MOLECULAR CHARACTERIZATION OF RESISTANCE AND VIRULENCE IN METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS (*MRSA) FROM THE PRIVATE SECTOR IN KWAZULU-NATAL, SOUTH AFRICA

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Submitted in fulfillment of the requirements for the degree of Masters in Medical Sciences in the School of Health Science, University of KwaZulu-Natal

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A dissertation submitted to the School of Health Sciences, College of Health Science, University of KwaZulu-Natal, Westville, for the degree of Master of Medical Science.

This is a dissertation by manuscript with an overall introduction and final summary.

This is to certify that the content of this dissertation is the original research work of Mr. Daniel Gyamfi Amoako, supervised by;

Supervisor: Signed: ----------Name: **Prof. S. Y. Essack Date: ……**

Co-Supervisor: Signed:  Name: **Dr. L.A. Bester Date:** *07.03.2016*

DECLARATION

I, Mr. **Daniel Gyamfi Amoako**, declare as follows:

1. That the work described in this dissertation has not been submitted to UKZN or any other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

2. That my contribution to the project was as follows:
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   - This dissertation does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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Signed: 

DG. Amoako

Student Number 214583994

Date: 07/03/16

DEDICATION

This work is dedicated to my late mother Paulina Ampofo and late aunty Maltida Oduro Poku who gave me the chance to get education.

This work is dedicated to my supervisor Dr. Linda Antionette Bester for being my inspiration and the drive towards achieving this degree.

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Any omissions and shortcomings that may be identified in this piece of work remain the sole responsibility of the researcher.

DG Amoako

Durban

2016

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LIST OF ABBREVIATI ONS AND ACRONYMS

CLSI	Clinical Laboratory Standards Institute
CDD	Cefoxitin disc diffusion
GIs	Genomic Islands
IgG	Immunoglobulin G
KZN	KwaZulu-Natal
Mbps	Megabase pairs
MDR	Multidrug resistance
MGEs	Mobile genetic elements
MICs	Minimum inhibitory concentrations
MLS_B	Macrolide-lincosamide-streptogramin B
MLST	Multi locus sequence typing
MRSA	Methicillin resistance *Staphylococcus aureus*
MSCRAMMS	Microbial surface components recognising adhesive matrix molecules
ORFs	Open reading frames
PBP2A	Penicillin-Binding Protein 2A
PCR	Polymerase chain reaction
PEARLS	Pan-European antimicrobial resistance using local surveillance
PFGE	Pulse field gel electrophoresis
PVL	Panton-valentine leukocidin

SCC	Staphylococcal cassette chromosome
SCVs	Small colony variants
SNPs	Single nucleotide polymorphisms
Spa	Staphylococcal protein A
TSST-1	Toxic shock syndrome toxin-1
WGS	Whole genome sequencing

APPENDICES

Appendix 1 Biomedical research ethics committee (BREC) approval letter

Appendix 2 Submission confirmation from the International Journal of Infectious Diseases

SUPPLEMENTARY MATERIAL

A Compact Disc (CD) with the electronic copy of this dissertation in portable digital format (pdf).

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) causes nosocomial, community and livestock-associated infections. MRSA strains harbor diverse mobile genetic elements (MGEs), including plasmids, pathogenicity islands, transposons, integrons and prophages, which comprise 15-25% of the genome encoding resistance and virulence genes. We investigated resistance and virulence genes in the plasmids of 27 MRSA clinical isolates from the private healthcare sector in Durban, South Africa.

MRSA was confirmed by *mecA* gene identification on plasmids extracted using a commercial plasmid extraction kit. The isolates were subjected to antimicrobial susceptibility testing and molecular characterization of 4 common resistance encoding genes and four frequently encountered virulence factors: *blaZ, aac (2')-aph (6''), ermC, and tetK, and, hla, hld, eta* and *LukS/F-PV* by PCR using plasmid DNA as the template. The genetic relatedness between the isolates was determined by pulsed field gel electrophoresis (PFGE).

All MRSA isolates contained plasmids, and were resistant to ampillicin, while 85.2% were resistant to ciprofloxacin, 74.1% to gentamicin, 70.4% to rifampicin, 66.7% to tetracycline, 63.0% to erythromycin, and 11.1% to clindamycin. They were also all susceptible to daptomycin, linezolid, vancomycin, tigecycline and fusidic acid. Multidrug resistance (MDR) was found in 74.1% (20/27) of the MRSA isolates. The frequency of the resistance genes *blaZ, aac (2')-aph (6'')* and *ermC* were 100%, 92.6%and 48.2% respectively, while *tetK* was not found in any of the MRSA isolates. The prevalence of virulence genes *hla and hld* were 96.3% and 92.6% respectively, however, *eta and LuKS/F-PV* were not detected. PFGE analysis revealed 10 pulsotypes, designated A–J, which correlated with the resistance profile and mechanism of the isolates in each group. 85.2% (23/27) of the isolates clustered into six major PFGE types, giving an indication of similar circulating MRSA clones. Type F was the major pulsotype (29. 6%) and was found in eight of the 27 MRSA isolates. Hospital centers 1 and

10 were found to contain pulsotypes C and H, whiles identical pulsotypes F and G were identified in nine of the 15 centers, indicating the possibility of inter health centers spread of MRSA in the province.

The complexity and diversity of molecular resistance and virulence profiles poses a challenge for MRSA infection management. A comprehensive understanding of the molecular epidemiology is essential to inform treatment and contain dissemination.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Staphylococcus aureus has persisted as one of the fundamental human pathogens, causing a variety of infections in hospital and community settings. *S. aureus* possess a remarkable number of resistance and virulence factors, and a unique capacity to adapt and survive under different host conditions, making it successful as a pathogen (1). Although Staphylococci were naturally susceptible to penicillin G, the distribution of penicillinase-encoding plasmids in the 1950s caused the rapid emergence of resistance. Penicillinase production has become a species-related trait of most staphylococcal strains (2), the main challenge being depicted by methicillin-resistance (MR), mediated by the acquisition of the penicillin-binding protein 2A (PBP2A), which the *mecA* gene encodes. This PBP2A enables the staphylococci to escape inhibition by methicillin and other penicillinase-stable beta-lactams by taking over the functions of the other PBPs (3). Methicillin-resistant *Staphylococcus aureus* (MRSA) strains also harbor other mobile genetic elements (MGEs), including plasmids, pathogenicity islands, transposons, integrons and prophages, which comprise 15-25% of the genome. MGEs carry the majority of the genes, through which strains of staphylococcal vary from each other, such as resistance and virulence genes (4). MGEs play a significant role in bacterial survival and adaptability, as they encode many antibiotic resistance determinants and virulence factors, hence understanding their composition will broaden our knowledge on their genetic determinants (5).

Glycopeptide vancomycin is currently used as a primary treatment for MRSA, as the number of therapeutic options has decreased over time. Predictably, the increase in vancomycin usage has caused the emergence of resistance, with the resulting reduction in its efficacy against MRSA. Reports of infection by strains resistance to the newest drugs available (daptomycin, vancomycin, teicoplanin), and their rapid dissemination, are of major concern as they introduce new challenges in the therapeutic and diagnostic fronts. Ascertaining potential risk factors for

MRSA acquisition, and completely characterizing the molecular epidemiology and clinical properties of these strains are vital for effectively managing infections (6).

Epidemiological and pathogenicity studies on MRSA in most African countries are limited, with South Africa being no exception (7). The Pan-European Antimicrobial Resistance Using Local Surveillance (PEARLS) study showed resistance to methicillin to be 33.3% for South African strains isolated during the 2001–2002 national survey (8). Prevalence studies of MRSA in specific South African settings have been conducted, with the varying rates being influenced by the geographical location, study population and clinical practices (9, 10). MRSA infection increases health care cost and treatment complications, and is associated with higher mortality rates (11, 12). All efforts should be geared towards preventing the spread of resistant and virulent MRSA worldwide clones.

1.2 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, facultative anaerobic, coagulase- and catalase-positive, coccus-shaped bacterium in the family *Micrococcaceae*. *S. aureus* is an opportunistic pathogen that can be found in human as well as animals, such as cats, dogs, pigs and horses. A typical 24-h Staphylococcal colony is large, β-haemolytic and cream-yellow pigmented on blood agar (13, 14). It is protected against many toxic molecules by an outer layer, the cell wall consisting of teichoic acids and peptidoglycan, which are connected to the cytoplasmic membrane (15). In addition, the staphylococcal cell wall is connected to many supporting surface proteins. The majority of all clinical *S. aureus* strains are protected by a polysaccharide capsule, with 11 unique putative capsule serotypes (16).The genome of the *S. aureus* is composed of a single circular chromosome with transposons, insertion sequences, genomic islands and plasmids. Prophages and pathogenicity islands are also considered to play vital roles in the virulence and evolution of *S. aureus* (22). Complete Staphylococcal genomes have been obtained through various genome sequencing methods (23).

