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Genomic Characterisation of Methicillin-Resistant and Methicillin-Susceptible *Staphylococcus aureus* Implicated in Bloodstream Infections, KwaZulu Natal, South Africa

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Abstract: *Staphylococcus aureus* is an opportunistic human pathogen and a leading cause of bloodstream infections. It can acquire different antibiotic resistance genes, leading to treatment failure. Aim: We elaborate on the genomic characteristics; antibiotic resistance, virulence, pathogenicity, phylogenomics and clonal diversity of *S. aureus* implicated in bloodstream infections. Six multidrug-resistant (MDR) *S. aureus*, three methicillin-resistant *S. aureus* (MRSA) and three methicillin-sensitive *S. aureus* obtained from blood cultures underwent whole genome sequencing and bioinformatics analysis. All isolates carried different permutations and combinations of resistance genes including, *blaZ, mecA, aac(6')-aph (2'')*, *ant(9)-Ia, ant(6)-Ia, mepR, fosB, norA, norC, lmrS, arlS, arlR, mgrA, kdpD and sdrM*. We found 6 *spa* types (t9475, t355, t045, t1265, t1257, and t7888) with varying profiles of virulence genes responsible for immune invasion, enterotoxins, adhesion/biofilm, haemolysins, and leukotoxins. Panton-Valentine leukocidin (*Luk-PV*) was found in one MSSA isolate. Two *SCCmec* types *IVd(2B)* and *I(1B*) were identified. Isolates belonged to four multilocus sequence types (MLSTs), the most common of which was ST5 (n=3). The STs were clustered into two clonal complexes CC5 and CC8. We found two MRSA clones typed as ST5-CC5-t045-SCC*mec*_I(1B), and the human-associated MRSA endemic clone ST612-CC8-t1257-SCC*mec*_IVd(2B). The insertion sequences IS30 and IS6 associated with virulence were found in two isolates. The presence of virulent MDR *S. aureus* in bloodstream infections poses a clinical concern because of limited treatment options and increased risk of mortality.

Keywords: *Staphylococcus aureus*; bloodstream infections; whole-genome sequencing; antibiotic resistance; virulence

1. Introduction

Staphylococcus aureus is a Gram-positive bacterium inhabiting healthy individuals' nostrils and skin. However, it has become an important opportunistic pathogen in communities and hospitals [1]. It causes severe skin infections, pneumonia, endocarditis, and bloodstream infections (BSIs) [2]. BSIs caused by *S. aureus* infections have high morbidity and mortality if not treated timeously [3]. The most significant risk factors for *S. aureus* BSIs are intravascular devices, surgical procedures, and a debilitated immune system [4].

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Methicillin-resistant *S. aureus* (MRSA) has become a significant cause of BSIs. MRSA poses a major public health threat because of multidrug resistance to different antibiotic classes that limit treatment options [5]. Resistance is mediated by the *mecA* gene, found on a mobile genetic element (MGE) known as the staphylococcal cassette chromosome *mec* (SCC*mec*) [6]. Methicillin-susceptible *S. aureus* (MSSA) is also emerging as a causative agent of BSIs [7] and has been reported to display high virulence and multidrug resistance [8].

The pathogenicity of *S. aureus* depends on its ability to produce a wide array of virulence factors involved in adhesion, invasion of host tissues, immune system evasion, and biofilm formation [9,10]. Virulence factors and multiple resistance genes can be transmitted by horizontal gene transfer (HGT) [11] on diverse MGEs, amongst which plasmids are reported as the primary sources for dissemination [4].

The epidemiology of *S. aureus* strains indicates that its molecular characteristics continually change over time, resulting in new clones, which vary by region. In a study in the United States, ST5 and ST8 were the most prevalent sequence types [12]. In South Africa, ST612 is dominant in the hospital environment [13]. The ST612-IV [2B], belonging to spa type t1257, was identified as a typical clone in clinical settings [14] and sporadically in poultry settings [15]. The ST5 and ST8 clones are commonly associated with BSIs and the pandemic lineages of *S. aureus*, such as the clonal complex CC8 and CC5 [16]. Notably, the sequence types ST612, ST5, ST8, and ST72 have displayed high resistance to most antibiotic drug classes and are challenging to treat [16].

Multidrug-resistant (MDR) *S. aureus* infections pose a serious clinical concern. A high incidence of pathogenic MDR MRSA has been reported, and the data suggest that its prevalence is increasing in Africa [17]. A recent South African study investigating the genetic relatedness of hospital-acquired-associated MRSA isolates in two hospitals revealed that all isolates were resistant to aminoglycosides and β-lactams. All the isolates carried the *aacA-aphD* and *mecA*-resistant genes and clusters of virulence genes [18]. We elaborate on the genomic characteristics, antibiotic resistance, virulence, pathogenicity, phylogenomics, and clonal diversity of six *S. aureus* clinical strains implicated in bloodstream infections at a regional hospital in the KwaZulu-Natal province in South Africa

2. Results

2.1. Patient Demographics and Characteristics

The 6 isolates investigated in this study were obtained from patients who visited a regional hospital in the uMgungundlovu District in the KwaZulu-Natal Province. Three of the six isolates were recovered from the neonatal ICU (n=3, 50%), two from surgical wards and one isolate from the paediatric ward. Four patients were males, while 2 were females. The age distribution of patients ranged from 0 to 33 years old, and the mean age was 8.83 years (Table 1). The demographic details of the source participants of the isolates that were selected for WGS are shown in **Supplementary Table 1.**

2.2. Antibiotic Susceptibility Test Results

The isolates displayed varying phenotypic resistance profiles with most being resistant to penicillin G (n=6), tetracycline (n=5), doxycycline (n=5), clindamycin (n=5), moxifloxacin (n=5), rifampicin (n=4), and erythromycin (n=3). The lowest resistance was against nitrofurantoin, tigecycline, and chloramphenicol (n=1) (Table 1).

2.3. Phenotypic and Genotypic Identification of MRSA Isolates

MRSA isolates were confirmed by phenotypic resistance to cefoxitin (Table 1), and the detection of the mecA gene using polymerase chain reaction (PCR).

