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Antimicrobial resistance and virulence characteristics in 3 collections of staphylococci from bovine milk samples

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ABSTRACT

Mastitis is a prevalent disease in dairy cattle, and staphylococci are among the most common causative pathogens. Staphylococci can express resistance to a range of antimicrobials, of which methicillin resistance is of particular public health concern. Additionally, *Staphylococcus aureus* carries a variety of virulence factors, although less is understood about the virulence of non-*aureus* staphylococci (NAS). The aim of our study was to identify and characterize 3 collections of staphylococcal isolates from bovine milk samples regarding antimicrobial resistance, with emphasis on methicillin resistance, and their carriage of virulence genes typically displayed by *Staph. aureus*. A total of 272 staphylococcal isolates collected in Norway and Belgium in 2016 were included, distributed as follows: group 1, Norway, 100 isolates; group 2, Flanders, Belgium, 64 isolates; group 3, Wallonia, Belgium, 108 isolates. Species identification was performed by use of MALDI-TOF mass spectrometry. Phenotypic resistance was determined via disk diffusion, and PCR was used for detection of methicillin resistance genes, *mecA* and *mecC*, and virulence genes. Antimicrobial resistance was common in *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* from all different groups, with resistance to trimethoprim-sulfonamide frequently occurring in *Staph. epidermidis* and *Staph. haemolyticus* as well as in *Staph. aureus*. Resistance to penicillin was most frequently observed in group 1. Ten Belgian isolates (1 from group 2, 9 from group 3) carried the methicillin resistance determinant *mecA*: 5 *Staph. aureus* from 2 different farms and 5 NAS from 3 different farms. Almost all *Staph. aureus* isolates were positive for at least 3 of the screened virulence genes, whereas, in total, only 8 NAS isolates harbored any of the same genes. Our study contributes to the continuous need for knowledge regarding staphylococci from food-producing animals as a basis for better understanding of occurrence of resistance and virulence traits in these bacteria.

Key words: *Staphylococcus aureus*, non-*aureus* staphylococci, antimicrobial resistance, virulence genes, bovine mastitis

INTRODUCTION

Mastitis is a common disease in dairy cattle, affecting animal health and welfare, and dairy farm profitability (Halasa et al., 2007; Rollin et al., 2015). Staphylococci are among the most recognized udder pathogens, of which *Staphylococcus aureus* is an important cause of subclinical and clinical mastitis in dairy cattle, with its importance in the context of mastitis and milk quality varying by region and farm (Østerås et al., 2006; Olde Riekerink et al., 2008; Gao et al., 2017). In a Belgian study, *Staph. aureus* was isolated from 7.3% of the clinical mastitis cases in Flemish dairy herds (Verbeke et al., 2014), whereas in a Norwegian study *Staph. aureus* was found in 45.8% of the clinical mastitis cases from heifers (Waage et al., 1999). In contrast, NAS are the most frequently isolated bacteria causing subclinical mastitis. Their role as mastitis-causing pathogens is debatable, but in general they are associated with moderately increased SCC (Piepers et al., 2007; De Vliegher et al., 2012; De Visscher et al., 2016; Valckenier et al., 2019). A negative effect on milk production after infection has been reported (Heikkilä et al., 2018), although others have reported differently (Valckenier et al., 2019). The distribution of NAS species in milk

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samples is described in several studies; however, the results are not directly comparable due to a variety of study designs. A recent study from Belgium concluded that the 5 most prevalent NAS species recovered from quarter milk samples were *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus equorum*, *Staphylococcus hominis*, and *Staphylococcus cohnii* (Wuytack et al., 2020). In Norway, *Staphylococcus epidermidis* and *Staph. aureus* are currently regarded as the most common staphylococcal udder pathogens (Dalen et al., 2019).

Most of the antimicrobial usage in dairy production concerns udder health (Saini et al., 2012; Nobrega et al., 2017). The largest proportion is administered for the intramammary treatment of clinical mastitis and dry cow therapy (De Briyne et al., 2014; Stevens et al., 2016; Stevens et al., 2018). In general terms, treatment with antimicrobials is regarded as the main driver for antimicrobial resistance, although the association between antimicrobial use and corresponding resistance is less compelling in the case of mastitis (Tacconelli, 2009; Oliver and Murinda, 2012).

Methicillin-resistant *Staph. aureus* (**MRSA**) is classified as a high-priority pathogen by WHO and has gained most attention among the resistant staphylococci. However, methicillin resistance has also been described in several species of the NAS group (Feßler et al., 2010; Gindonis et al., 2013), and NAS are thought to be a reservoir for numerous resistance genes that could be transferred into the more pathogenic *Staph. aureus* (Becker et al., 2014). Methicillin-resistant staphylococci are resistant to almost all β-lactams, and infections caused by these bacteria result in limited treatment options, delayed initiation of effective treatment, and poorer outcomes (Yaw et al., 2014). When it comes to MRSA as cause of mastitis, Norway may be regarded as a naive country, as MRSA has been associated with bovine mastitis on only 1 occasion (NORM/NORM-VET, 2015). This contrasts with the current situation in Belgium, where MRSA is an established cause of clinical mastitis. Bardiau et al. (2013) found MRSA in 4.4% of milk samples from bovine mastitis, and Vanderhaeghen et al. (2010) found 9.3% of *Staph. aureus* isolates to be MRSA in milk samples from farms experiencing *Staph. aureus* mastitis.

Staphylococci are well known for their carriage of a range of other resistance determinants in addition to the methicillin resistance trait, often occurring in different combinations, giving rise to multidrug-resistant isolates (Kadlec et al., 2012; Wendlandt et al., 2015; Schoenfelder et al., 2017). An important aspect of the antimicrobial resistance challenge is that resistant staphylococci from animals may pose a threat to public health due to the possible transfer from their different reservoirs to humans (Lee, 2003), and the possible transmission and spread of resistance genes between staphylococci (Levy and Marshall, 2004; Hanssen and Ericson Sollid, 2006). VetPath, a pan-European antimicrobial susceptibility monitoring program, has recently reported data on antimicrobial susceptibility of 9 udder pathogens from bovine mastitis in Europe from 2015 to 2016, and on an overall level they report low resistance to antimicrobials and a low prevalence of MRSA (El Garch et al., 2020).

Mastitis-related *Staph. aureus* are also associated with a variety of virulence factors that play an important role in the pathogenesis of mastitis. These include, among others, cell wall–associated factors, different enzymes, and exotoxins that facilitate the infection pathway. The NAS species lack the aggressive potential of *Staph. aureus*, and less is known about the virulence of these species (Becker et al., 2014; Naushad et al., 2019; Wuytack et al., 2020). Knowledge regarding virulence of both *Staph. aureus* and NAS species associated with bovine mastitis, especially in combination with resistance patterns, is important for designing efficient prophylaxis and treatment guidelines.

The aim of this study was to gain knowledge on resistance and virulence characteristics of 3 groups of mastitis-associated *Staph. aureus* and NAS isolates from bovine milk samples collected in Norway and Belgium as part of an international collaboration. The objectives were (1) to characterize the isolates with regard to their antimicrobial resistance properties, with emphasis on methicillin resistance, (2) to determine carriage of genes encoding known *Staph. aureus* virulence factors in all 3 *Staph. aureus* and NAS isolate collections, and (3) to analyze for correlation patterns of resistance and of virulence of the collected isolates.

MATERIALS AND METHODS

Collection of Staphylococcal Isolates

The isolates were collected as 3 unique collections, all being part of a larger European collaboration. The 3 different sampling strategies, ensuring diversity of the material and at the same time conveniently adapted to the national structures of dairy production, the framework, and the resources of the collaborative project, are described in Table 1. Table 1 also contains information about characteristics of the sampled farms. The aim was to collect a total of 300 staphylococcal isolates: 100 from Norway and 200 from Belgium. The ratio between *Staph. aureus* and NAS was defined a priori to be 1:5. All sampling was performed in 2016.