Although *S. aureus* has been regarded as an extracellular pathogen, various studies have shown that it can survive in different eukaryotic cells, including osteoblasts, fibroblasts, epithelial cells, endothelial cells and keratinocytes. Staphylococci also survive within neutrophils and

human monocyte-derived macrophages (17), which contributes to the recurrent and/or persistent nature of certain infections. *S. aureus* mutants, known as 'small colony variants' (SCVs), are slow-growing colonies that are 10-fold smaller than normal bacteria, and have characteristics such as reduced haemolytic activity, low coagulase activity, decreased pigment formation, decreased toxin production and resistance to aminoglycosides (18). SCVs show similar metabolic characteristics in the central metabolism regardless of the fundamental auxotrophism, and have the ability to persist intracellularly as well as to cause persistent and recurrent infections (19).

1.2.1 Infections

Staphylococcus aureus causes many infections, ranging from soft skin to lethal conditions. The most common infection by *S. aureus* is superficial skin inflammation, which presents as a boil or furuncle. Common subcutaneous and skin infections caused by *S. aureus* include impetigo, folliculitis, carbuncles, mastitis and cellulitis. *S. aureus* can also cause chronic skin infections within human populations with some major disorders such as. More severe *S. aureus* infections include myocarditis, arthritis, pericarditis, osteomyelitis, endocarditis, scalded skin syndrome, pneumonia, and bacteraemia (20, 21). *S. aureus* bacteraemia often emanates from various infection sources, such as deep tissue abscesses, osteomyelitis, pneumonia and septic pulmonary emboli (22, 23). Foreign bodies, such as endoprostheses and intravenous catheters, can also originate *S. aureus* bacteraemia (20). Several infections caused by Staphylococcal are toxin-mediated, including toxic shock syndrome (TSS), impetigo, food poisoning and necrotising pneumonia. TSS is caused by a potent super-antigen called toxic shock syndrome toxin 1, he symptoms including rash, hypotension, high fever and the involvement of multiple organ systems (24). Self-limiting staphylococcal food poisoning is caused by ingested enterotoxins via contaminated food, with the symptoms including headache, vomiting and diarrhea (25).

S. aureus bacteraemia in the era of pre-antibiotics was mostly fatal, with reported mortality rates observed over a seven year period in the early 1940s in Boston City Hospital being 82% among 122 consecutive patients, and 98% in people aged over 50 years (26). *S. aureus* remains

a main cause of hospital-associated infections, with a large proportion of these being caused by MRSA (27). The mortality rate associated with MRSA infections differs noticeably between studies in various locations (28). In a large cohort study in Europe, the duration and mortality of bloodstream infections attributable to *S. aureus* were evaluated. The outcomes demonstrated the clinical significance of *S. aureus* invasive bacteria, and highlighted the extra burden imposed by resistance for 30 day mortality (29). In a Canadian study (2000-2006), *S. aureus* bacteraemia mortality rate was 4–6 deaths per 100 00 persons (30). In Africa, few studies targeting only *S. aureus* infections have been reported, with the percentage of *S. aureus* bacteraemia differing considerably between countries, with as high as 52% in Egypt, 45% in Algeria, 44% in Botswana and 19% in Morocco (31, 32). The prevalence rate of *S. aureus* bacteraemia within South Africa varies, based on the population studied and geographical setting (33-35).

1.2.2 Carriage

S. aureus colonises multiple sites in the body, with the anterior nares of the nose being the most consistent carriage site in humans. Other typical sites for *S. aureus* colonisation include the skin, pharynx, gastrointestinal tract, axillae, perineum and vagina (36). Recent studies suggests that approximately 25–35% of healthy humans carry *S. aureus* on the mucous or skin membranes (36). Predictions from careful estimations based on the US and Dutch prevalence data indicate that approximately 53 million individuals carry MRSA (37). In a longitudinal carrier studies on *S. aureus*, three carrier patterns have been assigned: non-carriers, intermittent carriers and persistent carriers, with various criteria for assigning an individual to specific carriage patterns. Most studies use a cross-sectional study design with a single nasal culture, with Van Belkum and co-workers in their 2009 studies predicting persistent and non-persistent as the only two types of *S. aureus* nasal carriers (38). Persistent nasal carriage is known as the main risk factor for causing infections in different locations. It is also the major risk factor in patients with intravascular devices and human immunodeficiency virus (HIV) infection, and those undergoing haemodialysis and surgery. The prevalence of nasal colonisation with *S. aureus* varies among countries, with a study in Lebanon during 2006 and 2007 reporting rates

of 38.4% (39). In the USA, similar studies conducted in 2008 reported a decrease in *S. aureus* nasal colonisation from 32.4% to 26.8% (40).

1.2.3 Genome Composition

The *S. aureus* genome has a comparatively low G+C content and a size of approximately 2.5 - 2.9 Megabase pairs (Mbp), with up to 3 additional-chromosomal plasmids. It also harbors between 2600 and 2750 genes (41). The complete structure of the *S. aureus* genome is made up of a core and accessory genomes, which are well conserved (4) with only the accessory genomes being explored in this study.

The accessory genome of *S. aureus* was acquired through lateral gene transfer from other bacterial species, and accounts for approximately 25% of any *S. aureus* genome (5). The accessory genome mainly consists of mobile genetic elements that transfer horizontally between strains and make *S. aureus* a notorious hospital pathogen. These mobile genetic elements that will be explored further include plasmids, transposons, chromosome cassettes, pathogenecity and genomic islands (4). Many of these genetic determinants carry genes with antibiotic resistance, as well as virulence molecules and functions. Mobile genetic elements identification and characterization provides insight into infection, pathogenesis and the evolution of *S. aureus*. The horizontal transfer and distribution of these determinants can therefore have significant clinical consequences (42).

a. Staphylococcal Cassette Chromosome (SCC): SCC is a fundamental mobile genetic element that serves as the medium for gene transmission within the staphylococcal species. It belongs to the SCC family and specialises as a methicillin resistance carrier. It is a genomic island fixed at the 3‘end of open reading frame X (orf X), and is found near the origin of replication of *S. aureus*. The structure of SCC*mec* consists of the *ccr* and the mec gene complexes. The *ccr* gene complex encodes the recombinases responsible for its mobility and contains two site specific recombines genes, *ccrA and ccrB*. The mec gene complex encodes methicillin resistance (43).

b. Pathogenecity Islands of S. aureus: *S. aureus* pathogenicity islands are a group of 15-27 kb genetic elements that usually carry one or more super-antigen genes, encoding toxic shock syndrome toxin 1 (TSST-1) and/or enterotoxins. Pathogenecity Islands of *S. aureus* are firmly integrated at distinct chromosomal sites, but can be moved following infection by certain staphylococcal bacteriophages or by inducting endogenous prophages (44). *S. aureus* pathogenicity islands are believed to be formed by integrating extra chromosomal DNAs (plasmids) or by incorporating bacteriophages carrying toxin genes, which may transform a non-toxic into toxic strain (45).

c. Plasmids in S. aureus: Plasmids are significant genetic vehicles that carry antibiotic resistance and virulence genes. Various forms of plasmids encoding for specific genes has been reported in staphylococci. For example, the plasmid pUB110 is found embedded into the type II SCCmec of some strains of *S. aureus* and encodes resistance to tobramycin, kanamycin, aminoglycosides and bleomycin. The plasmid pI258 encodes resistance to heavy metals and penicillins, while the pT181 plasmid also encodes tetracycline resistance (46, 47).

d. Transposons in S. aureus: Transposons (Tn) are minute transferable fragments of DNA that are located in the chromosome of *S. aureus*. They inactivate or modify certain cellular functions by transposing beside or into the genes involved in the function (48). Macrolide-lincosamide-streptogramin B (MLS_B) and spectinomycin resistance is located on the *Tn 554*. Transposons carrying genes responsible for resistance to gentamycin, tobramycin and kanamycin are the *Tn552* containing the *blaZ* pencillinase gene and *Tn4001* containing *aacA-aphD* gene (42, 49).

e. Genomic islands (GIs): This is a term given to non-SCC genomic and non-phage islands (20-30 kb in size) found exclusively in *S. aureus* (50). These GIs frequently encode virulence determinants inserted at a specific locus in the chromosome. In *S.aureus* each genomic island is found in various allelic forms with diverse sets of resistance or virulence genes. As each allelic form of GIs encodes different properties, identifying the allelic set of GIs that the isolate

carries provides information on the overall properties of the isolate, i.e. its pathogenic potential and the antibiotic susceptibility patterns (51).