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2.4. Genomic Features

The genome size of our draft genomes ranged from 2.7Mb to 2.9Mb. The genomic characteristics of the sequences, in relation to G+C content (%), number of RNAs, number of coding sequences, size, N50, L50, coverage and are shown in **Supplementary Table 2.**

Table 1. Antibiotic susceptibility profiles, age and demographic characteristics of patients with BSIs attributed to *S. aureus.*

Isolate ID	**Species**	**Sex**	**Ward**	**Age**	**Antibiotics**																	
					PEN	**AMP**	**FOX**	**CIP**	**MXF**	**LEV**	**GEN**	**AMK**	**ERY**	**CLI**	**TET**	**DOX**	**TGC**	**CHL**	**NIT**	**SXT**	**VAN**	**RIF**	**LZD**	**TEC**
S11	MRSA	F	Surgical ward	17 years	R	R	R	R	R	R	R	R	R	R	R	R	R	I	S	R	S	R	R	R
S29	MRSA	M	Paediatric ward	<1 year	R	R	R	R	R	R	R	R	I	R	R	R	S	I	S	R	S	I	R	R
S31	MRSA	F	Surgical ward	3 years	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	R	S	R	R	R
S24	MSSA	M	ICU	33 years	R	S	S	R	R	R	S	S	R	R	R	I	S	I	R	R	S	R	I	I
S13	MSSA	M	ICU	<1 year	R	S	S	R	R	R	I	R	I	R	I	R	S	S	S	I	S	S	I	R
S34	MSSA	M	NICU	<1 year	R	S	S	R	R	R	I	R	I	R	R	R	S	I	S	S	S	I	S	S

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Key: PEN, penicillin; AMP, ampicillin; FOX, cefoxitin; CIP, ciprofloxacin; MXF, moxifloxacin; LEV, levofloxacin; GEN, gentamicin, AMK, amikacin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; DOX, doxycycline; TGC, tigecycline; CHL, chloramphenicol; NIT, nitrofurantoin; SXT, trimethoprim-sulfamethoxazole; VAN, vancomycin; RIF, rifampicin; LZD, linezolid and TEC, teicoplanin. R, resistant; I, intermediate; S, susceptible; M, male; F, female; NICU, neonatal intensive care unit; ICU, intensive care unit.

Isolates harboured various permutations and combinations of ARGs which included ARGs against β-lactams [blaZ, mecA], aminoglycosides [aac(6')-aph(2''), aad(6'), ant(9)-la, ant(6)-Ia, aph(2'')-Ia, aph(3')-IIa, kdpD sat-4], trimethoprim [dfrG, dfrC], macrolides [erm(C), erm(A)], tetracycline [tet(K), tet(M), mepR, mepA], flouroquinolones [parE, parC, mgrA, arlS, arlR, grlA, gyrA, norA, norC, sdrM (multidrug efflux pumps)], rifampicin [rpoB] and fosfomycin [fosB, murA], (Table 2). There was a good concordance between ARGs and phenotypic profiles for blaZ, mecA, and aminoglycoside resistance genes in MRSA and MSSA isolates.

Table 2. Genotypic characteristics of the of *S. aureus* implicated in BSIs.

Isolate ID	**MRSA/MSSA**	**MLST**	***spa* Type**	**Resistome**	**Plasmid replicon type**	**Insertion sequences**	**Confirmed CRISPRs (CAS)**	**Clonal complex**	***SCC*mec type**	**agr type** [b]	**ACME type**	**Pathogenicity score**
S11	MRSA	ST8	t9475	*blaZ, mecA, aac(6')-aph(2''), parC, dfrG, erm(C), grlA, tetK, mepR, mepA, norA, norC, fosB, arlR, arlS, mgrA, sdrM, kdpD*	rep10, rep7a, rep7c	-	6 (0)	CC8	-	Type I	Type II	0.982 (882)
S29	MRSA	ST5	t045	*blaZ, mecA, aph(3')-III, aac(6')-aph(2''), ant(6)-Ia, ant(9)-Ia, aad(6'), erm(C), erm(A), qacA, mepR, fosB, arlR, arlS, norA, norC, mgrA, sat-4*	rep10, rep21	IS6, IS256	12 (0)	CC5	*SCCmec* type I(1B)	Type II	-	0.98 (914)

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S31	MRSA	ST612	t1257	*blaZ, mecA, aac(6')-aph(2'')-Ia, aph(2'')-Ia, aad(6'), ant(6)-Ia, ant(9)-Ia , tet(M), mepR, mepA, dfrC, parC, erm(C), parE, gyrA, rpoB, fosB, arlR, arlS, norA, norC, murA, sdrM, kdpD*	rep7c, rep20	IS256, IS6	7 (0)	CC8	SCC*mec* type IVd(2B)	Type I	-	0.976 (978)
S24	MSSA	ST152	t355	*blaZ, dfrG, mepR, mgrA, arlR, kdpD, norC, sdrM, murA*	rep16, rep5a	-	8 (0)	-	-	Type IV	-	0.975(225)
S13	MSSA	ST5	t1265	*blaZ, norA, norC, arlR, arlS, sdrM, mgrA, fosB, kdpD*	rep20	-	9 (0)	CC5	-	Type II	-	0.985 (844)
S34	MSSA	ST5	t7888	*blaZ, norA, norC, sdrM, mepR, arlS, alrR, kdpD, mgrA, fosB, lmrS*	rep19, rep16, rep20, rep5a	1S6	7 (0)		-	Type II	-	0.983 (871)

*SCCmec typing was predicted with the SCCmecFinder, MSSA –Methicillin-susceptible Staphylococcus aureus, MRSA- Methicillin-resistant Staphylococcus aureus2.5. In Silico ARGs Analysis.

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We identified known mutations in the gyrA, gyrB, parC, and parE genes found in the quinolone resistance-determining region (QRDR) known to confer fluoroquinolone resistance in some isolates (supplementary material). Also, the major facilitator superfamily (MFS) antibiotic efflux pump (norA, norC), which can also confer resistance to fluoroquinolones, was identified in most isolates. We detected two known mutations (H481N, I527M), and a putatively novel mutation in the rpoB gene (F737Y) conferring resistance to rifampicin in one isolate (S31).