Table 1. Information about number of isolates, farms sampled, sampling strategies, and characteristics of farms for the 3 different collections of staphylococcal isolates studied

Item	No. of *Staphylococcus aureus* isolates	No. of NAS isolates	No. of farms sampled and total number of isolates	Sampling strategy	Housing of animals and milking systems
Group 1, Norway	20	80	100 farms Total number of isolates = 100, i.e., 1 isolate per farm.	Staphylococci were isolated from milk samples submitted to the routine diagnostics of the TINE Norwegian Dairies Mastitis Laboratory. Only *Staph. aureus* isolated in pure culture from milk samples of cows with clinical mastitis were included in the collection. The NAS isolates were collected from samples with rich growth of NAS in pure culture, sent to the laboratory because of high SCC or clinical mastitis.	Not available per farm, but Norwegian dairy production is characterized by varying housing conditions, including tiestall barns (40%) and loose housing with automatic milking system (47%) or milking parlor (13; Mikalsen et al., 2019).
Group 2, Flanders, Belgium	11	53	NAS isolates were collected on 2 farms with 56 and 49 lactating cows at the time of sampling (n = 105), respectively (new sampling). No *Staph. aureus* were found in this new sampling. *Staph. aureus* isolates were collected in 8 different herds (De Visscher et al., 2016). Total number of isolates = 64.	All quarters from lactating cows in 2 herds with a high *Staphylococcus* prevalence, as measured in a previous study (De Visscher et al., 2016), were sampled during one new cross-sectional sampling and quarter SCC (qSCC) were measured. *Staph. aureus* isolates were retrieved from the collection of the Mastitis and Milk Quality Research Unit of Ghent University and qSCC measurements were available.	Cows were housed in freestall barns with slatted floors and cubicles with mattresses. Conventional milking parlor.
Group 3, Wallonia, Belgium	14	94	The 3 herds had 118, 147, and 94 cows at the time of sampling. Total number of isolates = 108.	Three herds with a subclinical mastitis problem were chosen from 300 farms previously included in another project (MammiScan project, Service Public Wallonie, Division Générale de l'Agriculture RNE; A. S. Rao and L. Théron, Faculty of Veterinary Medicine, University of Liège, Belgium, unpublished data). All cows with a cow somatic cell count (cSCC) >300,000 cell/mL on the last DHI sampling were selected (n = 114), and all quarters were sampled.	One herd as freestall barn with straw, 2 herds with slatted floor. Conventional milking parlor.
Total	45	227	272		

Isolation and Identification of Staphylococcus Isolates

First, 10 μL of each quarter milk sample were plated on a quadrant of a Columbia agar supplemented with 5% sheep blood (Oxoid) for samples from group 2 and 3, and Difco heart infusion agar with 5% washed bovine erythrocytes (BD Biosciences) for samples from group 1, both nonselective media. For groups 2 and 3, modified Chapman's agar or mannitol salt agar, a semiselective medium, were also used for the recovery of staphylococci (Oxoid). Plates were examined after 24-h aerobic incubation at 37°C. If more than 2 phenotypically different colony types were present on blood agar, the quarter milk sample was considered contaminated and rejected. If not, for samples from group 2 and 3 all phenotypically different colony types were counted on mannitol salt agar and subcultured (1 colony per colony type) on blood agar (Columbia agar with 5% sheep blood, Oxoid), whereas for group 1 all suspected staphylococcal colonies on blood agar, based on colony morphology and catalase test, were subcultured (1 colony per colony type) on blood agar (Difco heart infusion agar with 5% washed bovine erythrocytes, BD Biosciences) to obtain pure cultures (aerobic incubation at 37°C, a half plate per colony). The previously mentioned procedures were performed at the laboratories at the place of collection (TINE Norwegian Dairies Mastitis Laboratory, Ghent University, and University of Liège). Pure cultures were examined as described by De Visscher et al. (2013). All suspected staphylococci were stored at −80°C in Microbank cryovials (Pro-Lab Diagnostics) and identified at the species level using MALDI-TOF MS and a validated and updated library for bovine-related NAS species (Cameron et al., 2017, 2018). If no identification could be assigned using MALDI-TOF MS, DNA was subjected to sequencing of the *rpoB* gene (Supré et al., 2009). The examination of pure cultures and species identification with MALDI-TOF MS (or the *rpoB* gene) for all groups were performed at Ghent University.

DNA Extraction

Bacterial DNA was extracted using the following procedure, based on Unal et al. (1992): 1 or 2 colonies of each *Staphylococcus* isolate were mixed with 3 mL of brain heart infusion broth (Oxoid) and aerobically incubated at 37°C overnight. Then 100 μL of each bacterial suspension was centrifuged at 16,500 × *g* for 3 min (Thermo Scientific Heraeus Pico 21 microcentrifuge). The supernatant was removed, and the pellet was resolved with 50 μL of lysostaphin (50 μg/mL). The tubes were heated at 37°C for 10 min before adding 50 μL of proteinase K (100 μg/mL) and 150 μL of 0.1 *M* Tris (pH 7.5), and then heated at 37°C for 10 min and 95°C for 5 min. The extracted DNA was stored at −20°C before analyses.

Phenotypic Antimicrobial Resistance Testing

Antimicrobial resistance was determined by means of the disk diffusion method by Bauer et al. (1966) and according to the methodology recommended by the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org, accessed Aug. 2018), except that Mueller Hinton broth was used instead of saline water for the bacterial suspension, corresponding to 0.5 McFarland for the panel of antimicrobial agents. The following antimicrobial agents were included in the test panel: ampicillin (10 μg), amoxicillin and clavulanic acid (20 + 10 μg), ciprofloxacin (5 μg), clindamycin (10 μg), erythromycin (15 μg), gentamicin (10 μg), linezolide (10 μg), penicillin (1 U), trimethoprim (5 μg), sulfonamide and trimethoprim (19:1, 25 μg), and tetracycline (30 μg). Cefoxitin (30 μg) was used for determination of phenotypic methicillin resistance, as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org). Categorization of the isolates as resistant or intermediate (assigned as resistant) versus susceptible was based on clinical breakpoints determined by EUCAST; these breakpoints are prepared for human strains and not mastitis pathogens. Species-specific breakpoints for the NAS group are scarce, so the general breakpoints for coagulase-negative staphylococci were used.

Genotypic β-Lactam Resistance Testing

The PCR for detection of *mecA* and *mecC* was carried out as described by Stegger et al. (2012) to test for β-lactam resistance (methicillin resistance). Genes, primer sequences, amplicon sizes, and annealing temperatures are listed in Table 2. A *mecA*-positive *Staph. aureus* isolate previously confirmed using DNA sequencing (NMBU2664/16, Routine Microbiology Laboratory, Faculty of Veterinary Medicine, Norwegian University of Life Science) and *Staph. aureus* NTCT 13552 were included as positive controls in the *mecA* and *mecC* PCR, respectively. Sterile water was used as negative control.

Virulence Genes

Nine virulence genes common in *Staph. aureus* were selected for PCR analysis, based on a review of relevant articles available in the PubMed database in November and December 2016. The chosen genes were *clfA* (clumping factor), *fnbpA* and *fnbpB* (fibronectin-bind-

Table 2. Genes, proteins, primer sequences, amplicon sizes, and annealing temperatures for all virulence and resistance genes analyzed in the collection of 272 staphylococcal isolates