1.2.4 Virulence factors and pathogenesis

The ability of bacteria to cause an infection in humans is largely due to an evasion of the host's immune system, with *S. aureus* playing a role in disease pathogenesis by expressing a number of distinct virulence factors. The complex interaction between the virulence factors of the *S. aureus* infecting strain and host defence mechanisms determines the severity and form of the infection. Each virulence factor may have various functions in pathogenesis, and multiple virulence factors may execute an identical function. Pathogenesis in Staphylococci is multifactorial, involving three factors that are directly or indirectly injurious: cell surface components, cell-surface-bound proteins, and secreted proteins. Secreted proteins, including tissue-degrading enzymes (lipases, proteases), cytotoxins [e.g. α, β, γ, δ–haemolysin, Panton-Valentine leukocidin (PVL)], and super-antigens [e.g. enterotoxins A-D, exfoliatins A-B, toxic shock syndrome toxin-1 (TSST-1)], allow bacteria to penetrate and destroy the local structural and cellular elements of host organs and tissues (52-54).

Two genes are found on the prophage encode the PVL toxin (55), which is harmful to neutrophils causing tissue necrosis. It is associated with severe necrotising pneumonia as well as soft and skin tissue infections (56). The release of PVL has also been associated with community-acquired methicillin-resistant *S. aureus* (CA-MRSA) strains (57). Microbial surface components recognising adhesive matrix molecules (MSCRAMMs) are among the numerous surface proteins found in *S. aureus,* and include collagen-binding, fibrinogen-binding and fibronectin-binding protein, as well as adhesins (58). MSCRAMMs play an active role in mediating their attachment to bacterial cells, host tissues, the inert and extracellular surfaces. They also aid in initiating prosthetic-device, endovascular, joint and bone infections (17, 53).

The Staphylococcal protein A (Spa) is the best known surface protein of *S. aureus,* being first isolated in 1972 from *S. aureus* after lysostaphin digestion (59). It is made up of five closely

related immunoglobulin G (IgG)-binding domains, a polymorphic region X and C- terminal cell wall attachment sequence (60, 61), with the X region containing a highly polymorphic sequence of 24 bp repeats (62). Spa blocks the normal function IgG by binding to its Fc region, which causes an inhibition of the phagocytosis that are disguising the bacterium from the innate immune system by preventing opsonisation-dependent activation of the complement cascade (63). Due to the highly variable X-region, spa typing is a universally used genotyping method for comparing *S. aureus* isolates (64). Each cell surface component has a different role in the pathogenesis of *S. aureus*, with the mucoid capsule inhibiting phagocytosis by covering C3b complement factor bound to the cell wall (65). However, the function of the capsules involved in the pathogenesis of *S. aureus* is controversial.

A complex regulatory network controls the staphylococcal virulence factors encoding genes. The surface protein genes are released shortly after the pre-exponential growth phase during the establishment of an infection. This enables adherence to the host tissues and protects the bacteria from host defenses, such as complement-mediated killing and opsonisation-phagocytosis. Other genes encoding secreted enzymes and cytotoxins are released during the post-exponential growth phase to aid in spreading the bacteria, acquiring nutrients and killing phagocytes (52). The accessory gene regulation (agr) locus is the main global regulatory system of *S. aureus* (66). Additionally, three other two-component signalling modules are known to be involved in staphylococcal virulence genes regulation. Environmental factors, such as temperature, CO_2, O_2 and pH levels, also affect the overall regulatory system by helping the bacterium to recognise and respond appropriately to its local environments (52, 66).

1.3 Molecular detection

Many molecular based techniques are available to identify MRSA, the 'gold standard' being *mecA* gene detection. Multiplex PCR assays that concurrently target *mecA* gene and that are specific for some *S. aureus*, including *spa, coa, femA, nuc* or all staphylococci, e.g. 16S rDNA conserved genus sequences, have been developed (67-69). Genomic DNA extracts from broth culture lysates or bacterial colonies are usually used to perform these assays. However, these

methods can produce false positive results if the culture contains a mixture of methicillin-susceptible and methicillin resistant coagulase negative staphylococci. Recently, MRSA was detected within one to six hours directly from screening swabs using commercially available real-time PCR assays.

1.4 Typing methods

Phenotypic or genotypic characters are generated by typing strains within a species to predict the routes and sources of spread of the micro-organisms. Typing contributes to understanding the study bacterial genetic population structures and infectious diseases pathogenesis. Data from typing help to define the relatedness of isolates implicated in an outbreak and elucidates its chain of transmission (70). Microbial typing systems should be evaluated and validated with respect to different performance criteria, including typeability, discriminatory power, reproducibility, stability, convenience criteria and epidemiological concordance (71). It can be used at various levels: (i) globally, through international surveillance networks; (ii) nationally/regionally, in reference laboratories, or (iii) locally at hospital or health-care institutions. The choice of methods and quality assessment depends solely on the level at which typing is performed, with phenotypic characters showing limited performance, reliability and specificity. In genotyping, comparisons based on the sequence polymorphism or genomic DNA fragments are the most preferred techniques. Typing systems can be grouped as definitive ('library' system) or comparative, which are often the same 'typing run', and enable comparisons of isolates in the same laboratory. The latter can be enough for an outbreak investigation, but it poses difficulties when comparing results with future or past data samples. In contrast, a definitive typing system has the advantage to compare typing data produced in various laboratories, by different investigators at a range of time intervals, and generates standardised and reproducible data. The most frequently used molecular typing methods are pulsed field gel electrophoresis, multi locus sequence typing, SCC*mec* typing and spa typing (72).

1.4.1 Pulsed-field gel electrophoresis (PFGE)

This typing methodology employs a restriction enzyme (*SmaI* in the case of *S. aureus*) for digesting chromosomal DNA and agarose gel electrophoresis using an alternating voltage gradient. A software package was used to analyze the banding patterns, this being one of the most popular methods, which helps to detect genetic variation between staphylococcal strains (73). However, PFGE is less reproducible and does not involve a selective amplification step, hence it has a limited use for long-term epidemiology surveillance or for studying the phylogenetic and evolutionary relationship among *Staphylococcus aureus* strains (74).

1.4.2 Multi locus sequence typing (MLST)

MLST is a genotypic technique for bacterial strain characterization that uses DNA sequencing to reveal allelic variants in various conserved genes (75). MLST offers the advantages of high degrees of typeability, unambiguous identification and reproducibility based on the nucleotide sequences of 450-500bp internal fragments of seven housekeeping genes. Data obtained by MLST also permits an investigation of the population structure, and the development and testing of evolutionary hypotheses (76). The main advantage of this technique is its ability for sequence data to be compared and contrasted between different laboratories through the MLST website, making it suitable for local and global epidemiology of *S. aureus* and other bacterial pathogens (77).

1.4.3 Spa typing

Spa typing is based on variations of the polymorphic X region of staphylococcal protein A (spa) locus (78). The highly polymorphic X region is suited directly upstream of the region encoding the C-terminal cell wall attachment sequence, and is characterized by a variable number of 24 bp repeats (70, 73). Its discriminatory power falls between that of MLST and PFGE. Spa typing involves sequencing only one locus, which makes the technique simple compared to MLST. Another advantage over MLST is its ability to investigate both hospital outbreaks of MRSA and molecular evolution (79). It also allows laboratories to use diverse sequencing platforms and specialised software (Bionumerics software) to interpret the chromatograms of resulting sequence (72).

1.4.4 SCCmec Typing

This is a technique used to investigate the structure of SCC*mec*. A multiplex PCR assay for identifying *mecA* and various loci on SCC*mec* was developed to determine the specific SCC*mec* types, which are based on the allotype of *ccr* genes and the *mec* gene complex (80-82).

1.4.5 Whole Genome Sequencing (WGS)

WGS is a technology that is advancing our understanding of MRSA evolution, especially during epidemics, with various WGS studies of MRSA isolates from outbreaks having been published (83-85). This method includes sequencing DNA fragments and aligning them to a complete and well-annotated reference genome sequence. It can be used to detect lineage, as well as single nucleotide polymorphisms (SNPs) variation in the core genome (86). A few studies have also investigated additional DNA found in the test isolates that are not found in the reference genome, indicating that it is also a reliable method for identifying mobile genetic elements (MGE) variation. Bioinformatic data analysis is currently complex, and user friendly algorithms to identify spa, MLST type and the presence of key virulence and resistance genes are being developed. There are good prospects that this technology could be widely incorporated into infection control outbreak investigation in real time (87).

