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MOLECULAR CHARACTERIZATION OF ANTIBIOTIC-RESISTANT *STAPHYLOCOCCUS AUREUS* IN AN INTENSIVE PIG PRODUCTION SYSTEM IN KWAZULU-NATAL, SOUTH AFRICA

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

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DECLARATION

I, **Ncomeka Sineke,** declare that:

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DEDICATION

This research work is dedicated to My Parents, Mr. and Mrs. Nogwaza, thank you for the love and support and always making sure I never lacked. I wish nothing but continuous blessings over your lives and I hope I continue to make you proud.

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LIST OF ABBREVIATIONS

ACTB	Actin beta
ADAM	A disintergrin metalloprotease
AdsA	Adenosine synthase A
ADP	Adenosine diphosphate
AME	Aminoglycoside modifying enzyme
AMP	Adenosine monophosphate
AMR	Antimicrobial resistance
ATP	Adenosine triphosphate
CA-MRSA	Community associated methicillin-resistant *Staphylococcus aureus*
CCR	Cassette chromosome recombinase
CDC	Centers for Disease Control
CHIPS	Chemotaxis inhibitory protein of staphylococcus
CLFA	Clump A factor
CLFB	Clump B factor
dAdo	Deoxyadenosine
dAMP	Deoxyadenosine monophosphate
DNA	Deoxyribonucleic acid
EBP	Elastin binding protein
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FNBP	Fibronectin binding protein
GADPH	Glyceraldehyde-3-phosphate dehydrogenase

HA- MRSA	Hospital acquired methicillin-resistant *Staphylococcus aureus*
HGT	Horizontal gene transfer
LA-MRSA	Livestock associated methicillin-resistant *Staphylococcus aureus*
MDR	Multi-drug resistance
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
NIH	National Institutes for Health
OIE	World Organization for Animal Health
ORFX	Open reading frame X
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
PIA	Polysaccharide intercellular adhesin
PNAG	Poly-N-acetyl-glucosamine
PVL	Panton Valentine leucocidin
REP-PCR	Repetitive element palindromic PCR
RNA	Ribonucleic acid
RPP	Ribosomal protection protein
RRN18S	18s ribosomal RNA
SAK	Staphylokinase

Sbi	Staphylococcal binder of immunoglobulin
SCC	Staphylococcal cassette chromosome
SCIN	Staphylococcal complement inhibitor
Spa	Staphylococcal protein A
TSST	Toxic shock syndrome toxin
VISA	Vancomycin intermediate *Staphylococcus aureus*
VRSA	Vancomycin resistant *Staphylococcus aureus*
VWBP	Von Willebrand factor binding
WHO	World Health Organization

ABSTRACT

The increase in antibiotic resistance in food animals and food of animal origin has been attributed to the extensive use of antibiotics during animal husbandry giving rise to multidrug-resistant bacteria. *Staphylococcus aureus* is a major threat in veterinary medicine, the agricultural sector and public health because of its zoonotic potential. Despite significant research on *S. aureus* in food animals in other parts of the world, in-depth studies outside healthcare facilities are limited in South Africa. This study characterized the molecular epidemiology of antibiotic resistant *S. aureus* from farm-to-fork in an intensive pig production chain in the uMgungundlovu district, Kwa-Zulu Natal, South Africa.

A total of 333 samples collected along a pig production chain on the farm (faecal, litter and slurry samples) during transport (truck samples) and at the abattoir (caeca, carcass swabs, carcass rinsate and retail meat samples) were investigated for the presence *S. aureus* using selective media and biochemical tests. Confirmation was done by using PCR targeting the *nucA* gene. Antibiotic susceptibility patterns were investigated by the Kirby Bauer disk diffusion according to CLSI guidelines against the WHO-AGISAR recommended panel of antibiotics. Selected resistance and virulence genes were detected using PCR. REP-PCR was used to evaluate the molecular relatedness of isolates across the pig production chain.

Of the 333 samples, 141 (43%) yielded staphylococci isolates. After molecular confirmation, 97(69%) isolates were confirmed *S. aureus* and 44(31%) as other staphylococcal species. Isolates displayed resistance to erythromycin (85%), clindamycin (85%), penicillin-G (81%), tetracycline (79%), doxycycline (77%), vancomycin (69%), ampicillin (61%), trimethoprim/sulfamethoxazole (57%), rifampicin (57%), teicoplanin (52%), linezolid (51%), chloramphenicol (51%), nitrofurantoin (47%), moxifloxacin (33%), cefoxitin (20%), ciprofloxacin (15%), tigecycline (10%), levofloxacin (8%), gentamicin (8%), and amikacin (2%). Multidrug resistance (MDR) was recorded in 84% (80/97) of isolates with 56 different antibiograms. Resistance genes