Gene, protein	Primer name	Primer sequence	Amplicon size	Annealing temperature	Source
hla	hla-F	GGT TTA GCC TGG CCT TC	534	57°C	Wang et al., 2016
α-Hemolysin	hla-R	CAT CAC GAA CTC GTT CG			
hlb	hlb-F	GCC AAA GCC GAA TCT AAG	833	57.5°C	Wang et al., 2016
β-Hemolysin	hlb-R	CGC ATA TAC ATC CCA TGG C			
fnbpA	fnbpA-F	GCG GAG ATC AAA GAC AA	1,279	48°C	Wang et al., 2016
Fibronectin-binding protein A	fnbpA-R	CAT CTA TAG CTG TGT GG			
fnbpB	fnbpB-F	GGA GAA GGA ATT AAG GCG	812	45°C	Wang et al., 2016
Fibronectin-binding protein B	fnbpB-R	GCC GTC GCC TTG AGC GT			
tsst01	tsst-1-F	GCT TGC GAC AAC TGC TAC AG	599	61°C	Wang et al., 2016
Toxic shock syndrome toxin-1	tsst-1-R	TGG ATC CGT CAT TCA TTG TTA T			
ssl7 (*set1*)	ssl7-F	GGT TTA TTC ATA GCG CAG TAT C	879	58°C	Salasia et al., 2004
Staphylococcal superantigen-like protein	ssl7-R	CAA CGT TTC ATC GTT AAG CTG C			
clfA	clfA-F	GGC AAC GAA TCA AGC TAA TAC AC	719	58°C	Wang et al., 2016
Clumping factor A	clfA-R	TTG TAC TAC CTA TGC CAG TTG TC			
mecA[1]	mecA-P4	TCC AGA TTA CAA CTT CAC CAG G	162	55°C	Stegger et al., 2012
Penicillin-binding protein 2a	mecA-P7	CCA CTT CAT ATC TTG TAA CG			
mecC[1]	mecC-F	GAA AAA AAG GCT TAG AAC GCC TC	138	55°C	Stegger et al., 2012
Penicillin-binding protein 2a	mecC-R	GAA GAT CTT TTC CGT TTT CAG C			
cap5	cap5-H	ATG AGG ATA GCG ATT GAA AA	518	49.7°C	Ote et al., 2011
Capsular polysaccharide 5 synthesis enzyme	cap5-R	CGC TTC TTA ATC ACT TTT GC			
cap8	cap8-H	ATC GAA GAA CAT ATC CAA GG	834	46.4°C	Ote et al., 2011
Capsular polysaccharide 8 synthesis enzyme	cap8-R	TTC ATC ACC AAT ACC TTT TA			

[1]Multiplex PCR.

ing proteins), *hla* and *hlb* (hemolysins), *tsst01* (toxic shock syndrome toxin-1), *ssl7* (*set1*, staphylococcal superantigen-like protein), *cap5* (CP5 capsule synthesis enzyme), and *cap8* (CP8 capsule synthesis enzyme; Salasia et al., 2004; Ote et al., 2011; Wang et al., 2016). The PCR were carried out using the protocol of Wang et al. (2016) for *clfA*, *fnbpA*, *fnbpB*, *hla*, *hlb*, and *tsst01*, according to Salasia et al. (2004) for *ssl7*, and according to Ote et al. (2011) for *cap5* and *cap8*.

Genes, primer sequences, amplicon sizes, and annealing temperatures are listed in Table 2. Because positive controls were not available for any of the virulence genes except *cap5* and *cap8*, the amplified PCR products from 1 isolate positive for all the remaining tested virulence genes was confirmed by DNA sequencing, using Sanger sequencing (GATC, Eurofins Genomics) of each gene, and the DNA from the confirmed isolate was further used as positive control. For *cap5* and *cap8*, previously published positive strains B34 and B79 were used as positive controls (Ote et al., 2011). Sterile water was used as negative control.

Correlation Plots

Correlation plots were created in R 3.6.2 (R Core Team, 2013) using the corrplot (Wei and Simko, 2017) package v. 0.84. We considered 2 variables with a phi coefficient greater than 0.7 to be strongly correlated. The correlations that were calculated were correlations between resistance genes in *Staph. aureus*, between resistance genes in NAS, and between virulence genes in *Staph. aureus*. Correlation between virulence genes in NAS was not calculated, due to the low number of virulence genes detected.

RESULTS

Staphylococcus Species Identification

A total of 272 *Staphylococcus* isolates from 319 cattle, collected in Norway (group 1, 100 isolates/100 cattle), Flanders (group 2, 64 isolates/105 cattle), and Wallonia (group 3, 108 isolates/114 cattle), were collected, and all were included in the study. After analysis with MALDI-TOF MS, the following *Staphylococcus* species were most frequently (≥10%) identified: in group 1 *Staphylococcus simulans* (n = 29), *Staph. chromogenes* (n = 25), *Staph. aureus* (n = 20), and *Staph. epidermidis* (n = 13); in group 2: *Staph. chromogenes* (n = 15), *Staph. haemolyticus* (n = 11), *Staph. aureus* (n = 11), and *Staph. cohnii* (n = 8); and in group 3: *Staph. epidermidis* (n = 19), *Staph. chromogenes* (n = 17), *Staph. haemolyticus* (n = 15), and *Staph. aureus* (n = 14). The full species distribution in relation to each group is shown in Figure 1.

Phenotypic Antimicrobial Resistance

Species distribution and number of isolates categorized as resistant toward the panel of antimicrobials tested are shown in Table 3. Antimicrobial resistance was most common in *Staph. haemolyticus* and *Staph. epidermidis* in all 3 sample groups.

Group 1. All 3 *Staph. haemolyticus* isolates and 77% of the *Staph. epidermidis* isolates (n = 10) from group 1 showed resistance to at least 1 of the tested antimicrobials. Resistance to penicillin was more frequently observed, except in the *Staph. simulans* isolates, in which resistance to trimethoprim was most common. *Staphylococcus epidermidis* was the only species of which more than 50% of the isolates were resistant, as 54% were resistant to penicillin. However, no resistance to cefoxitin was observed in this collection. Neither were any isolates categorized as multidrug resistant, defined as resistance to 3 or more of the tested classes of antimicrobials. Isolates fully susceptible toward the tested antimicrobials were distributed as follows: *Staph. chromogenes* 17/25, *Staph. aureus* 16/20, *Staph. simulans* 14/29, *Staph. epidermidis* 3/13, *Staphylococcus hyicus* 4/4, and *Staphylococcus devriesei* 2/2.

Group 2. Eleven *Staph. haemolyticus* isolates (91%) and the single *Staph. epidermidis* isolate in group 2 showed resistance to at least 1 of the tested antimicrobials. Resistance to trimethoprim and trimethoprim-sulfonamide were most common, as, in most species including trimethoprim and trimethoprim-sulfonamide–resistant isolates, at least 50% of the isolates were categorized as resistant. Resistance toward cefoxitin was observed in 3 different species: *Staph. cohnii*, *Staph. aureus*, and *Staphylococcus sciuri*; and 18 isolates were multidrug resistant. The multidrug-resistant isolates included the following species: *Staph. cohnii* (n = 5), *Staph. chromogenes* (n = 3), *Staph. equorum* (n = 3), *Staphylococcus arlettae* (n = 2), *Staph. haemolyticus* (n = 2), *Staph. sciuri* (n = 2), and *Staph. aureus* (n = 1). Isolates fully susceptible toward the tested antimicrobials were distributed as follows: *Staph. chromogenes* 5/15, *Staph. aureus* 3/12, and *Staph. haemolyticus* 1/11.

Group 3. All *Staph. haemolyticus* isolates (n = 15) and all *Staph. epidermidis* isolates (n = 19) in group 3 showed resistance to at least 1 of the tested antimicrobials. Also, in group 3, resistance to trimethoprim and trimethoprim-sulfonamide were most observed. Resistance toward cefoxitin was observed in 9 different species, including *Staph. aureus*, *Staph. chromogenes*, *Staph. epidermidis*, and *Staph. haemolyticus*. A total of 24 isolates were multidrug resistant, represented by the