1.5 Justification for the study

Although research has been done on MRSA in South Africa, few have been done on its genetic determinants, with little information being available on the content and resistance of MRSA plasmids in the private health sector in South Africa. There is therefore a need to delineate antibiotic resistance patterns, compare their plasmids profiles and establish the possible genetic relatedness of MRSA in the private sector, as this will increase the evidence base to optimize infection management, and inform control policies and practices.

1.6 Aim

The study aims to establish the clonality and characterize the plasmid-encoded antibiotic resistance and virulence profile of 27 clinical MRSA isolates collected from a private pathology laboratory in Durban, South Africa.

1.7 Objectives

1. To verify the identity of the clinical isolates using cefoxitin disc diffusion (CDD) test and polymerase chain reaction (PCR) - based *mecA* gene detection technique.
2. To determine the minimum inhibitory concentration (MIC's) to the following antibiotics: ampicillin, ciprofloxacin, gentamicin, erythromycin, clindamycin, tetracycline, linezolid, daptomycin, fusidic acid, tigecycline, rifampicin and vancomycin.
3. To establish the presence of resistance genes (*blaZ, tetK, ermC, aac-aph*) and specific virulence genes (PVL genes (lukS/F-PV, *eta*, *hla*, *hld*) on plasmids DNA using PCR.
4. To study the clonal relatedness of the isolates using pulse field gel electrophoresis (PFGE).
5. To establish possible correlations between particular plasmid types, antimicrobial susceptibility and the clonal lineages of isolates for their genetic relatedness using PFGE.

1.8 Outline

This study is presented in three chapters, the first outlining the rationale for the research, as well as the Aim and Objectives. As this dissertation is presented in manuscript format, it contains the following two chapters:

Chapter 2 outlines the characterization of plasmid-mediated resistance and virulence genes in clinical MRSA isolates in private sector in KZN province and their genetic relatedness. This section has been submitted to the International Journal of Infectious Diseases and formatted according to the journal's standards.

Chapter 3, the final chapter presents the conclusions, limitations and recommendations arising from the study.

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CHAPTER TWO

This dissertation is in a manuscript format (as is the requirement of the College of Health Sciences at the University of KwaZulu-Natal) as follows:

Daniel G. Amoako, Linda A. Bester, Anou M. Somboro, Sooraj Baijnath, Chetna N. Govind, Sabiha Y. Essack; Molecular characterization of resistance and virulence in Methicillin Resistant *Staphylococcus aureus (*MRSA) from the private sector in KwaZulu-Natal, South Africa**,** Submitted to International Journal of Infectious Diseases.

Contributions:

- Mr. Daniel G. Amoako, as investigator, worked on the study design with the assistance of my supervisors, developed the protocols for the study, executed the laboratory work and wrote the manuscript.

- Professor Sabiha Y. Essack, as main supervisor, conceptualized the study and undertook critical revision of the manuscript.

- Dr. Linda Bester, as co-supervisor, designed the study, facilitated data acquisition, laboratory work and data analysis, and contributed to the writing and critical revision of the manuscript.

- Dr. Chetna N. Govind of Lancet Laboratories provided clinical data and isolates for the study.

- Mr. Anou M. Somboro and Mr. Sooraj Baijnath assisted in the development of the protocols and the analysis of the results from the study.

Molecular Characterization of Resistance and Virulence in Methicillin Resistant *Staphylococcus aureus* (MRSA) from the Private Sector in KwaZulu-Natal, South Africa

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Abstract

Objectives: There is limited information on the plasmid content of MRSA strains from South Africa. We investigated resistance and virulence genes in the plasmids of 27 MRSA clinical isolates from the private healthcare sector in Durban, South Africa and their genetic relatedness.

Methods: MRSA was confirmed by *mecA* gene identification in plasmids extracted using a commercial plasmid extraction kit. The isolates were subjected to antimicrobial susceptibility testing and molecular characterization of four common resistance encoding genes and four frequently encountered virulence factors: *blaZ, aac (2')-aph (6''), ermC, tetK, hla, hld, eta* and *LukS/F-PV* respectively by PCR using plasmid DNA as the template. The genetic relatedness between the isolates was determined by pulsed field gel electrophoresis (PFGE).

Results: All MRSA isolates contained plasmids. The isolates were 100% resistant to ampillicin, 85.2% were resistant to ciprofloxacin, 74.1% to gentamicin, 70.4% to rifampicin, 66.7% to tetracycline, 63.0% to erythromycin, and 11.1% to clindamycin. They were all susceptible to daptomycin, linezolid, vancomycin, tigecycline and fusidic acid. Multidrug resistance (MDR) was found in 74.1% (20/27) of the MRSA isolates. The frequency of the resistance genes *blaZ, aac (2')-aph (6'')* and *ermC* were 100%, 92.6% and 48.2% respectively, but *tetK* was not found in any of the MRSA isolates. The prevalence of virulence genes *hla and hld* were 96.3% and 92.6% respectively, however, *eta and LuKS/F-PV* were not detected. PFGE analysis revealed 10 pulsotypes, designated A–J, which correlated with the resistance profile and mechanism of the isolates in each group. Of note, 85.2% (23/27) of the isolates clustered into six major PFGE types giving an indication of similar circulating MRSA clones. Type F was the major pulsotype (29. 6%) and was found in eight of the 27 MRSA isolates. Hospital centers 1 and 10 contained pulsotypes C and H, whiles identical pulsotypes F and G were spread across nine of the 15 facilities, intimating the possibility of inter-health center spread of MRSA in the province.

Conclusions: The complexity and diversity of the molecular resistance and virulence profiles poses a challenge for managing MRSA infections. A comprehensive understanding of the molecular epidemiology is essential to inform treatment and contain dissemination.

Keywords: MRSA, plasmids, PCR, PFGE

1. Introduction

The World Health Organization (WHO) and the African Health Observatory (AHO) have increasingly recognized the significance of tracking antibiotic resistance, specifically the resistance mechanisms and their dissemination, to optimize managing infections and provide the basis for evaluating the effectiveness of infection control programmes.[1, 2] One of the fundamental human pathogens is *Staphylococcus aureus*, an adaptive bacterium that causes superficial, deep and fatal diseases. The ability of *S. aureus* strains to cause infection depends on various resistance and virulence factors that contribute to its colonization and disease development in the host.[3] *S. aureus* has gradually developed resistance towards all the main classes of antibiotics to which it was once susceptible.[4]

MRSA strains, whose definitive characteristic is the *mecA* carrier element called staphylococcal cassette chromosome *mec* (SCCmec), confer resistance to methicillin, causing nosocomial, community and livestock-associated infections that have resulted in major public health, economic and social problems worldwide [5, 6] These strains also harbor mobile genetic elements (MGEs), including plasmids, pathogenicity islands, transposons, integrons and prophages, which comprise 15-25% of the genome. MGEs carry the majority of the genes, through which strains of staphylococcal may be differentiated from each other.[7] As MGEs play a significant role in bacterial survival and adaptability because they encode many resistance and virulence genes, understanding their composition will broaden our knowledge on the genetic determinants of antibiotic resistance.[8]

The most significant MGEs are plasmids, which aid antibiotic resistance genes transmission and carriage. Plasmids of staphylococcal vary in size from 1 to over 200 kb, and are classified according to their mutual incompatibility, size and replication mechanism.[9] *S. aureus* possess a wide variety of plasmid-borne genes, with many of the currently sequenced plasmids containing open reading frames (ORFs) that have not been described.[10]

Although research has been conducted on MRSA in Africa, the recently available information on the plasmid content of these strains from African countries is comparatively scarce, and

data on their resistance in the South Africa private sector being lacking. A study of this nature is a necessity, as understanding antibiotic resistance patterns, comparing the plasmids profiles, and assessing the possible relatedness of MRSA isolates is useful for epidemiological studies and evaluating the effectiveness of infection control. The aim of this study was to ascertain the clonality, and characterize the plasmid-encoded antibiotic resistance and virulence profile of 27 clinical MRSA isolates obtained from a private laboratory in Durban, KwaZulu-Natal Province, South Africa.

2. Materials and Methods

2.1. MRSA isolates and mecA gene confirmation

A total of 27 consecutive non-repetitive MRSA isolates were obtained from a pathology laboratory based in Durban, South Africa, over a three months period, from June to August, 2015, that caters for the private healthcare sector. The isolates were identified using Vitek 2 (bioMerieux, Durham, NC, USA) and confirmed by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF/MS). The cefoxitin disc diffusion (CDD) test was used to identify putative MRSA[11, 12], which were confirmed by PCR detection of the *mecA* gene.[13] *S. aureus* ATCC 29213 (susceptible to methicillin) and *S. aureus* ATCC 43300 (resistant to methicillin) were used as controls.

