Molecular Characterization of *Staphylococcus aureus* Isolated from Bovine Mastitis and Close Human Contacts in South African Dairy Herds: Genetic Diversity and Inter-Species Host Transmission

Tracy Schmidt [1,2]*, Marleen M. Kock [2,3] and Marthie M. Ehlers [2,3]

[1] Allerton Provincial Veterinary Laboratory, KwaZulu-Natal Department of Agriculture and Rural Development, Pietermaritzburg, South Africa, [2] Department of Medical Microbiology, University of Pretoria, Pretoria, South Africa, [3] Tshwane Academic Division, National Health Laboratory Service, Pretoria, South Africa

Staphylococcus aureus is one of the most common etiological agents of contagious bovine mastitis worldwide. The purpose of this study was to genetically characterize a collection of *S. aureus* isolates (bovine = 146, human = 12) recovered from cases of bovine mastitis and nasal swabs of close human contacts in the dairy environment. Isolates were screened for a combination of clinically significant antimicrobial and virulence gene markers whilst the molecular epidemiology of these isolates and possible inter-species host transmission was investigated using a combination of genotyping techniques. None of the isolates under evaluation tested positive for methicillin or vancomycin resistance encoding genes. Twenty seven percent of the bovine *S. aureus* isolates tested positive for one or more of the pyrogenic toxin superantigen (PTSAg) genes with the *sec* and *sell* genes predominating. Comparatively, 83% of the human *S. aureus* isolates tested positive for one or more PTSAg genes with a greater variety of genes being detected. Genomic DNA macrorestriction followed by pulsed-field gel electrophoresis (PFGE) of the bovine isolates generated 58 electrophoretic patterns which grouped into 10 pulsotypes at an 80% similarity level. The majority of the bovine isolates, 93.2% (136/146), clustered into four major pulsotypes. Seven sequence types (ST) were identified among the representative bovine *S. aureus* isolates genotyped, including: ST8 (CC8), ST97 (CC97), ST351 (CC705), ST352 (CC97), ST508 (CC45), ST2992 (CC97) and a novel sequence type, ST3538 (CC97). Based on PFGE analysis, greater genetic diversity was observed among the human *S. aureus* isolates. Bovine and human isolates from three sampling sites clustered together and were genotypically indistinguishable. Two of the isolates, ST97 and ST352 belong to the common bovine lineage CC97, and their isolation from close human contacts suggests zoonotic transfer. In the context of this study, the third isolate, ST8 (CC8), is believed to be a human

clone which has transferred to a dairy cow and has subsequently caused mastitis. The detection of indistinguishable *S. aureus* isolates from bovine and human hosts at three of the sampling sites is suggestive of bacterial transmission and supports the need for vigilant monitoring of staphylococcal populations at the human-animal interface.

Keywords: *Staphylococcus aureus*, bovine mastitis, PFGE, MLST, inter-species transmission

INTRODUCTION

Staphylococcus aureus is a versatile pathogen responsible for a variety of infections in humans and animals (Hata et al., 2008). In humans *S. aureus* is responsible for a number of conditions ranging from superficial skin infections to life-threatening diseases, such as endocarditis and hemolytic pneumonia (Lindsay and Holden, 2004). Additionally, through the production of toxins, *S. aureus* can cause specific toxin-mediated conditions, such as scalded skin syndrome, staphylococcal food poisoning and toxic shock syndrome (Becker et al., 2015). In animals, *S. aureus* is a common etiological agent of mastitis, an infectious disease condition responsible for significant financial losses to dairy farmers worldwide (Petrovski et al., 2006; Fitzgerald, 2012).

The success of *S. aureus* as a pathogen is attributable, in part, to the diverse range of virulence factors produced (Gordon and Lowy, 2008). The virulence factors facilitate the invasion and colonization of host tissue, evasion of the hosts' immune defence mechanisms, aid in acquisition of nutrients and dissemination of the bacteria within the host tissue (Ferry et al., 2005; Haveri et al., 2008). Among the vast array of virulence factors produced are numerous enzymes and cytotoxins, such as coagulase, collagenase, exfoliative toxins, hemolysins, hyaluronidase, leukocidins, lipases, nucleases and staphylokinase (Smeltzer et al., 2009). Most *S. aureus* strains are able to produce one or more pyrogenic toxin superantigens (PTSAgs) which includes staphylococcal enterotoxins and the structurally related protein, toxic shock syndrome toxin-1 (TSST-1) (Wright and Novick, 2003). All of these toxins exhibit superantigenic activity by interacting with antigen-presenting cells and T-lymphocytes irrespective of the antigen specificity of the cells (Akineden et al., 2001; Argudín et al., 2010). This interaction leads to a massive proliferation of T-cells and the uncontrolled release of pro-inflammatory cytokines that can lead to clinical signs in the host that include, fever, hypotension and shock (Plata et al., 2009; Argudín et al., 2010). The production of enterotoxins is particularly significant from a public health standpoint as the ingestion of preformed toxins is a major cause of food poisoning worldwide (Le Loir et al., 2003; Srinivasan et al., 2006). The expression of most staphylococcal virulence factors occurs in a co-ordinated manner and is strictly controlled by a series of regulatory genes which operate under the control of the accessory gene regulator (*agr*) (Ben Ayed et al., 2008; Lowy, 2013).

Staphylococcus aureus is a contagious mastitis pathogen with the milk from infected mammary glands serving as the primary reservoir of the bacterium which may be transferred to other animals in the herd during milking (Capurro et al., 2010). Several epidemiological studies have suggested that a few specialized *S. aureus* strains are responsible for the majority of bovine intramammary infections (IMIs) worldwide (Kapur et al., 1995; Zadoks et al., 2002; Sakwinska et al., 2011; Budd et al., 2015). However, the occurrence of herd-specific strains has also been reported (Joo et al., 2001; Sommerhäuser et al., 2003; Rabello et al., 2007; Oliveira et al., 2011). Moreover, it has been demonstrated that different strains are associated with different clinical outcomes in the host including severity and persistence of infections and response to antimicrobial treatment (Haveri et al., 2005, 2007; Lundberg et al., 2014). A better understanding of the epidemiology, including transmission and virulence characteristics of *S. aureus* strains may assist with the development of strategies to control the spread of the bacterium within herds (Haveri et al., 2005, 2008).

In the dairy environment documented evidence of methicillin-resistant *S. aureus* transmission between cows and close human contacts exists (Juhász-Kaszanyitzky et al., 2007; Haenni et al., 2010). Further, reports of livestock-associated MRSA (LA-MRSA) ST398 causing mastitis have created additional public health concerns regarding production animals, including dairy cows, as a source of antimicrobial-resistant bacteria which can spill-over into humans (Monecke et al., 2007; Feßler et al., 2010; Fitzgerald, 2012; Tenhagen et al., 2014). It is now commonly accepted that persons working in close contact with animals are at a higher risk of being colonized or even infected with zoonotic bacteria carried by animals than individuals who do not interact with animals (Graveland et al., 2011). It is therefore recommended that surveillance at the interface between human and animal hosts is monitored in order to detect any potential, emerging human health risks (Fitzgerald, 2012; Cuny et al., 2015).

A number of genotyping methods have been used to characterize *S. aureus* isolates including virulence gene profiling, *agr* typing, pulsed-field gel electrophoresis (PFGE), *spa* sequence typing and multilocus sequence typing (MLST) (Hata et al., 2010; Kadlec et al., 2015). The application of whole genome sequencing (WGS) has gained traction during the past decade as technological advances have improved the accessibility and cost-effectiveness of high-throughput sequencing (Trees et al., 2015). At present however, WGS still remains unaffordable in resource-limited settings.