2.5.1. MLST, spa typing, and Clonal Complex

MLST typing revealed a total of four sequence types, ST5 (n=3), ST152 (n=1), ST612 (n=1), and ST8 (n=1). Two MRSA isolates belonged to CC8 (n=2), and CC5 (n=1), while one MSSA isolate belonged to CC5. Two MSSA strains belonging to ST152 and ST5 that could not be classified into a CC. The genetic diversity of the isolates was confirmed by spa typing which revealed six different spa types: t9475, t1265, t355, t045, t1257, t7888 (Table 2). CC and spa type combinations were CC8-t9475, CC8-t1257, CC5-t045 among MRSA isolates, and CC5-t1265 belonging to one MSSA isolate. There was no association observed between STs, spa type, and CC. The grouping of the STs, and spa-types yielded six genotypes, i.e., ST8-t9475, ST152-t355, ST5-t045, ST5-t1265, ST612-1257, ST5-t7888 indicating that isolates were not clonally related.

The SCCmecFinder analysis identified two SCCmec types, i.e., IVd (2B), and I (1B) among the MRSA isolates (Table 2). One MRSA isolate was non-typeable (NT) for SCCmec. The combination of MLST, CC, spa, and SCCmec yielded the ST612-CC8-t1257-SCCmec_IVd (2B), and ST5-CC5-t045-SCCmec_I (1B), clones both of which have been reported in South Africa.

2.5.2. Mobilome (Plasmids, Insertion Sequences, Intact Prophages, and SCCmec Elements)

Analysis of the six isolates genomes identified various MGEs, including plasmid replicons, IS's, prophages, and SCCmec elements. A total of eight different plasmid replicons were detected of which rep20 (n=3), was the most prevalent (Table 2). There were no associations between plasmid replicons and STs. However, the rep7c was found in CC8 isolates in addition to other plasmid replicons, while rep16 and rep5a were found in isolates with the non-typeable CC. The rep20 plasmid replicon was associated with CC5 and CC8 isolates. The rep10 was carried in CC8 and CC5 isolates, while the re7a and rep21 were carried in CC8 and CC5 isolates, respectively. IS6 and IS256 were identified in three isolates, and their occurrence was not associated with any STs or CC (Table 2). A total of six intact prophages were detected, of which the most identified were PHAGE_Staphy_phi2958PVL (n=2), and PHAGE_Staphy_P282 (n=2) (Table S5). PHAGE_Staphy_phiJB was associated with the dfrG gene.

2.5.3. Virulome and Pathogenicity of S. aureus Strains

A total of 82 virulence genes were detected across the isolates **(Table S3)**. The virulence genes belonged to the five main virulence determinant classes of S. aureus: adherence factors, immune evasion, enzymes (exoenzymes), toxins and the secretion system. It is noteworthy that the most prevalent toxins were hemolysins i.e., gamma (hlg), delta (hld), alpha (hly/hla), staphylococcal enterotoxins (se, set, sel) genes, and leucocidin genes (lukD/E), while lukS-PV, lukF-PV genes were detected in two isolates (S24 & S29). The prediction of isolates pathogenicity towards humans yielded a high average probability score (Pscore ≈ 0.980).

2.6. Genetic Environment of the ARGs and Virulence Genes

The co-carriage of ARGs, and virulence genes was evident across the isolates. Using NCBI annotation, we identified blaZ genes on five isolates in parallel with cacD, virulence genes, and type 1 toxin-antitoxin system. Across the isolates, most blaZ genes were associated with regulator genes blaR and blaI and frequently found with either a recombinase, integrase, cadmium resistance (cadD) gene, or type I toxin-antitoxin system (Table 3). A similar genetic context was detected in S13 isolate, where blaZ, blaR, and blaI were flanked by IS6, cadD, a type I toxin-antitoxin system, on a contig with the closest nucleotide homology to a plasmid from S. aureus pER10678.3A.1 (CP051928.1),

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suggesting that ARGs, heavy metal resistance genes (HMRGs), and virulence genes may be mobilised by plasmids (Table 3). It is noteworthy that the IS1182 was associated with the mecA, mecI and mecR1 genes together with recombinases, while IS6 bracketed the mecA gene and its regulatory genes (mecI and mecR) in one isolate (S11). Most ARGs, including erm(A), ant(9)-Ia, dfrG, tet(M) were associated with a recombinase and integrase. One isolate was found harbouring the dfrG gene bracketed by ISL3, and recombinases.

2.6.1. Regulatory Genes

The accessory gene regulator system (agr) involved in the regulation and expression of toxins, exoenzymes, and biofilm was detected in all isolates. Isolates carried agr type I and II. The distribution of the agr group in MRSA was: agr I (n=1), agr II (n=2), while in MSSA agr I (n=2), and agr II (n=1).

2.7. Phylogenomics

Phylogenetic trees generated from genomes that were obtained from BV-BRC (coloured in purple) (Figure 1), including isolates from the present study (coloured in green).

Table 3. Genetic context of virulence genes in *S. aureus* isolates.

Strain (MLST)	**Strain**	**Contig**	**Synteny of virulence genes and MGEs**	**Plasmid/chromosomal sequence with closest nucleotide homology (accession number)**
S11 (ST8)	MRSA	4	pmtC:pmtB:pmtA:***eap***::***scn***::***sak***:::sph::***lukG***::***lukH***::integrase:::agrB	*S. aureus* strain Laus385 chromosome (CP071350.1)
		6	***icaR***::***icaD***:***icaB***:***icaC***:vraD:vraE:vraH::IS30:vraH::recombinase:IS6	*S. aureus* strain TF3198 chromosome, complete genome (CP023561.1)
		10	***lukE:lukD***::splA::epiE::***splA***:splB:splC:splD:splE:splF::pepA1:transposase	*S. aureus* strain 82 chromosome, complete genome (CP031661.1)
S29 (ST5)	MRSA	53	type I toxin-antitoxin system:IS6:cadD	*S. aureus* strain MIN-175 chromosome (CP086121.1)