2.2. Antimicrobial agents and MIC determinations

The minimum inhibitory concentration (MIC) was determined for 12 antibiotics by the broth microdilution method.[14] They included ampicillin (β-lactams), ciprofloxacin (fluoroquinolones), erythromycin (marcolides), gentamicin (aminoglycosides), tetracycline (tetracyclines), rifampicin (ansamycins), clindamycin (lincosamides), linezolid, daptomycin (lipopeptides), fusidic acid, vancomycin (glycopeptides) and tigecycline (glycylcyclines). The Clinical and Laboratory Standards Institute (CLSI) Guideline[14] was used for interpreting the results, and *Staphylococcus aureus* ATCC 29213 was used as the control. Isolates resistant to β-lactams, and at least three classes of antibiotics, were defined as multidrug resistant (MDR).

2.3. Molecular characterization of plasmid-borne resistance and virulence determinants by PCR

A plasmid DNA extraction kit (GeneJET Plasmid Miniprep kit, Thermoscentific) was used to purify the plasmid DNA from all 27 MRSA strains, according to the manufacturer's instructions. The presence of resistance genes conferring resistance to ampicillin-penicillin (*blaZ*), aminoglycoside (*aac (6')–aph* (2'')), macrolide-lincosamide-streptogramins B [MLS_B] (*ermC*) and tetracycline (*tetK*) were determined using PCR.[15] The virulence determinants encoding the bio-component Panton-Valentine leukocidin (*LukS/F-PV* gene), exfoliative toxin (*eta*), alpha and delta hemolysin genes (*hla and hld*) were also ascertained by PCR.[16] All specific primers and programs for detecting antibiotic resistance and virulence determinants can be found in Table 1.[15,16]. Positive and negative controls were included in all PCR assays.

2.4. Pulsed-field gel electrophoresis (PFGE)

PFGE was used to determine the genetic relationship between the isolates[17]. The Bionumerics software version 6.6 (Applied Maths NV, Belgium) using the Dice coefficient, and represented by unweighted pair group method with arithmetic mean (UPGMA) with optimization settings and position tolerance set at 0.5% and 1% respectively was used to analyze the electrophoretic patterns. Clusters were defined using the criterion of a difference of ≤ 6 bands, as described by Tenover et al [18], and a similarity cut-off of ≥70.0%. *Salmonella* serotype *Braenderup* strain H9812 was used as the quality control strain for normalization between gels.

3. Results

3.1. Patient demographics and clinical sources of infection

All 27 laboratory-confirmed *mec*A-positive MRSA isolates constituted the study sample. Of the available isolates, 22 had complete data, such as hospital center, specimen source, ward type, date of sample collection, gender and age (Table 3). Demographic data reflected 10 males (37.0%) and 13 females (48.2%), while four patients had no record for gender. The median age was 43.8 (SD 26.7) years, ranging from day 0 to 86 years, and patient age was missing in four

isolates. Five (18.5%) were classified as paediatric isolates, and 18 isolates (66.7%) as adult isolates. Nineteen isolates (70. 4%) were collected from in-patients, three (11.1%) from outpatients, with five (18.5%) being unknown. The intensive care unit (ICU) yielded nine (33.3%) isolates, that largest number of all ward types.

3.2. Antimicrobial resistance pattern

The resistance patterns of the 27 MRSA isolates and the MIC distributions of the tested antimicrobial agents are shown in Tables 2 and 3. Ampicillin showed no activity against MRSA isolates, while 85.2% (23/27) were resistant to ciprofloxacin, 74.1% (20/27) to gentamicin, 70.4% (19/27) to rifampicin, 66.7% (18/27) to tetracycline, 63.0% (17/27) to erythromycin, and 11.1% (3/27) to clindamycin. All isolates were susceptible to daptomycin, linezolid, fusidic acid, tigecycline and vancomycin. Multidrug resistance (MDR) was determined in 74.1% (20/27) of the MRSA isolates.

3.3. Prevalence of the genetic determinants of resistance and virulence

The prevalence of four resistance genes and four virulence factors on extracted plasmids of the 27 clinical MRSA isolates are shown in Table 3. The penicillin resistance gene, *blaZ,* was amplified from all 27 MRSA isolates. The *aac (6')–aph* (2'') gene for aminoglycoside resistance and the *ermC* gene for macrolide-lincosamide-streptogramins [MLS_B] resistance were amplified from 25 (92.6%) and 13 (48.2%) isolates respectively. The tetracycline resistance gene (*tetK*) was not detected in any of the 27 MRSA isolates. The most prevalent virulence gene was alpha hemolysin *hla,* which was identified in 26 (96.3%) isolates, followed by delta hemolysin *hld*entified in 25 (92.6%) of isolates. However, Panton-Valentine leukocidin *lukS/F-PV* and exfoliative toxin *eta* were not detected.

3.4. Genetic relationship by PFGE

The PFGE profiles and the dendrogram of the MRSA isolates are shown in Figure 3. PFGE analysis grouped the 27 isolates into 10 pulsotypes designated A-J, displaying 70.0% similarity, and correlating with their resistance profile and the genetic determinants tested in this study. Of note, 85.2% of the isolates were clustered into six major PFGE types: pulsotypes

F (8/27 strains; 29.6%), G (5/27; 18.5%), C, I (3/27; 11.1%) and A, H (2/27; 7.4 %). Pulse types B, D, E and J were each represented by single isolates. PFGE types F and G were subdivided into five and three subtypes respectively. Hospital centers 1 and 10 were found to contain pulsotypes C and H, while identical pulsotypes F and G were spread across nine centers, intimating the possibility of inter-health centers spread of MRSA in the province.

4. Discussion

Antibiotic resistance is a global health problem and MRSA remains a prominent multi-drug resistant bacterial species, which presents a challenge to clinicians, as the introduction of new classes of antibiotics is usually followed by the emergence of resistant pathogenic forms. Antibiotic resistance surveillance and delineating resistance and virulence genes in MRSA isolates is essential to understand their genetic basis and inform their management.

The majority of the MRSA isolates in this study were resistant to ciprofloxacin, gentamicin, rifampicin, tetracycline and erythromycin. Notwithstanding the small sample size of 27 isolates, resistance rates in this study were in some cases lower than those seen in another KZN study on 61 confirmed MRSA isolates by Shittu *et al*[19], particularly for gentamicin (74.1% vs. 96.7%), rifampicin (70.4% vs. 73.8%), tetracycline (66.7% vs. 90.2%) and erythromycin (63.0% v. 82.0%). Resistance to clindamycin of 11.1% was also much lower in this investigation than the rates of 82%[19], 62.5%[20], 34%[21] reported in other studies conducted on MRSA isolates in KZN and South Africa. Only the ciprofloxacin resistance rate in our study was notably higher (85.2% v. 18%[19]), with its resistance on MRSA isolates in South Africa having been reported to 69.7% and 88.7% in the private sector.[20] Multidrug resistance rate was lower (74.1% vs. 87%) than those reported by Shittu *et al*[19] but similar to a study by Heysell *et al*[22] in KZN with a rate of 79% on 19 clinical MRSA.

All MRSA isolates were susceptible to daptomycin, vancomycin, linezolid, fusidic acid and tigecycline, while only ampillicin showed 100% resistance. The susceptibility patterns of the isolates in this study were comparable to studies conducted on MRSA in South Africa[19, 20, 23] and Nigeria[24], which were fully susceptible to daptomycin, vancomycin, linezolid, fusidic acid

and tigecycline. The full susceptibility of MRSA to these antibiotics observed in this study confirms their use as treatment options for infections in South Africa.