ermC, *blaZ*, *tetK*, *tetM*, *msrA*, *aac'6*, *mecA* were evident in 82%, 73%, 58%, 28%, 15%, 5%, and 53% respectively and not all resistance phenotypes were genotypically confirmed. The *hla* (39%), *hld* (23%), *seb* (3%), *sed* (2%), *etb (*1%), *LukS/F-PV* (30%) and *tst* (11%) virulence genes encoding hemolysin, cytotoxins, staphylococcal enterotoxins (*sea and seb*), exfoliative toxins, PVL pore-forming toxin and toxic shock syndrome toxin-1 were detected. Genetic fingerprinting revealed the diversity of MRSA isolates in the pig production chain with the major REP-types constituting isolates from different sources within the farm, suggesting transmission within the farm environment with no evidence of transmission across the production chain.

This study highlights the phenotypic and genotypic diversity of the virulence and resistance profiles of *S. aureus* isolated across the pig production chain. Resistance to antibiotics used as growth promoters was evident and the high prevalence of MDR isolates with elevated MAR index values >0.2, specifically at farm level indicates exposure to environments of high antibiotic use, necessitating antibiotic stewardship and proper infection control measures in pig husbandry and intensive pig production**.**

CHAPTER ONE – INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Staphylococcus aureus is an opportunistic bacterium that exists as both a commensal and pathogen in animals and humans. It has been described as one of the leading causes of nosocomial infections in healthcare facilities, causing diseases such as sepsis, pneumonia, infective endocarditis and bloodstream infections (Boswihi & Udo, 2018; Grema *et al*., 2015; Verkade & Kluytmans, 2014). *S. aureus* has also been implicated as a cause of toxin-mediated diseases inclusive of toxic- shock syndrome and scalded-skin syndrome as well as food poisoning (Boswihi & Udo, 2018). Its ability to resist a wide range of antibiotics has led to limited therapeutic options (Reddy *et al*., 2017). In food animals, *S. aureus* has been implicated in persistent and recurring mastitis infections in cows, septicemia in small ruminants and dermatitis in goats, In poultry and pigs *S. aureus* has been associated with abscesses, septic arthritis, gangrenous dermatitis and septicemia amongst others (Haag *et al*., 2019). The ability of *S. aureus* to cause a wide spectrum of diseases in a variety of animal hosts and their reservoirs in animals facilitates transmission to humans and thus a significant public health impact.

S. aureus throughout the years has acquired the ability to resist most penicillin antibiotics inclusive of methicillin and all anti-Gram-positive β-lactam antibiotics, hence termed methicillin-resistant *Staphylococcus aureus* (MRSA). Gram-positive bacteria contain a structurally significant protective layer called peptidoglycan that constitutes the cell wall. It provides cell integrity and facilitates cell division and subsequently, growth. The final steps of peptidoglycan synthesis are facilitated by enzymes collectively called penicillin-binding proteins (PBPs). The transglycosylase domain of the PBPs produce glycan strands while the transpeptidase domain link the peptides to form a three-dimensional network (Liu & Breukink, 2016). Penicillins, cephalosporins and other β-lactams harbor a four-membered ring that facilitates bacterial cell degradation

by binding with the PBP active site, due to structural similarities. They inhibit its cross-linking activity, preventing cell wall formation and subsequently bacterial cell death (Fishovitz *et al*., 2014).