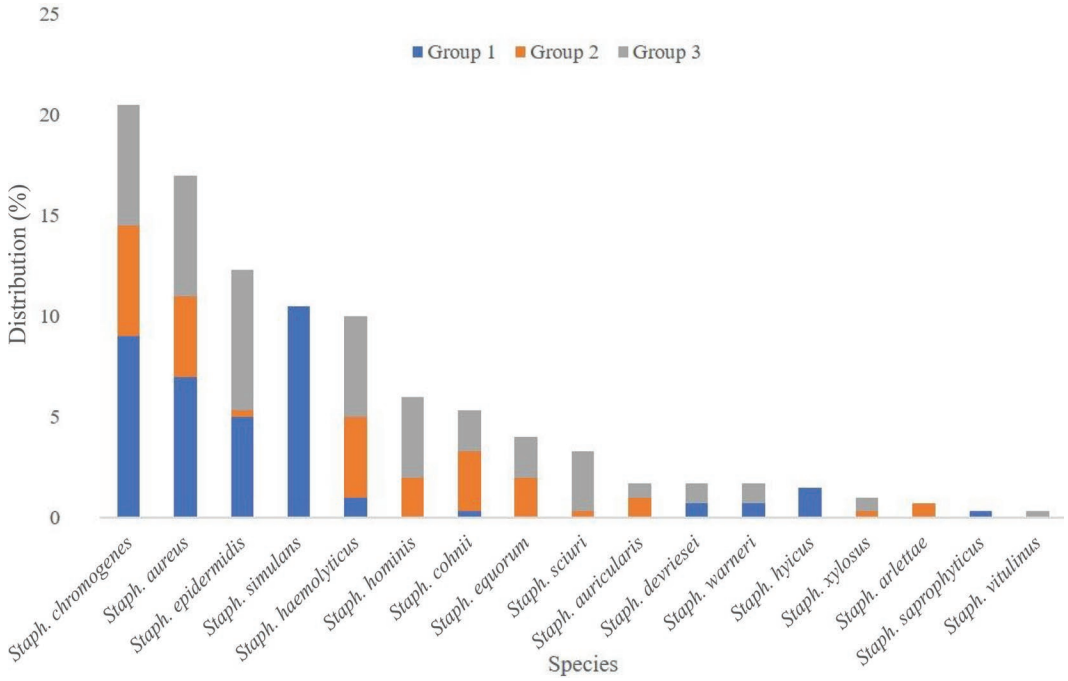

Figure 1. *Staphylococcus* species distribution for the collection of 272 staphylococcal isolates by group, shown as percentage of the total number of isolates. Group 1: collected in Norway; milk samples from quarters with mastitis or high SCC, all different herds. Group 2: collected in Flanders, Belgium; 53 NAS isolates from milk samples from all quarters from all cows (105 dairy cows) from 2 different herds, and 11 *Staphylococcus aureus* isolates from 8 additional herds. Group 3: collected in Wallonia. Belgium; milk samples from all quarters from 114 dairy cows with composite cow SCC >300,000 cells/mL from 3 different herds.

following species: *Staph. epidermidis* (n = 5), *Staph. aureus* (n = 4), *Staph. haemolyticus* (n = 4), *Staph. sciuri* (n = 4), *Staph. cohnii* (n = 3), *Staphylococcus warneri* (n = 3), and *Staph. hominis* (n = 1). Of these, originating from the same farm, 1 *Staph. epidermidis* isolate had 9 and 2 *Staph. aureus* isolates had 8 observations of resistance toward the panel of tested antimicrobials. This *Staph. epidermidis* isolate was the only isolate phenotypically resistant toward linezolid (Table 3). Isolates fully susceptible toward the tested antimicrobials were distributed as follows: *Staph. aureus* 4/16 and *Staph. chromogenes* 3/17.

Figure 2 and Figure 3 show the correlations between the different observed resistances for *Staph. aureus* and the NAS species, respectively. The correlation plots revealed a strong correlation between resistance to trimethoprim and trimethoprim-sulfonamide and between resistance to clindamycin and erythromycin. In addition, we found a stronger correlation between several resistance observations in *Staph. aureus* compared with the NAS species as a group.

Characterization of Isolates with mec Genes

Group 1. No isolates in group 1 were positive for the *mec* genes.

Group 2. The only *mecA*-positive isolate in group 2 was a *Staph. aureus* isolate, which showed resistance to cefoxitin on disk diffusion, as well as resistance to penicillin, ampicillin, and amoxycillin-clavulanic acid, as well as 4 other antimicrobials, as shown in Table 4. No isolates were positive in the *mecC* PCR.

Group 3. In group 3, 4 *Staph. aureus* isolates and 5 NAS isolates were positive in the PCR for the *mecA* gene, but none in the *mecC* PCR. These *mecA*-positive *Staph. aureus* isolates (hereafter referred to as MRSA) originated from the same farm and were thus epidemiologically related. They showed resistance to cefoxitin on disk diffusion; 3 showed resistance to ampicillin; and 2 showed resistance toward amoxicillin-clavulanic acid. These isolates were also multidrug resistant and showed resistance toward 6 or more of the tested antimicrobials. Many of the observed resistances in *Staph. aureus* isolates were traced to these 5 isolates.

The 5 *mecA*-positive NAS belonged to 5 different species: *Staph. epidermidis*, *Staph. haemolyticus*, *Staph. sciuri*, *Staphylococcus vitulinus*, and *Staphylococcus xylosus*. The *Staph. haemolyticus* originated from the same farm as the MRSA, whereas *Staph. epidermidis* came from another farm, and the *Staph. sciuri*, *Staph. vitulinus*, and *Staph. xylosus* from the same third farm. Only the *Staph. haemolyticus* and *Staph. epidermi-*

Table 3. Species distribution and antimicrobial resistance. Species distribution and number of isolates categorized as resistant toward the panel of antimicrobials tested[1]

Species[2]	Total	Ampicillin	Amoxycillin-clavulanic acid	Ciprofloxacin	Clindamycin	Erythromycin	Gentamicin	Linezolid	Penicillin	Trimethoprim-sulfonamide	Trimethoprim	Tetracycline
Group 1												
Staphylococcus aureus	20	1	0	0	0	0	1	0	1	0	1	0
Staphylococcus chromogenes	25	0	0	0	0	2	0	0	4	2	2	0
Staphylococcus epidermidis	13	0	0	0	0	0	0	0	7	5	4	0
Staphylococcus simulans	29	0	0	2	0	1	1	0	1	2	12	0
Staphylococcus hyicus	4	0	0	0	0	0	0	0	0	0	0	0
Staphylococcus haemolyticus	3	0	0	0	0	0	0	0	3	0	0	0
Staphylococcus devriesei	2	0	0	0	0	0	0	0	0	0	0	0
Staphylococcus warneri	2	0	0	0	0	0	1	0	1	0	0	3
Staphylococcus cohnii	1	0	0	0	0	1	0	0	1	0	0	0
Staphylococcus saprophyticus	1	0	0	0	0	0	0	0	1	0	0	0
Group 2												
Staph. aureus	11	1	1	0	0	0	1	0	1	5	8	1
Staph. chromogenes	15	0	0	0	3	3	0	0	5	9	10	3
Staph. haemolyticus	11	0	0	1	2	2	0	0	1	7	9	2
Staph. cohnii	8	0	0	0	0	5	0	0	5	7	8	4
Staphylococcus equorum	5	1	0	0	2	3	0	0	0	3	5	2
Staphylococcus hominis	5	0	0	0	0	0	0	0	1	4	4	2
Staphylococcus auricularis	3	0	0	0	0	0	0	0	0	3	3	0
Staphylococcus arlettae	2	0	0	0	2	2	0	0	2	0	1	0
Staphylococcus sciuri	2	0	0	0	0	0	0	0	2	2	2	2
Staph. epidermidis	1	1	0	0	0	0	0	0	1	0	0	0
Staphylococcus xylosus	1	0	0	0	0	0	0	0	1	0	1	0
Group 3												
Staph. aureus	14	3	2	4	4	4	1	0	4	7	9	0
Staph. epidermidis	19	0	0	2	1	4	3	1	12	18	19	3
Staph. chromogenes	17	0	0	0	0	0	0	0	2	13	13	3
Staph. haemolyticus	15	1	0	0	3	3	0	0	6	15	15	2
Staph. hominis	12	0	0	1	0	1	2	0	0	12	12	4
Staph. sciuri	8	0	0	0	2	0	4	0	4	7	7	4
Staph. cohnii	6	0	0	1	3	3	0	0	5	5	6	1
Staph. equorum	6	0	0	0	0	2	1	0	0	6	6	0
Staph. devriesei	3	0	0	0	0	0	0	0	1	2	3	0
Staph. warneri	3	0	0	0	3	3	0	0	3	0	0	3
Staph. auricularis	2	0	0	0	0	0	0	0	0	2	2	0
Staph. xylosus	2	0	0	0	0	0	0	0	2	1	1	0
Staphylococcus vitulinus	1	0	0	0	0	0	1	0	0	1	1	0

[1]Grey boxes indicate resistant isolates.

