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Diversity and antimicrobial susceptibility profiling of staphylococci isolated from bovine mastitis cases and close human contacts

T. Schmidt,*†[1] M. M. Kock,*‡ and M. M. Ehlers*‡
*Department of Medical Microbiology, University of Pretoria, Pretoria 0001, South Africa
†Allerton Provincial Veterinary Laboratory, KwaZulu-Natal Department of Agriculture and Rural Development, Cascades 3202, South Africa
‡Tshwane Academic Division, National Health Laboratory Service, Pretoria 0001, South Africa

ABSTRACT

The objectives of this study were to examine the diversity of *Staphylococcus* spp. recovered from bovine intramammary infections and humans working in close contact with the animals and to evaluate the susceptibility of the staphylococcal isolates to different antimicrobials. A total of 3,387 milk samples and 79 human nasal swabs were collected from 13 sampling sites in the KwaZulu-Natal province of South Africa. In total, 146 *Staph. aureus* isolates and 102 coagulase-negative staphylococci (CNS) were recovered from clinical and subclinical milk samples. *Staphylococcus aureus* was isolated from 12 (15.2%) of the human nasal swabs and 95 representative CNS were recovered for further characterization. The CNS were identified using multiplex-PCR assays, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and *tuf* gene sequencing. Seven *Staphylococcus* spp. were identified among the CNS of bovine origin, with *Staph. chromogenes* (78.4%) predominating. The predominant CNS species recovered from the human nasal swabs was *Staph. epidermidis* (80%) followed by *Staph. chromogenes* (6.3%). The antimicrobial susceptibility of all staphylococcal isolates was evaluated using disk diffusion and was supplemented by screening for specific antimicrobial resistance genes. Ninety-eight (67.1%) *Staph. aureus* isolates of bovine origin were pansusceptible; 39 (26.7%) isolates were resistant to a single class, and 7 (4.8%) isolates were resistant to 2 classes of antimicrobials. Two *Staph. aureus* (1.4%) isolates were multidrug-resistant. Resistance to penicillin was common, with 28.8% of the bovine and 75% of the human *Staph. aureus* isolates exhibiting resistance. A similar observation was made with the CNS, where 37.3% of the bovine and 89.5% of the human isolates were resistant to penicillin. Multidrug-resistance was common among the human CNS, with 39% of the isolates exhibiting resistance to 3 or more classes of antimicrobials. The antimicrobial susceptibility results suggest that resistance among staphylococci causing bovine intramammary infections in South Africa is uncommon and not a significant cause for concern. In contrast, antimicrobial resistance was frequently observed in staphylococcal isolates of human origin, highlighting a possible reservoir of resistance genes. Continued monitoring of staphylococcal isolates is warranted to monitor changes in the susceptibility of isolates to different classes of antimicrobials.

Key words: *Staphylococcus* spp., bovine mastitis, antimicrobial resistance, close human contact

INTRODUCTION

Bovine mastitis, or inflammation of the mammary gland, is one of the most common and economically important diseases affecting the dairy industry worldwide (Barkema et al., 2006). Mastitis primarily occurs in response to microbial infections, although chemical, physical, or traumatic factors may also lead to inflammation. A variety of bacteria have been implicated in bovine IMI (Gentilini et al., 2000), with staphylococci being considered one of the most significant and prevalent groups (Pyörälä et al., 2011).

Staphylococcus aureus is a contagious udder pathogen that readily spreads between cows at milking (Akineden et al., 2001). The main source of the bacterium is milk from infected quarters, with milking machine teat liners playing a significant role in the transmission of the bacteria among cows and mammary quarters (Zadoks et al., 2002). It is imperative that infected cows are promptly identified so that appropriate control measures can be implemented to curb bacterial transmission (Barkema et al., 2006). Other *Staphylococcus* spp., the so-called CNS, have traditionally been regarded as opportunistic pathogens of minor importance. This perception has arisen because mastitis caused by CNS is very mild and usually remains subclinical (Tenhagen et al., 2006). However, the significance of the CNS is being

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[1]Corresponding author: Tracy.Schmidt@kzndard.gov.za

reassessed because, in many countries including South Africa, CNS have become the most common bacteria isolated from bovine IMI (Petzer et al., 2009; Taponen and Pyörälä, 2009; Piessens et al., 2011; Sampimon et al., 2011).

At present, the CNS group comprises 51 species and 25 subspecies (LPSN, 2015). In routine mastitis diagnostic laboratories, CNS are usually not identified to the species level but are reported as a single group (Taponen et al., 2012). Consequently, limited knowledge is available regarding the epidemiology, pathogenicity, and relative importance of different species in this group. Research emanating primarily from European countries has suggested that some of the CNS species recovered from IMI are specifically adapted to the udder, whereas other species are environmental opportunists that only sporadically cause infections (Taponen, 2008; Thorberg et al., 2009; Piessens et al., 2011). Accurate identification of CNS is paramount in order for the epidemiology of the different species to be elucidated and appropriate control strategies to be implemented in dairy herds where CNS mastitis is a problem (Sawant et al., 2009). Although the importance of species-level identification of CNS is recognized, the heterogeneity of the species within this group presents challenges in diagnostic laboratories. Biochemical identification of staphylococcal isolates is labor intensive because of the large number of tests required to differentiate the number of species within the genus (Vanderhaeghen et al., 2015). Furthermore, commercial phenotyping systems have been found to have limited accuracy when identifying CNS of veterinary origin, presumably due to the limited number of veterinary isolates incorporated into the databases (Zadoks and Watts, 2009). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (**MALDI-TOF MS**) has been used successfully to identify mastitis pathogens (Barreiro et al., 2010; El Behiry et al., 2013); however, the capital costs of the instrumentation limit the use of MALDI-TOF MS to specialized research facilities (Wieser et al., 2012). Molecular methods, such as PCR and sequencing of housekeeping genes, have been shown to have higher discriminatory power, reproducibility, and typeability than conventional phenotyping methods and are now recommended for the accurate identification of CNS (Zadoks and Watts, 2009). Amplification and sequencing of the *tuf* gene, which encodes the elongation factor Tu, has been used successfully to differentiate CNS species (Capurro et al., 2009).

Antimicrobials are commonly used for the treatment of cows with IMI or as a preventative measure during the dry-cow period (Sawant et al., 2009). *Staphylococcus aureus* is intrinsically susceptible to most classes of antimicrobials but has the extraordinary potential to develop, or acquire, resistance to virtually any antimicrobial to which it is exposed (Chambers and DeLeo, 2009). Many of the genes encoding antimicrobial resistance determinants reside on mobile genetic elements, which can be exchanged between bacteria of the same or different species occupying the same ecological niche (Lindsay and Holden, 2006). This is of great clinical significance, as the transfer of resistance genes can lead to the emergence of more resistant and virulent bacterial strains (Lindsay and Holden, 2006). Although less pathogenic than *Staph. aureus*, CNS often exhibit greater resistance to antimicrobials and also have a greater tendency to develop multidrug resistance (**MDR**) (Taponen and Pyörälä, 2009). The CNS are believed to serve as reservoirs of antimicrobial resistance genes, which can transfer and integrate into the genome of *Staph. aureus* (Otto, 2013; Vitali et al., 2014).

Evaluating the antimicrobial susceptibility of staphylococcal isolates recovered from IMI is important to guide therapeutic treatment of infected animals (Barkema et al., 2006). Moreover, monitoring the antimicrobial resistance of *Staphylococcus* spp. in animals is important from a public health perspective. The proximity of humans and animals in the dairy environment presents opportunities for the transmission of bacteria between animal and human hosts (Juhász-Kaszanyitzky et al., 2007). The recent description of livestock-associated methicillin-resistant *Staph. aureus* (**LA-MRSA**; Graveland et al., 2011) and *Staph. aureus* strains harboring a novel methicillin-resistance gene, *mecC* (García-Álvarez et al., 2011), have renewed public concerns regarding the role of animals, particularly livestock, as a reservoir and source of antimicrobial-resistant bacteria, which can infect humans (Fitzgerald, 2012). Both LA-MRSA and *Staph. aureus* strains harboring the *mecC* gene have been recovered from bovine milk samples in several studies (Vanderhaeghen et al., 2010; García-Álvarez et al., 2011; Unnerstad et al., 2013). Inasmuch as the zoonotic transfer of bacteria is of concern from a human health perspective, the reverse scenario—the anthroponotic transfer of bacteria from humans to animals—warrants equal consideration (Fitzgerald, 2012). It is therefore important that bacterial populations at the animal–human interface should be monitored.