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		40	***clfA***:vwb:***emp***	*S. aureus* strain ER02693.3 chromosome, complete genome (CP030605.1)
S31(ST612)	MRSA	11	pmtD:pmtC:pmtB:pmtA::***eap***:***scn***::***sak***	*S. aureus* strain 2395 USA500, complete genome (CP007499.1)
		15	***lukE***:***lukD***::::***splA***:***splB***:***splC***:***splF***::type I restriction-modification system	*S. aureus* strain NRL 02/947 chromosome, complete genome (CP103850.1)
		19	***lukG***:***lukH***:pathogenicity island:intergrase::phenol-soluble modulin:agrB	*S. aureus* strain 2395 USA500, complete genome (CP007499.1)
		22	***seq***:***sek***:integrase::::***emp***:***clfA***	*S. aureus* strain 2395 USA500, complete genome (CP007499.1)
		33	recombinase::universal stress protein:::cadD::***seq***:***sek***:integrase:::***emp***:***clfA***	*S. aureus* plasmid SAP017A, complete sequence (GQ900382.1)
		64	***sea***:putative holin-like toxin	*S. aureus* strain R50 chromosome, complete genome (CP039167.1)
S13 (ST5)	MSSA	4	***sbi***:***hlgA***:***hlgC***:***hlgB***	*S. aureus* strain AR462 chromosome, complete

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				genome (CP029086.1)
		5	scpA:::**eap**::***scn***:***sak***::::::intergrase:sph:***lukH***:***sbi***:***hlgA***:***hlgC***:***hlgB***	*S. aureus* strain pt239 chromosome, complete genome (CP049467.1)
		15	IS6::cadD:::***sed***:***sej***:***ser***::recombinases:cpA::eap::***scn***:**sak**::integrase:sph:***lukH***	*S. aureus* strain ER10678.3 plasmid pER10678.3A.1 (CP051928.1)
S24 ST152)	MSSA	8	arsB::crcB::***scn***:***sak***:::recombinase::type II toxin-antitoxin system toxin:intergrase	*S. aureus* strain UMCG579 chromosome, complete genome (CP091066.1)
		21	cadD:type toxin-antitoxin::integrase	*S. aureus* strain GHA13 chromosome (CP043911.1)
		11	BrxA/BrxB:::msrA:msrB:::norD::cspA:cvfB	*S. aureus* strain NGA84b chromosome, complete genome (CP051165.2)
S34 (ST5)	MSSA	7	***eap***/***map***::***scn***:***sak***::::***sea***:::type II toxin-antitoxin:integrase:sph:***lukG***:***lukH***	*S. aureus* strain HPV107 chromosome, complete genome (CP026074.1)
		8	clfA:vwb:emp::thermonuclease protein:::s**ek**:***seq***::pathogenicity island	*S. aureus* strain B4-59C chromosome, complete genome (CP042153.1)
		12	**sem**:**sei**:**seu**:**sen**:**seg**:::***lukE***:***lukD***::splA:splB:splC:splD:splF	*S. aureus* strain ER03588.3

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		chromosome, complete genome (CP030595.1)
14	isdB:isdA:isdC:isdD:isdE:isdF::isdG::***ecb*::*efb*:*scb***	*S. aureus* strain B3-17D chromosome, complete genome (CP042157.1)
20	SSL13:SSL12:**hyl**	*S. aureus* strain NAS_AN_239 chromosome, complete genome (CP062409.1)

*Virulence gene(s) in bold.