There was a relationship between resistance to methicillin and to other antibiotics, as noted in other investigations[25-27]. The presence of *mecA* is an absolute requirement for *S. aureus* to express methicillin resistance[28]. The structural component of *mecA* encodes the penicillin-binding protein 2a (PBP2a) that establishes resistance to methicillin, other semisynthetic penicillinase-resistant beta-lactams that are frequently co-carried with genes conferring resistance to aminoglycosides, macrolide-lincosamide-streptogramin B [MLS_B] and spectinomycin[29]. All the isolated plasmids of the 27 MRSA isolates contained the *mecA and blaZ* resistance genes, indicating the correlation between MICs and the presence of genes encoding resistance against beta-lactams. The gentamicin resistance gene *aac (6')–aph* (2'') was identified in 92.6% of the isolates, which varied from the phenotypic resistance profile of 74.1%, indicating that gene carriage does not necessarily translate into the resistance phenotype. This result was similar to the studies conducted in Turkey by Duran *et al*[30], where 17 gentamicin-susceptible isolates were found to be positive, in terms of one or more gentamicin resistance genes mostly *aac (6')–aph* (2''). The *ermC* gene responsible for macrolide-lincosamide-streptogramins B [MLS_B] resistance was amplified in 48.2% (13/27) of the MRSA isolates, while it was not found in those that were susceptible to both erythromycin and clindamycin. The 23.5% (4/17) with phenotypic resistance to MLS_B that did not contain the *ermC* gene indicates the occurrence of other resistance mechanism, *ermA, ermB and msrA,* which was not investigated in this study but have been previously reported.[31] This confirms that the incidence of MLS_B phenotypes and genotypes vary according to country, patterns of infections and drug use.[31, 32] Although there was high tetracycline resistance, the *tetK* gene was not detected, indicating that it this may be due to different mechanisms and not mediated by active drug efflux, as *tetK* resistance has so far not been reported in clinical MRSA studies in South Africa.

The prevalence of virulence factors in all isolated plasmids showed a similar trend, with the hemolysin genes, *hla* and *hld* being the most abundant, with frequencies of 96.3% (26/27) and

92.6% (25/27) respectively. Comparatively, this was similar to other studies conducted from Iran[16], Uganda[33] and United States[34], with either *hla* being more frequent than the *hld* genes, or both showing 100% co-dominance. The prevalence rate of *eta* in our study was 0%, which was similar to studies conducted in Japan[35] and China[36], where no *eta* was detected in 197 and 62 clinical MRSA isolates respectively. However, the prevalence of *eta* differed among studies, which could be associated with a variety of geographical and health conditions[37]. LuKS/F-PV was not detected in any of the 27 clinical MRSA isolates, which was comparable to a study conducted in South Africa on 320 clinical MRSA isolates with only one positive LuKS/F-PV gene being detected[38]. Generally, the resistance and virulence profiles showed a similar trend in all the plasmids, indicating closely related MRSA isolates.

PFGE, which is the gold standard for strain typing, was able to predict the genetic relatedness of the MRSA isolates.[17] The analysis revealed 10 pulsotypes, designated A–J, which correlated with the resistance profile and mechanism of the isolates in each group (Fig. 2). Isolates of group G were all resistant to the five antibiotics, and were clustered in 3 subtypes. These isolates also clustered at a similarity of 0.75 when analyzed by *smaI* PFGE. Pulsotype C showed susceptibility to both tetracycline and rifampicin. Of note was that 85.2% (23/27) of the isolates clustered into six major PFGE types, indicating similar circulating MRSA clones in health centers in the province, as predicted by Shittu *et al*[39] and Moodley *et al*[38] in their study in KZN and South Africa respectively. Although the sample size was too small to show a definite correlation, the assertion of similar circulating clones in the province was supported by our study, as the PFGE analysis revealed some form of association between pulsotypes and the centers of sample collection. Centers 1 and 10 were found to contain pulsotypes C and H, while identical pulsotypes F and G were spread across nine of the 15 centers, intimating the possibility of similar clones of MRSA within the health care centers in the province. However, the study could not relate the pulsotypes to specific reported clones, as *spa*, SCC*mec* and multi locus sequence typing (MLST) are needed in tandem, with the PFGE for to predict the population clonal structure not being performed in this study.

To the best of our knowledge this is the first study of clinical MRSA isolates in the private sector in KZN Province characterizing the plasmid-mediated resistance and virulence genes. The study provides a private sector perspective of antibotic suscepbility patterns, and strongly affirms reports of interhealth centres spread of identical and closely related clones of MRSA in Durban, South Africa, highlighting the need for implementing efficeint and effective infection control programs.

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Ethical approval

Permission to carry out this study was granted by the Biomedical Research Ethics Committee (BREC) (**REF/No: BE394/15**) of the University of KwaZulu-Natal (UKZN).

Conflict of interest

Professor SY Essack is a member of the Global Respiratory Infection Partnership sponsored by Reckitt and Benckiser.

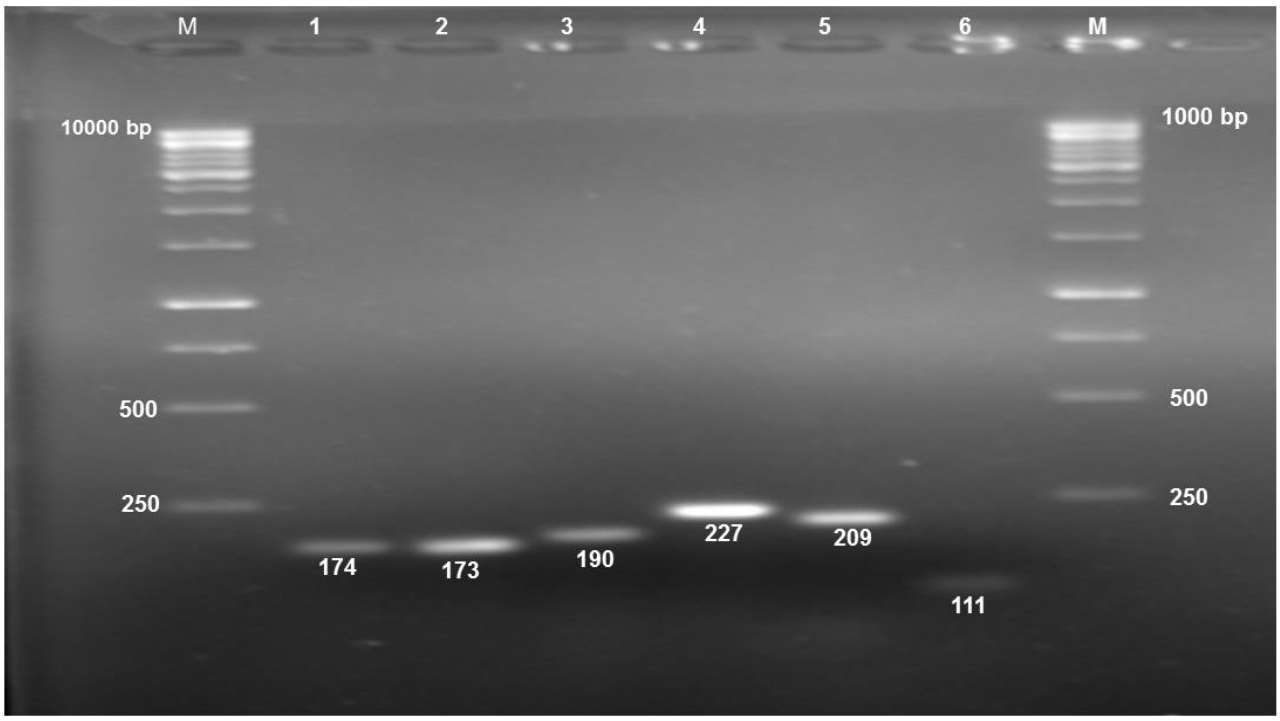

Figure 1. Patterns of agarose gel electrophoresis showing PCR products for isolated MRSA plasmid genes. Lanes M: DNA molecular size maker (1-Kb ladder; Thermo Fisher Scientific Inc, Massachusetts, USA) Lane 1: *mecA*; Lane 2: *blaZ*; Lane 3: *ermC*; Lane 4: *aac (6')-aph (2'')* Lane 5: *hla* Lane 6: *hld*