In a previous study our group investigated the diversity and antimicrobial susceptibility of staphylococci responsible for causing bovine IMIs (Schmidt et al., 2015). Concurrent sampling and characterization of staphylococci from close contact workers facilitated a comparison between isolates from different hosts. To date no genotyping studies have been undertaken in South Africa to investigate the genetic diversity of *S. aureus* strains causing IMIs and the potential public health risk posed by these

bacterial populations. Using our previous work as a platform the purpose of the present study was to (i) screen isolates for clinically significant antimicrobial and virulence gene markers including: antimicrobial resistance (*bla*Z, *mec*A, *mec*C, *van*A, *van*B), exfoliative toxin (*eta*, *etb*, *etd*), hemolysin (*hla*, *hlb*, *hld*, *hlg*, *hlg2*), leukocidin (*luk*ED, *luk*M, *luk*S/F) and pyrogenic superantigen toxins (*sea* to *see*, *seg* to *sei*, *selj* to *selr*, *selu* and *tst*) among bovine and human *S. aureus* isolates; (ii) investigate the diversity of *S. aureus* genotypes causing mastitis in dairy herds through genomic macrorestriction and PFGE analysis of isolates; (iii) relate the occurrence of local genotypes to global *S. aureus* lineages, and (iv) evaluate the genetic relatedness of *S. aureus* isolates causing mastitis and isolates recovered from humans working in close contact with the animals.

MATERIALS AND METHODS

Ethics Statement

Ethical clearance for this investigation was obtained from the Animal Ethics Committee (Faculty of Veterinary Science, H010-13) and the Research Ethics Committee (Faculty of Health Sciences, 295/2013) of the University of Pretoria. Participation in this study was voluntary and written informed consent was obtained from all animal owners and human contacts prior to sampling.
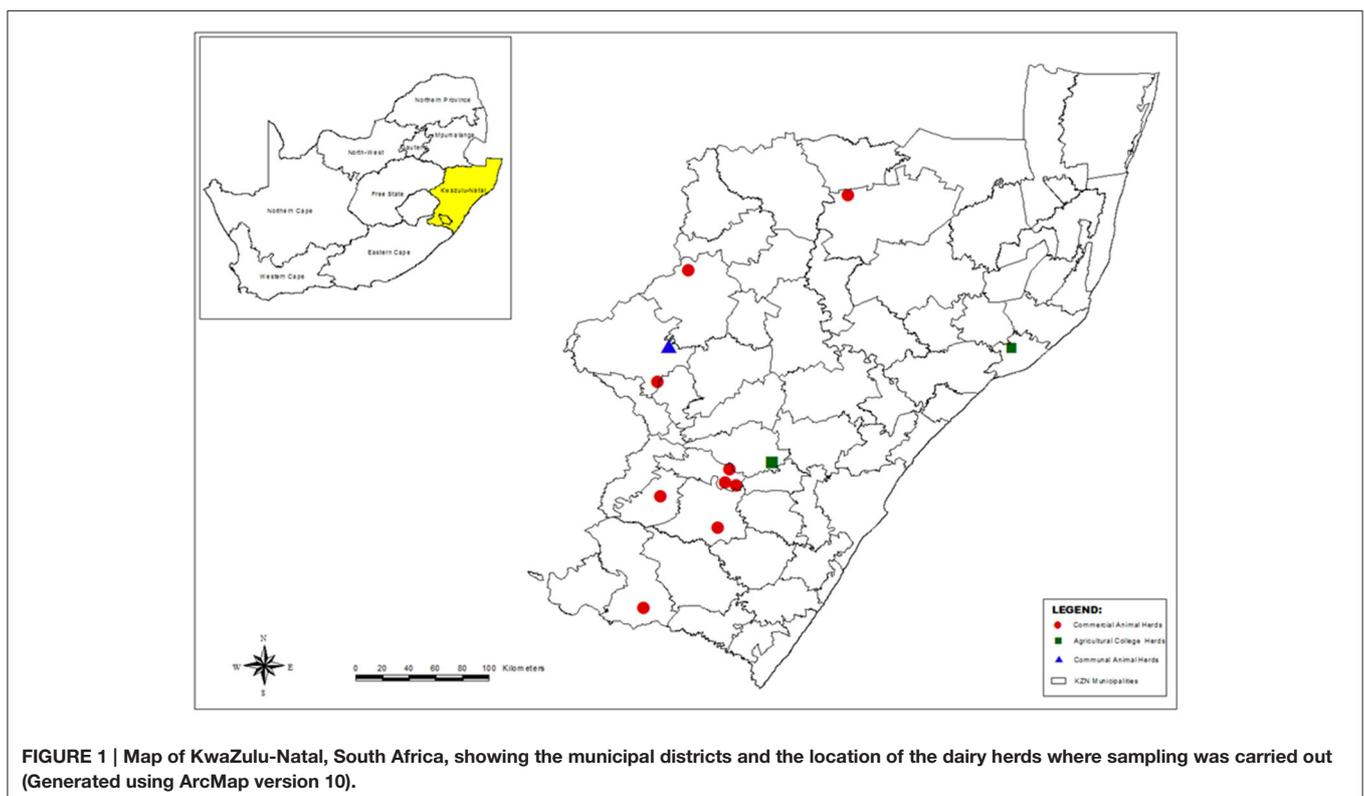
Bacterial Isolates

A total of 146 *S. aureus* isolates from milk and 12 isolates from human nasal swabs were characterized in this study. The samples originated from 13 sampling sites (identified by letters A to J and L to M) located in the province of KwaZulu-Natal (KZN), South Africa (**Figure 1**). At the time of sampling animal owners were asked to provide details regarding the: average size of their dairy herd; intramammary preparations used; number of laborers working in the dairy; and the source of new heifers.

Sample collection, processing and bacteriological analyses are described in detail elsewhere (Schmidt et al., 2015). All *Staphylococcus aureus* isolates were identified by means of standard phenotyping methods including coagulase production using rabbit plasma (Bio-Rad, USA) and the detection of clumping factor, fibronectin-binding proteins and protein A using the Pastorex™ StaphPlus latex agglutination test kit (BioRad, USA). Phenotypic results were confirmed using a multiplex-PCR (M-PCR) assay (Schmidt et al., 2015).

Detection of Virulence Factors and Immune Evasion Cluster Genes

The genomic DNA of all test isolates was extracted using the DNeasy blood and tissue kit (Qiagen, Germany) in accordance with the manufacturer's instructions. Isolates were evaluated using nine M-PCR assays (AMR1, VGP1 to VGP8) to screen for antimicrobial resistance (*bla*Z, *mec*C, *van*A, and *van*B), exfoliative (*eta*, *etb*, *etd*), hemolysin (*hla*, *hlb*, *hld*, *hlg*, *hlg2*), leukocidin (*luk*M, *luk*ED) and PTSAg (*sea* to *see*, *seg* to *sei*, *selj* to *selr*, *seu*, *tst*) genes. Multiplex PCR assays were carried out using the Qiagen Multiplex PCR kit (Germany) according to the manufacturer's instructions. The presence of the human immune

FIGURE 1 | Map of KwaZulu-Natal, South Africa, showing the municipal districts and the location of the dairy herds where sampling was carried out (Generated using ArcMap version 10).

evasion cluster (IEC) genes, *chp*, *sak* and *scn* were assayed using separate uniplex PCR assays. Two microliters of each DNA extract was added to PCR reaction mixtures comprising of 1X PCR buffer (Promega, USA), 1.5 mM MgCl$_2$(Promega, USA), 0.4 mM dNTPs (Promega, USA), 0.5 μM of each primer and 2.5 IU HotStart Taq (Promega, USA).

Details of all the oligonucleotide primers, PCR assay conditions and positive control isolates used are summarized in **Table 1**. All PCR reactions were cycled in a MJ Mini thermocycler (BioRad, USA) with appropriate positive (**Table 1**) and negative (deionized water) controls. Amplification products were electrophoresed in a 1.8% (m/v) agarose gel (SeaKem, Lonza, USA) in a 1X Tris-borate-EDTA (TBE) buffer (Melford, UK). Following staining in a 1 μg/mL ethidium bromide solution (10 mg/mL) (BioRad, USA) the PCR products were visualized and documented (UVIPro, Uvitec, UK). A 100 bp molecular weight marker (MWM) (Solis BioDyne, Estonia or Thermo Scientific, USA) was electrophoresed alongside samples to enable the size of the PCR products to be determined.

Accessory Gene Regulator (*agr*) typing

The *agr* allele types (I-IV) of all *S. aureus* isolates were determined as described by Gilot et al. (2002). The multiplex-PCR assay comprised of a forward primer, Pan, common to all *agr* groups and four reverse primers each one specific to each *agr* group (Gilot et al., 2002). Amplification products were electrophoresed as described in Section Detection of Virulence Factors and Immune Evasion Cluster Genes.