[2]Group 1: collected in Norway; milk samples from quarters with mastitis or high SCC, all different herds. Group 2: collected in Flanders, Belgium; 53 NAS isolates from milk samples from all quarters from all cows (105 dairy cows) from 2 different herds, and 11 *Staphylococcus aureus* isolates from 8 additional herds. Group 3: collected in Wallonia, Belgium; milk samples from all quarters from 114 dairy cows with composite cow SCC >300,000 cells/mL from 3 different herds.

dis isolates showed resistance to cefoxitin. The *Staph. haemolyticus* isolate also showed resistance toward ampicillin, whereas the 4 others were susceptible, and all 5 NAS were phenotypically susceptible toward amoxicillin-clavulanic acid. These isolates also showed resistance toward 4 or fewer of the tested antimicrobials, including trimethoprim-sulfonamide (4/5), penicillin (2/5), gentamicin (1/5), and ampicillin (1/5).

Table 4 shows an overview of the resistance patterns of all *Staph. aureus* isolates.

Virulence Factors

The percentages of *Staph. aureus* isolates in each sample group harboring the different virulence genes are illustrated in Figure 4. All *Staph. aureus* isolates, except one with 2 virulence genes, carried at least 3 of the selected virulence genes. Table 5 shows the presence of virulence genes in a total of 8 NAS isolates.

Group 1. In group 1, 80% of *Staph. aureus* isolates carried at least 4 selected virulence genes. The *cap5* gene was the least frequent gene, followed by the *tsst01* and *fnbpB* genes. One isolate carried 8 of the 9 examined virulence genes, lacking only *cap5*. Only one NAS isolate, a *Staph. chromogenes* isolate, carried any of the virulence genes, namely the *tsst01* gene.

Group 2. A total of 73% of the *Staph. aureus* isolates in group 2 carried 5 or more of the selected virulence genes. The *tsst01* and *fnbpB* genes were the least frequent virulence genes; however, the *tsst01* gene was also carried by the only NAS isolate from group 2 positive for any of the virulence genes tested. This was a *Staph. chromogenes* isolate.

Group 3. All *Staph. aureus* isolates carried 5 or more of the selected virulence genes, of which the *tsst01* and *fnbpB* genes were the least frequent. Three isolates from the same farm carried 7 virulence genes, missing the *tsst01* and *cap8* genes. In this group, 6 NAS iso-

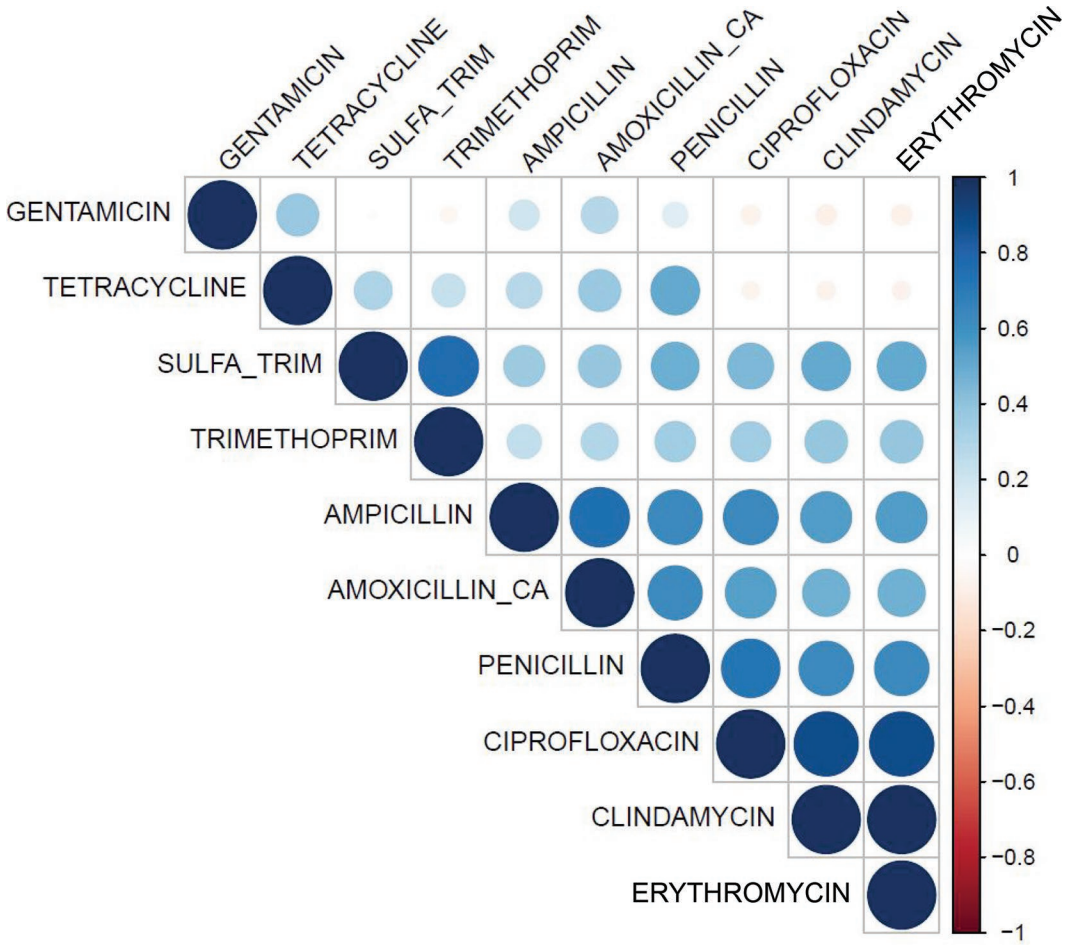

Figure 2. Correlation plot for observed antimicrobial resistances for *Staphylococcus aureus* isolates. Two variables with a phi coefficient >0.7 were considered to be strongly correlated. SULFA_TRIM = sulfonamide and trimethoprim; amoxicillin_ca = amoxicillin and clavulanic acid.

lated carried 1, 5, or 6 of the selected virulence genes, although none with the same profile. This NAS group included 4 *Staph. epidermidis* isolates, 1 *Staph. sciuri* isolate, and 1 *Staph. hominis* isolate.

Figure 5 shows the correlation between the different virulence genes for *Staph. aureus* isolates. We detected little correlation between the virulence genes in *Staph. aureus* isolates. The least frequent virulence genes (*tsst01* and *fnbpB*) as well as the *cap5* gene showed less correlation with other virulence genes. We found a negative correlation between the 2 *cap* genes, *cap5* and *cap8*, in *Staph. aureus* isolates.

Resistance and Virulence

The *Staph. aureus* isolate from group 1 with 8 virulence genes was completely susceptible toward the tested panel of antimicrobials. The 3 isolates from group 3 with 7 virulence genes were multidrug resistant, showing resistance toward 6 or more of the tested antimicrobials, and all 3 harbored the *mecA* gene. The remaining 2 *mecA*-positive *Staph. aureus* isolates carried 6 virulence genes and showed resistance toward 7 of the tested antimicrobials. Out of the 5 NAS that carried 1 virulence gene, one isolate was completely susceptible and the others showed resistance toward 1 to 3 of the tested antimicrobials. However, 1 of these isolates was a *mecA*-positive *Staph. epidermidis*. The other 4 *mecA*-positive NAS did not harbor any of the tested virulence genes. The NAS with 5 virulence genes showed resistance to 4 antimicrobials, and the 2 isolates with 6 virulence genes showed resistance toward 2 or 3 antimicrobials.