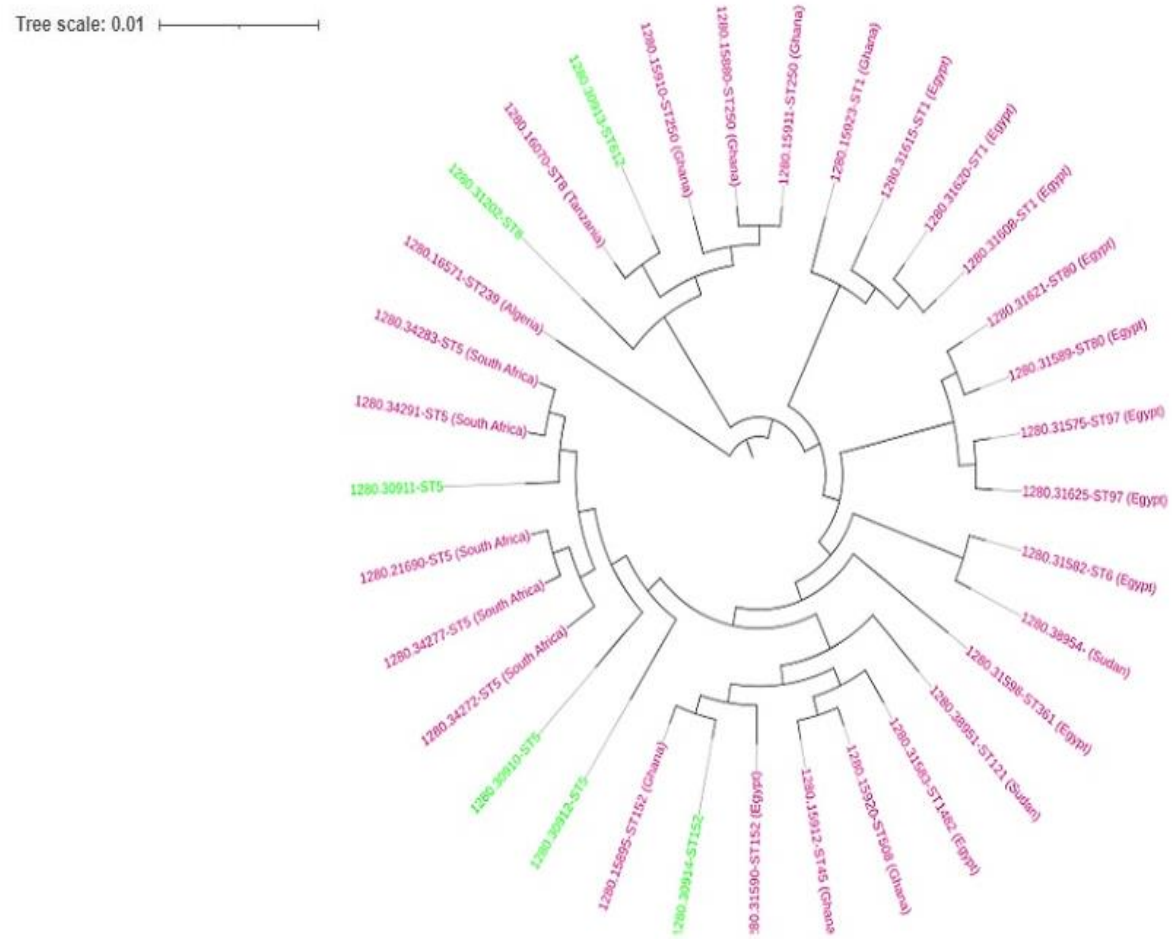

Figure 1. Circular phylogenetic tree with colour annotations depicting the relationship between S. aureus isolates from this study coloured in green and African blood culture isolates coloured in purple.

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3. Discussion

We studied the genomic characteristics of six MDR *S aureus* isolates implicated in BSIs. The study analysed the resistome, virulome, mobilome, phylogeny, and genetic environment of the resistance genes using WGS and bioinformatics. The genomes analysed herein were predominantly recovered from patients ≤1-year-old.

There was a diversity of ARGs encoding resistance to different antibiotics and good concordance between the observed phenotypic and genotypic resistance. The incidence of ARG's encoding resistance to β-lactams, aminoglycosides, macrolides, fosfomycin, trimethoprim, tetracycline, and genes coding multidrug resistance (MDR) efflux pumps (*nor*A, *mep*R, *arl*R, *mgr*A, and *lmr*S) was not dependent on the clonal type. Resistance genes found in this study included *erm* genes which mediate macrolide–lincosamide–streptogramin B (MLSB) resistance, the aminoglycoside-modifying enzymes, MFS antibiotic efflux pump (*msrA*, *nor*A, *nor*C), which also confers resistance to fluoroquinolones [25]. The *erm*(C) and *erm*(A) genes that are commonly found in MLS_B-resistant *S. aureus* were found in erythromycin and clindamycin resistant isolates (Table 2), which was expected since resistance to erythromycin co-selects resistance to other antibiotics, such as streptogramin B (MLS_B) and lincosamides [26]. The *erm*C gene is among the primary *erm* type which facilitates ribosome methylation of the 23S rRNA, triggering conformational changes, resulting in drug binding inhibition [27], and have been reported in clinical *S. aureus* isolates from South Africa [28]. In this study, the *erm*C encoding macrolide resistance was carried on a plasmid, on a contig that had the closest nucleotide homology to plasmids from *S. epidermidis* strain TMDU-137 plasmid p5, complete sequence (CP093178.1), implying the likelihood of horizontal transfer of *erm*C genes in clinical *S. aureus* isolates. The *erm*C are often plasmid-mediated, resulting in high resistance to macrolides in *S. aureus* [29].

The *bla*Z gene which inactivates penicillin through hydrolysis of the beta-lactam ring was observed in all six isolates that were phenotypically resistant to penicillin. The *blaZ* genes have also been isolated in clinical isolates of Staphylococci in South Africa [30]. In this study, the *bla*Z genes were found on contigs with closest homology to either chromosomes or plasmids. This agrees with a study done in Spain that analysed ARGs presence in chromosomes and plasmids from the genomes of *S. aureus*. WGS analysis of *S. aureus* revealed that *bla*Z (n=2) were located on chromosomic contigs, while *blaZ* was found in plasmid contigs in three isolates [31]. It is important to note that most *bla*Z and associated MGEs from isolates belonging to ST5 (S13, S34) isolated from the intensive care unit (ICU), and paediatric ward (S29) were located on contigs that had the closest homology to plasmids, implying that plasmids play a crucial role in mobilizing the *bla*Z gene in clinical *S. aureus* isolates. The S29 isolate belonging to t045-CC5 lineage, carried assortment of ARGs encoding resistance to different antibiotics (Table 4). Similar ARGs in MRSA lineage t045-CC5-MRSA were also reported in a study conducted in South Africa, where t045-CC5 MRSA lineages obtained from different clinical samples from South Africa and Nigeria reported that t045 lineages were MDR, suggesting that this lineage is hospital-associated, and their multidrug resistance nature may compromise treatment [32].

Also, the *blaZ* genes, heavy metal genes and associated MGEs were carried on either plasmid or chromosome. The *bla*Z and *cad*AC genes were found on the genetic element recombinase:*bla*I:*bla*R1:*bla*Z:*cad*C:*cad*A for isolates S24 (MSSA) that was from the ICU, and S29 (MRSA) from the paediatric ward, suggesting co-selection of heavy metal resistance dissemination and adaptation in different wards. The *cad*A gene confers a high resistance to cadmium and other heavy metals like zinc and lead in *S. aureus* isolates [33]. The *cad*A was associated with a plasmid, similar to the findings of a study that was done by Al-Trat et al. (2023) in Malaysia who used WGS to analyse the plasmid content of clinical MRSA isolates, and reported that heavy metal resistance plasmids harboured cadmium resistance genes with the majority being *cad*AC [34]. The HMRGs have been reported to trigger co-selection mechanism with antibiotics, which may complicate treatment [35]. This may pose a challenge especially among patients in the ICU where broad-spectrum antibiotics are often used.

Tetracycline resistance genes (*tetK, tetM*) were observed in two isolates. Isolate S11 carried *tet(K)* associated with a genetic context: plasmid recombination:*tet(K)* that had a high similarity to *S.*

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epidermidis BPH0662, plasmid: 1 (LT614820.1), which could be significant in mobilising TET resistant genes. Also, the *tet*(M) was bracketed by integrase and IS256 in isolate S31. The IS*256* is a retrotransposon that can mobilize the resistance genes through a copy-and-paste mechanism and has been shown to confer a robust genomic plasticity in MRSA strains [36]. We found mutations in *gyrA, gyrB parC*, *parE*, and *rpoB*, implicated in fluoroquinolone and rifampicin resistance. However, the detection of double mutations of gyrA/parC could be associated with high levels of resistance to fluoroquinolones [37].