Genomic Macrorestriction and Pulsed-Field Gel Electrophoresis of *Staphylococcus aureus* Isolates

All *S. aureus* isolates were characterized by macrorestriction of genomic DNA using *Sma*I followed by PFGE of the restriction products. The procedure followed is based on protocols described by Graves and Swaminathan (2001) and McDougal et al. (2003) but with several modifications. *Staphylococcus aureus* ATCC 12600 was processed and electrophoresed together with each batch of test samples. Bacterial cell suspensions were prepared in TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0, Sigma-Aldrich, USA) and the turbidity adjusted to 1.2–1.6 using a microplate reader at 630 nm (ELx 800, BioTEK, USA). Twenty microliters of lysozyme (20 mg/mL; Sigma-Aldrich, USA) was added to 400 μL of each cell suspension and incubated (LabNet International Inc., USA) at 56°C for 20 min. Following incubation, 20 μL proteinase K (20 mg/mL; Roche, Germany) and 5 μL lysostaphin (1 mg/mL; Sigma-Aldrich, USA) was added to each sample before an equal volume of molten 1.2% (m/v) SeaKem® LE agarose (Lonza, USA) was added. Each sample suspension was gently mixed before being transferred to a re-usable plug mold (Biometra, Germany) and allowed to solidify. Sample plugs were transferred to separate 15 mL conical tubes (Falcon, México) containing 5 mL cell lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA, pH 8.0, 1% sodium lauryl sarcosine, Sigma-Aldrich, USA) and 25 μL proteinase K (20 mg/mL) (Roche, Germany). Sample tubes were incubated (Stuart®, Bibby

Scientific Ltd., UK) for 18–24 h at 51°C with constant agitation at 170 rpm. Following incubation the plugs were washed as described by Graves and Swaminathan (2001). Appropriately sized slices were cut from each plug and digested with 50 IU *Sma*I (New England BioLabs, USA) at 37°C for 2 h. Plug slices were sealed in the wells of a 1.2% (m/v) SeaKem® LE agarose gel (Lonza, USA) and electrophoresed in 0.25X TBE buffer (pH 8.3; Sigma-Aldrich, USA) using the Rotaphor® system (Biometra, Germany). The electrophoretic parameters were set as follows: switch times of 5–40 s; angle 120° constant; 220 V linear to 200 V for 25 h at 13°C. Following electrophoresis the gel was stained in a 0.25 μg/mL ethidium bromide solution (10 mg/mL; Sigma-Aldrich, USA) for 15 min and de-stained in deionized water for 30 min. Gels were visualized and captured using a gel documentation system (UVPro, Uvitec, UK). All gel images were analyzed using the GelCompar *II* software program (Applied Maths, Belgium). Similarity co-efficients were calculated and a dendrogram constructed using the Dice coefficient and UPGMA. A band position tolerance of 1.0% was used and the cluster (pulsotype, PT) cut-off was set at an 80% similarity level which corresponds to the Tenover et al. (1995) criteria of four to six band differences between related isolates (McDougal et al., 2003; Faria et al., 2008). All pulsotypes were assigned Arabic numerals and were classified as being either major pulsotypes (≥5 isolates), minor pulsotypes (two to three isolates) or singletons. Sixteen bovine isolates, representative of all pulsotypes, and 10 isolates of human origin were genotyped further using sequence analysis of the *spa* gene and MLST.

Sequence Typing of the Staphylococcal Protein A Gene of *Staphylococcus aureus* Isolates

The polymorphic X-Region of the *spa* gene was amplified according to the protocol of Harmsen et al. (2003) and the PCR amplicons were submitted to Inqaba Biotechnical Industries (South Africa) for sequencing. Sequence files were assembled using CLC Main Workbench Version 6.0 (CLCbio, USA) before being imported and analyzed in DNAgear (201203012225). Novel repeat sequence profiles and trace data files were submitted to the Ridom Spa server database curator for evaluation and assignment of new *spa* types.

Multilocus Sequence Typing Analysis of *Staphylococcus aureus* Isolates

The internal fragments of seven *S. aureus* housekeeping genes (*arc*C, *aro*E, *glp*F, *gmk*, *pta*, *tpi*, and *yqi*L) were amplified as previously described (Enright et al., 2000). The PCR amplicons were submitted to Inqaba Biotechnical Industries (South Africa) for DNA sequencing of both the forward and reverse strands. Following assembly of the DNA sequences in CLC Main Workbench version 6.0 (CLCbio, USA) the sequence of each locus was compared to allele sequences in the MLST database (http://saureus.mlst.net). The combination of alleles at the seven loci was used to define the allelic profile for each isolate and assigned to a sequence type (ST). The eBURST algorithm (version 3.0; last previous update 24/9/2015) was used to assign MLST

TABLE 1 | Oligonucleotide primer sequences and details for the PCR-based assays used in this study to characterize *S.aureus*.

Gene	Primer name	Oligonucleotide sequence (5′–3′)[†]	Size of PCR amplicon (bp)	Positive control	References
16S rRNA*	Staph 756F	AACTCTGTTATTAGGGAAGAACA	756	None	McClure et al., 2006
	Staph 750R	CCACCTTCCTCCGGTTTGTCACC			
AMR1[a]					
blaZ	blaZ-F	AAGAGATTTGCCTATGCTTC	517	*S. aureus*	Vesterholm-Nielsen et al., 1999
	blaZ-R	GCTTGACCACTTTTATCAGC		ATCC 29213	
mecC	mecA$_{LGA251}$ MultiFP	GAAAAAAAGGCTTAGAACGCCTC	138	*S. aureus* field isolate 13ANR24389_RV	Stegger et al., 2012
	mecA$_{LGA251}$ MultiRP	GAAGATCTTTTCCGTTTTCAGC			
vanA	vanA-F	CATGAATAGAATAAAAGTTGCAATA	1,030	*Enterococcus faecalis*	Clark et al., 1993
	vanA-R	CCCCTTTAACGCTAATACGATCAA		ATCC 70021	
vanB	vanB-F	GTGACAAACCGGAGCGAGGA	433	*E. faecalis*	
	vanB-R	CCGCCATCCTCCTGCAAAAAA		ATCC 51299	
VGP1[a]					
sea	GSEAR-1	GGTTATCAATGTGCGGGTGG	102	*S. aureus* field isolate 00V08510	Mehrotra et al., 2000
	GSEAR-2	CGGCACTTTTTTCTCTTCGG			
seb	GSEBR-1	GTATGGTGGTGTAACTGAGC	164	*S. aureus* field isolate 00V08510	
	GSEBR-2	CCAAATAGTGACGAGTTAGG			
sec	GSECR-1	AGATGAAGTAGTTGATGTGTATGG	451	*S. aureus* field isolate	
	GSECR-2	CACACTTTTAGAATCAACCG		13ANR24389_RV	
sed	GSEDR-1	CCAATAATAGGAGAAAATAAAAG	278	*S. aureus* field isolate	
	GSEDR-2	ATTGGTATTTTTTTTCGTTC		13ANR12816	
see	GSEER-1	AGGTTTTTTCACAGGTCATCC	209	*S. aureus* field isolate	
	GSEER-2	CTTTTTTTTTCTTCGGTCAATC		Jena_FLI W56	
VGP2[a]					
seg	SEG-1	AAGTAGACATTTTTGGCGTTCC	287	*S. aureus* field isolate	Omoe et al., 2002, 2005
	SEG-2	AGAACCATCAAACTCGTATAGC		13ANR12816	
seh	SEH-1	GTCTATATGGAGGTACAACACT	213	*S. aureus* field isolate	
	SEH-2	GACCTTTACTTATTTCGCTGTC		05-Student-18	
sei	SEI-1	GGTGATATTGGTGTAGGTAAC	454	*S. aureus* field isolate	
	SEI-2	ATCCATATTCTTTGCCTTTACCAG		13ANR12816	
selj	SEJ-1	ATAGCATCAGAACTGTTGTTCCG	152	*S. aureus* field isolate	
	SEJ-2	CTTTCTGAATTTTACCACCAAAGG		13ANR12816	
selp	SEP-3	TGATTTATTAGTAGACCTTGG	396	*S. aureus* field isolate	
	SEP-4	ATAACCAACCGAATCACCAG		13ANR12816	
VGP3[a]					
selk	SEK-1	TAGGTGTCTCTAATAATGCCA	293	*S. aureus* field isolate 00V08510	Omoe et al., 2005
	SEK-2	TAGATATTCGTTAGTAGCTG			
selm	SEM-1	GGATAATTCGACAGTAACAG	379	*S. aureus* field isolate	
	SEM-2	TCCTGCATTAAATCCAGAAC		13ANR12816	
selo	SEO-1	TGTGTAAGAAGTCAAGTGTAG	214	*S. aureus* field isolate	
	SEO-2	TCTTTAGAAATCGCTGATGA		13ANR12816	
tst1	GTSSTR-1	ACCCCTGTTCCCTTATCATC	326	*S. aureus* field isolate	Mehrotra et al., 2000
	GTSSTR-2	TTTTCAGTATTTGTAACGCC		05-Student-18	
VGP4[a]					
sell	SEL-1	TAACGGCGATGTAGGTCCAGG	383	*S. aureus* field isolate	Omoe et al., 2005
	SEL-2	CATCTATTTCTTGTGCGGTAAC		07V33069	
selq	SEQ-1	AATCTCTGGGTCAATGGTAAGC	122	*S. aureus* field isolate 00V08510	
	SEQ-2	TTGTATTCGTTTTGTAGGTATTTTCG			