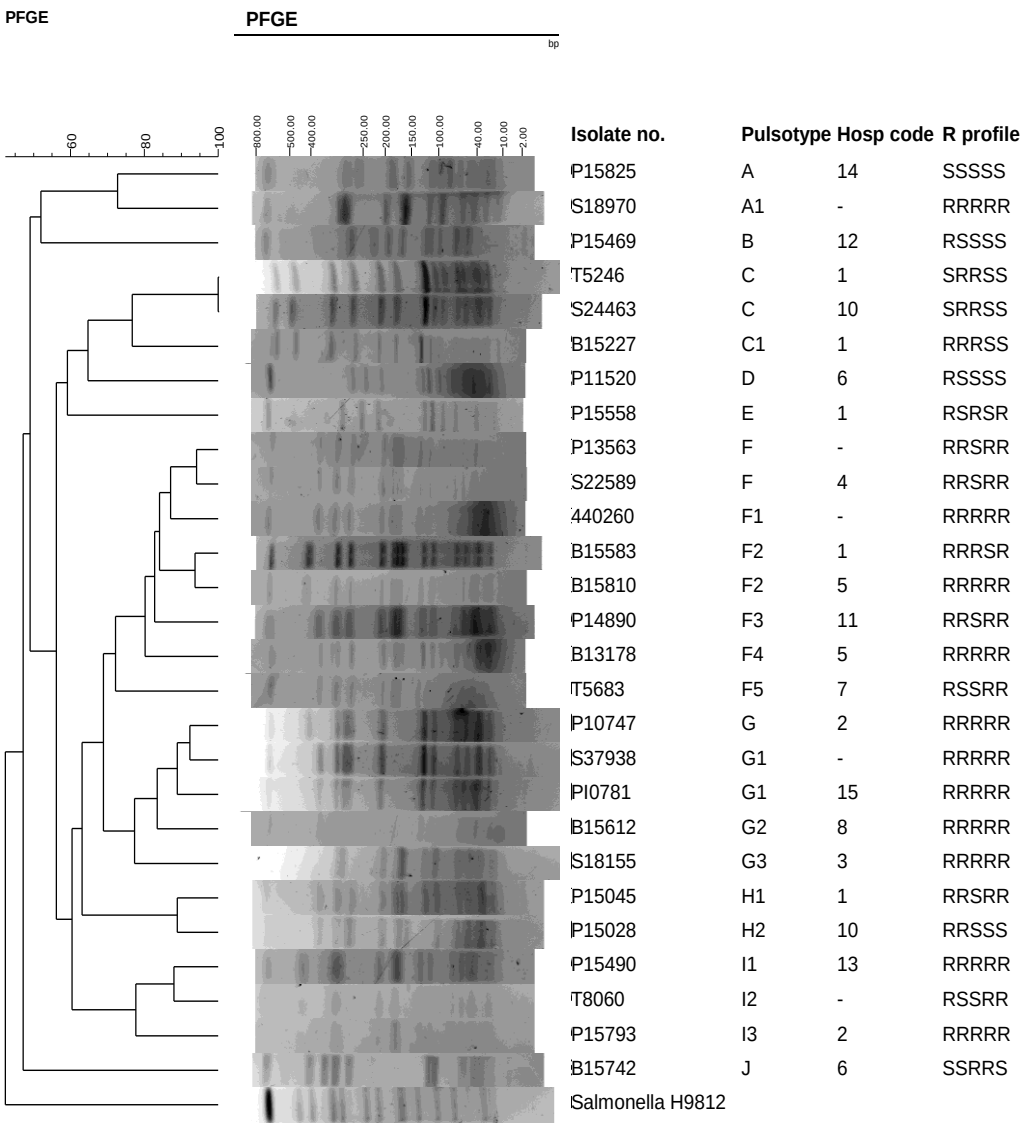

Figure 2: PFGE *Smal* genotypic types generated from 27 clinical MRSA isolates from private sector in KZN. Pretested *Salmonella* serotype *Braenderup* strain H9812 was used as the quality control strain. The R and S indicate resistance or susceptibility for ciprofloxacin, gentamicin, erythromycin, tetracycline and rifampicin respectively. The alphabets A –J shows the main pulsotype and subtype of each isolate. The numbers 1 – 15 indicates codes of the hospital centers where the MRSA isolates were collected.

Table 1

PCR primers and cycling parameters for genes presented in this study

Gene	Primer/sequence	PCR conditions	PCR size (bp)	Reference
mecA	**F**-AACAGGTGAATTATTAGCACTTGTAAG **R**-ATTGCTGTTAATATTTTTTGAGTTGAA	30 s 94 °C, 30 s 55 °C, 1 min 72 °C	174	[15]
blaZ	**F**-ACTTCAACACCTGCTGCTTT **R**-TGACCACTTTTATCAGCAAC	30 s 94 °C, 30 s 55 °C, 1 min 72 °C	173	[15]
ermC	**F**-CTTGTTGATCACGATAATTTCC **R**-ATCTTTTAGCAAACCCGTATTC	30 s 94 °C, 30 s 55 °C, 1 min 72 °C	190	[15]
aac(6')-aph(2'')	**F**-TAATCCAAGAGCAATAAGGGC **R**-GCCACACTATCATAACCACTA	30 s 94 °C, 30 s 55 °C, 1 min 72 °C	227	[15]
tetK	**F**-TCG ATA GGA ACA GCA GTA **R**-CAG CAG ATC CTA CTC CTT	30 s 94 °C, 30 s 55 °C, 1 min 72 °C	169	[15]
hla	**F**-CTGATTACTATCCAAGAAATTCGATTG **R**-CTTTCCAGCCTACTTTTTTATCAGT	30 s 95 °C, 45 s 58 °C, 1 min 72 °C	209	[16]
hld	**F**-AAGAATTTTTATCTTAATTAAGGAAGGAGTG **R**-TTAGTGAATTTGTTCACTGTGTCGA	30 s 95 °C, 45 s 58 °C, 1 min 72 °C	111	[16]
eta	**F**-GCAGGTGTTGATTTAGCATT **R**-AGATGTCCCTATTTTTGCTG	30 s 95 °C, 45 s 54 °C, 1 min 72 °C	93	[16]
LukS/F-PV	**F**-ATCATTAGGTAAAATGTCTGGACATGATCCA **R**-GCATCAAGTGTATTGGATAGCAAAAGC	30 s 95 °C, 45 s 60 °C, 1 min 72 °C	443	[16]

Table 2

Minimum inhibitory concentration (MIC) distributions of antimicrobial agents for 27 MRSA isolates

Antibiotic	Resistance pattern, n (%)		Distribution of MIC (mg/ ml)											
	R	S	<0.25	0.5	1	2	4	8	16	32	64	128	256	>512
Ampicillin	27 (100)	0	0	0	0	0	0	0	0	0	0	1	1	25
Ciprofloxacin	23 (85.2)	4 (14.8)	0	2	2	0	4	1	1	0	2	5	8	2
Gentamicin	20 (74.1)	7 (25.9)	2	3	1	0	1	0	2	4	7	7	0	0
Erythromycin	16 (59.3)	11 (40.7)	0	8	2	1	0	2	6	4	4	0	0	0
Rifampicin	19 (70.4)	8 (29. 6)	7	1	0	0	0	0	0	0	1	5	8	5
Tetracycline	18 (66.7)	9 (33.3)	6	1	1	2	0	0	1	1	8	5	2	0
Clindamycin	3 (11.1)	24 (88.9)	24	0	1	1	0	0	0	0	0	0	0	1
Daptomycin	0	27 (100)	7	16	4	0	0	0	0	0	0	0	0	0
Vancomycin	0	27 (100)	1	10	16	0	0	0	0	0	0	0	0	0
Linezolid	0	27 (100)	0	0	4	23	0	0	0	0	0	0	0	0
Fusidic acid	0	27 (100)	26	1	0	0	0	0	0	0	0	0	0	0
Tigecycline	0	27 (100)	27	0	0	0	0	0	0	0	0	0	0	0

R, resistant; I, intermediate; S, susceptible, All intermediate MIC values were taken as resistant.

Table 3

Clinical data, minimum inhibitory concentrations (MIC), and results of PCR for 27 MRSA isolates