Antimicrobial-resistant *Staph. aureus*, particularly methicillin-resistant *Staph. aureus* (**MRSA**), represent a significant public health burden in both hospital and community settings worldwide (Chambers and DeLeo, 2009). The treatment options for infected patients are complicated by the fact that MRSA strains are MDR

because they carry other resistance genes on the cassette chromosome harboring the *mecA* gene (Holmes and Zadoks, 2011). Resistance to frontline antimicrobials such as the glycopeptide vancomycin has been documented and the alarming lack of new antimicrobials is a cause of great concern (Pantosti et al., 2007). Significant efforts are therefore aimed at conserving existing antimicrobials by promoting good antimicrobial stewardship in both human healthcare and veterinary medicine (Morley et al., 2005).

The purpose of this research was to investigate the diversity of *Staphylococcus* spp. responsible for IMI in South African dairy cows and to assess the susceptibility of the different species to antimicrobials commonly used in the veterinary field as well as antimicrobials relevant to human medicine. Concomitantly, individuals working in close contact with the animals were sampled and the diversity and susceptibility profiles of staphylococcal isolates determined and compared with staphylococcal species of animal origin.

MATERIALS AND METHODS

Sample Collection and Processing

Ethical clearance for this investigation was obtained from the Animal Ethics Committee, Faculty of Veterinary Science, University of Pretoria (H010–13) and the University of Pretoria, Faculty of Health Sciences Research Ethics Committee (No.295/2013).

KwaZulu-Natal (**KZN**) is 1 of 9 provinces in the Republic of South Africa. Commercial dairy herds (n = 9; identified by the letters A, B, C, E, F, G, I, J, and L), communal animal herds (n = 2; collectively identified by the letter M), and agriculture college herds (n = 2; identified by the letters D and H) in KZN were recruited to participate in this study through private and state veterinarians in the province. Participation was voluntary. For commercial dairy herds, the sole criterion for inclusion in this study was a history of staphylococcal mastitis. Because of the limited number of agriculture college herds in the province and the limited accessibility to communal animals, no specific selection criteria could be implemented. Herd sizes ranged from 95 to 1,450 (commercial herds), 40 to 50 (agriculture college herds), and 3 to 8 (communal herds). A single sampling was carried out at each site between October 2013 and March 2014.

Milk samples were aseptically collected into sterile sample tubes following cleaning and disinfection of the teat ends and stripping of the first 2 to 3 squirts of milk. At each of the sampling sites, close-contact workers including animal owners, dairy laborers, and veterinary practitioners were approached and asked to participate in the study by providing a self-collected nasal swab (Copan, Brescia, Italy). Following collection, all milk samples and nasal swab specimens were packed on ice and transported to the laboratory.

All milk samples were visually inspected following receipt in the laboratory. Milk samples showing any visual evidence of abnormality, such as the presence of clots, flakes or blood, were classed as clinical. No SCC was performed on clinical samples. All milk samples with a normal appearance were analyzed by flow cytometry using a Somacount instrument (Bentley Instruments, Chaska, MN), and the number of somatic cells per milliliter of sample was determined.

Microbiological Analysis of Milk Samples and Nasal Specimens

Ten microliters of each milk sample was inoculated onto one quadrant of a Columbia blood agar plate (Oxoid, Basingstoke, UK) supplemented with 5% ovine blood. Culture plates were incubated (NuAire, Plymouth, MN) at 37°C for 48 h. Nasal specimens were transferred to brain heart infusion broth (Oxoid) and incubated at 37°C overnight before being streaked onto Columbia blood agar (Oxoid) supplemented with 5% ovine blood, BBL Columbia nalidixic acid agar (Becton Dickinson, Sparks, MD), and oxacillin-resistance screening agar (Oxoid). All plates were incubated at 37°C for 48 h before being examined for the presence of staphylococcal-like colonies (Quinn et al., 1999). Preliminary testing of staphylococcal isolates included Gram staining and testing for catalase production. All catalase-positive, gram-positive, coccus-shaped bacterial isolates were tentatively identified as *Staphylococcus* spp. and tested for coagulase production using rabbit plasma (Bio-Rad, Hercules, CA) and for clumping factor using a commercial latex agglutination kit (Pastorex StaphPlus kit, Bio-Rad (Quinn et al., 1999).

Only staphylococcal isolates recovered from milk samples with an SCC $\geq$200,000 cells/mL were selected for further characterization. Additional selection criteria for CNS included the occurrence of isolates in pure growth and the presence of 5 or more colonies ($\geq$500 cfu/mL of milk) on primary culture. When more than one type of CNS was isolated from different quarters of the same cow, based on phenotypic appearance, both isolates were retained for further testing. With respect to the staphylococcal isolates recovered from human nasal swabs, all *Staph. aureus* isolates were retained along with representative CNS colonies from each individual. All isolates were stored in 25% glycerol (Merck, Darmstadt, Germany) at −80°C (NuAire) until required.

Identification of Staphylococci and Screening for mecA and pvl Genes Using PCR

One or two bacterial colonies were suspended in 50 μL of sterile deionized water. Bacterial DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. All staphylococcal isolates were amplified using a multiplex PCR assay (multiplex 1) to confirm genus identification, to identify *Staph. aureus* isolates, and to simultaneously screen isolates for the methicillin resistance gene, *mecA*, and the Panton-Valentine leukocidin encoding gene, *pvl*. Each PCR assay was carried out in a 25-μL volume comprising 1× PCR buffer (Promega, Madison, WI), 0.3 m*M* deoxynucleotide triphosphate mixture (Promega), 3 m*M* $MgCl_2$ (Promega), 0.2 μ*M* of each primer, 1.5 U of HotStart GoTaq (Promega) and 2 μL of DNA extract. Reactions were cycled in a MJ Mini thermocycler (BioRad, Chaska, MN) with the following parameters: initial denaturation (95°C for 2 min) followed by 30 cycles of amplification (94°C for 45 s, 51°C for 90 s, and 72°C for 90 s) and ending with a final extension step at 72°C for 10 min. A positive control and negative control (deionized water) was set up with every batch of PCR samples amplified. Details of all oligonucleotide primers and PCR controls are summarized in Table 1. The PCR products were resolved in a 1.5% agarose gel (SeaKem, Lonza, Rockland, ME), in 1× Tris-borate-EDTA (TBE) buffer (Melford, Ipswich, UK). Following staining in a 1 μg/mL ethidium bromide solution (BioRad), the PCR products were visualized and documented (UVIPro, Uvitec, Cambridge, UK).

Speciation of CNS Using PCR, MALDI-TOF MS, and tuf Gene Sequencing

Following genus-specific identification, CNS were identified to the species level using a combination of methods. All test isolates were amplified using 1 of 2 multiplex PCR assays (multiplex 2 and 3) based on the origin of the isolates. Reactions were prepared in 25-μL volumes using the Qiagen Multiplex PCR kit and amplified according to the manufacturer's instructions. The PCR products were analyzed as described above with the exception that a 1.8% agarose gel (SeaKem, Lonza) was used to resolve the PCR amplicons.

With the exception of one bovine CNS isolate that failed to grow, overnight cultures of all isolates were preserved in chromatography-grade ethanol (Merck) and submitted for MALDI-TOF MS analysis (Bruker Daltonics GmbH, Bremen, Germany). Coagulase-negative staphylococcal isolates that could not be identified using the multiplex-PCR assay or MALDI-TOF MS or where discordant species identifications were obtained using the 2 methods were analyzed further using *tuf* gene sequencing. The *tuf* gene was amplified according to the method described by Heikens and coworkers (2005), and PCR amplicons were submitted to Inqaba Biotechnical Industries (Pretoria, South Africa) for sequencing. Consensus sequences were analyzed in GenBank and cut-off values for the percentage of identity were adjusted to ≥97% sequence similarity.