(Continued)

TABLE 1 | Continued

Gene	Primer name	Oligonucleotide sequence (5′–3′)[†]	Size of PCR amplicon (bp)	Positive control	References
selr	SER-1	GGATAAAGCGGTAATAGCAG	166	*S. aureus* field isolate	
	SER-4	GTATTCCAAACACATCTAAC		13ANR12816	
VGP5[b]					
seln	Mp-sen-1	ATGAGATTGTTCTACATAGCTGCAAT	680	*S. aureus* field isolate	Jarraud et al., 2002
	Mp-sen-2	AACTCTGCTCCCACTGAAC		13ANR12816	
selu	SEU-F	ATCAGAAACAAACATTAAAGCCA	500	*S. aureus* field isolate	Park et al., 2011
	SEU-R	TGACCATTTCCTTCGATAAACTTTAT		13ANR12816	
VGP6[b]					
hla	HLA-1	CTGATTACTATCCAAGAAATTCGATTG	209	*S. aureus* field isolate	Jarraud et al., 2002
	HLA-2	CTTTCCAGCCTACTTTTTTATCAGT		05-Student-18	
hlb	HLB-1	GTGCACTTACTGACAATAGTGC	309	*S. aureus* field isolate	
	HLB-2-2	GTTGATGAGTAGCTACCTTCAGT		11ANR780898	
hld	HLD-1	AAGAATTTTTATCTTAATTAAGGAAGGAGTG	111	*S. aureus* field isolate	
	HLD-2	TTAGTGAATTTGTTCACTGTGTCGA		05-Student-18	
hlg	mpHLG-1	GTCAYAGAGTCCATAATGCATTTAA	535	*S. aureus* verified field isolate UP-MR40	
	mpHLG-2	CACCAAATGTATAGCCTAAAGTG			
*hlg*2	mpHLG2-1	GACATAGAGTCCATAATGCATTYGT[d]	390	*S. aureus* verified field isolate UP-MR42	
	mpHLG2-2	ATAGTCATTAGGATTAGGTTTCACAAAG			
VGP7[a]					
eta	GETAR-1	GCAGGTGTTGATTTAGCATT	93	*S. aureus* field isolate	Mehrotra et al., 2000
	GETAR-2	AGATGTCCCTATTTTTGCTG		07V33069	
etb	GETBR-1	ACAAGCAAAAGAATACAGCG	226	*S. aureus* field isolate	
	GETBR-2	GTTTTTGGCTGCTTCTCTTG		07V33069	
etd	ET-14	AACTATCATGTATCAAGG	376	*S. aureus* field isolate	Yamaguchi et al., 2002
	ET-15	CAGAATTTCCCGACTCAG		04V16073	
VGP8[b]					
*luk*M	lukM-1	TGGATGTTACCTATGCAACCTAC	780	*S. aureus* field isolate	Jarraud et al., 2002
	lukM-2	GTTCGTTTCCATATAATGAATCACTAC		RF122	
*luk*ED	lukDE-1	TGAAAAAGGTTCAAAGTTGATACGAG	269	*S. aureus* field isolate	
	lukDE-2	TGTATTCGATAGCAAAAGCAGTGCA		RF122	
UNIPLEX ASSAYS[c]					
chp		TTTTTAACGGCAGGAATCAGTA	404	*S. aureus* verified field isolate UP-MR40	Sung et al., 2008
		TGCATATTCATTAGTTTTTCCAGG			
sak		TGAGGTAAGTGCATCAAGTTCA	403	*S. aureus* field isolate 00V08510	
		CCTTTGTAATTAAGTTGAATC CAGG			
scn		ATACTTGCGGGAACTTTAGCAA	320	None	
		TTTTAGTGCTTCGTCAATTTCG			
ACCESSORY GENE REGULATOR TYPING[c]					
agr	agr1	GTCACAAGTACTATAAGCTGCGAT	441	*S. aureus*	Gilot et al., 2002 Smyth et al., 2009
	PAN	ATGCACATGGTCGACATGC		ATCC 16600	
	Agr2	TATTACTAATTGAAAAGTGGCCATAGC	575	*S. aureus*	
	PAN	ATGCACATGGTCGACATGC		ATCC 29213	
	Agr3	GTAATGTAATAGCTTGTATAATAATACCCAG	323	*S. aureus*	
	PAN	ATGCACATGGTCGACATGC		ATCC 25923	
	Agr4	CGATAATGCCGTAATACCCG	659	*S. aureus* field isolate	
	PAN	ATGCACATGGTCGACATGC		07V33069	
SEQUENCE TYPING OF THE STAPHYLOCOCCAL PROTEIN A GENE[c]					
spa	1095F	AGACGATCCTTCGGTGAGC	Variable	None	Shopsin et al., 1999

(Continued)

TABLE 1 | Continued

Gene	Primer name	Oligonucleotide sequence (5'–3')[†]	Size of PCR amplicon (bp)	Positive control	References
	1517R	GCTTTTGCAATGTCATTTACTG			Harmsen et al., 2003
MULTILOCUS SEQUENCE TYPING[c]					
*arc*C	*arc*-Up	TTGATTCACCAGCGCGTATTGTC	456	None	Enright et al., 2000
	arc-Dn	AGGTATCTGCTTCAATCAGCG			
*aro*E	*aro*E-Up	ATCGGAAATCCTATTTCACATTC	456	None	
	*aro*E-Dn	GGTGTTGTATTAATAACGATATC			
*glp*F	*glp*F-Up	CTAGGAACTGCAATCTTAATCC	465	None	
	*glp*F-Dn	TGGTAAAATCGCATGTCCAATTC			
gmk	*gmk*-Up	ATCGTTTTATCGGGACCATC	429	None	
	gmk-Dn	TCATTAACTACAACGTAATCGTA			
pta	*pta*-Up	GTTAAAATCGTATTACCTGAAGG	474	None	
	pta-Dn	GACCCTTTTGTTGAAAAGCTTAA			
tpi	*tpi*-Up	TCGTTCATTCTGAACGTCGTGAA	402	None	
	tpi-Dn	TTTGCACCTTCTAACAATTGTAC			
*yqi*L	*yqi*L-Up	CAGCATACAGGACACCTATTGGC	516	None	
	*yqi*L-Dn	CGTTGAGGAATCGATACTGGAAC			

[†] *All oligonucleotides were synthesized and purified by Inqaba Biotechnical Industries, South Africa with the exception of GSEAR-1, GSEAR-2, GSEBR-1, GSEBR2, GSECR-1, GSECR-2, GSEDR-1, GSEDR-2, GSEER-1, GSEER-2, GETAR-1, GETAR-2, GETBR-1, GETBR-2, GTSSTR-1 GTSSTR-2, lukDE-1, lukDE-2, LukM-1, and lukM-2, which were synthesized by Integrated DNA Technologies (IDT$^{®}$) (California, USA).*
*The 16S primer pair was included in the following M-PCR assays: AMR1, VGP1, VGP2, VGP3, VGP4, VGP 6, and VGP7.
[a] *Annealing temperature 57°C.*
[b] *Annealing temperature 51°C.*
[c] *Annealing temperature 55°C.*
[d] *Y = T or C.*

clonal complexes, with the minimum number of common alleles set at six of seven (Feil et al., 2003). Trace files of putative novel alleles and the allelic profiles of novel STs were submitted to the curator of the database for evaluation, assignment of allele or ST number and entry into the database.