DISCUSSION

Methicillin-resistant *Staph. aureus* is a well-recognized and dreaded bacterium threatening both human

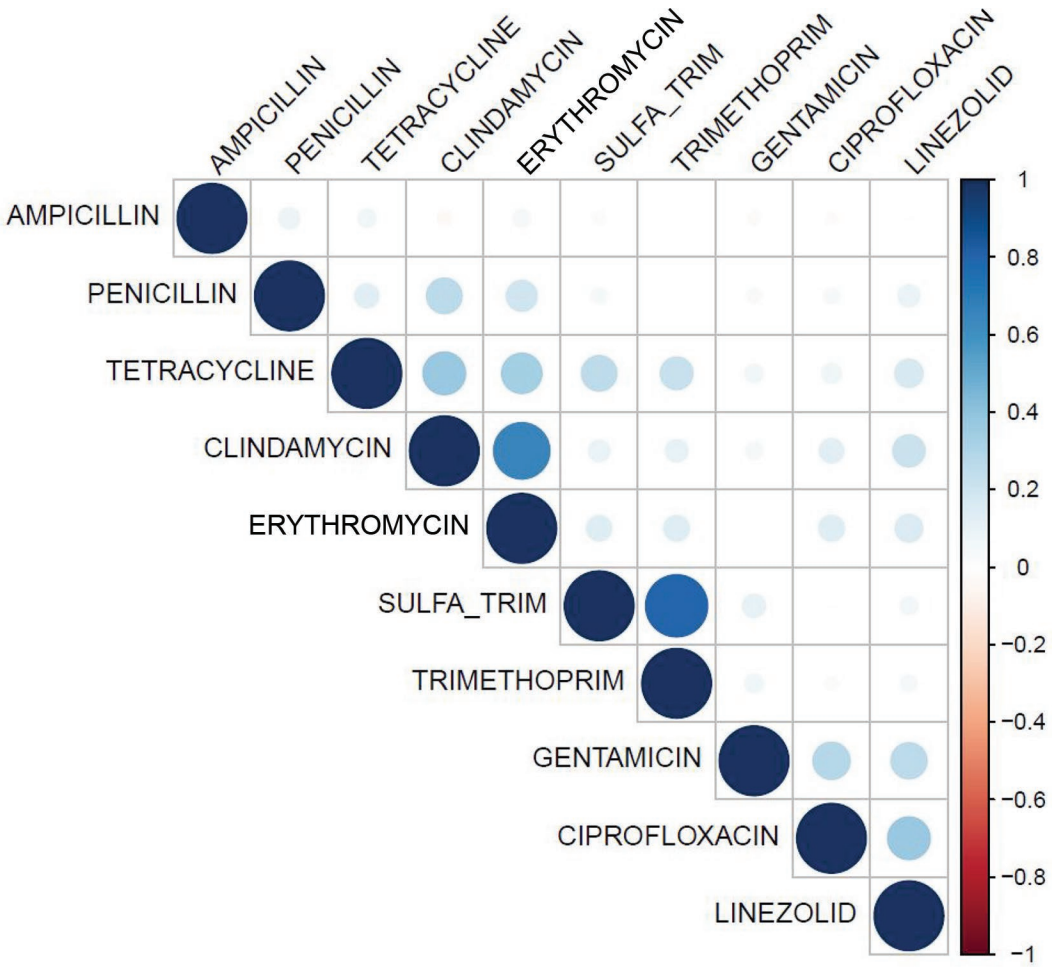

Figure 3. Correlation plot for observed antimicrobial resistances for NAS isolates. Two variables with a phi coefficient greater than 0.7 were considered to be strongly correlated. SULFA_TRIM = sulfonamide and trimethoprim.

Table 4. Number of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staph. aureus* (MSSA) isolates categorized as resistant toward the panel of antimicrobials tested[1]

Species and origin[2]	Total n	Ampicillin	Amoxycillin-clavulanic acid	Ciprofloxacin	Clindamycin	Erythromycin	Gentamicin	Linezolid	Penicillin	Trimethoprim-sulfonamide	Trimethoprim	Tetracycline
MRSA												
Group 1[3]	0	0	0	0	0	0	0	0	0	0	0	0
Group 2	1	1	1	0	0	0	1	0	1	1	1	1
Group 3	4	3	2	4	4	4	0	0	4	4	4	0
MSSA												
Group 1	20	1	0	0	0	0	1	0	1	0	1	0
Group 2	10	0	0	0	0	0	0	0	0	4	7	0
Group 3	10	0	0	0	0	0	1	0	0	3	5	0

[1]Grey boxes indicate resistant isolates.

[2]Group 1: collected in Norway; milk samples from quarters with mastitis, all different herds. Group 2: collected in Flanders, Belgium; 53 NAS isolates from milk samples from all quarters from all cows (105 dairy cows) from 2 different herds, and 11 *Staph. aureus* isolates from 8 additional herds. Group 3: collected in Wallonia, Belgium; milk samples from all quarters from 114 dairy cows with composite cow SCC >300,000 cells/mL from 3 different herds.

[3]No isolates of this species.

and animal health, causing potentially serious infections and limited treatment options. In addition, NAS are regarded as a potential reservoir for antimicrobial resistance genes that can be utilized by the more pathogenic *Staph. aureus* (Becker et al., 2014). This study provides findings of MRSA that are both multidrug resistant and harboring several virulence genes, and detection of several resistance traits in NAS isolates belonging to different species. By using 3 distinct collections of staphylococci from bovine milk samples of diverse origins, we were able once again to highlight the presence of antimicrobial resistance characteristics, as well as virulence genes, contributing to an increased knowledge base on this important bacterial group.

Descriptive analyses of antimicrobial resistance characteristics in all 3 sample groups showed that these were more widespread in several NAS species compared with *Staph. aureus*, apart from the MRSA isolates. This distribution corresponds with previous findings in Norwegian and Dutch surveillance systems for use of antimicrobial agents and occurrence of antimicrobial resistance (Mevius et al., 2007, 2008; NORM/NORM-VET, 2015). Non-*aureus* staphylococci are believed to represent an important reservoir for antimicrobial resistance (Becker et al., 2014), and NAS isolates from group 2 and group 3 in this study were more frequently multidrug resistant compared with their group-corresponding *Staph. aureus* isolates. Antimicrobial resistance was frequently observed in *Staph. epidermidis* and *Staph. haemolyticus* regardless of sample group, the "worst case" being the one *Staph. epidermidis* isolate from group 3 that was resistant to 9 of the 11 tested antimicrobials and the only isolate phenotypically resistant to linezolid. However, this isolate was not *mecA* positive. The finding of multidrug-resistant *Staph. epidermidis* has been shown previously (Nobrega et al., 2018). Although the milk food chain is not regarded as a major transfer route for antimicrobial-resistant bacteria because the pasteurization process will kill vegetative bacteria, the fact that *Staph. epidermidis* and *Staph. haemolyticus* are common causes of nosocomial infections in humans (Spanu et al., 2003; Huang et al., 2005; Shin et al., 2011) suggests that resistant NAS from dairy cattle could potentially be a public health hazard.

The high prevalence of resistance toward trimethoprim and trimethoprim-sulfonamide in groups 2 and 3 was surprising. This contrasts with findings from other European studies on staphylococci, where resistance toward penicillin is most prevalent (Botrel et al., 2010; Persson Waller et al., 2011; Taponen et al., 2016). A survey conducted among 3,000 practitioners in 25 European countries reported that β-lactams, mainly penicillin, are the drugs of choice when treating bovine