Antimicrobial Susceptibility Testing of Staphylococcal Isolates

The antimicrobial susceptibility of the staphylococcal test isolates to 15 antimicrobials was carried out using the agar disk-diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, 2008). The antibiotics tested (Oxoid) included amoxicillin and clavulanate (20 μg/10 μg), ampicillin (10 μg), cefoxitin (30 μg), ceftaroline (30 μg), cephalothin (30 μg), clindamycin (2 μg), erythromycin (15 μg), gentamicin (10 μg), linezolid (30 μg), moxifloxacin (5 μg), penicillin (10 U), streptomycin (10 μg), tetracycline (30 μg), and trimethoprim/sulfamethoxazole (1.25/23.75 μg). The clindamycin and erythromycin disks were placed within 25 mm of each other on the agar surface to facilitate the detection of inducible clindamycin resistance or the "D-effect" (Fiebelkorn et al., 2003). A control isolate, *Staph. aureus* ATCC 25923, was tested together with each batch of field isolates to ensure the validity of the test results. The zone measurements for all antimicrobials, with the exception of fusidic acid and streptomycin, were compared with CLSI breakpoints based on data derived from human medicine (CLSI, 2008, 2014). In the case of fusidic acid, the breakpoint recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2014) was used and for streptomycin a breakpoint of ≥14 mm was used as described by Kim and coworkers (2004) and Shittu and Lin (2006). The production of β-lactamase by *Staph. aureus* isolates was determined by examining the edge of the zone of inhibition surrounding the penicillin disk. Isolates showing abrupt, heaped edges were recorded as being resistant to penicillin, irrespective of the zone diameter (CLSI, 2014). Beta-lactamase production by CNS isolates was determined using nitrocefin-impregnated sticks (Oxoid) in accordance with the manufacturer's instructions.

Resistance categories were defined based on recommendations made by Schwarz and coworkers (2010). Briefly, isolates showing susceptibility to all classes of antimicrobials tested were termed pansusceptible; isolates showing resistance to 1 or 2 classes or anti-

Table 1. Details of PCR primers used for the identification and characterization of *Staphylococcus* isolates

Gene target	Primer sequence (5′ – 3′)[1]	Size of PCR product (bp)	Multiplex PCR assay	Positive control	Reference
16S rRNA	AACTCTGTTATTAGGGAAGAACA CCACCTTCCTCCGGTTTGTCACC	756	1, 2, 3, 4, 5		McClure et al. (2006)
nuc	TCGCTTGCTATGATTCTGG GCCAATGTTCTACCATAGC	359	1	*Staph. aureus* ATCC 25923	Sasaki et al. (2010)
luk S/F	ATCATTAGGTAAAATGTCTGGACATGATCCA GCATCAAGTGTATTGGATAGCAAAAGC	433	1	*Staph. aureus*[2]	McClure et al. (2006)
mecA	GTAGAAATGACTGAACGTCCGATAA CCAATTCCACATTGTTTCGGTCTAA	310	1	*Staph. aureus*[2]	McClure et al. (2006)
Staph. chromogenes sodA	GCGTACCAGAAGATAAACAAACTC CATTATTTACAACGAGCCATGC	222	2	*Staph. chromogenes*[2]	Shome et al. (2011)
Staph. haemolyticus groESL	GGTCGCTTAGTCGGAACAAT CACGAGCAATCTCATCACCT	271	2, 3	*Staph. haemolyticus* ATCC 29970	Chiang et al. (2012)
Staph. xylosus groESL	CGCCATATTACTTATACCTCC AAGGCGATAATGTAGTATTTCA	180	2	*Staph. xylosus* ATCC 29971	Chiang et al. (2012)
Staph. simulans gap	AGCTTCGTTTACTTCTTCGATTGT AAAAGCACAAGCTCACATTGAC	472	2	*Staph. simulans* ATCC 27851	Shome et al. (2011)
Staph. epidermidis rdr	AAGAGCGTGGAGAAAAGTATCAAG TCGATACCATCAAAAAGTTGG	130	2, 3	*Staph. epidermidis* ATCC 12228	Shome et al. (2011)
Staph. warneri nuc	CGTTTGTAGCAAAACAGGGC GCAACGAGTAACCTTGCCAC	999	2	*Staph. warneri* ATCC 49454	Hirotaki et al. (2011)
Staph. hominis nuc	TACAGGGCCATTTAAAGACG GTTTCTGGTGTATCAACACC	177	3	*Staph. hominis*[2]	Hirotaki et al. (2011)
Staph. capitis nuc	ACTACGCCTATGATTATTGC GAYGCTTCTTTACCATAGGG	525	3	*Staph. capitis* ATCC 35661	Hirotaki et al. (2011)
tuf	GCCAGTTGAGGACGTATTCT CCATTTCAGTACCTTCTGGTAA	412	Uniplex	None	Heikens et al. (2005)
blaZ	AAGAGATTTGCCTATGCTTC GCTTGACCACTTTTATCAGC	517	4	*Staph. aureus* ATCC 29213	Vesterholm-Nielsen et al. (1999)
mecC	GAAAAAAAGGCTTAGAACGCCTC GAAGATCTTTTCCGTTTTCAGC	138	4	*Staph. aureus*[2]	Stegger et al. (2012)
vanA	CATGAATAGAATAAAAGTTGCAATA CCCCTTTAACGCTAATACGATCAA	1,030	4	*Enterococcus faecalis* ATCC 700221	Clark et al. (1993)
vanB	GTGACAAACCGGAGCGAGGA CCGCCATCCTCCTGCAAAAAA	433	4	*E. faecalis* ATCC 51299	Clark et al. (1993)
tetM	AGTGGAGCGATTACAGAA CATATGTCCTGGCGTGTCTA	158	5	*Staph. aureus*[2]	Strommenger et al. (2003)
tetK	GTAGCGACAATAGGTAATAGT GTAGTGACAATAAACCTCCTA	360	5	*Staph. aureus*[2]	Strommenger et al. (2003)
aacA-aphD	TAATCCAAGAGCAATAAGGGC GCCACACTATCATAACCACTA	227	5	*Staph. aureus*[2]	Strommenger et al. (2003)

[1]All primers were synthesized by Inqaba Biotechnical Industries (Pretoria, South Africa).

[2]Verified field isolate.

microbials were termed resistant; and isolates showing resistance to 3 or more classes of antimicrobials were termed MDR.

PCR Screening of Staphylococcal Isolates for Antimicrobial Resistance Determinants

Staphylococcal isolates were screened for a combination of 7 antimicrobial resistance genes using 2 multiplex PCR assays. Reactions were prepared in 25-μL volumes using the Qiagen Multiplex PCR kit and included primers specific for the *bla*Z, *mecC*, *vanA*, and *vanB* (multiplex 4) and *tetK*, *tetM*, and *aacA-aphD* (multiplex 5) genes. Amplification of PCR reactions was carried out in a MJ Mini thermocycler (BioRad) using the thermocycling conditions recommended by the kit manufacturer. Electrophoresis was carried out as described above.

RESULTS

Microbiological Analysis

A total of 3,387 milk samples from 1,374 cows and 79 human nasal swab specimens were collected from 13 dairy herds. One hundred forty-six *Staph. aureus* and 102 CNS isolates cultured from the milk samples fulfilled the selection criteria and were retained for further analysis. *Staphylococcus aureus* was recovered from 12 (15.2%) of the human nasal swabs. All 12 *Staph. aureus* isolates and the 95 CNS isolates recovered from the nasal swabs were retained for analysis.

Identification of Staphylococci and Screening for mecA and pvl Genes by PCR

All of the analyzed staphylococcal isolates were genotypically identified as *Staphylococcus* spp. using the first multiplex PCR assay. Furthermore, all of the isolates phenotypically identified as *Staph. aureus* were confirmed to be *Staph. aureus* using the same multiplex PCR assay. None of the bovine or human staphylococcal isolates was positive for the Panton-Valentine leucocidin-encoding gene, *pvl*. Fifteen CNS of human origin tested positive for the *mecA* gene.

Speciation of CNS Using PCR, MALDI-TOF MS, and tuf Gene Sequencing

A combination of 2 multiplex PCR assays and MALDI-TOF MS was used to identify the 102 CNS isolates of bovine origin and 95 CNS isolates of human origin. Five bovine isolates originating from 2 farms were not identified using either the multiplex PCR assay or MALDI-TOF MS and were analyzed further using *tuf* gene sequencing. The consensus *tuf* DNA sequences of the 5 isolates showed 99 to100% agreement with sequences already deposited in GenBank (accession no. EU571021.1). The sequences corresponded to an unidentified *Staphylococcus* species recovered from bovine mastitis cases deposited by Capurro and coworkers (2009).

A further 9 CNS were analyzed using *tuf* gene sequencing to confirm the species identification obtained using MALDI-TOF MS. The percentage distribution of CNS species isolated from both the milk samples and human specimens is presented in Table 2. Seven different *Staphylococcus* spp. were identified among the CNS isolates of bovine origin, with the majority of the isolates being identified as *Staph. chromogenes* (78.4%). Other species identified included *Staph. xylosus* (4.9%), *Staph. hyicus* (3.9%), *Staph. simulans* (3.9%), *Staph. haemolyticus* (1.9%), *Staph. lugdunensis* (0.9%), and *Staph. epidermidis* (0.9%). Nine species were identified among the CNS recovered from the human specimens, with *Staph. epidermidis* (80%), *Staph. chromogenes* (6.3%), and *Staph. haemolyticus* (5.3%) predominating.