RESULTS

The distribution of samples and *S. aureus* isolates recovered at each of the sampling sites is summarized in **Table 2**. For further details pertaining to sampling and selection of bacterial isolates the reader is referred to Schmidt et al. (2015).

Phenotypic and Molecular Identification of *Staphylococcus aureus* Isolates

All *S. aureus* isolates tested positive for coagulase production using rabbit plasma. With the exception of 10 isolates from sampling site F, all *S. aureus* isolates tested positive with the Pastorex^{TM} StaphPlus agglutination test kit. The phenotypic identification of *S. aureus* isolates was confirmed using the species-specific multiplex-PCR assay.

Virulence Gene Profiling of *Staphylococcus aureus* Isolates

The distribution of virulence genes between the different *S. aureus* pulsotypes is shown in **Table 3**. None of the bovine or human *S. aureus* isolates tested positive for the presence of either of the methicillin resistance genes, *mec*A or *mec*C, or the

vancomycin resistance genes, *van*A or *van*B. The *bla*Z gene was detected in 28.8% (42/146) and 75% (8/12) of the bovine and human *S. aureus* isolates respectively. All *S. aureus* isolates tested positive for the hemolysin genes, *hla* and *hld*. The *hlg* and *hlg2* genes were detected in all the bovine isolates and 75% (9/12) and 67% (8/12) of the human isolates respectively. The β-hemolysin gene was detected in 86% (126/146) of the bovine isolates and only 25% (3/12) of the human isolates. None of the *S. aureus* isolates tested positive for the Panton-Valentine leukocidin toxin gene, *luk*S/F. All bovine *S. aureus* isolates tested positive for *luk*ED whilst this leukocidin gene was found in only 75% (8/12) of the isolates of human origin. Comparatively, the *luk*M gene was detected in 56.2% (82/146) of the bovine isolates and only a single human isolate. None of the bovine isolates tested positive for the exfoliative toxin genes *eta*, *etb*, or *etd*. Only a single human *S. aureus* isolate tested positive for the *eta* gene.

Overall 27% (39/146) of the bovine *S. aureus* isolates and 83.3% (10/12) of the *S. aureus* isolates of human origin tested positive for one or more PTSAg genes. The PTSAg gene positive bovine *S. aureus* were isolated from only three of the sampling sites namely, commercial dairy herds E, F and L. The *sec* and *sell* genes were the most common genes detected among the bovine isolates with the *sec-sell* gene combination occurring in 71.8% (28/39) of the PTSAg-positive *S. aureus* isolates. Comparatively, a more diverse range of PTSAg toxin genes were detected among the human isolates including: *sea* (n = 1), *sec* (n = 3), *sed* (n = 1), *seg* (n = 5), sei (n = 3), *selj* (n = 3), *selk* (n = 1), *sell* (n = 3), *selm* (n = 3), *seln* (n = 2), *selo* (n = 3), *selq* (n =

TABLE 2 | Distribution of *Staphylococcus aureus* isolates and pulsotypes from bovine intramammary infections and human nasal swabs collected from 13 sampling sites in the province of KwaZulu-Natal, South Africa.

Sampling sites	Average size of milking herd	Bovine milk samples		Human nasal swab specimens	
		Percentage of sampled cows positive for *S.aureus*	Pulsotypes* (number of isolates)	Percentage of specimens positive for *S.aureus*	Pulsotypes* (number of isolates)
COMMERCIAL DAIRY HERDS (*n* = 9)					
A	400	5.6 (5/89)	PT7 (5)	12.5 (1/8)	PT14 (1)
B	700	25.9 (14/54)	PT7 (12), PT8 (2)	25 (2/8)	PT17 (2)
C	300	4.8 (3/63)	PT7 (3)	7.1 (1/14)	PT12 (1)
E	1,100	10.5 (29/277)	**PT2** (29)	12.5 (1/8)	**PT2** (1)
F	500	18.3 (22/120)	PT7 (12), PT10 (10)	14.3 (1/7)	PT13 (1)
G	95	14.5 (18/124)	PT1 (9), PT2 (7), PT7 (1), PT8 (1)	0 (0/4)	–
I	500	21.4 (21/98)	PT1 (12), PT3 (1), PT4 (3), PT6 (1), PT7 (4)	0 (0/6)	–
J	600	0 (0/281)	–	27.3 (3/11)	PT11 (2), PT15 (1)
L	1,450	7.8 (16/206)	PT5 (1), PT7 (14), **PT9** (1)	14.3 (1/7)	**PT9** (1)
AGRICULTURE COLLEGE DAIRY HERDS (*n* = 2)					
D	40	39.5 (15/38)	**PT7** (15)	50 (1/2)	**PT7** (1)
H	50	37.5 (3/8)	PT7 (3)	0 (0/2)	–
COMMUNAL ANIMAL HERDS (*n* = 2)					
M	Unknown	0 (0/16)	–	50 (1/2)	PT16 (1)

*Pulsotypes common to both bovine and human S. aureus are shown in bold.

1), *selr* (*n* = 3), *selu* (*n* = 2), and *tsst*-1 (*n* = 2). None of the bovine or human *S. aureus* isolates tested positive for the *seb*, *sed*, *see*, *seh*, or *selp* genes. The immune evasion cluster genes, *chp* and *sak*, which code the chemotaxis inhibitory protein of *S. aureus* (CHIPS) and the staphylokinase (SAK) enzyme, were detected in 0.7% (1/146) and 13% (19/146) of the bovine *S. aureus* isolates evaluated. Among the human isolates the *chp* and *sak* genes were detected in 50% (6/12) and 66.7% (8/12) of the isolates evaluated. None of the isolates carried the *scn* gene encoding the staphylococcal complement inhibitor protein (SCIN).

Accessory Gene Regulator (*agr*) typing

The *agr* of all *S. aureus* isolates could be characterized using the described *agr* M-PCR assay of Gilot et al. (2002). Among the isolates of bovine origin *agr* type I was the dominant type observed, with 93.2% (136/146) of the isolates found to be of this type. The remaining 6.8% (10/146) of the bovine isolates were *agr* type II. Among the 12 *S. aureus* isolates recovered from the human nasal swabs, 58.3% (7/12), 16.7% (2/12), and 25% (3/12) were identified as *agr* types I, II and III respectively (**Table 3**). No *S. aureus* isolates were found to have a type IV accessory gene regulator.

Genomic Macrorestriction and Pulsed-Field Gel Electrophoresis

All *S. aureus* isolates could be typed using macrorestriction followed by PFGE. Digestion of genomic DNA with *Sma*I produced eight to 13 fragments which ranged in size from <76 kb to approximately 674 kb. The *S. aureus* isolates of bovine origin generated 58 electrophoretic patterns which grouped into