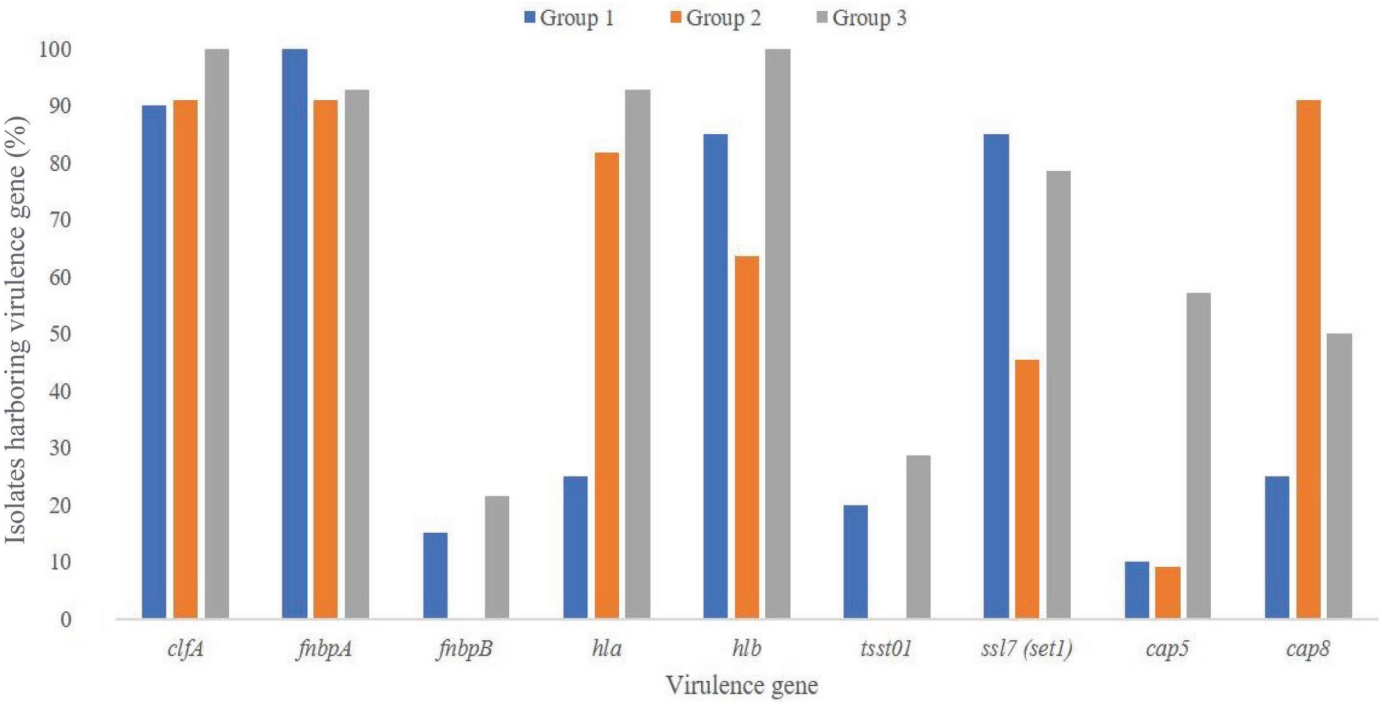

Figure 4. Percentage of *Staphylococcus aureus* isolates harboring the different virulence genes. Group 1: collected in Norway; milk samples from quarters with mastitis or high SCC, all different herds. Group 2: collected in Flanders, Belgium; 53 NAS isolates from milk samples from all quarters from all cows (105 dairy cows) from 2 different herds, and 11 *Staph. aureus* isolates from 8 additional herds. Group 3: collected in Wallonia, Belgium; milk samples from all quarters from 114 dairy cows with composite SCC >300,000 cells/mL from 3 different herds.

mastitis (De Briyne et al., 2014). In the European Surveillance of Veterinary Antimicrobial Consumption report of 2017 on sales of veterinary antimicrobial agents for food-producing animals, measured in milligrams per population-correction unit (mg/PCU), penicillin is the most sold drug in Norway and Belgium, followed by tetracyclines and sulfonamides in Belgium, and sulfonamides, amphenicols, and aminoglycosides in Norway

Table 5. Presence of virulence genes in single non-*aureus* staphylococcal isolates[1]

Species, origin[2]	*cap5*	*cap8*	*clfA*	*fnbpA*	*fnbpB*	*hla*	*hlb*	*tsst01*	*ssl7* (*set1*)
Staphylococcus chromogenes Group 1	−	−	−	−	−	−	−	+	−
Staph. chromogenes Group 2	−	−	−	−	−	−	−	+	−
Staphylococcus sciuri Group 3	+	−	−	−	−	−	−	−	−
Staphylococcus epidermidis Group 3	−	−	−	−	−	+	−	−	−
Staph. epidermidis Group 3	+	−	−	−	−	−	−	−	−
Staph. epidermidis Group 3	−	+	+	+	−	+	+	−	−
Staph. epidermidis Group 3	−	+	+	+	−	−	+	+	+
Staphylococcus hominis Group 3	−	+	+	+	−	+	+	+	−

[1]Grey boxes indicate presence of virulence genes in individual isolates.

[2]Group 1: collected in Norway; milk samples from quarters with mastitis or high SCC, all different herds. Group 2: collected in Flanders, Belgium; 53 NAS isolates from milk samples from all quarters from all cows (105 dairy cows) from 2 different herds, and 11 *Staphylococcus aureus* isolates from 8 additional herds. Group 3: collected in Wallonia, Belgium; milk samples from all quarters from 114 dairy cows with composite cow SCC >300,000 cells/mL from 3 different herds.

(European Medicines Agency, 2019), although the use of amphenicols in Norway is mostly related to farmed fish (Lillehaug et al., 2018). The survey from De Briyne et al. (2014) indicates that most practitioners report prescribing sulfonamides for pigs, not cattle. However, there is not always a simple link between the use of one antimicrobial and subsequent development of resistance toward the same antimicrobial. Studies of *E. coli* from horses and calves have shown that treatment with penicillin leads to increased phenotypic resistance to multiple unrelated antimicrobials (Grønvold et al., 2010; Grønvold et al., 2011). If similar, and still cryptic, relations between use and resistance also holds true for staphylococci, it may be one explanation of the high prevalence of observed trimethoprim and trimethoprim-sulfonamide resistance. Resistance toward penicillin was most common in group 1, except in the *Staph. simulans* isolates, and second most common in groups 2 and 3, more in accordance with reported prescription patterns. Some of the observed differences in antimicrobial resistance between group 1 and groups 2 and 3 might be related to differences between the countries, as Norway generally has a low prevalence of antimicrobial resistance in dairy herds (NORM/NORM-VET, 2017).

The strong correlation between erythromycin and clindamycin resistance observed in this study is consistent with what has been shown in other studies (Lüthje and Schwarz, 2006; Li et al., 2015). This is most likely due to *erm* genes that generally confer resistance toward both macrolides and lincosamides, as well as streptogramin B. These genes are commonly found in staphylococci and are often located on mobile genetic elements, which could allow for horizontal spread of the genes (Feßler et al., 2018). It is interesting to note that stronger correlations seem to exist between the observed resistances in the *Staph. aureus* isolates compared with the NAS isolates. This could possibly be related to the plasticity of the *Staph. aureus* pan genome, with mobile elements readily exchanged between *Staph. aureus* isolates, and resistance genes possibly

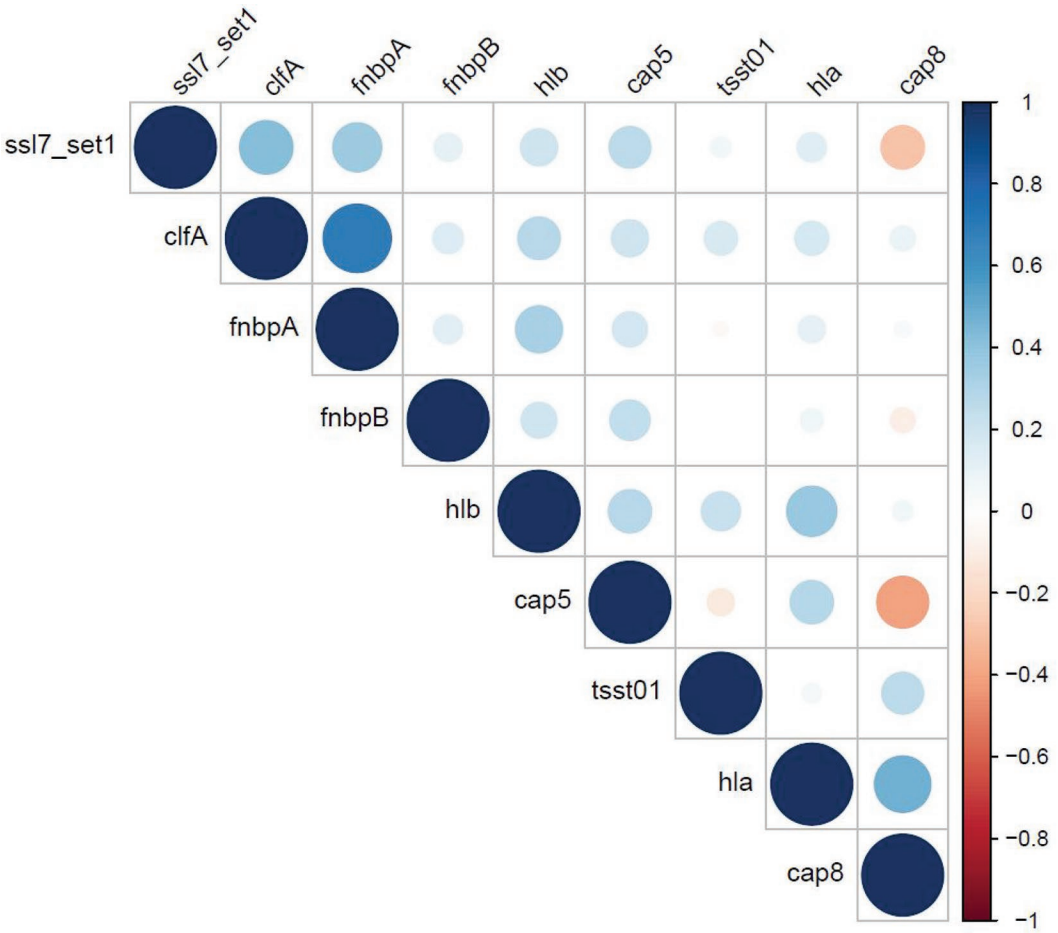

Figure 5. Correlation plot of virulence genes for *Staphylococcus aureus* isolates. Two variables with a phi coefficient >0.7 were considered to be strongly correlated.