Antimicrobial Susceptibility Testing of Staphylococcal Isolates

All staphylococcal isolates were evaluated for susceptibility to antimicrobials used for the treatment of bovine IMI as well as antimicrobials relevant to human medicine. The antimicrobial susceptibility profiles of the *Staph. aureus* isolates of bovine and human origin are summarized in Table 3. Isolates showing intermediate susceptibility were grouped together with isolates showing resistance to the antimicrobial evaluated.

The *Staph. aureus* isolates of bovine origin were divided into 8 antibiotypes based on their susceptibility patterns to the different classes of antimicrobials evaluated. Ninety-eight (67.1%) *Staph. aureus* isolates of bovine origin were susceptible to all of the antimicrobial classes evaluated (pansusceptible). Thirty-nine (26.7%) isolates were resistant to a single class of antimicrobials, whereas 7 (4.8%) were resistant to 2 classes. Two (1.4%) *Staph. aureus* isolates, from different herds, exhibited MDR. One of these isolates was resistant to the β-lactams penicillin and ampicillin, as well as tetracycline and potentiated sulfonamides. The second MDR isolate showed resistance to penicillin and ampicillin, tetracycline, and streptomycin. The *Staph. aureus* isolates of human origin were divided into 4 antibiotypes. Three (25%) of the 12 *Staph. aureus* isolates were pansusceptible; 4 *Staph. aureus* isolates (33.3%)

Table 2. Phenotypic resistance (no.; % in parentheses) of bovine and human CNS to the different antimicrobials evaluated

Origin and *Staphylococcus* spp. identification	Total no. (%) of isolates	No. (%) of MDR[1] isolates	Antimicrobial evaluated[2]													
			PEN	AMP	AMC	FOX	KF	TET	SXT	MXF	DA	E	GEN	S	FD	LZD
Bovine																
Staph. chromogenes	80 (78.4)	2 (2.5)	33 (41.3)	33 (41.3)	0	0	0	6 (7.5)	0	0	0	0	0	4 (5.0)	0	0
Staph. epidermidis	1 (0.9)	0	1 (100)	1 (100)	0	0	0	0	1 (100)	0	0	0	0	0	0	0
Staph. haemolyticus	2 (1.9)	0	1 (50)	1 (50)	0	0	0	0	0	0	0	0	0	0	0	0
Staph. hyicus	4 (3.9)	0	0	0	0	0	0	0	0	0	0	0	0	0	1 (25)	0
Staph. lugdunensis	1 (0.9)	0	1 (100)	1 (100)	0	0	0	0	0	0	0	0	0	0	0	0
Staph. simulans	4 (3.9)	0	0	0	0	0	0	1 (25)	0	0	0	0	0	1 (25)	0	0
Staph. xylosus	5 (4.9)	0	0	0	0	0	0	2 (40)	0	0	0	0	0	0	1 (20)	0
Staphylococcus spp.	5 (4.9)	0	2 (40)	2 (40)	0	0	0	0	0	0	0	0	0	0	0	0
Total	102 (100)	2 (1.9)	38 (37.3)	37 (36.3)	0	0	0	9 (8.8)	1 (1)	0	0	0	0	5 (4.9)	2 (2)	0
Human																
Staph. capitis	1 (1.1)	0	1 (100)	1 (100)	0	0	0	1 (100)	0	0	0	0	0	0	0	0
Staph. chromogenes	6 (6.3)	1 (16.7)	5 (83.3)	5 (83.3)	0	0	0	0	0	0	1 (16.7)	1 (16.7)	0	1 (16.7)	0	0
Staph. epidermidis	76 (80)	29 (38.2)	71 (93.4)	71 (93.4)	4 (5.3)	12 (15.8)	0	51 (67.1)	30 (39.5)	3 (3.9)	6 (7.9)	15 (19.7)	3 (3.9)	1 (1.3)	0	0
Staph. haemolyticus	5 (5.3)	5 (100)	5 (100)	5 (100)	0	5 (100)	0	5 (100)	5 (100)	4 (80)	0	2 (40)	0	0	0	0
Staph. hominis	1 (1.1)	1 (100)	1 (100)	1 (100)	0	0	0	1 (100)	0	0	0	1 (100)	0	0	0	0
Staph. lugdunensis	1 (1.1)	0	1 (100)	1 (100)	0	0	0	1 (100)	0	0	0	0	0	0	0	0
Staph. pseudintermedius	1 (1.1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staph. sciuri	2 (2.1)	1 (50%)	1 (50)	0	0	0	0	0	0	0	2 (100)	0	0	0	1 (50)	0
Staph. simulans	2 (2.1)	0	0	0	0	0	0	2 (100)	0	0	0	0	0	0	0	0
Total	95 (100)	37 (39.0)	85 (89.5)	84 (88.4)	4 (4.2)	17 (17.9)	0	61 (64.2)	35 (36.8)	7 (7.4)	9 (9.5)	19 (20.0)	3 (3.2)	2 (2.1)	1 (1.1)	0

[1]Multidrug resistance (resistant to ≥3 antimicrobial classes).

[2]PEN = penicillin; AMP = ampicillin; AMC = amoxicillin/clavulanic acid; FOX = cefoxitin; KF = cephalothin; TET = tetracycline; SXT = trimethoprim and sulfamethoxazole; MXF = moxifloxacin; DA = clindamycin; E = erythromycin; GEN = gentamicin; S = streptomycin; FD = fusidic acid; LZD = linezolid.

Table 3. Phenotypic susceptibility[1] of *Staphylococcus aureus* isolates recovered from bovine and human hosts

Species, farm of origin (no. of isolates)	No. (%) of isolates with phenotypic pattern	No. of classes of antimicrobials to which resistance was detected	Antimicrobial evaluated[2]														
			PEN	AMP	AMC	FOX	KF	TET	SXT	MXF	DA	E	GEN	S	FD	LZD	CPT
Bovine																	
A (3), C (3), D (15), E (28), F (20), G (9), H (3), I (4), L (13)	98 (67.1)	0	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
A (1)	1 (0.7)	1	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S
A (1), E (1), F (2)	4 (2.7)	1	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S
B (13), G (7), I (14)	34 (23.3)	1	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
I (3), L (1), G (1), B (1)	6 (4.1)	2	R	R	S	S	S	S	S	S	S	S	S	R	S	S	S
L (1)	1 (0.7)	2	S	S	S	S	S	R	S	S	S	S	S	R	S	S	S
G (1)	1 (0.7)	3	R	R	S	S	S	R	S	S	S	S	S	R	S	S	S
L (1)	1 (0.7)	3	R	R	S	S	S	R	R	S	S	S	S	S	S	S	S
No. of isolates	146 (100)		42 (28.8)	42 (28.8)	0	0	0	4 (2.7)	1 (0.7)	0	0	0	0	12 (8.2)	0	0	0
Human																	
D (1), E (1), M (1)	3 (25)	0	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
A (1), B (1), C (1), J (1)	4 (33.3)	1	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S
B (1), F (1), J (2)	4 (33.3)	2	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S
L (1)	1 (8.3)	3	R	R	S	S	S	R	R	S	S	S	S	S	S	S	S
No. of isolates	12 (100)		9 (75)	9 (75)	0	0	0	5 (41.6)	1 (8.3)	0	0	0	0	0	0	0	0

[1]S = susceptible; R = resistant.

[2]PEN = penicillin; AMP = ampicillin; AMC = amoxicillin/clavulanic acid; FOX = cefoxitin; KF = cephalothin; TET = tetracycline; SXT = trimethoprim and sulfamethoxazole; MXF = moxifloxacin; DA = clindamycin; E = erythromycin; GEN = gentamicin; S = streptomycin; FD = fusidic acid; LZD = linezolid; CPT = ceftaroline.

were resistant to a single class of antimicrobials; and a further 4 isolates (33.3%) were resistant to 2 classes of antimicrobials. A single *Staph. aureus* isolate was found to be MDR, showing resistance to penicillin and ampicillin, as well as tetracycline and the potentiated sulfonamides. None of the *Staph. aureus* isolates of bovine or human origin showed resistance to cefoxitin (surrogate for methicillin and other penicillinase-resistant β-lactams), ceftaroline, cephalothin, clindamycin, erythromycin, fusidic acid, gentamicin, linezolid, or moxifloxacin.