10 pulsotypes (PT1 to PT10) at an 80% similarity level (**Figure 2**). The majority of the bovine isolates, 93.2% (136/146), grouped into four major pulsotypes [PT1 (*n* = 21, 14.4%), PT2 (*n* = 36, 24.7%), PT7 (*n* = 69, 47.3%), and PT10 (*n* = 10, 6.8%)]. At five of the sampling sites (A, C, D, E, and H) only a single *S. aureus* pulsotype was recovered from the mastitic milk samples. Two *S. aureus* pulsotypes were identified at sites B and F, whilst four pulsotypes were identified amongst the isolates from site G and five *S. aureus* pulsotypes were identified at site I. Three *S. aureus* isolates (E/CPS1h; D/CPS16h; and L/CPS1h) recovered from human nasal swabs were assigned to pulsotypes common to bovine isolates namely PT2, PT7, and PT9. The nine remaining human isolates were allocated to seven separate pulsotypes (PT11–PT17).
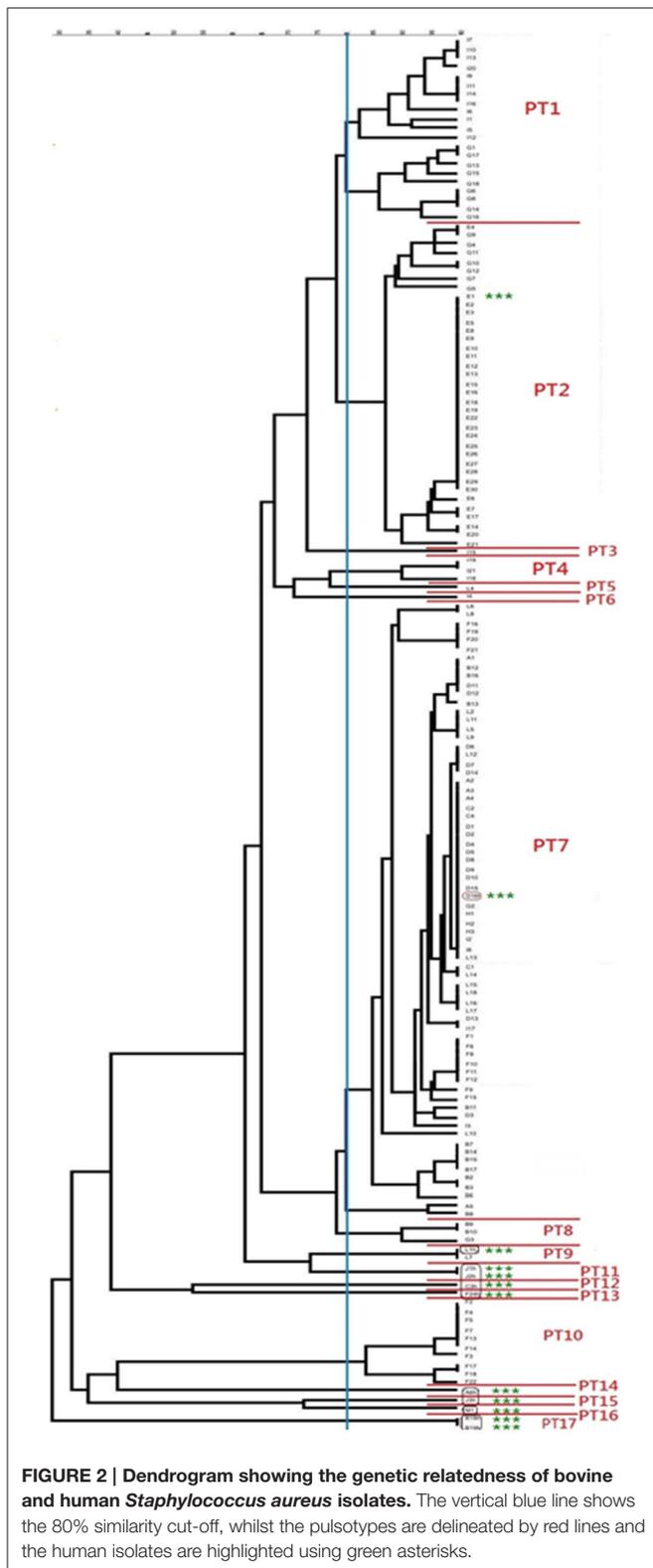
Genotyping of *Staphylococcus aureus* Isolates

The 16 representative *S. aureus* isolates of bovine origin were assigned to 11 different *spa* types which varied in length between 1 and 11 repeats. Four new *spa* types, t15533 (*n* = 1), t15536 (*n* = 1), t15538 (*n* = 1) and t15539 (*n* = 4) were identified among the bovine isolates in this study. Other *spa* types detected include: t015 (*n* = 1), t064 (*n* = 1), t189 (*n* = 1), t529 (*n* = 1), t693 (*n* = 1), t730 (*n* = 1), and t1201 (*n* = 3). Among the ten representative human *S. aureus* isolates a diverse range of *spa* types were detected including: t012 (*n* = 1), t015 (*n* = 1), t064 (*n* = 1), t189 (*n* = 1), t360 (*n* = 1), t581 (*n* = 1), t1201 (*n* = 1), t1476 (*n* = 1), t2172 (*n* = 1), and t7023 (*n* = 1). MLST of isolates revealed a limited number of predominant sequence types among the 16 bovine isolates typed with ST352 (CC97) (*n*

TABLE 3 | Genotypic characteristics of *S. aureus* isolates from bovine intramammary infections and close human contacts.

PT	Origin of isolates	Sampling site (no. of isolates)	Spa type*	ST* (CC)	agr type	Antimicrobial resistance genes	Immune evasion cluster genes†	Cytotoxin genes detected†	PTSAg genes detected†
PT1 (major)	Bovine	G (9)	**t15533**	ST2992 (CC97)	I	blaZ	chp[1], sak	hla, hlb, hld, hlg, hlg2, lukED	
		I (12)	**t15539**	ST2992 (CC97)	I	blaZ		hla, hlb[1], hld, hlg, hlg, hlg2, lukED	
PT2 (major)	Bovine	E (29)	t189	ST97 (CC97)	I	Negative		hla, hlb, hld, hlg, hlg, hlg2, lukED	sec[28], sel[28]
		G (7)					Negative		hla, hlb, hld, hlg, hlg, hlg2, lukED	
	Human	E (1)	t189	ST97 (CC97)	I	Negative		hla, hlb, hld, hlg, hlg, hlg2, lukED	sec, sell
PT3	Bovine	I (1)	**t15539**	ST2992 (CC97)	I	blaZ	sak	hla, hld, hlg, hlg2, lukED	
PT4 (minor)	Bovine	I (3)	**t15539**	ST2992 (CC97)	I	blaZ	sak	hla, hld, hlg, hlg2, lukED	
PT5	Bovine	L (1)	**t15536**	**ST3538** (CC97)	I	blaZ	sak	hla, hld, hlg, hlg2, lukED	
PT6	Bovine	I (1)	**t15539**	ST2992 (CC97)	I	blaZ	sak	hla, hld, hlg, hlg2, lukED	
PT7 (major)	Bovine	A (5)	**t15538**	ST352 (CC97)	I	Negative		hla, hlb, hld, hlg, hlg, hlg2, lukED, lukM	
		B (12)					blaZ		hla, hlb, hld, hlg, hlg, hlg2, lukED, lukM	
		C (3)	t730	ST352 (CC97)	I	Negative		hla, hlb, hld, hlg, hlg, hlg2, lukED, lukM	
		D (15)	t1201	ST352 (CC97)	I	Negative		hla, hlb, hld, hlg, hlg, hlg2, lukED, lukM	
		F (12)	t1201	ST352 (CC97)	I	Negative		hla, hlb, hld, hlg, hlg, hlg2, lukED, lukM	
		G (1)					Negative		hla, hlb, hld, hlg, hlg, hlg2, lukED, lukM	
		H (3)	t1201	ST352 (CC97)	I	Negative		hla, hlb, hld, hlg, hlg, hlg2, lukED, lukM	
		I (4)					Negative		hla, hlb[3], hld, hlg, hlg, hlg2, lukED, lukM	
		L (14)					Negative		hla, hlb[13], hld, hlg, hlg, hlg2, lukED, lukM	
	Human	D (1)	t1201	ST352 (CC97)	I	Negative		hla, hlb, hld, hlg, hlg, hlg2, lukED, lukM	
PT8 (minor)	Bovine	B (2)	t693	ST352 (CC97)	I	blaZ		hla, hlb, hld, hlg, hlg, hlg2, lukED, lukM	
		G (1)	t015	ST508 (CC45)	I	Negative		hla, hlb, hld, hlg, hlg, hlg2, lukED, lukM	
PT9	Bovine	L (1)	t064	ST8 (CC8)	I	blaZ	sak	hla, hld, hlg, hlg2, lukED	sea
	Human	L (1)	t064	ST8 (CC8)	I	blaZ	sak	hla, hld, hlg, hlg2, lukED	sea
PT10 (major)	Bovine	F (10)	t529	ST351 (CC705)	II	Negative		hla, hlb, hld, hlg, hlg, hlg2, lukED, lukM	sec, seg, seli, sell, seln, selu, tst
PT11 (minor)	Human	J (2)	t1476	ST8 (CC8)	I	blaZ	chp, sak	hla, hld, hlg, hlg2, lukED	selj, selr
PT12	Human	C (1)	t360	ST15 (CC15)	II	blaZ	chp	eta, hla, hld, hlg, hlg2, lukED	
PT13	Human	F (1)	t581	ST5 (CC5)	II	blaZ		hla, hlb, hld, hlg, hlg, hlg2, lukED	sed, seg, sei, selj selm seln, selo, selr, seu
PT14	Human	A (1)	t2172	ST707 (CC707)	III	blaZ	chp, sak	hla, hld, hlg, hlg2, lukED	selk, selq, tst
PT15	Human	J (1)	t7023	ST45 (CC45)	I	blaZ	chp, sak	hla, hld, hlg, hlg2, lukED	sec, selg, seli, sell, selm, seln, selo, selu
PT16	Human	M (1)	t015	ST508 (CC45)	I	Negative	chp, sak	hla, hld, hlg	sec, seg, seli, sell, selm, selo, tst
PT17 (minor)	Human	B (2)	t012	ST30 (CC30)	III	blaZ	sak	hla, hld	seg

*New spa types and sequence type are shown in bold font; †In some cases not all of the isolates in the group presented with the indicated genes. Where this occurred the number of isolates positive for the gene is indicated by a superscript; CC, Clonal complex; PT, Pulsotype; PTSAg, pyrogenic toxin superantigen; ST, sequence type.