We found that ARGs, and virulence genes were associated with MGEs, which may enable their transfer within and between plasmids and chromosomes [38]. In this study, the *m*ecA gene was located on IS1182 in two isolates, surrounded by recombinase in genetic context *mecA:mecR1*::IS1182::recombinase. The insertion sequence IS1182 was present in 2/3 MRSA strains that contained *mecA*. IS1182 has been shown to occur close to the SCC*mec* element and increase resistance through inactivating *lytH* gene encoding a putative lytic enzyme in pathogenic MRSA isolates [39].

MLST typing, clonal complex, *spa* typing, and SCC*mec* typing were used to analyse the molecular characteristics of the *S. aureus* isolates. Four ST types and two clonal clusters (CCs) were found among the six clinical isolates in this study, with ST5, the most predominant complex clonal CC5 and CC8. Generally, clonal lineages ST5, ST8, ST152, and ST612 are among the most commonly reported in hospital environments, along with other sequence types of *S. aureus* [40]. *S. aureus* ST5, belonging to CC5, was predominant in this study and was previously reported among patients with bloodstream infections at Ruijin Hospital in Shanghai [3]. The detection of clonal complexes CC5 and CC8 agrees with a study by Smith et al. [16], which also found CC8, and CC5 were predominant in a study that analysed the genomic epidemiology of MRSA and MSSA from bloodstream infections in the USA. Their results revealed that the MDR phenotype observed in strains belonging to CC5 and CC8 was responsible for the occurrence of multidrug and methicillin resistance in the *S. aureus* population. MRSA strains belonging to CC8 and CC5 are frequently associated with global outbreaks and have been identified in Africa [41].

The *spa* typing revealed six different spa types, suggesting a non-clonal MRSA and MSSA distribution. The detection of *spa* types t1257, t045 and t355 agrees with a study conducted in South Africa, which analysed the diversity of SCC*mec* elements and spa types in *S. aureus* isolates from blood culture in the Gauteng, KwaZulu-Natal, Free State, and Western Cape provinces [14], in which t037 and t1257 were the most common and predominated throughout the seven-year study period. In this study, some antibiotic resistance genes were associated with specific MRSA clones belonging to *spa* types t1257 and t045, t9475. Shittu et al. (2021) found the *spa* types t045 and t1257 to be the most prevalent and associated with genes conferring resistance to aminoglycosides, trimethoprim, macrolides and tetracycline in clinical isolates of *S. aureus* from South Africa and Nigeria.

The analysis of SCC*mec* types revealed the presence of SCC*mec* type IVd (2B) and SCC*mec* type I (B) carrying the *mecA* gene, which occurred in tandem with *mecR1* in both isolates. However, one MRSA (S11) isolate had a non-typeable SCC*mec* element cassette due to the missing cassette chromosome recombinase (*ccr*) gene complex [42]. The *ccr* gene complex is an essential component required to facilitate the integration or excision of the SCC*mec* element in the staphylococcal chromosome, and their loss has also been reported [43]. The SCC*mec* IV detected in our study is associated with the spa type t1257, previously reported in South Africa in *S. aureus* obtained from poultry isolates [15], implying its possible transfer between humans and animals.

We found different MRSA genotypes ST612-t1257-CC8, ST8-t9475-CC8, and ST5-t045-CC5, suggesting that MRSA isolates were not clonally and epidemiologically related. The ST612-t1257-CC8 identified in this study, is an endemic MRSA clone which have been reported in animal and clinical settings [14,15]. The ST5-I-MRSA, known as the pandemic British EMRSA-3 clone, was detected in the paediatric ward. This is similar to a study conducted in South Africa, where the t045-MRSA strain occurred in paediatric patients [18]. The isolation of t045-ST5-MRSA strain could confirm its successful persistence in the hospital and its capacity to cause infections in neonatal and paediatric wards [44].

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Several virulence factors, including adherence, immune invasion, toxins, and exoenzymes associated with invasive infections, were detected in our isolates. The virulence genes encoding clumping factor proteins (*clfA* and *clfB*) are involved in the pathogenesis of *S. aureus,* including bacteremia [9]. Consistent with pathogenic *S. aureus* strains isolated in various environments globally, our isolates were characterized by *icaADBC* operon and (*sdrC*, *sdrD*, *sdrE*) involved in biofilm-forming genes [45]. Most strains haboured genes, including the alpha and gamma-hemolysin genes (*hlg*A, *hlg*B, *hlg*C, *hly/hla*, *hlb*), and the *ica* operon associated with pathogenicity and adhesion. Additionally, our isolates were characterised by various toxins, including *lukE/D* genes, and panton-valentine leukocidin (PVL) *lukS-PV/lukF-PV* genes in one MSSA, and MRSA strains. The expression of these PVL toxin genes in *S. aureus* isolates, lyses host cells and promotes virulence of the bacteria [46], which might worsen the outcomes *S. aureus* infection. Consistent with clinical *S. aureus* strains, our isolates were characterised by a capsular polysaccharide (CP) serotype 8, which shields the bacterial pathogen from host immune defence mechanisms associated with increased virulence in BSIs [47].

Most virulence genes including those encoding SEs, *sak*, *hlg*, *luk*, *scn clfA*, *sbi*, and associated MGEs were carried on chromosomes in the majority of isolates. The *ica* gene operon and *vra* genes were found to be associated with ISs (IS30, IS6) and recombinase for S11 (ST8) isolate from the surgical ward. The *ica* genes *vra*DEH genes have been shown to play an important role in biofilm formation [48], daptomycin resistance in *S. aureus* [49]. which could enhance antibiotic resistance traits and chronic infection. The occurrence of ST8-t9475 MRSA strains co-harbouring *ica* genes and genes encoding daptomycin resistance in ST8 MRSA could be advantageous to the ST8-t9475 colonization, invasion, and survival in the surgical ward. The virulence genes encoding SEs, *eap, scn, sak, sph*, *lukH,* and *cad*A, were found on a contig that had high sequence similarity to *S. aureus* strain ER10678.3 plasmid pER10678.3A.1 (CP051928.1), implying that they are mobilized by plasmids. Virulence genes, including those encoding hla/hld, toxin production, and biofilm formation, are plasmid-mediated [50], thus could easily facilitate their transfer resulting in highly pathogenic strains that may be difficult to treat.