co-localized on these mobile elements (Holden et al., 2004; Lindsay and Holden, 2004). Because this study mostly tested phenotypic resistance, it is also possible that some isolates carry resistance mechanisms conferring resistance to multiple antimicrobial classes (Wendlandt et al., 2015). Although one may assume that these genes and elements spread between staphylococcal species, it is possible that the more pathogenic *Staph. aureus* is more often exposed to antimicrobial treatments and therefore more frequently exchanges mobile elements containing resistance genes. However, because NAS are often found as commensals, it could be expected that these staphylococci are constantly exposed to systemically administered antimicrobial agents; this has also been proposed in another study (Stevens et al., 2018). This assumption may contribute to explain the development of NAS as a reservoir for antimicrobial resistance.

A previous study of Belgian farms suffering from *Staph. aureus* mastitis indicated that about 10% of these *Staph. aureus* isolates were MRSA (Vanderhaeghen et al., 2010). As documented, MRSA strains display resistance to a variety of antimicrobial agents, in addition to their resistance to β-lactams (Vanderhaeghen et al., 2010; Bardiau et al., 2013). Our results are consistent with these observations, as the 5 MRSA showed resistance to 6 to 8 of the tested antimicrobials. The high prevalence of antimicrobial resistance in MRSA is a serious problem, limiting treatment options and threatening both animal and human health.

The 5 MRSA isolates were concordantly resistant to cefoxitin on disk diffusion, yet only 2 of the *mecA*-positive NAS showed this resistance. The possibility exists that these NAS isolates may carry a variant of the *mecA* gene that does not confer resistance to cefoxitin. Studies regarding the evolution of *mecA* and the mobile genetic cassette that carries the gene in staphylococci have found that most *Staph. sciuri* harbor a gene, *mecA1*, with 80% homology to *mecA* in *Staph. aureus*. However, this gene generally does not confer methicillin resistance (Couto et al., 1996; Wu et al., 1996). It is also possible that the *mecA* gene is truncated or not expressed in these isolates. In addition, 15 NAS isolates were phenotypically resistant to cefoxitin without a positive PCR for the *mecA* gene. The explanation of why some of the NAS isolates did not show phenotypic cefoxitin resistance despite harboring a *mecA* gene and others showed resistance without being *mecA*-positive on PCR is not known at this stage and warrants further genetic studies. For example, some reports have found a plasmid-encoded *mecB* gene in *Staph. aureus* isolates, which, if present in the cefoxitin-resistant NAS, could explain the negative PCR for *mecA* and *mecC* (Becker et al., 2018).

We found a high prevalence of several virulence genes in *Staph. aureus* in all groups, especially *clfA*, *fnbpA*, and *hlb*, as well as a low occurrence of *fnbpB*. These patterns are in accordance with other studies of both bovine and human *Staph. aureus* isolates (Booth et al., 2001; Salasia et al., 2004). We observed a higher frequency of *hlb* compared with *hla* in both groups of staphylococci collected from cows with mastitis or high cow SCC (groups 1 and 3). This contrasts with findings from clinical human isolates, were *hla* appears more common (Booth et al., 2001). However, Aarestrup et al. (1999) found the *hlb* gene to be significantly more prevalent in bovine isolates compared with human isolates. This indicates that *hlb* might play a more important role in the pathogenicity of *Staph. aureus* in bovine mastitis, which is also proposed in another study (Resch et al., 2013). We detected a strong correlation between several different virulence genes, especially *ssl7* (*set1*), *hlb*, *clfA*, and *fnbpA*, further indicating that these genes are important determinants in the virulence of *Staph. aureus* associated with the bovine udder. Adherence to extracellular matrix proteins is believed to be crucial for the ability of *Staph. aureus* to colonize and invade tissue (Cremonesi et al., 2013). Fibronectin-binding proteins play a significant role in bacterial adhesion and invasion of the bovine mammary gland (Lammers et al., 1999), and the *clfA* and *clfB* genes are also associated with the initial adherence of *Staph. aureus* to the teat canal (da Costa et al., 2014). This makes *Staph. aureus* harboring *clfA* and *fnbpB* especially fit for invasion (Cremonesi et al., 2013). The *ssl7* (*set1*) gene represents a group of genes encoding staphylococcal superantigen-like proteins that shares homology with other superantigens (Langley et al., 2005). The gene encodes a protein that contributes to bacterial immune evasion, such as inhibition of phagocytosis and cytokine and chemokine secretion (Wines et al., 2011). The role of superantigens and superantigen-like proteins in the pathogenesis of bovine mastitis is not fully known. However, indications exist that these proteins play a role in bovine mastitis, inducing tissue damage and inflammation, as well as immunosuppression and immune evasion (Wilson et al., 2018). This study further supports the virulence potential of *Staph. aureus*, with many of the isolates carrying several virulence genes. It is worth noting that the 5 *mecA*-positive *Staph. aureus* isolates harbored 7 or 8 of the screened virulence genes, in addition to showing phenotypic resistance toward 6 or more of the tested antimicrobials. These isolates pose serious challenges for the management of individual udder infections, as the bacteria have a high pathogenic potential and are difficult to treat.

The screened virulence genes were derived from studies on *Staph. aureus*, potentially leading to a lower de-

tection of these genes in NAS species. Compared with *Staph. aureus*, less is known about the pathogenicity of NAS, but they possess fewer virulence properties than *Staph. aureus* (Becker et al., 2014). In more recent studies, virulence genes in NAS have been identified by whole-genome sequencing of different collections of isolates (Åvall-Jääskeläinen et al., 2018; Naushad et al., 2019), but no conclusive findings about gene content and virulence have been made.

The isolates in this study were collected from dairy farms in Norway and 2 regions (Flanders and Wallonia) in Belgium, but, as the sampling regimens were different for the 3 geographical areas, the results are not comparable between regions. The main reasons for the variability in sampling strategies were the different structures of the dairy farm industry in Norway versus Belgium, and the accessibility to farms. These differences in sampling strategies might have affected the distribution of NAS species in the different groups. In group 1, NAS species were isolated from samples primarily sent to the laboratory due to high SCC or clinical mastitis, whereas in groups 2 and 3 NAS were collected from samples from all quarters of each animal. This could explain why *Staph. simulans* was more frequently isolated in group 1 and *Staph. cohnii* more prevalent in groups 2 and 3, as *Staph. simulans* has been shown to be associated with a higher SCC compared with several other NAS species and *Staph. cohnii* more frequently associated with low SCC (Supré et al., 2011; Condas et al., 2017). There is also a possibility that isolates collected from different cows in groups 2 and 3 are part of the same strain, so-called copy strains, as *Staph. aureus* is considered a contagious pathogen (Kirkeby et al., 2019) transmitted from cow to cow.

CONCLUSIONS

By using 3 distinct collections of staphylococci from bovine milk samples of diverse origins, we were able once again to highlight the presence of antimicrobial resistance characteristics, as well as virulence genes, contributing to an increased knowledge base of this important bacterial group. Antimicrobial resistance characteristics in all 3 sample groups were more widespread in several NAS species compared with *Staph. aureus*, apart from the MRSA isolates. In all groups, antimicrobial resistance was common in *Staph. epidermidis* and *Staph. haemolyticus.* Only a few NAS isolates carried any of the virulence genes typically displayed by *Staph. aureus.* Even though the results for the MRSA in this study are concerning with regard to multidrug resistance, most of the *Staph. aureus* collected did not carry the *mecA* gene and showed relatively little resistance, giving hope that it is still possible to slow the development and spread of MRSA among dairy cattle.

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