Isolate No.	Clinical data					MIC (mg/l)[b]											PCR								
	Hos cod	Source	Ward type	Sex	Age	AP	CP	GT	ET	RF	TT	CM	DP	VM	LZ	FA	TG	*mecA*	*blaZ*	*ermC*	*aac-aph*	*tetK*	*hla*	*hld*	*eta*	*lukS/F-PV*
B11970	1	Blood	Neo ICU	F	NB	>512	0.5	32	8	≤0.25	2	≤0.25	1	1	2	≤ 0.25	≤0.25	+	+	+	+	-	+	+	-	-
P10781	15	Nasal	OPD	M	86	>512	256	64	32	512	256	≤0.25	0.5	1	2	≤0.25	≤0.25	+	+	+	+	-	+	+	-	-
P10747	2	CVP	ICU	F	66	>512	4	>64	64	512	128	≤0.25	0.5	0.5	1	≤0.25	≤0.25	+	+	+	+	-	+	+	-	-
S37938	-	-	-	-	-	>512	256	16	32	256	64	2	0.5	1	2	≤0.25	≤0.25	+	+	+	+	-	+	+	-	-
S18155	3	ETT	ICU	F	76	>512	256	64	64	256	128	≤0.25	0.25	0.5	2	≤0.25	≤0.25	+	+	-	+	-	+	-	-	-
B13178	5	Blood	LW	F	26	>512	256	>64	64	512	128	≤0.25	0.5	1	2	≤0.25	≤0.25	+	+	+	+	-	+	+	-	-
440260	-	-	-	-	-	>512	>512	>64	64	256	128	≤0.25	0.5	1	2	≤0.25	≤0.25	+	+	+	+	-	+	+	-	-
S18970	-	-	-	-	-	>512	256	64	32	512	64	≤0.25	0.5	0.5	2	≤0.25	≤0.25	+	+	-	+	-	+	+	-	-
P11520	6	Pus	OPD	M	62	512	>512	0.25	0.5	≤0.25	≤0.25	≤0.25	0.5	1	2	≤0.25	≤0.25	+	+	-	-	-	+	+	-	-
T5683	7	Nasal	OPD	F	43	>512	8	0.5	0.5	256	32	≤0.25	0.5	1	1	≤0.25	≤0.25	+	+	-	-	-	+	+	-	-
B15227	1	Blood	Neo ICU	F	NB	>512	4	64	8	≤0.25	≤0.25	≤0.25	1	1	1	≤0.25	≤0.25	+	+	+	+	-	+	+	-	-
P13563	-	-	-	M	49	>512	128	>64	0.5	128	16	≤0.25	0.5	1	2	≤0.25	≤0.25	+	+	-	+	-	+	+	-	-
S22589	4	Sputum	ICU	M	49	>512	128	>64	0.5	128	64	≤0.25	1	1	2	≤0.25	≤0.25	+	+	-	+	-	+	+	-	-
B15612	8	Blood	ICU	M	46	>512	128	>64	16	512	256	≤0.25	1	1	2	≤0.25	≤0.25	+	+	-	+	-	+	-	-	-
B15810	5	Pus	Surgical	M	41	>512	256	32	16	128	64	≤0.25	0.5	1	2	0.5	≤0.25	+	+	+	+	-	+	+	-	-
B15583	1	Blood	ICU	F	37	>512	16	>64	2	64	2	≤0.25	0.5	1	2	≤0.25	≤0.25	+	+	-	+	-	+	+	-	-
S24463	10	ETT	ICU	F	59	512	1	32	1	≤0.25	≤0.25	1	0.5	0.5	2	≤0.25	≤0.25	+	+	+	+	-	+	+	-	-
P15045	1	Wound	Surgical	F	47	>512	64	64	16	256	64	≤0.25	0.25	0.25	2	≤0.25	≤0.25	+	+	-	+	-	+	+	-	-
P15028	10	Eye	Nursery	F	NB	512	4	16	0.5	0.5	≤0.25	≤0.25	0.25	1	2	≤0.25	≤0.25	+	+	-	+	-	-	+	-	-
P14890	11	Wound	ICU	F	41	512	256	64	0.5	256	128	≤0.25	0.5	1	2	≤0.25	≤0.25	+	+	-	+	-	+	+	-	-
P15558	1	CVP	Medical	F	94	512	>512	0.12	1	256	≤0.25	>512	0.5	0.5	1	≤0.25	≤0.25	+	+	+	+	-	+	+	-	-
P15469	12	Humerus	General	F	68	128	64	1	0.5	≤0.25	0.5	≤0.25	0.25	0.5	2	≤0.25	≤0.25	+	+	-	+	-	+	+	-	-
P15490	13	Bone	General	M	63	>512	128	32	16	128	64	≤0.25	0.25	0.5	2	≤0.25	≤0.25	+	+	+	+	-	+	+	-	-
P15742	6	cheek	Trauma	M	29	256	0.5	0.5	16	≤0.25	64	≤0.25	0.25	0.5	2	≤0.25	≤0.25	+	+	+	+	-	+	+	-	-
P15825	14	Buttock	Paediatri	M	5	512	1	0.5	0.5	≤0.25	0.25	≤0.25	0.25	1	2	≤0.25	≤0.25	+	+	-	+	-	+	+	-	-
P15793	2	Head	Surgical	M	10	512	256	64	32	256	32	≤0.25	0.5	0.5	2	≤0.25	≤0.25	+	+	+	+	-	+	+	-	-
T8060	-	-	-	-	-	512	4	4	16	128	64	≤0.25	0.5	0.5	2	≤0.25	≤0.25	+	+	-	+	-	+	+	-	-

a. ETT, Endotracheal tube; CVP, Central venous cathete; ICU, Intensive/High care unit; LW, Labour ward: OPD, outpatient department, NB, Newborn (day 0), -, No information.

b AP, ampicillin; CP, ciprofloxacin; GT, gentamicin; ET, erythromycin; RF, rifampicin; TT, tetracycline; CM, clindamycin; DP, daptomycin; VM, vancomycin; LZ, linezolid; FA, fusidic acid; TG, tigecycline.

c. The numbers 1 – 15 indicates codes of the hospital centers where the MRSA isolates were collected.

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CHAPTER 3

This study describes the antibiotic resistance pattern, characterizes plasmid-mediated resistance and, virulence genes and assesses the genetic relatedness of 27 clinical MRSA isolates from the private sector in KZN, South Africa.

3.1 Conclusions

The following conclusions were drawn from the study with respect to the study objectives:

- All MRSA isolates contained the plasmid-mediated *mecA* gene.
- The isolates were all (100%) resistant to ampillicin, 85.2% were resistant to ciprofloxacin, 74.1% to gentamicin, 70.4% to rifampicin, 66.7% to tetracycline, 63.0% to erythromycin, and 11.1% to clindamycin.
- All MRSA isolates showed no resistance to daptomycin, linezolid, vancomycin, tigecycline and fusidic acid.
- Multidrug resistance (MDR) defined as resistant to β-lactams along with at least three classes of antibiotics was found in 74.1% (20/27) of the MRSA isolates.
- The frequency of the resistance genes *blaZ, aac (2')-aph (6'')* and *ermC* were 100%, 92.6% and 48.2% respectively but *tetK* was not found in any of the MRSA isolates.
- The prevalence of virulence genes *hla and hld* were 96.3% and 92.6% respectively, however *eta and LuKS/F-PV* were not detected.
- PFGE analysis revealed 10 pulsotypes; designated A–J with 70.0% similarity which correlated with the resistance profile isolates in each group.
- Type F was the major pulsotype (29. 6%). It was found in 8 of the 27 MRSA isolates. subdivided into 5 subtypes
- Of note, 85.2% (23/27) of the isolates clustered into 6 major PFGE types giving an indication of similar circulating MRSA clones in the KZN province.
- The hospital centers 1 and 10 were found to contain similar pulsotypes C and H, whiles identical pulsotypes of F and G were spread across 9 out 15 different centers intimating the possibility of inter health centers spread of MRSA in the KZN province.

3.2 Limitations

The following limitations are acknowledged for this study:

- The short collection period of isolates yielded a small sample size which may have under- and/or over-estimated results.
- The study sample for molecular profiling was limited to isolates from only one private laboratory in eThekwini Municipality hence the results cannot be extrapolated to KZN or to South African private health sector in general.
- Detailed patients' records were unavailable making it difficult to discuss the PFGE results appropriately.

3.3 Recommendations

That the following recommendations are made as a result of the findings from this study:

- Further molecular studies should be conducted to investigate the mechanism of tetracycline resistance, including the role of efflux proteins (*TetA, TetB, TetC, TetL*), ribosomal protection (*TetO, TetM*) and modification proteins (*Tet37, TetX*).
- Additional mechanisms of macrolide-lincosamide resistance, including the role of efflux pumps mediated by *mrsA*, the acquisition of ribosomal methylase enzyme (*ermA, ermB*) and drug modification (*lnuA, lnuB)* should be investigated.
- The mechanisms of rifampicin and fluroquinolone resistance need to be studied in similar isolates.
- Additional virulence factors, such as enterotoxins (*sea-see, seg-seo, and seq*), gamma hemolysins (*hlg*), toxic shock syndrome toxin (*tst*) and adhesive proteins (*clfA/B, fib, fnbA/B*) should be investigated to ascertain the repertoire of virulence genes.
- Other molecular typing techniques that work in tandem with PFGE, such as *spa*, SCC*mec* and multi locus sequence typing (MLST) must be performed to determine the clonal clusters (CC).
- Further studies involving larger sample sizes from geographically distinct areas in South Africa should be conducted to investigate the clonal evolution of MRSA overtime.

APPENDIX

BREC approval Letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

11 November 2015

Mr DG Amoako (214583994)
Department of Pharmaceutical Sciences
School of Health Sciences
dasticky2010@gmail.com

Dear Mr Amoako

Protocol: Molecular characterization of genetic determinants of resistance and virulence in methicillin resistant staphylococcus aureus (MRSA) from the private sector in KwaZulu-Natal, South Africa.
Degree: MMedSc
BREC reference number: BE394/15

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 02 September 2015.

The conditions have been met and the study is given full ethics approval.

This approval is valid for one year from **11 November 2015.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its meeting taking place on **08 December 2015.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor J Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

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Submission confirmation from the International Journal of Infectious Diseases