Phylogenomic analyses revealed that the clinical isolates in this study clustered mainly with clinical isolates from hospital patients (Figure 2). ST5 study isolates were closely related to clinical isolates from South Africa suggesting possible dissemination of ST5 strains and adaptation in hospital environments. Furthermore, ST152 isolate was closely related to ST152 strains from Egypt and Ghana, implying a possible spread and epidemiological linkage between these isolates. ST152-PVL-producing *S. aureus* isolates is particularly frequent and widespread in West and Central Africa [51], and livestock [52]. The ST152- PVL-positive MSSA, has also been reported from cutaneous abscesses among mine workers at a gold mine in Gauteng, South Africa [53]. Identifying ST152 in livestock and humans suggests animal–human transmission, which requires further investigation. ST8 and ST612 isolates were closely related to ST8 isolated from Tanzania, indicating that ST612 is a double-locus variant of ST8. ST8 and ST612 isolates are potentially multidrug-resistant and highly virulent strains associated with hospital outbreaks [54].

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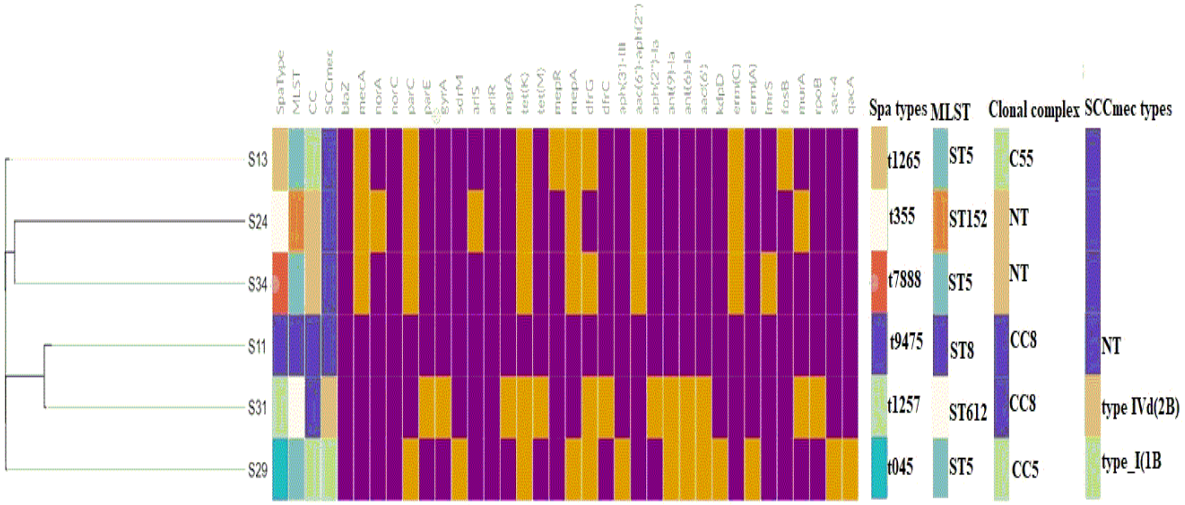

Figure 2. The phylogenetic branch and metadata [spa type, sequence type (ST), clonal complex, SCCmec types and ARGs coupled using Phandango (https://github.com/jameshadfield/phandango/wiki) in S. aureus isolates.

4. Materials and Methods

4.1. Ethical Consideration

The study isolates were part of a larger surveillance study using the Global Antimicrobial Resistance and Use Surveillance System (GLASS) guidelines. Ethical approval for the study was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal under the following reference number BCA444/16.

4.2. Sample Collection and Bacterial identification

Forty-five putative *Staphylococcus* isolates were collected from blood cultures sourced from hospitals within the uMgungundlovu district in the KwaZulu-Natal province from November 2017 to December 2018. Isolates were identified by the National Health Laboratory Services (NHLS) using the automated VITEK 2 system (BioMérieux, MarcyL'Etoile, France). The WGS study sample consisted of a subset of 10 MDR isolates, the selection of isolates was based on their antibiograms. However, 4 isolates were excluded during the quality control process.

4.3. Antimicrobial Susceptibility Testing and MRSA detection

Antibiotic susceptibility of the *S. aureus* isolates was determined by the Kirby–Bauer disk-diffusion and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical and Laboratory Standards Institute (CLSI) breakpoints. The following antibiotics were tested: penicillin G (10 µg), ampicillin (10 µg), cefoxitin (30 µg) tigecycline (15 µg), nitrofurantoin (300µg), (interpreted using EUCAST breakpoints) (EUCAST, 2017), ciprofloxacin (5 µg), levofloxacin (5 µg), moxifloxacin (5 µg), erythromycin (15 µg), gentamicin (10µg), amikacin (30 µg), chloramphenicol (30µg), tetracycline (30µg), doxycycline (30µg), sulphamethoxazole/trimethoprim (1.25 µg + 23.75 µg), teicoplanin (30 µg), linezolid (30 µg), clindamycin (2 µg), rifampicin (5 µg) (interpreted using CLSI breakpoints) (CLSI, 2017). All antibiotic discs were from Oxoid (Oxoid, Basingstoke, UK). *S. aureus* ATCC 29213, was used as the control. Multidrug resistance (MDR) was defined as resistance to one or more antibiotics belonging to three or more different antibiotic classes [21].