Staphylococci, particularly MRSA harbor a defining feature, a gene cassette known as a staphylococcal cassette chromosome (*SCC*) mec which is a mobile genetic element that encompasses a methicillin resistance determinant encoded by *mecA*. *SCCmec* contain similar elements including a *mec* gene complex, cassette chromosome recombinase *(ccr)* gene complex, integration site sequence for SCC and a junkyard region comprising of three regions, i.e. the region between *ccr* and chromosomal region, the region between *mec* and *ccr*, and the region between *orfX* (open reading frame X) and *mec*. Although similarities may be observed, SCC*mec* types are structurally diverse and thus classified based on differences in *mec*, *ccr*, and subtyping and junkyard regions. To date 13 SCCmec types have been described for *S. aureus* namely types Ⅰ, Ⅱ, Ⅲ, Ⅳ, Ⅴ, Ⅵ, Ⅶ, Ⅷ, Ⅸ, Ⅹ, Ⅺ, Ⅻ, and XIII (Liu *et al*., 2016; Singh-Moodley *et al*., 2020). Additionally, these SCC*mec* types are differentiated based on the ccr and *mec* class present, for example, type (1A) stipulates a SCC*mec* containing a type 1 *ccr* and a class A *mec* gene complex. This nomenclature has been considered more informative and specific. SCC*mec* types are then further differentiated into subtypes based on the polymorphism on J region variation within the same *ccr* and *mec* gene complex combination. The methods used to describe SCC*mec* subtypes are mainly: (1) expression of J1 region denoted in small letters, e.g. type Ⅳ (a), (2) expression of the differences of mobile genetic elements as capital letters, e.g. type Ⅳ (B) and (3) expression of differences in J1, J2. J3 regions and denoted in Arabic numerals in the order of discovery (IWG-SCC 2009). The acquisition and insertion of this genetic element into methicillin-susceptible strains accounts for the emergence and existence of methicillin-resistant lineages (Liu *et al*., 2016). A variant *mecA* gene, named *mecC* was described in 2015. This gene has a higher affinity for oxacillin and presents an alternative mechanism for β-lactam resistance (Abdulgader *et al*., 2015). Some *mecC* lineages show resistance to cefoxitin but might show susceptibility to oxacillin. This may pose diagnosis

complications due to misidentification as methicillin-sensitive, with adverse consequences for individual patients (Kim et al., 2012; Paterson *et al.*, 2014).

MRSA was discovered and reported in healthcare facilities as a nosocomial pathogen and termed hospital-acquired methicillin-resistant *Staphylococcus aureus* (HA-MRSA). However, MRSA since been discovered in community settings among healthy individuals with no history of hospital admission, hence termed community-associated methicillin-resistant *S. aureus* (CA-MRSA). In addition, MRSA can colonize and infect a wide range of host species inclusive of livestock, wildlife and companion animals. Its significance has been noted not only in animal welfare and economic growth but in its role as a reservoir for zoonotic infections in humans (Paterson *et al*., 2014). This led to a third epidemiological classification group termed livestock-associated MRSA (LA-MRSA) (El-Hamid *et al*., 2019). The evident spread and transmission of this pathogen between humans, animals and the environment is a matter of concern.

After the discovery of this resistance phenotype in *S. aureus* and its zoonotic implications in the 1900s, MRSA has gained interest inthe veterinary sector due to the increase in MRSA infections and limited treatment options (Garipcin & Seker, 2015). It has been identified in pigs, cattle, poultry, and humans, specifically occupationally exposed workers such as farmworkers, veterinarians (Cuny *et al*., 2015; Dahms et al., 2014; Herold *et al.*, 1998; Lee, 2003; Rich & Roberts, 2004). The use of antibiotics as growth promoters for animal farming and food production facilitates the emergence and transmission of resistant bacteria, including multi-drug resistant organisms such as MRSA. Sequence type 398 (ST398) which belongs to the clonal complex 398 (CC398) is the predominant strain linked to LA-MRSA, carrying SCC*mec* genes including but not limited to type **IV** and **V**.CC398 was initially discovered in pigs but has since been isolated in other animal species such as wildlife and companion animals (Cuny *et a*l., 2010; Voss *et al*., 2005). A German study conducted in 2004 determined the prevalence of MRSA among regional pig farmers and pigs, following a 6-month-old girl testingpositive to MRSA prior to thoracic surgery with no history of travelling or hospital stay.

Although consecutive decolonization had been done through administering treatment; the girl remained infected. It was then discovered that the girl lived on a farm, and the parents tested positive after screening as well. Forty pigs and twenty-six farmers were then screened for MRSA. The study reported that out of the 40 pigs, one was positivefor MRSA, and six farmers were MRSA- positive. Overall, three different MRSA strains were identified. It wasthen concluded that the primary pig production system was potentially an important reservoir for LA-MRSA, suggesting possible transmission between humans and animals (Voss *et al*., 2005).

According to the Food and Agriculture Organization of the United Nations (FAO) in 2016, the overuse and improper use of antimicrobials in many parts of the world is the driving force for the emergence and spread of antimicrobial resistance (AMR) (FAO, 2016a). Further, based on available data, it is estimated that antimicrobial use (AMU) in livestock will continue to increase in low and middle- income countries in the next decade. This global attention of AMR and its health significance has raised concerns on the epidemiological relationship of AMU and the occurrence of AMR in food animals, food products as well as exposure to humans.

Only a few studies have been conducted in South Africa on antibiotic-resistant *Staphylococcus* spp. in animals. Little is known about the incidence of S. *aureus* infection and/or colonization in pigs and occupationally exposed workers nor the transmission of antibiotic resistance from pigs to pig products through the production chain to consumers. This is a cause for concern for a country without an active surveillance program with antimicrobial resistance (AMR) reported as being a major threat globally. This study describes the molecular epidemiology of antibiotic-resistant *Staphylococcus* spp. in an intensive pig production system in KwaZulu-Natal, South Africa.

