC 35984 and the nonbiofilm-producer \textit{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

### Table 1. Polymerase chain reaction results for detection of the \textit{bap}, \textit{icaA}, and \textit{mec}A genes in 366 bovine isolates belonging to 22 CNS species

<table>
<thead>
<tr>
<th>CNS species group</th>
<th>Isolates (no.)</th>
<th>Origin (no.)</th>
<th>No. of PCR-positive isolates from milk/environment (% of [sub]total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Milk</td>
<td>Environment</td>
</tr>
<tr>
<td><strong>IMI-causing</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Staph. chromogenes}</td>
<td>28</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>\textit{Staph. epidermidis}</td>
<td>24</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>\textit{Staph. haemolyticus}</td>
<td>131</td>
<td>20</td>
<td>111 \textsuperscript{2}</td>
</tr>
<tr>
<td>\textit{Staph. simulans}</td>
<td>37</td>
<td>5</td>
<td>32</td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td>220</td>
<td>54</td>
<td>166</td>
</tr>
<tr>
<td><strong>Environmental</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Staph. equorum}</td>
<td>34</td>
<td>5</td>
<td>29</td>
</tr>
<tr>
<td>\textit{Staph. sciuri}</td>
<td>31</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>\textit{Staph. xylosus}</td>
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<td>0</td>
<td>9</td>
</tr>
<tr>
<td>\textit{Staph. devriesii}</td>
<td>8</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
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<td>7</td>
<td>3</td>
<td>4</td>
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<td>3</td>
<td>4</td>
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<td>4</td>
<td>3</td>
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<td>0</td>
<td>6</td>
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<td>1</td>
<td>4</td>
</tr>
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<td>\textit{Staph. lentus}</td>
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<td>2</td>
</tr>
<tr>
<td>\textit{Staph. nepalensis}</td>
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<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td>146</td>
<td>23</td>
<td>123</td>
</tr>
<tr>
<td><strong>Overall</strong></td>
<td>366</td>
<td>77</td>
<td>289</td>
</tr>
</tbody>
</table>

\textsuperscript{1}An 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.

\textsuperscript{2}Including 7 isolates originating from teat apices.
staining solution was carefully removed, the plate was washed 2 times with Milli Q water (Millipore, Billerica, MA) and 150 μL 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 × 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 μg/mL for cephalothin, 4 μg/mL for enrofloxacin, 8 μg/mL for erythromycin, 8 μg/mL for gentamicin, and 0.5 μg/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 μL/well), which were inoculated within 2 h after preparation. Subsequently, bacterial suspensions of the 10^{-2} dilution (~3.10^6 cfu/mL) were diluted 2-fold in the designated interfering substance (10% skim milk; Oxoid Ltd.), and 40 μL 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-μL quantities of the test suspensions were transferred to plates containing 180 μL of neutralizer per well. After 5 min of neutralization at room temperature, 20-μL quantities were transferred
to plates containing D/E neutralizing broth (200 μL/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. fleurettii, 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.
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 \( \text{mecA} \)-positive and \( \text{mecA} \)-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 \textit{Staph. haemolyticus} (90.6%) and \textit{Staph. simulans} (90.0%). Twenty-nine isolates showed resistance to a single compound, either erythromycin (22.0%) or oxacillin (13.4%), and 1 \textit{Staph. sciuri} isolate was resistant to both. Another \textit{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 proportion in \textit{Staph. equorum} (90.0%) and \textit{Staph. epidermidis} (30.0%). The oxacillin-resistant isolates belonged to 3 species: 8 \textit{Staph. sciuri}, 3 \textit{Staph. epidermidis}, and 2 \textit{Staph. chromogenes}, but carriage of \( \text{mecA} \) was confirmed in only 10 (7 \textit{Staph. sciuri} and 3 \textit{Staph. epidermidis}) out of 13.
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 1](image1.png)

**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).

![Figure 2](image2.png)

**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>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>No. of isolates with MIC</th>
<th>No. (%) of resistant isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>58</td>
<td>7</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>35</td>
<td>26</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>80</td>
<td>2</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>41</td>
<td>9</td>
</tr>
</tbody>
</table>

AB bioMérieux (Marcy l'Etoile, France). Intermediate resistant. Resistant.
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. haemolyticus).

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**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)

<table>
<thead>
<tr>
<th>MBC(^1) of iodine (ppm)</th>
<th>Isolates (no.)</th>
<th>IMI-causing species(^2) [no. (%)]</th>
<th>Environmental species (^3) [no. (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
<td>30 min</td>
<td>Staph. chromogenes (n = 10)</td>
</tr>
<tr>
<td>75</td>
<td>75</td>
<td>44</td>
<td>1 (10.0)</td>
</tr>
<tr>
<td>112.5</td>
<td>75</td>
<td>4</td>
<td>1 (10.0)</td>
</tr>
<tr>
<td>150</td>
<td>75</td>
<td>3</td>
<td>1 (10.0)</td>
</tr>
<tr>
<td>150</td>
<td>125.5</td>
<td>1</td>
<td>1 (10.0)</td>
</tr>
<tr>
<td>150</td>
<td>150</td>
<td>28</td>
<td>9 (90.0)</td>
</tr>
<tr>
<td>300</td>
<td>150</td>
<td>1</td>
<td>1 (10.0)</td>
</tr>
<tr>
<td>450</td>
<td>225</td>
<td>1</td>
<td>1 (10.0)</td>
</tr>
</tbody>
</table>

\(^1\)MBC = minimum biocidal concentration.

\(^2\)An 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.

\(^3\)Bactericidal 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 (Moretrø 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 (Moretrø 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 (Moretrø 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 (Tsukakishita 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. epidermidis, 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 (Liithje 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. fleurentii, 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.

ACKNOWLEDGMENTS

This research was funded by the agency for Innovation by Science and Technology in Flanders (IWV-Vlaanderen, grant no. 60714). The authors thank Ann Vanhee and Katrien Verheyen (Institute for Agricultural and Fisheries Research, Melle, Belgium) for their excellent laboratory support.

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