The antimicrobial susceptibility of the different CNS species of bovine and human origin are summarized in Tables 2 and 4. Phenotypic resistance to penicillin and ampicillin was observed in 37.3% of the bovine CNS isolates and 89.5% of the human CNS isolates. Among the 102 bovine CNS isolates evaluated, only 2 *Staph. chromogenes* (1.9%) isolates showed MDR. None of the bovine CNS isolates showed phenotypic resistance to cefoxitin, cephalothin, clindamycin, erythromycin, gentamicin, linezolid, or moxifloxacin. By comparison, 37 (39%) of the human CNS isolates were MDR. The resistance profiles of the MDR isolates are detailed in Table 4. None of the CNS isolates of human origin showed phenotypic resistance to cephalothin or linezolid. The 15 human CNS isolates, which tested positive for the presence of the *mecA* gene using the first multiplex PCR assay and were subsequently identified as *Staph. epidermidis* (n = 12) and *Staph. haemolyticus* (n = 5), were found to be phenotypically resistant to cefoxitin.

PCR Screening of Staphylococcal Isolates for Antimicrobial Resistance Determinants

The *blaZ* gene was detected in 172 of the 355 (48.5%) staphylococcal isolates examined. All 172 isolates were found to be resistant to penicillin when tested phenotypically. Two isolates, 1 *Staph. sciuri* (human origin) and 1 *Staph. chromogenes* (bovine origin), that were found to be phenotypically resistant to penicillin did not test positive for the *blaZ* gene using the multiplex PCR assay. None of the staphylococcal isolates tested positive for the presence of the *tetM* gene. The *tetK* gene was detected in 76 of 79 (96.2%) isolates that were phenotypically resistant to tetracycline. Three of the staphylococcal isolates that were phenotypically resistant to tetracycline did not carry either the *tetK* or *tetM* gene. Nine isolates of human origin (4 *Staph. epidermidis*, 5 *Staph. haemolyticus*) tested positive for the presence of the aminoglycoside resistance determinant *aacA-aphD*. None of the bovine isolates were found to be carrying this specific gene. None of the staphylococcal isolates were positive for the *mecC* gene or the vancomycin resistance determinants *vanA* and *vanB*.

DISCUSSION

Staphylococci are a common cause of bovine IMI worldwide, including South Africa (Petzer et al., 2009). This study was not designed to carry out a prevalence study but rather to facilitate the collection of a representative number of staphylococcal isolates from different dairy operations in KZN for further characterization. Consequently, the selection of participating commercial dairy herds was based on the criterion that each of the herds had a history of IMI caused by *Staphylococcus* spp.

A combination of methods was used to identify the CNS isolates of bovine and human origin. This approach was used to improve the accuracy of bacterial identification at the species level. The predominant CNS species recovered from the milk samples analyzed in this study was *Staph. chromogenes*, which accounted for 78.4% of the isolates examined. The proportion of different *Staphylococcus* species recovered from bovine IMI has varied between studies carried out in different countries. In general, *Staph. chromogenes*, *Staph. haemolyticus*, *Staph. epidermidis*, *Staph. simulans*, and *Staph. xylosus* are usually among the most commonly isolated species (Vanderhaeghen et al., 2015). In Sweden, Persson-Waller and coworkers (2011) found *Staph. chromogenes* (24%) and *Staph. epidermidis* (22%) to be the most common CNS species identified among a collection of isolates assembled from 2 national mastitis surveys. A study in Switzerland reported *Staph. xylosus* (36.0%) and *Staph. chromogenes* (16.8%) to be among the most common CNS causing mastitis (Frey et al., 2013). In the United States, Park and coworkers (2011) identified 11 CNS species from cases of mastitis, with *Staph. chromogenes* (72.2%), *Staph. xylosus* (9.1%), and *Staph. haemolyticus* (6.1%) being the most common species identified. It has been suggested that the distribution of CNS species causing IMI is herd-specific and may be influenced by specific management practices that may vary between countries (Thorberg et al., 2009; Supré et al., 2011). Species-level epidemiological studies conducted in European dairy herds have shown that *Staph. chromogenes* is a more specific udder pathogen than the other CNS and tends to cause persistent IMI (Supré et al., 2011). *Staphylococcus chromogenes* was also found to have a significant effect on the SCC of infected quarters, comparable to counts induced by *Staph. aureus* infections (Supré et al., 2011).

Five of the CNS isolates remained unidentified following PCR, MALDI-TOF MS, and sequencing of the *tuf* gene. All 5 of the isolates had the same *tuf* gene sequence and in all likelihood are the same species. The *tuf* gene sequence showed 99 to 100% similarity with sequence data deposited by Capurro and cowork-

Table 4. Antimicrobial profiles of CNS isolates showing multiple drug resistance[1]

Origin and species	Farm of origin[2]	No. of classes of antimicrobial resistance detected	Antimicrobial evaluated[3]													
			PEN	AMP	AMC	FOX	KF	TET	SXT	MXF	DA	E	GEN	S	FD	LZD
Bovine																
Staphylococcus chromogenes	D (2)	3	R	R	S	S	S	R	S	S	S	S	S	R	S	S
Human																
Staph. chromogenes	F (1)	4	R	R	S	S	S	S	S	S	R	R	S	R	S	S
Staph. epidermidis	A (1)	2	R	R	S	R	S	R	S	S	S	S	S	S	S	S
	A (1), C (3), D (1), J (1), L (1), M(1)	3	R	R	S	S	S	R	R	S	S	S	S	S	S	S
	A (1), J (1)	3	R	R	S	R	S	R	S	S	S	R	S	S	S	S
	A (1)	3	R	R	S	S	S	S	R	S	S	R	S	S	S	S
	A (1)	3	R	R	S	S	S	S	R	S	S	S	R	S	S	S
	E (1)	3	R	R	S	R	S	R	S	S	S	S	S	R	S	S
	F (1)	3	R	R	S	S	S	R	S	R	S	S	S	S	S	S
	H (1)	3	R	R	R	S	S	R	R	S	S	S	S	S	S	S
	J (1)	3	R	R	S	R	S	R	R	S	S	S	S	S	S	S
	A (1)	4	R	R	S	S	S	R	R	S	S	R	S	S	S	S
	A (1), C (1)	4	R	R	S	S	S	R	S	S	R	R	S	S	S	S
	E (1), J (1)	4	R	R	S	R	S	R	R	S	S	R	S	S	S	S
	E (1)	4	R	R	S	S	S	S	R	S	R	R	S	S	S	S
	E (1)	4	R	R	S	S	S	R	S	S	R	R	S	S	S	S
	I (1)	4	R	R	R	R	S	S	R	R	S	S	R	S	S	S
	J (1)	4	R	R	S	R	S	R	R	S	S	R	S	S	S	S
	J (1)	5	R	R	S	R	S	R	R	S	R	R	S	S	S	S
	J (1)	5	R	R	S	S	S	R	R	S	R	R	S	S	S	S
	H (1)	6	R	R	S	R	S	R	R	R	S	R	R	S	S	S
Staph. haemolyticus	A (1), H (1), L (1)	4	R	R	S	R	S	R	R	R	S	S	S	S	S	S
	H (1)	4	R	R	S	R	S	R	R	S	S	R	S	S	S	S
	M (1)	5	R	R	S	R	S	R	R	R	S	R	S	S	S	S
Staph. hominis	C (1)	3	R	R	S	S	S	R	S	S	S	R	S	S	S	S
Staph. sciuri	E (1)	3	R	R	S	S	S	S	S	S	R	S	S	S	R	S

[1]S = susceptible; R = resistant.

[2]Number of isolates in parentheses.

[3]PEN = penicillin; AMP = ampicillin; AMC = amoxicillin/clavulanic acid; FOX = cefoxitin; KF = cephalothin; TET = tetracycline; SXT = trimethoprim and sulfamethoxazole; MXF = moxifloxacin; DA = clindamycin; E = erythromycin; GEN = gentamicin; S = streptomycin; FD = fusidic acid; LZD = linezolid.

ers (2009), which interestingly belonged to an isolate recovered from mastitic milk samples in Finland. In recent years, several new *Staphylococcus* species have been described. Supré and coworkers (2010) described a novel *Staphylococcus* species, *Staph. devriesei*, which had been isolated from bovine teat apices as well as milk from study herds in Belgium and the Netherlands. Taponen and coworkers (2012) published details describing a new, previously unidentified *Staphylococcus* species, named *Staph. agnetis*. Isolates representing this newly described species were recovered from bovine milk samples (Taponen et al., 2012). Further genotyping studies will be undertaken to identify the unidentified *Staphylococcus* spp. recovered from the milk samples analyzed in the current study.