1.2 Staphylococcus aureus

S. aureus is a Gram-positive, cocci. It is a member of the Firmicutes, under the Bacilli class, Bacillales order and Staphylococcaceae (Micrococcaceace) family. This micro-organism is found as a commensal on the skin, nose, throat, anus and mucous membranes in animals and humans (Pirolo *et al*., 2019). Sites for MRSA screening in animals include nose, skin, the perineum and the rectum (Khanna *et al.*,2008). Screening sites for humans are the nose, throat and hands (Grundmann *et a*l., 2006; Peacock *et al*.,2001; Senn *et a*l., 2012).

Apart from the well-known and clinically significant S. aureus, more than 50 species and subspecies of Staphylococci exist inclusive of, *S. epidermidis, S. capitis, S. caprae, S. lentus, S. lugdunensis, S. massiliensis, S. simulans* amongst others (Parte, 2018). However, *S. aureus* and *S. epidermidis* are frequently isolated in hospitals as nosocomial pathogens and therefore remain of major clinical importance (Foster, 1996). However, in animals' species prevalence varies according to host. For example, *S. felis* is predominantly isolated from healthy felines or those presenting with ocular disease or those with signs of lower urinary tract disease. Furthermore, *S. pseudintermedius* has been isolated from several domestic animals while *S. agnetis* is found frequently in cows and *S. carprae* in dairy goats amongst many others (Rossi *et al.,* 2020).

S. aureus is catalase-positive and coagulase-positive bacteria, it is cocci shaped and shows β- hemolysis on blood agar and mannitol formation in mannitol salt agar. This organism can grow in a wide range of temperatures from 7°C to 48.5°C with an optimum temperature of 30 to 37°C. *S. aureus* can resist adverse conditions with its ability to survive in a wide range of pH between 4.2 to 9.3 with 7 to 7.5 being optimal. Growth of *S. aureus* occurs in the presence of NaCl of up to 15%. This organism is considered a desiccation-tolerant species, which is the ability to survive in dry environments such as skin, nose. It is resistant to freezing

and survives up to -20°C; however, viability is reduced at temperatures between -10°C to 0°C (Kadariya *et al*., 2014; Missiakas & Schneewind, 2013). Phenotypic methods used to identify *S. aureus* include Gram staining, colony morphology, pigment formation, anaerobic growth or catalase and coagulase tests (Karthy *et al.*, 2009). Resistance to cefoxitin is indicative of MRSA.

1.3 Molecular Detection of MRSA

Molecular methods have been used to confirm MRSA through the detection of *mec* genes. The gold standard for determination of MRSA is the detection of *mecA* gene by polymerase chain reaction (PCR). Several PCR assays such as multiplex PCR, real-time PCR amongst others have been used for *S. aureus* identification by targeting specific genes inclusive of *spa, coa, femA, nuc* and 16S rDNA (Stegger *et al*., 2012). Genomic DNA from broth bacterial cultures or bacterial colonies influences the performance of these essays; therefore, cultures with mixed colonies might yield a false-positive result. Real-time PCR assay allows the amplification and detection of MRSA genes by screening directly from swabs with a convenient turnaround time of one to six hours. This method also differentiates different SCC*mec* types making it a method of choice, although it may be costly (Pournajaf *et al*., 2014).

1.4 Typing Techniques

Bacterial typing methods are used to clarify population dynamics and monitor the spread of microorganisms. Molecular methods such as PCR, pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST) and Staphylococcus protein A (*spa)* typing are typical methods for strain typing (Stefani *et al*., 2012; Stryjewski & Corey, 2014). MRSA strains isolated from livestock are non-typeable by standard PFGE method

due to the presence of a DNA methylation enzyme that protects against DNA digestion by *Sma*I endonuclease (Bens *et al.*, 2006). However, several molecular methods are available for the identification of LA-MRSA such as REP-PCR, MLST and *spa* typing and SCC *mec* typing.

MLST uses sequence analysis of internal fragments of housekeeping genes such as RRN18S (18S ribosomal RNA), ACTB (actin beta), GAPDH (glyceraldehyde-3-phosphate dehydrogenase) amongst many others. DNA sequences are then compared to those previously identified alleles at each locus on the MLST database (Curina *et al*., 2017; Eisenberg & Levanon, 2013). Although this method has increased reproducibility and speed in obtaining results, it is highly expensive and time-consuming.