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4.4. Whole-Genome Sequencing (WGS) and bioinformatic analysis

The genomic DNA was extracted from the *S. aureus* isolates using the GenElute Bacterial Genomic DNA kit (Sigma Aldrich, St. Louis, USA) according to the manufacturer's instructions. The quantity and quality of the extracted gDNA was analysed using NanoDrop 8000c (Thermo Scientific, Waltham, MA, USA). The Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) was used for library preparation. WGS was conducted using an Illumina NextSeq Machine (Illumina, San Diego, CA, USA). Good quality trimming of raw reads was done using Sickle v1.33 (https://github.com/najoshi/sickle). The raw reads were assembled spontaneously using the SPAdes v3.6.2 assembler (https://cab.spbu.ru/software/spades/). Subsequently, all contiguous sequences were submitted to NCBI and assigned accession numbers under BioProject

4.5. Genomic Analysis and Annotation

The draft genomes were submitted to GenBank and assigned accession numbers under the BioProject PRJNA400143. Analysis including antibiotic resistance genes, virulence factors and pathogenicity, mobile genetic elements (MGEs), plasmid replicons, and genotyping including MLST, spa, and *SCCmec* typing were performed the Centre for Genomic Epidemiology (CGE) (https://www.genomicepidemiology.org/services/), and their respective databases. Antibiotic resistance genes (ARGs) and other ABR determinants were identified using the CGE ResFinder 4.1 (https://cge.cbs.dtu.dk/servic es/ResFinder/), and the comprehensive antibiotic resistance database (https://card.mcmaster.ca/analyze/rgi). Virulence factors were identified using the virulence factor database (VFDB: http://www.mgc.ac.cn/VFs/main.htm. PHASTER was used to identify prophage elements (https://phaster.ca/). The accessory gene regulator (*agr*) typing was conducted through nucleotide BLAST. GenBank accession numbers AFS50129.1, AFS50128.1, AFS50130.1, and AFS50131.1 were used as reference sequences for agr type I to IV, respectively [22]. The synteny and genetic environment of ARGs and associated MGEs were investigated using GenBank's general feature format (GFF3) files. The GFF3 files were imported into Geneious Prime 2020. 2 (https://www.geneious.com) for analysis [23]. The arginine catabolic mobile element (ACME) is a genomic island in Staphylococci that contains virulence factors including an arginine deiminase (*arc*) pathway and an oligopeptide permease (*opp-3*) system, which contribute to enhanced pathogenicity [24]. The ACME genes within the genomes were detected and aligned. Alignment of the ACME components made up of the arc operon, the *opp-3* operon, and the kdp operon, was used to classify the ACME components as follows: arc and *opp-3* operons (type I), the arc operon only (type II), the *opp-3* operon only (type III), the *arc* and *kdp* operons (type IV), and all three *arc*, *opp*, and *kdp* operons (type V), using Pathosystems Resource Integration Center (PATRIC) (https://www.patricbrc.org/) annotations.

4.6. Single Nucleotide Polymorphism (SNP) Analysis in Antibiotic Resistance Strains

Mutations conferring resistance to fluoroquinolones and rifampicin were determined using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Briefly, *gyrA*, *gyrB*, *parC*, *parE*, and *rpoB* genes in a reference susceptible *S. aureus* ATCC 29213 were aligned with the corresponding genes from resistant isolates in this study with BLASTn to call for SNPs in those genes using the Clustal Omega tool (European Molecular Biology Laboratory). Mutations in the genomes of the study isolates were manually curated and tabulated.

4.7. Phylogenomic Analysis

Whole-genome sequences of *S. aureus* isolates from blood culture curated at the bacterial and viral bioinformatics resource center (BV-BRC) online platform (https://www.bv-brc.org/) from Northern Africa (Egypt, Algeria, Sudan), Western Africa (Ghana), and Eastern Africa (Tanzania) were downloaded and used together with our study's isolates for phylogeny analysis. The selection of isolates was based on the sample source (blood culture). A phylogenomic tree of *S. aureus* was built using BV-BRC's Phylogenetic Tree Building tool, using the nucleotide and amino acid sequences from

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1,000 shared genes (https://www.bv-brc.org/). The generated phylogenetic trees were visualized, annotated, and edited using iTOL (https://itol.embl.de/) and Figtree (http://tree.bio.ed.ac.uk/software/figtree/).

4.8. Nucleotide Sequence Accession Number

The nucleotide sequences of MRSA (S29, S11, S31) and MSSA (S13, S24, S34) isolates were submitted to the NCBI GenBank database under the following accession numbers; JADQTH000000000, JADIXB000000000, JADIXC000000000, JADIXA000000000, JADIXE000000000, JADIXD000000000.

5. Conclusions

The study presents an insight into ARGs, virulence genes, MGEs, and genetic diversity of *S. aureus* collected from a public hospital in uMgungundlovu. We observed high diversity of spa types, STs, predominance of CC8, and CC5 indicating the genetic variability of *S. aureus* in hospital settings. The occurrence of pathogenic and MDR strains in the hospital setting, especially in ICU can pose a serious threat that limits the therapeutic options available. Here, we demonstrate that while MRSA displayed multidrug resistance, MSSA reflect potentially increasing resistance to the antibiotics used for treatment. Continuous surveillance and monitoring of MRSA and MSSA strains circulating in hospital environments is needed.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Patient demographics; Table S2: Genomic characteristics of *S. aureus* strains; Table S3: Virulence genes identified in MSSA and MRSA isolates in this study; Table S4: Distribution of insertion sequences and plasmid replicon among the *Staphylococcus aureus* strainsDistribution of intact prophage region among the *Staphylococcus aureus* strains.**Author Contributions:** Conceptualization, B.H., DGA and S.Y.E; methodology, B.H. A.I. and A.L.K.A; formal analysis, B.H., D.G.A., J.M., J.A. and A.L.KA; investigation, B.H.; resources, A.I., D.G.A. and S.Y.E ; writing—original draft preparation, B.H.; writing—review and editing, B.H., J.A., J.M., A.I., A.L.K.A., D.G.A., and S.Y.E; supervision, S.Y.E., A.L.K.A and D.G.A. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethical approval for the study was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal under the following reference number BCA444/16. The study isolates were part of a larger surveillance study using the Global Antimicrobial Resistance and Use Surveillance System (GLASS) guidelines.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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