The predominant CNS recovered from the human nasal swab samples was *Staph. epidermidis*. This is not surprising as *Staph. epidermidis* is a known commensal forming part of the natural microflora on human skin (Thorberg et al., 2006). Molecular typing studies conducted by Thorberg and coworkers (2006) in Sweden showed that *Staph. epidermidis* isolates recovered from cases of mastitis were genotypically identical to those recovered from the skin of milkers. This observation led them to suggest that *Staph. epidermidis* is a zoonotic organism. In contrast to the high prevalence of *Staph. epidermidis* in the human swab samples analyzed in the current study, only one of the bovine isolates tested was identified as *Staph. epidermidis*. This may indicate that *Staph. epidermidis* is not a significant causative agent of IMI in our study area.

Overall, 15.2% of the humans sampled in this study were found to harbor *Staph. aureus*. It is reported that approximately 20% of healthy human individuals are persistent carriers of *Staph. aureus*, 30% are intermittent carriers, and 50% of individuals are never colonized by *Staph. aureus* (Kluytmans and Struelans, 2009; Graveland et al., 2011). Nasal carriage of *Staph. aureus* is recognized as a risk factor for acquiring infections and additionally plays a role in the dissemination of *Staph. aureus* in hospitals and the community (Zriouil et al., 2012).

The β-lactam antibiotics penicillin and ampicillin are used extensively for the prevention and treatment of IMI in dairy cows (IVS, 2015). In South Africa, more than half of the intramammary preparations available for use comprise penicillin or ampicillin with or without dihydrostreptomycin (IVS, 2015). Furthermore, many of the preparations are available to farmers over the counter, making it difficult to monitor and control antimicrobial usage. In our study, resistance to penicillin was the most commonly detected resistance, with 28.8% of *Staph. aureus* and 37.3% of CNS isolates showing phenotypic resistance to penicillin. This scenario parallels reports from other countries. Pyörälä and coworkers (2011) indicated that generally between one-third and two-thirds of bovine *Staph. aureus* isolates are resistant to penicillin, with lower percentages being reported in countries where strict antimicrobial policies have been implemented. Differences in the levels of bacterial resistance documented between studies are influenced by sampling strategies and bacterial populations but also by differences in test methodology and the use of different interpretation criteria (Barkema et al., 2006). It is therefore suggested that comparisons between studies be restricted to studies that have used the same methodology and interpretation criteria (Schwarz et al., 2010).

It is of great significance, from both an animal and a public health perspective, that none of the staphylococcal isolates of bovine origin exhibited resistance to cefoxitin, the in vitro surrogate for examining the phenotypic susceptibility of isolates to methicillin (CLSI, 2014). Several studies have reported on the presence of methicillin-resistant staphylococci in milk samples, raising concerns over milk as a source of methicillin-resistant bacteria. In Switzerland, 47% of the CNS isolates evaluated in vitro were found to be resistant to oxacillin (Frey et al., 2013). Vanderhaeghen and coworkers (2010) found that almost 10% of herds sampled in Belgium to have cases of mastitis caused by MRSA. The in-herd prevalence of MRSA varied between 0 and 7.4%. Furthermore, all strains had characteristics typical of the zoonotic LA-MRSA strains belonging to clonal complex (CC) 398.

In South Africa, antimicrobial susceptibility data are sparse. Three studies have reported on the occurrence of MRSA from cases of bovine mastitis with prevalence rates varying between 1.1 and 93.2% (Petzer et al., 2007; Ateba et al., 2010; Schmidt, 2011). The high prevalence of 93.2% noted in one study is largely attributable to the sampling approach and the small sample size (Ateba et al., 2010). None of the studies confirmed the presence of the *mecA* gene in the isolates exhibiting phenotypic resistance. Generally, it is accepted that screening for *mecA* is the most reliable method for the detection of methicillin resistance in staphylococcal isolates. Performing only phenotypic tests has previously led to the reporting of both false-positive and false-negative results (Vanderhaeghen et al., 2010).

Phenotypic resistance to antimicrobials other than penicillin and ampicillin was not common among the bovine staphylococcal isolates evaluated in our study. A low percentage of *Staph. aureus* (8.2%) and CNS (4.9%) isolates exhibited phenotypic resistance to streptomycin. Resistance to tetracycline was also noted, with 2.7% of the *Staph. aureus* and 8.8% of the CNS isolates showing phenotypic resistance to this antimicrobial.

The observed resistance coincides with the availability of both of these antimicrobials in intramammary preparations in South Africa (IVS, 2015). None of the bovine *Staph. aureus* or CNS isolates exhibited resistance to cephalothin, clindamycin, erythromycin, linezolid, or moxifloxacin, and only 2 isolates, 1 *Staph. hyicus* and 1 *Staph. xylosus*, were found to be phenotypically resistant to fusidic acid. The aforementioned antimicrobials have limited application in veterinary medicine but are used in human medicine to treat different types of staphylococcal infections (Lowy, 2013).

Multidrug resistance was more common among the CNS than the *Staph. aureus* isolates and more common among human isolates than bovine isolates. In total, 37 (39%) of the CNS isolates of human origin were MDR, whereas only 2 *Staph. chromogenes* isolates of bovine origin were MDR. Two *Staph. aureus* isolates of bovine origin were MDR, whereas only a single *Staph. aureus* isolate from the human nasal swabs was found to be MDR. Interestingly, the human MDR *Staph. aureus* was isolated from a farm laborer on the same farm as the MDR bovine *Staph. aureus* isolate and displayed the same antimicrobial susceptibility profile. Future genotyping studies will be undertaken to determine the genetic relatedness of the bovine and human isolates and investigate possible bacterial transmission between hosts.

Antimicrobial susceptibility testing of isolates was supplemented by screening for specific antimicrobial resistance genes. None of the staphylococcal isolates evaluated were shown to carry the *vanA* and *vanB* genes, which confer vancomycin resistance to isolates. Considering the importance and reliance on vancomycin for the treatment of MRSA infections in human medicine, the lack of vancomycin resistance markers in both the bovine and human staphylococci analyzed in our study is therefore positive.

The correlation between the phenotypic susceptibility of isolates to penicillin with the presence of the *blaZ* gene was not absolute. A *Staph. sciuri* isolate of human origin and a *Staph. chromogenes* isolate of bovine origin were found to be phenotypically resistant to penicillin but negative for the presence of the *blaZ* gene. A possible explanation for this discrepancy could be a mutation of the primer-annealing site that prevented amplification. All staphylococcal test isolates were also screened for the presence of 2 tetracycline resistance determinants: *tetK* and *tetM*. The *tetK* gene was detected in the majority of the isolates showing phenotypic resistance to tetracycline, whereas *tetM* was not detected. Three isolates, 2 *Staph. chromogenes* and 1 *Staph. haemolyticus*, that were phenotypically resistant to tetracycline tested negative for the presence of both *tetK* and *tetM*. This discrepancy could be due to the presence of other tetracyclin-resistance encoding genes, such as *tetL* or *tetO*, or due to a mutation in the primer-annealing site. The discrepancies observed between the phenotypic susceptibility of our test isolates and PCR screening for resistance genes highlight some of the shortcomings associated with the molecular detection of antimicrobial resistance. In many cases, antimicrobial resistance can be mediated by several different genes and mechanisms, some of which are still undescribed (Fluit et al., 2001).

One of the specific limitations associated with the determination of the antimicrobial susceptibility of veterinary bacterial isolates is the fact that there are currently very few antimicrobial breakpoints validated specifically for use with isolates of veterinary origin (Schwarz et al., 2010). At present, interpretive data are only available for the testing of mastitis pathogens against ceftiofur, penicillin-novobiocin, and pirlimycin (CLSI, 2008). The latter antimicrobial is currently not registered for use in South Africa (IVS, 2015). Because of the lack of data, it is common practice to make use of breakpoints derived from human medicine to interpret in vitro data. This is not ideal and should be considered when interpreting results.

CONCLUSIONS

This study provides insight into the diversity of *Staphylococcus* spp. causing IMI in cows in South African dairy herds and communal animals. The predominance of *S. chromogenes* among the CNS isolates warrants further investigation to elucidate the epidemiology and virulence characteristics of strains circulating within herds. This study further demonstrated that antimicrobial resistance among *Staphylococcus* spp. causing IMI was generally low. These results suggest the responsible usage of antimicrobials on dairy farms or the existence of management practices that do not favor the accumulation of resistance genes, or both. Because of the propensity of staphylococci to acquire antimicrobial resistance through the genetic exchange of DNA with compatible strains occupying the same microbiological niche, continued monitoring of bacterial populations is necessary.