Spa typing is the typing of a single locus zone in the polymorphic region X of *S. aureus* A which involves duplication and mutation in variable repeats. It is faster, cheaper and highly discriminatory, however, it is not recommended for large epidemiological studies (Alkharsah *et a*l., 2019).

Repetitive element palindromic PCR (REP-PCR) is a DNA based technique that discriminates bacteria at subspecies or strain level by observing genomic fingerprinting patterns. It infers genetic relatedness of isolates by comparing banding patterns of amplicons and is a preferred method for tracking the pathway of contamination for *S. aureus* (Han *et al*., 2019). PCR based techniques have a shorter turnaround time, are reproducible and simple to use.

1.5 MRSA Epidemiological Classification

MRSA is divided into three epidemiological categories: hospital acquired-MRSA (HA-MRSA), community associated-MRSA (CA-MRSA) and livestock associated-MRSA (LA-MRSA) (Petersen *et al*., 2013) as follows:

- HA-MRSA is termed as an infection caused by MRSA 48 hours or more post-hospital admission (Rajan, 2012). HA- MRSA is usually genetically associated with type I, II and III strains that are multi-drug resistant to antibiotics (Armand-Lefevre *et al*., 2005)
- CA- MRSA is an infection that occurs at hospital admission or within 48 hours of hospital admission with no history of hospitalization, surgery or long-term care in a healthcare facility (DeLeo *et a*l., 2010). Genetically, CA-MRSA carries smaller sized SCC*mec* types IV, V and VI, which are usually susceptible to non- β-lactam antibiotics (Boswihi & Udo, 2018).
- LA- MRSA are distinct strains usually of animal origin but can be isolated from humans frequently exposed to colonized animals. As early as 2003, strains were reported to carry SCCmec type IV and V associated with CC398 strain. However, lineages in humans are described as ST9, ST97 and ST433, amongst others. CC398 has been reported in animals (livestock) and humans due to occupational exposure with an increasing rate of infection and colonization among various countries (Grema *et al.,* 2015; Witte *et al*., 2007).

1.6 Mechanisms of Antibiotic Resistance in *S. aureus*

S. aureus was first reported to be resistant to penicillin and methicillin, then vancomycin, linezolid and daptomycin (Reygaert, 2013). Its resistance mechanisms encompass one or more of enzymatic inactivation of antibiotics, alteration of the target PBPs, and decreasing affinity of the drug to bacterial target, efflux and inhibition of antibiotic uptake (impermeability). Resistance occurs through horizontal gene transfer of mobile genetic elements carrying antibiotic resistance genes as well as mutations that alter drug binding sites (Foster, 2017; Pantosti *et a*l., 2007).

Horizontal gene transfer (HGT) allows the incorporation of multiple resistance determinants within and between bacterial species by mobile genetic elements (MGE's) such as plasmids, transposons, integrons,

gene cassettes, integrative and conjugative elements and insertion sequence common regions (Stokes & Gillings, 2011). This allows the re-arrangement, selection or the modification of expression of various resistance determinants. Gene transfer may also be accompanied by the selection of resistance to various unrelated antimicrobials resulting in MDR species as well as resistance to other compounds such as biocides and metal ions (Amabile-Cuevas, 2013). HGT is by far the most successful mechanism of resistance acquisition by most bacterial species (Woods *et al*., 2020).

1.6.1 Penicillin Resistance

In animal production, specifically swine, penicillins are used for the treatment of urinary tract infection, skin and soft tissue infections and prophylaxis. The penicillins are usually administered through drinking water over a prolonged period (Lekagul *et al*., 2019) and include amoxycillin, ampicillin, penicillin-G and phenoxymethyl penicillin. Penicillins function by targeting cell wall biosynthesis, facilitated by PBPs. *S. aureus* has four PBPs; PBP1, PBP2, PBP3, PBP4, however, MRSA harbors a fifth PBP known as PBP2a. PBP2a is a structurally unique transpeptidase that is not inhibited by β-lactam antibiotic function; hence peptidoglycan synthesis continues (Fishovitz *et a*l., 2014). *S. aureus* harbors a β-lactamase gene known as *blaZ* that facilitates penicillin resistance. *Blaz* produces β-lactamase which inactivates penicillin by hydrolyzing its β-lactam ring. This gene is carried by transposon Tn 552 located on a plasmid pI524 integrated into the bacterial chromosome (Foster, 2017).