FIGURE 2 | Dendrogram showing the genetic relatedness of bovine and human *Staphylococcus aureus* isolates. The vertical blue line shows the 80% similarity cut-off, whilst the pulsotypes are delineated by red lines and the human isolates are highlighted using green asterisks.

novel sequence type, ST3538 ($n = 1$) with an allelic profile of 357-1-378-1-1-5-3. The human isolates typed as ST5 (CC5) ($n = 1$), ST8 (CC8) ($n = 2$), ST15 (CC15) ($n = 1$), ST30 (CC30) ($n = 1$), ST45 (CC45) ($n = 1$); ST97 (CC97) ($n = 1$), ST352 (CC97) ($n = 1$), ST508 (C45) ($n = 1$), and ST707 (CC707) ($n = 1$). The distribution of the different *S. aureus* sequence types and *spa* types are correlated with the pulsotypes in **Table 3**.

DISCUSSION

To investigate the specific objectives of this study a cross-section of farming operations were sampled including commercial dairy herds, government managed agriculture college herds and communal animals. The sampling approach ensured the collection of a diverse range of *S. aureus* isolates for characterization. One hundred and forty six bovine *S. aureus* isolates and 12 human isolates were characterized and genotyped using a combination of PCR and M-PCR assays, *agr* typing and PFGE, with representative isolates being evaluated further using *spa* typing and MLST.

Virulence Gene Profiling of Bovine and Human *Staphylococcus aureus*

Hemolysin genes were identified in all *S. aureus* isolates with bovine and human isolates testing positive for at least alpha- and delta-hemolysin. The beta-hemolysin gene was detected in the majority (87%) of the bovine isolates. The high occurrence of *hlb* among bovine isolates is in agreement with other reports (Zecconi et al., 2006; Monecke et al., 2007; Ikawaty et al., 2010). Comparatively, *hlb* was less frequently detected in human isolates. It is pertinent to relate the detection of *hlb* to the presence of the IEC genes, which are located on β-hemolysin converting bacteriophages (van Wamel et al., 2006), as the occurrence of IEC genes is accompanied by insertional inactivation of *hlb*. The IEC genes are considered a hallmark of human *S. aureus* isolates, although it has been demonstrated that not all human isolates carry these genes (Verkaik et al., 2011). In this study *sak* was detected in 67% (8/12) of the human isolates examined. This is not particularly high but may simply be a consequence of the limited number of human isolates evaluated in this study. Comparatively, *sak* was only sporadically detected among the bovine mastitis isolates in this study with 13% (19/146) of isolates found to carry the gene. With the exception of one of the 19 isolates, the *hlb* gene was not detected in any of the *sak*-positive bovine isolates.

All bovine isolates evaluated were positive for *luk*ED, this is consistent with the results of other studies which have reported a high prevalence of *luk*ED among bovine mastitis isolates (Fueyo et al., 2005; Monecke et al., 2007). Interesting to note was the distribution of *luk*M which was predominantly detected among isolates from two of the major bovine pulsotypes namely PT7 and PT10. Rainard et al. (2003) demonstrated that *luk*M was highly cytotoxic for bovine neutrophils leading the researchers to conclude that isolates producing this toxin would have a selective advantage in the bovine host enabling the bacteria to overcome the host immune system and establish infection

$= 6$) and ST2992 (CC97) ($n = 5$) being the most common. Other sequence types detected include: ST8 (CC8) ($n = 1$), ST97 (CC97) ($n = 1$), ST351 (CC705) ($n = 1$), ST508 (CC45) ($n = 1$) and a

(Rainard et al., 2003; Hata et al., 2006, 2010; Vrieling et al., 2015). This observation has been verified *in vivo* where Haveri et al. (2007) found *lukM*-positive *S. aureus* strains more likely to be associated with clinical mastitis cases instead of subclinical infections.

All *S. aureus* isolates were screened for a comprehensive range of PTSAg genes including the genes encoding the classical enterotoxins, enterotoxin A [SEA] to enterotoxin E [SEE], the newer enterotoxins [SEG, SEH, SEI], enterotoxin-like proteins [SElJ, SElK, SElL, SElM, SElN, SElO, SElP, SElQ SElR, SElU] and the toxic shock syndrome toxin-1 [TSST-1]. Although approximately 27% of the bovine *S. aureus* isolates in this study tested positive for one or more PTSAg genes, all of these isolates originated from only three herds namely, E, F, and L. The *sec-sell* gene combination was detected in the majority of the bovine isolates originating from herd E whilst in herd F the *sec* and *sell* genes presented together with *seg, seli, seln, selu,* and *tst*. Many of the genes encoding PTSAgs reside on mobile genetic elements, which accounts for the co-occurrence of particular genes as well as the horizontal dissemination of genes amongst compatible bacterial strains residing in the same ecological niche. The *sec* and *sell* genes have been shown to reside on the pathogenicity island SaPI3 (Fitzgerald et al., 2001; Holtfreter and Broeker, 2005) whilst the combination of *sec-sell* together with *tst* is characteristic of SaPI2 (Fitzgerald et al., 2001; Smyth et al., 2005). Enterotoxin C is recognized as an important virulence factor in bovine mastitis and SEC-producing *S. aureus* strains have been associated with cases of severe mastitis (Matsunaga et al., 1993; Ferens et al., 1998; Haveri et al., 2007).

The carriage of PTSAgs by *S. aureus* isolates recovered from cases of bovine mastitis has varied considerably between studies (Srinivasan et al., 2006; Haveri et al., 2007; Wang et al., 2009; de Freitas Guimarães et al., 2013). In addition to *S. aureus* strain variation, which may be influenced by environmental and management factors, the observed differences could also be attributable to herd selection criteria or the geographic location of the study (Srinivasan et al., 2006). One study conducted in the United States reported the occurrence of one or more PTSAgs in more than 93% of the *S. aureus* isolates investigated (Srinivasan et al., 2006). The high prevalence led the investigators to suggest that PTSAgs are important in the development and, or, maintenance of intramammary infections. Given the immunosuppressive effect PTSAgs have on the host's immune system it is certainly a reasonable conclusion. In general data from other studies have reported much lower PTSAg gene prevalences with figures ranging from 10 to 70% (Haveri et al., 2007; Oliveira et al., 2011; de Freitas Guimarães et al., 2013). Overall the wide range in prevalences suggest that PTSAgs may provide *S. aureus* strains with a competitive advantage but these toxins are not essential for the development of mastitis (Wang et al., 2009; de Freitas Guimarães et al., 2013). It is apparent that the exact role of each toxin in the pathogenesis of staphylococcal mastitis still requires clarification.

The low occurrence of PTSAgs detected among the bovine *S. aureus* isolates evaluated in this study should also be considered in terms of the public health impact. The milk from sub-clinical cases of mastitis will enter into the milk supply chain together with PTSAg-positive *S. aureus* strains. The risk of bacterial transfer to workers in the supply chain or the ingestion of raw or incorrectly pasteurized milk is likely to be minimal in light of the low prevalence of PTSAg gene-positive *S. aureus* detected in this study.