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REFERENCES

Akineden, Ö., C. Annemüller, A. Hassan, C. Lämmler, W. Wolter, and M. Zschöck. 2001. Toxin genes and other characteristics of *Staphylococcus aureus* isolates from milk of cows with mastitis. Clin. Diagn. Lab. Immunol. 8:959–964.

Ateba, C. N., M. Mbewe, M. S. Moneoang, and C. C. Bezuidenhout. 2010. Antibiotic-resistant *Staphylococcus aureus* isolated from milk in the Mafikeng area, North West Province, South Africa. S. Afr. J. Sci. 106. http://dx.doi.org/10.4102/sajs.v106i11/12.243.

Barkema, H. W., Y. H. Schukken, and R. N. Zadoks. 2006. Invited review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. J. Dairy Sci. 89:1877–1895.

Barreiro, J. R., C. R. Ferreira, G. B. Sanvido, M. Kostrzewa, T. Maier, B. Wegemann, V. Böttcher, M. N. Eberlin, and M. V. dos Santos. 2010. Short communication: Identification of subclinical cow mastitis pathogens in milk by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J. Dairy Sci. 93:5661–5667.

Capurro, A., K. Artursson, K. Persson Waller, B. Bengtsson, H. Ericsson-Unnerstad, and A. Aspán. 2009. Comparison of a commercialized phenotyping system, antimicrobial susceptibility testing, and tuf gene sequenced-based genotyping for species-level identification of coagulase-negative staphylococci isolated from cases of bovine mastitis. Vet. Microbiol. 134:327–333.

Chambers, H. F., and F. R. DeLeo. 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat. Rev. Microbiol. 7:629–641.

Chiang, Y.-C., H.-C. Lu, S.-C. Li, Y.-H. Chang, H.-Y. Chen, C.-W. Lin, and H.-Y. Tsen. 2012. Development of PCR primers and a DNA macroarray for the simultaneous detection of major *Staphylococcus* species using *groESL* gene. Foodborne Pathog. Dis. 9:249–257.

Clark, N. C., R. C. Cooksey, B. C. Hill, J. M. Swenson, and F. C. Tenover. 1993. Characterization of glycopeptide-resistant enterococci from U.S. hospitals. Antimicrob. Agents Chemother. 37:2311–2317.

CLSI (Clinical Laboratory Standards Institute). 2008. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals–Third Edition: Vol. 28, No. 8. Approved Standard M31–A3. Clinical Laboratory Standards Institute, Wayne, PA.

CLSI (Clinical Laboratory Standards Institute). 2014. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-fourth International Supplement. CLSI document M100–S24: Vol. 34, No. 1. Clinical Laboratory Standards Institute, Wayne, PA.

El Behiry, A., R. M. Zahran, E. Marzouk, and M. Al-Dabib. 2013. Phenotypical and mass spectral assessment methods for identification of some contagious mastitis pathogens. Am. J. Microbiol. 4:32–41.

EUCAST (European Committee on Antimicrobial Susceptibility Testing). 2014. Breakpoint tables for the interpretation of MICs and zone diameters. Version 4.0, 2014. http://www.eucast.org.

Fiebelkorn, K. R., S. A. Crawford, M. L. McElmeel, and J. H. Jorgensen. 2003. Practical disk diffusion method for detection of inducible clindamycin resistance in *Staphylococcus aureus* and coagulase-negative staphylococci. J. Clin. Microbiol. 41:4740–4744.

Fitzgerald, J. R. 2012. Livestock-associated *Staphylococcus aureus*: Origin, evolution and public health threat. Trends Microbiol. 20:192–198. http://dx.doi.org/10.1016/j.tim.2012.01.006.

Fluit, A. C., M. R. Visser, and F.-J. Schmitz. 2001. Molecular detection of antimicrobial resistance. Clin. Microbiol. Rev. 14:836–871.

Frey, Y., J. P. Rodriguez, A. Thomann, S. Schwendener, and V. Perreten. 2013. Genetic characterization of antimicrobial resistance in coagulase-negative staphylococci from bovine mastitis milk. J. Dairy Sci. 96:2247–2257.

García-Álvarez, L., M. Holden, H. Lindsay, C. Webb, D. Brown, M. Curran, E. Walpole, K. Brooks, D. Pickard, C. Teale, J. Parkhill, S. Bentley, G. Edwards, E. Girvan, A. Kearns, B. Pichon, R. Hill, A. Larsen, R. Skov, S. Peacock, D. Maskell, and M. Holmes. 2011. Methicillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine populations in the UK and Denmark: A descriptive study. Lancet Infect. Dis. 11:595–603.

Gentilini, E., G. Denamiel, P. Llorente, S. Godaly, M. Rebuelto, and O. DeGregorio. 2000. Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Argentina. J. Dairy Sci. 83:1224–1227.

Graveland, H., B. Duim, E. van Duijkeren, D. Heederik, and J. Wagenaar. 2011. Livestock-associated methicillin-resistant *Staphylococcus aureus* in animals and humans. Int. J. Med. Microbiol. 301:630–634.

Heikens, E., A. Fleer, A. Paauw, A. Florijn, and A. Fluit. 2005. Comparison of genotypic and phenotypic methods for species-level identification of clinical isolates of coagulase-negative staphylococci. J. Clin. Microbiol. 43:2286–2290.

Hirotaki, S., T. Sasaki, K. Kuwahara-Arai, and K. Hiramatsu. 2011. Rapid and accurate identification of human-associated staphylococci by use of multiplex PCR. J. Clin. Microbiol. 49:3627–3631.

Holmes, M. A., and R. N. Zadoks. 2011. Methicillin resistant *S. aureus* in human and bovine mastitis. J. Mammary Gland Biol. Neoplasia 16:373–382.

IVS. 2015. Index of Veterinary Specialities. Vol 53. Hirt and Carter, Durba, South Africa.

Juhász-Kaszanyitzky, É., S. Jánosi, P. Somogyi, Á. Dán, L. van der Graaf-van Bloois, E. van Duijkeren, and J. Wagenaar. 2007. MRSA transmission between cows and humans. Emerg. Infect. Dis. 13:630–632.

Kim, H. B., H.-C. Jang, H. J. Nam, Y. S. Lee, B. S. Kim, W. B. Park, K. D. Lee, Y. J. Choi, S. W. Park, M. D. Oh, E.-C. Kim, and K. W. Choe. 2004. In vitro activities of 28 antimicrobial agents against *Staphylococcus aureus* isolates from tertiary-care hospitals in Korea: A nationwide survey. Antimicrob. Agents Chemother. 48:1124–1127.

Kluytmans, J., and M. Struelans. 2009. Methicillin-resistant *Staphylococcus aureus* in the hospital. BMJ 338:b364.

Lindsay, J. A., and M. T. G. Holden. 2006. Understanding the rise of the superbug: Investigation of the evolution and genomic variation of *Staphylococcus aureus.* Funct. Integr. Genomics 6:186–201.

Lowy, F. 2013. Staphylococcal infections. Pages 159–170 in Harrison's Principles of Internal Medicine. 18th ed. D. L. Longo, A. S. Fauci, D. L., Kasper, S. L. Hauser, J. J. Jameson, and J. Loscalzo, ed. McGraw-Hill, New York, NY.

LPSN. 2015. List of Prokaryotic Names with Standing in Nomenclature. Accessed Apr. 8, 2015. http://www.bacterio.net/staphylococcus.html.

McClure, J.-A., J. M. Conly, V. Lau, S. Elsayed, T. Louie, W. Hutchins, and K. Zhang. 2006. Novel Multiplex PCR assay for detection of staphylococcal virulence marker Panton-Valentine leukocidin genes and simultaneous discrimination of methicillin-susceptible from –resistant staphylococci. J. Clin. Microbiol. 44:1141–1144.

Morley, P. S., M. D. Apley, T. E. Besser, D. P. Burney, P. J. Fedorka-Cray, M. G. Papich, J. L. Traub-Dargatz, and J. S. Weese. 2005. Antimicrobial drug use in veterinary medicine. J. Vet. Intern. Med. 19:617–629.

Otto, M. 2013. Coagulase-negative staphylococci as reservoirs of genes facilitating MRSA infection. BioEssays 35:4–11.

Pantosti, A., A. Sanchini, and M. Monaco. 2007. Mechanisms of antibiotic resistance in *Staphylococcus aureus.* Future Microbiol. 2:323–334.