1.6.2 Cephalosporin resistance

Cephalosporins have been used for the treatment of staphylococcal infections since the 19th century. They have been frequently used in human medicine for surgical prophylaxis to prevent surgical and post-surgical infections (Onuh & Timilehin, 2016). In veterinary medicine, cephalosporins are used in the treatment of

respiratory diseases in swine and ruminants e.g., fourth-generation cephalosporin cefquinome (Hornish & Kotarski, 2002). Cephalosporins have a mechanism of action identical to the penicillins (all are β-lactams). They disrupt peptidoglycan synthesis and cell wall formation. Resistance can be attributed to the presence of PBP2a and its interaction at an allosteric site within the peptidoglycan triggering conformational changes to reduce binding affinity (Duplessis & Crum-Cianflone, 2011; Villegas-Estrada *et al.*, 2008).

Second, third and fourth generation cephalosporins such as ceftazidime and cefepime are amongst a broad spectrum of antibiotics listed by the WHO as critically important in human medicine (World Health Organization 2017). Moreover, these antibiotics are also used for the treatment of bacterial infections in food animals. For example, ceftiofur, a third-generation cephalosporin is commonly used for the treatment of pneumonia, arthritis and septicemia and can be used for metaphylaxis or prophylaxis. To be noted, some of these generation cephalosporins (whether used in animas/ humans) may differ chemically but share the same mode of action and thus similar resistance mechanisms. The shared common use of third and fourth generation cephalosporins in humans and animals induces resistance especially in food borne pathogens with risk of transmission to humans. As a result the Food and Drug Admission (FDA) has since ordered prohibiting external label use in food animals (Hao *et al*., 2016). MRSA has exhibited resistance to all cephalosporins, including the fifth generation cephalosporins, ceftaroline and ceftobiprole (Duplessis, 2011; Long *et al*., 2014).

1.6.3 Vancomycin resistance

Vancomycin has been the drug of choice for MRSA infections over the last two decades. Vancomycin acts by interfering with peptidoglycan formation through forming bonds with D-Ala-D-Ala residues disrupting cell wall synthesis and resulting in bacterial cell death (Guo *et al*., 2020).

However, *S. aureus* has developed resistance known as vancomycin-resistant *S. aureus* (VRSA) and vancomycin-intermediate resistant *S. aureus* (VISA) which were initially discovered during the 1990s. VISA resistance is attributed to altering peptidoglycan synthesis and producing extra peptidoglycan with increased D-Ala-D-Ala residues which results in the thickening of the cell wall. These residues then bind vancomycin preventing it from reaching the bacterial target. Increased vancomycin resistance has also been associated with reduced cross-linking of peptidoglycan, dysfunction of *agr* system and reduced autolytic activity of enzymes responsible for cell wall turn-over (Cui *et al.*, 2000). VISA is facilitated by a variety of genes inclusive of*, walkR, vraSR, graSR* and occurs through the mutation of these genes encoding for molecules carrying out cell wall synthesis. VRSA is facilitated by a *VanA* gene cluster found on a plasmid originally belonging to *Enterococci* (VRE) located on a Tn 1546 transposon *(*Gardete & Tomasz, 2014; Moreno *et a*l., 2016). Acquisition of *VanA* gene by VRSA allows it to alter the terminal peptide of D-Ala-D-Ala to D-Ala-D-Lactate resulting in the reduced binding affinity of vancomycin (Appelbaum, 2006; Lowy, 2003).

Vancomycin resistance is not restricted to humans and has been reported in livestock animals such as pigs, goats, cattle (Moreno *et al*., 2016).

1.6.4 Gentamicin and Amikacin Resistance

Aminoglycosides function by disrupting bacterial protein synthesis by conformal changes and mistranslation of tRNA on the 16SrRNA of the 30S ribosome. They also act by inhibiting initiation and elongation during protein synthesis. *Staphylococcus aureus* has developed resistance to aminoglycosides by the inactivation of antibiotics by aminoglycoside modifying enzymes (AMEs) inclusive of *aac*, *aph* and *ant,* inactivating the antibiotic by acetylation, phosphorylation and or adenylation respectively. Modification of antibiotics prevents ribosomal binding. The above mentioned AMEs are the leading causes of the escalation and spread of resistance with more than a hundred AMEs having been reported. Gentamycin resistance is carried out by aminoglycoside-

6-N-acetyltransferase/2-O-phosphoryltransferase located in a Tn4001 transposon in pUB110 plasmid which is usually integrated into SCC*mec* type Ⅱ. According to a review by Ramirez in 2017, a newer semi-synthetic aminoglycoside to withstand resistance known as plazomicin has been approved for human use (Ramirez & Tolmasky, 2017; Shaeer *et al*., 2019; Watkins *et al*., 2019). Plazomicin, however, is not protected against other resistance mechanisms such as 16S rRNA methyltransferases that modify aminoglycoside target site but these enzymes have not been reported in *S. aureus (*Watkins *et al*., 2019*)*.