Diversity of Bovine Isolates within Herds and Relation to Global Clonal Lineages

The number of *S. aureus* pulsotypes causing IMIs in the dairy herds investigated ranged from one to five, with one ($n = 5$) or two ($n = 2$) pulsotypes being detected in the majority of herds. The most common pulsotype PT7, was detected in 75% (9/12) of the herds sampled. The limited genetic diversity observed within herds is consistent with the clonal nature of *S. aureus* and the contagious, lateral spread of the bacterium between cows in close contact (Capurro et al., 2010). The *S. aureus* isolates recovered from two herds, G and I, comprised of four and five *S. aureus* pulsotypes respectively. The owner of herd G indicated that cows and replacement heifers were sourced from other herds, in addition to rearing his own calves. This practice could account for the larger number of *S. aureus* pulsotypes observed amongst the mastitis isolates recovered from this herd. Middleton et al. (2002) reported that herds that used replacement heifers had more strains of *S. aureus* than closed herds which supplemented their dairy herds with their own stock. The same reason cannot be used to explain the high number of *S. aureus* pulsotypes detected in Herd I as the owner reportedly maintains a closed herd and rears his own replacement heifers. Other factors which are believed to influence genetic diversity and which may be contributing factors in this herd, include, herd size, milk parlor practices and specific management practices including treatment protocols and policies regarding the segregation and, or, the culling of *S. aureus* positive cows (Middleton et al., 2002; Middleton, 2013).

MLST of isolates belonging to the predominant bovine pulsotype PT7, and the second major pulsotype, PT2, identified the strains as ST352 (CC97) and ST97 (CC97) respectively. Both sequence types have been reported extensively from bovine mastitis cases in many countries including, but not limited to, Brazil, Chile, Italy, Japan, Norway, Spain, the Netherlands and the United States (Smith et al., 2005; Rabello et al., 2007; Hata et al., 2010). The expansive distribution of this successful lineage suggests that the strains are specifically adapted to the bovine host (Smith et al., 2005; Budd et al., 2015).

MLST of representative isolates from PT1, PT3, PT4, and PT6 were found to have a sequence type corresponding to *S. aureus* ST2992. Interestingly only a single report on the occurrence of *S. aureus* ST2992, a single-locus variant (SLV) of ST97, could be found in the literature. This ST was previously reported as a carriage isolate from a moose in a Polish study (http://saureus.mlst.net). All ST2992 isolates in this study presented with novel *spa* types. A novel *S. aureus* sequence type, ST3538, was identified from a milk sample collected at sampling site L. The sequence type, which also presented with a novel *spa* type, is a SLV of ST2992 (CC97). The detection of a new *S. aureus*

sequence type is further evidence supporting the diversification of CC97.

The *S. aureus* isolates comprising PT10 originated from a single herd, sampling site F, and were genotyped as ST351 (CC705). This cluster of isolates is interesting for a number of reasons. Firstly, these isolates were the only bovine *S. aureus* isolates found to have a type II accessory gene regulator. All other bovine *S. aureus* isolates in this study were found to have a type I accessory gene regulator; a finding which is consistent with other reports (Gilot and van Leeuwen, 2004; Buzzola et al., 2007). It has been demonstrated that *S. aureus* strains belonging to *agr* group I can invade epithelial cells and persist in higher numbers in mammary gland tissue than strains belonging to other *agr* groups (Buzzola et al., 2007), suggesting a functional adaptation to the udder milieu. The second point of interest regarding PT10/ST351 isolates is that all 10 isolates tested negative using a commercial slide agglutination test which is used for the rapid identification of *S. aureus* isolates. Stutz et al. (2011) reported a similar observation with bovine mastitis isolates when using a different commercial kit which was based on the same biological principles. The researchers attributed the negative results to genetic polymorphisms in the clumping factor (*clf* A) and fibronectin-binding (*fnb*A) genes as well as a premature stop codon in the *spa* gene. Subsequent genotyping of the isolates by Moser et al. (2013) found all isolates to be ST151 (CC151). *Staphylococcus aureus* ST351 is a double locus variant of ST151 and it would be interesting to establish if the molecular basis behind the agglutination-negative result is due to similar genetic polymorphisms. The practical implication of the false-negative test results reported with the latex agglutination test kit is quite considerable, especially in mastitis diagnostic laboratories relying on the assay to identify presumptive *S. aureus* isolates (Pyörälä et al., 2011; Stutz et al., 2011).

Genotypic Relatedness of Bovine and Human *Staphylococcus aureus* Isolates and Investigation of Transmission between Hosts

From the 13 sampling sites, *S. aureus* was simultaneously recovered from bovine milk and human nasal swabs at seven of the sites. The diversity and genetic relatedness of the bovine and human *S. aureus* isolates were initially examined through the use of PFGE. In comparison to the bovine isolates, the *S. aureus* strains recovered from close human contacts were far more genetically diverse. Nine of the 12 human isolates clustered separately from the bovine isolates in eight separate pulsotypes (PT11 to PT17) and comprised of multilocus sequence types not evident among the bovine isolates including ST5, ST15, ST30, ST45, and ST707.

At three of the sampling sites, D, E, and L, the *S. aureus* isolates recovered from both of the hosts were genetically indistinguishable. At sites D and E, the predominant bovine strains detected, PT7/ST352/CC97/t1201 and PT2/ST97/CC97/t189 respectively were also recovered from human nasal swabs collected at the sites. *Staphylococcus aureus* ST97 strains have been reported from human carriage and

clinical specimens (Lozano et al., 2011; Udo et al., 2011; Spoor et al., 2013). The presence of virulence markers such as *hlb*, *luk*ED, and *luk*M, which, although not exclusively found in bovine-associated strains, but are known to provide a selective advantage in the bovine host, suggest that the *S. aureus* isolates are bovine in origin. So does the lack of IEC-associated genes. Unfortunately, due to the fact that only a single round of sampling was done it was not possible to establish in the above cases whether the human contacts were transiently or permanently colonized with this *S. aureus* strain.

At sampling site L, genetically indistinguishable *S. aureus* strains belonging to PT9/ST8/CC8/t064 were recovered from both milk and human nasal swabs. The majority of the bovine isolates collected from this site were ST352; no other *S. aureus* ST8 isolates were detected. *Staphylococcus aureus* ST8 has been reported extensively from human carriage and clinical specimens (Sakwinska et al., 2009; Oosthuysen et al., 2014). This lineage also comprises a number of significant MRSA clones (Monecke et al., 2011). Whilst ST8 has been documented from cases of bovine mastitis (Sakwinska et al., 2011) the prevailing context in this herd, suggests the *S. aureus* isolate is of human origin and has been transferred to the bovine host.

The close contact between dairy cows and humans, particularly in the milking parlor creates the opportunity for the transmission of bacteria between hosts. Whilst evidence suggests that some *S. aureus* strains are adapted to colonize and infect certain host species, other lineages are non-specific. Irrespective hereof, *S. aureus* is a versatile pathogen and is readily able to adapt to new environments through gene mutation, transfer and decay (Spoor et al., 2013). It is primarily for this reason that the interface between animal populations and humans needs to be monitored in order to allow for changes in population dynamics to be timeously detected.

The low number of human *S. aureus* isolates recovered from the nasal swabs of close human contacts is considered one of the primary limitations of this study. The availability of only 12 human *S. aureus* isolates restricted the comparisons which could be made between isolates originating from bovine and human hosts. Further sampling at additional sites in KZN and other provinces of South Africa will potentially provide a larger number of isolates for characterization and will enable the preliminary observations made in this study to be confirmed.

CONCLUSION

This study presents the first insight into the virulence characteristics and genetic diversity of *S. aureus* strains causing mastitis in dairy herds in a province of South Africa. The absence of clinically significant antimicrobial resistance genes and virulence markers, such as the Panton Valentine leukocidin encoding gene (*pvl*), together with the low occurrence of PTSAg genes among bovine isolates suggests that the human and public health risks posed by the bovine *S. aureus* populations are low. Genotyping of bovine isolates revealed a limited number of clones with several new *spa* types and a new *S. aureus* sequence type being documented. A greater genetic diversity was observed

among the human isolates evaluated in this study. The detection of genotypically indistinguishable *S. aureus* isolates from bovine and human hosts at three of the sampling sites is suggestive of bacterial transmission and supports the need for vigilant monitoring of *S. aureus* populations at the interface between dairy cows and humans.

AUTHOR CONTRIBUTIONS

TS, ME, and MK conceived and designed the study. TS collected samples and performed all laboratory analyses. TS and MK analyzed the genotyping data. TS wrote the manuscript with critical appraisal and contributions received from all of the authors. All authors read and approved the final version of the manuscript.

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