Park, J.-Y., L. K. Fox, K. S. Seo, M. A. McGuire, Y. H. Park, F. R. Rurangirwa, W. M. Sischo, and G. A. Bohach. 2011. Comparison of phenotypic and genotypic methods for the species identification of coagulase-negative staphylococcal isolates from bovine intramammary infections. Vet. Microbiol. 147:142–148.

Persson Waller, K., A. Aspán, A. Nyman, Y. Persson, and U. G. Andersson. 2011. CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis. Vet. Microbiol. 152:112–116.

Petzer, I.-M., J. Karzis, T. J. van der Schans, J. C. Watermeyer, and M. Smither. 2007. Antibiotic efficacy against staphylococcal udder pathogens in dairy cows in South Africa from 2000 to 2006. Pages 33–49. Livestock Health and Production Group of the South African Veterinary Association Congress. Vetlink, Pretoria, South Africa.

Petzer, I.-M., J. Karzis, J. Watermeyer, T. J. van der Schans, and R. van Reenen. 2009. Trends in udder health and emerging mastitogenic pathogens in South African dairy herds. J. S. Afr. Vet. Assoc. 80:17–22.

Piessens, V., E. van Coillie, B. Verbist, K. Supre, G. Braem, A. van Nuffel, and L. de Vuyst. 2011. Distribution of coagulase-negative *Staphylococcus* species from milk and environment of dairy cows differs between herds. J. Dairy Sci. 94:2933–2944.

Pyörälä, S., H. Simojoki, and S. Taponen. 2011. News about mastitis-causing staphylococci. European Buiatrics Forum, Marseille, France.

Quinn, P. J., M. E. Carter, B. Markey, and G. R. Carter. 1999. Staphylococcus species. Pages 118–126 in Clinical Veterinary Microbiology. Mosby, Madrid, Spain.

Sampimon, O. C., T. Lam, D. Mevius, Y. Schukken, and R. Zadoks. 2011. Antimicrobial susceptibility of coagulase-negative staphylococci isolated from bovine milk samples. Vet. Microbiol. 150:173–179.

Sasaki, T., S. Tsubakishita, Y. Tanaka, A. Sakusabe, M. Ohtsuka, S. Hirotaki, T. Kawakami, T. Fukata, and K. Hiramatsu. 2010. Multiplex-PCR method for species identification of coagulase-positive staphylococci. J. Clin. Microbiol. 48:765–769.

Sawant, A. A., B. E. Gillespie, and S. Oliver. 2009. Antimicrobial susceptibility of coagulase-negative *Staphylococcus* species isolated from bovine milk. Vet. Microbiol. 134:73–81.

Schmidt, T. 2011. *In vitro* antimicrobial susceptibility of *Staphylococcus aureus* strains from dairy herds in KwaZulu-Natal. J. S. Afr. Vet. Assoc. 82:76–79.

Schwarz, S., P. Silley, S. Simjee, N. Woodford, E. van Duijkeren, A. Johnson, and W. Gaastra. 2010. Editorial: Assessing the antimicrobial susceptibility of bacteria obtained from animals. J. Antimicrob. Chemother. 65:601–604.

Shittu, A. O., and J. Lin. 2006. Antimicrobial susceptibility patterns and characterization of clinical isolates of *Staphylococcus aureus* in KwaZulu-Natal province, South Africa. BMC Infect. Dis. 6:125.

Shome, B. R., S. Das Mitra, M. Bhuvana, N. Krithiga, D. Velu, R. Shome, S. Isloor, S. B. Barbuddhe, and H. Rahman. 2011. Multiplex PCR assay for species identification of bovine mastitis pathogens. J. Appl. Microbiol. 111:1349–1356.

Stegger, M., P. Anderson, A. Kearns, B. Pichon, M. Holmes, G. Edwards, F. Laurent, C. Teale, R. Skov, and A. Larsen. 2012. Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either *mecA* or the new *mecA* homologue $mecA_{LGA251}$. Clin. Microbiol. Infect. 18:395–400.

Strommenger, B., C. Kettlitz, G. Werner, and W. Witte. 2003. Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in *Staphylococcus aureus*. J. Clin. Microbiol. 41:4089–4094.

Supré, K., S. De Vliegher, I. Cleenwerck, K. Engelbeen, S. Van Trappen, S. Piepers, O. S. Sampimon, R. N. Zadoks, P. De Vos, and F. Haesebrouck. 2010. *Staphylococcus devriesei* sp. nov., isolated from teat apices and milk of dairy cows. Int. J. Syst. Evol. Microbiol. 60:2739–2744.

Supré, K., F. Haesebrouck, R. N. Zadoks, M. Vaneechoutte, S. Piepers, and S. De Vliegher. 2011. Some coagulase-negative *Staphylococcus* species affect udder health more than others. J. Dairy Sci. 94:2329–2340.

Taponen, S. 2008. Bovine mastitis caused by coagulase-negative staphylococci. Doctoral Diss. Univ. Helsinki, Helsinki, Finland.

Taponen, S., and S. Pyörälä. 2009. Coagulase-negative staphylococci as cause of bovine mastitis – not so different from *Staphylococcus aureus?* Vet. Microbiol. 134:29–36.

Taponen, S., K. Supré, V. Piessens, E. Van Coillie, S. De Vliegher, and J. M. K. Koort. 2012. *Staphylococcus agnetis* sp. nov., a coagulase-variable species from bovine subclinical and mild clinical mastitis. Int. J. Syst. Evol. Microbiol. 62:61–65.

Tenhagen, B. A., G. Koster, J. Wallmann, and W. Heuwieser. 2006. Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. J. Dairy Sci. 89:2542–2551.

Thorberg, B. M., M. L. Danielsson-Tham, U. Emanuelson, and K. Persson Waller. 2009. Bovine subclinical mastitis caused by different types of coagulase-negative staphylococci. J. Dairy Sci. 92:4962–4970.

Thorberg, B. M., I. Kühn, F. Aarestrup, B. Brändström, P. Jonsson, and M.-L. Danielsson-Tham. 2006. Pheno- and genotyping of *Staphylococcus epidermidis* isolated from bovine milk and human skin. Vet. Microbiol. 115:163–172.

Unnerstad, H. E., B. Bengtsson, M. Horn af Rantzien, and S. Börjesson. 2013. Methicillin-resistant *Staphylococcus aureus* containing *mecC* in Swedish dairy cows. Acta Vet. Scand. 55:6 http://dx.doi.org/10.1186/1751-0147-55-6.

Vanderhaeghen, W., T. Cerpentier, C. Adriaensen, J. Vicca, K. Hermans, and P. Butaye. 2010. Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. Vet. Microbiol. 144:166–171.

Vanderhaeghen, W., S. Piepers, F. Leroy, E. Van Coillie, F. Haesebrouck, and S. De Vliegher. 2015. Identification, typing, ecology and epidemiology of coagulase negative staphylococci associated with ruminants. Vet. J. 203:44–51. http://dx.doi.org/10.1016/j.tvjl.2014.11.001.

Vesterholm-Nielsen, M., M. Larsen, J. Olsen, and F. Aarestrup. 1999. Occurrence of the *blaZ* gene in penicillin resistant *Staphylococcus aureus* isolated from bovine mastitis in Denmark. Acta Vet. Scand. 40:279–286.

Vitali, L. A., D. Petrelli, A. Lamikanra, M. Prenna, and E. O. Akinkunmi. 2014. Diversity of antibiotic resistance genes and staphylococcal cassette chromosome *mec* elements in faecal isolates of coagulase-negative staphylococci from Nigeria. BMC Microbiol. 14:106.

Wieser, A., L. Schneider, J. Jung, and S. Schubert. 2012. MALDI-TOF MS in microbiological diagnostics – identification of microorganisms and beyond (mini review). Appl. Microbiol. Biotechnol. 93:965–974.

Zadoks, R. N., W. B. van Leeuwen, D. Kreft, L. K. Fox, H. W. Barkema, Y. H. Schukken, and A. van Belkum. 2002. Comparison of *Staphylococcus aureus* from bovine and human skin, milking equipment, and bovine milk by phage typing, pulsed-field gel electrophoresis, and binary typing. J. Clin. Microbiol. 40:3894–3902.

Zadoks, R. N., and J. L. Watts. 2009. Species identification of coagulase-negative staphylococci: Genotyping is superior to phenotyping. Vet. Microbiol. 134:20–28.

Zriouil, S. B., M. Bekkali, and K. Zerouali. 2012. Epidemiology of *Staphylococcus aureus* infections and nasal carriage at the Ibn Rochd University hospital center, Casablanca, Morocco. Braz. J. Infect. Dis. 16:279–283.