1.6.5 Tetracycline resistance

Tetracyclines are used to treat human and animal infections aswell as being used for prophylaxis and growth promoters in animals (Michalova *et a*l., 2004; Trzcinski *et a*l.,2000). They function by binding on the 16S of the 30S ribosomal subunit and inhibit protein synthesis by preventing aminoacyl-tRNA accommodation on the receptor site during elongation (Grossman, 2016;Markley & Wencewicz, 2018).

Two mechanisms confer resistance to tetracycline: (1) active efflux of the drug due to acquisition of *tetK* and *tetL* on plasmid pT181 integrated within SCC*mec* type Ⅲ, and (2) ribosomal protection facilitated by *tetM* and *tetO* variants which are chromosomally located usually on transposon Tn916 and Tn1545 (Foster, 2017). Most MRSA isolates harbor both *tetK* and *tetM* genotype giving them the potential to become resistant to all tetracycline antibiotics. The tetM gene encodes the production of a ribosomal protection protein (RPP) that competes with tetracycline for binding on the 30s subunit (Reygaert, 2013). RPP interference with tetracycline confers resistance. Efflux proteins in the cytoplasmic membrane exchange a proton forming a magnesium-tetracycline complex which acts as an antiporter reducing the amount of antibiotic within the cytoplasm (Michalova *et al*., 2004).

1.6.6 Daptomycin Resistance

Daptomycin was approved for the treatment of skin, soft tissue infection; Staphylococcus right-sided endocarditis as well as bacteremia in 2003 and 2006 respectively. It is a potential alternative to vancomycin for the treatment of endocarditis and has been associated with skeletal muscle infection in small animals (Jacqueline *et a*l., 2011). Daptomycin is thought to bind to the target Gram-positive bacterial cell membrane via the interaction with phosphatidylglyceol, although this is poorly understood. It enters the cell reliant on calcium (Ca2+) resulting in depolarization and subsequent loss of other cell components such as potassium (K+) magnesium (Mg+) and ATP. The leakage of these components results in the dissipation of the cell membrane potential and cell death (Heidary *et al*., 2017). Resistance can be attributed to the mutation of the *mprf* gene, which encodes an enzyme that changes the charge of the peptidoglycan from negative to positive. The alteration in cell membrane charge reduces daptomycin binding affinity (Bayer *et al*., 2013; Reygaert, 2013). An alternative method that confers resistance by altering the cell membrane is the over-expression of *dlt* operon which facilitates the binding of positive amino acid- alanine to the cell wallteichoic acid thus increasing cell surface positive charge (Sabat *et al*., 2018).

A further resistance mechanism involves the change in membrane fluidity or decrease in the amount of phosphatidylglycerol in the membrane resulting in reduced affinity of daptomycin binding and subsequently oligomerization. It has been speculated that altering phosphatidylglycerol cardiolipin ratio also confers daptomycin resistance through mutations of *pgsA* and *cls2,* genes encoding enzymes that function in phosphatidylglycerol and cardiolipin synthesis respectively (Miller *et al.*, 2016; Peleg *et al.*, 2012).

1.6.7 Erythromycin and Clindamycin Resistance

Erythromycin resistance is usually associated with resistance to other macrolides, lincosamides and type B streptogramin or better known as MLS_B. Streptogramin was introduced for the treatment of MRSA infections in 1999 while another streptogramin antibiotic, virginiamycin is used an infeed in farm animals in the USA. Resistance has been reported in livestock-associated strains in China and Europe (Foster, 2017). Macrolides are bacteriostatic meaning that they halt bacterial growth rather than killing bacteria. This occurs through the inhibition of protein synthesis. Erythromycin bind to the 23S ribosomal RNA on the 50S subunit blocking the growth of peptide chain. Resistance occurs through four distinct mechanisms: (1) Use of energy-reliant efflux, that is encoded by the *msrA* gene (2) enzymes that facilitate drug inactivation such as macrolide esterases and macrolide phosphotransferases (3) alteration of 23S RNA methylase by plasmid integrated genes *ermA, ermB, ermC* encoding erythromycin methylase that facilitate methylation on the active site of the ribosome triggering conformational change resulting in drug binding inhibition (Lim *et al.*, 2012), and, (4) and most recently, ribosomal protection facilitated by ATP-binding proteins such as *msrE* (Golkar *et al*.,2018).

1.6.8 Chloramphenicol resistance

Chloramphenicol derivatives such as florfenicol and thiamphenicol, are used in food animals for the treatment of respiratory infections, e.g., pigs and cattle. They function by binding on the 50S subunit of the 70S ribosome and inhibit peptidyl transferase and subsequently protein synthesis, resulting in bacteriostatic effects (Lorenzo, 2019). Resistance is mediated by the acquisition of a plasmid that harbors chloramphenicol acetyltransferase gene, *cat*. This gene confers acetylation of the chloramphenicol antibiotic inhibiting it from binding to the 50S subunit (Reygaert, 2013). Ribosomal modification by changes in conformational structure confer resistance by inhibiting antibiotic binding (Schwarz *et al*., 2004).

1.6.9 Linezolid Resistance

Linezolid is an oxazolidinone drug used to treat fastidious MRSA infections and was approved for human use in almost two decades ago. Its mode of action is to prevent protein synthesis by binding to rRNA on the 30S and 50S subunits of target bacteria. This inhibits the formation of initiation complex resulting in reduced peptide chain length and a decrease in the rate of translation. In Gram-positive bacteria, it also prevents the expression ofvirulence factors resulting in decreased toxin production (Martinez-Olondris *et al.*, 2012). To date several resistance mechanisms have been described namely: (1) mutation in the domain V region of one or more ofthe 5 or 6 of the copies of 23S rRNA gene, where a guanine base is replaced by thymine through a point mutation known as G2576T (Gu *et al*., 2013; Morales *et al*., 2010) (2) acquisition of plasmid-mediated *cfr* gene hat encodes ribosomal methyltransferase, and (3) deletion or mutations in the L3 of the peptidyl transferase center (Gandham, 2014). However, newer semisynthetic oxazolidinones have been designed with improved properties based on the knowledge of the linezolid binding site and known resistance mechanisms (Long & Vester, 2012).

1.6.10 Ciprofloxacin Resistance

Fluoroquinolones are an antibiotic class that have been used in both humans and veterinary medicine (Redgrave *et al*., 2014). Ciprofloxacin is an antibiotic of the fluoroquinolone class together with norfloxacin, moxifloxacin, levofloxacin amongst many others. They are widely used in the treatment of respiratory tract infections and urinary infections in hospital and community settings suggesting frequent use. In veterinary medicine, they are approved for the treatment of urinary tract infections, respiratory tract infections, gastrointestinal infections, soft tissue infections and colibacillosis in poultry. They have also been recommended for prostatitis, deep-seated infections and non-therapeutic use in animal production.

Ciprofloxacin interferes with bacterial replication by targeting DNA gyrase and topoisomerase Ⅳ, enzymes that facilitate replication (Champion *et al*., 2004). DNA gyrases are constituted of two subunits *gyrA* and *gyrB* that function as replication initiators through the formation of negative supercoils ahead of the replication fork. Topoisomerase Ⅳ is composed of *grlA* and *grlB* that facilitate the replication of circular adult chromosome into two topologically linked daughter chromosomes or termed decatenation. The introduction of ciprofloxacin to these enzymes results in the inhibition of replication and consequently, DNA strand breakage. However, MRSA alters amino acid sequence in DNA gyrase*, gyrA* and topoisomerase *grlA* both facilitated by *gyrA* and *grlA* respectively. (Vestergaard *et al*., 2016). Resistance is also mediated through efflux pumps encoded by the *norA* gene. (Redgrave *et al*., 2014; Reygaert, 2013) that facilitates the removal of fluoroquinolones from the bacterial cytoplasm. In *S. aureus*, resistance to fluoroquinolones is conferred by the overexpression of *norA,* thus actively facilitating the pumping of the drug from the cytoplasm (Costa *et al*., 2019; David, 2001).

1.6.11 Trimethoprim/Sulfamethoxazole resistance

Trimethoprim/sulfamethoxazole is a combination antibiotic used in veterinary medicine for the treatment of skin and ear infections, coccidiosis and prostate infections. Sulfamethoxazole is a sulfonamide that inhibits the synthesis of dihydrofolate by competitive binding with p-aminobenzoic acid. Trimethoprim inhibits dihydrofolate reductase by competitive binding resulting in the halt production of folate from tetrahydrofolate, which is an essential component in the synthesis of purines used in DNA and protein synthesis (Wood *et al*., 2012).

The key determinants of resistance towards sulfamethoxazole is the mutation of dihydropteroate synthase enzyme that prevents the drug from binding to the enzyme (Foster, 2017). Trimethoprim resistance is conferred by the mutation of the chromosomal dihydrofolate reductase gene *dfr*B and the resistance gene

that encodes *dfhr* variants is found on exchangeable genetic elements. *dfrA* located on Tn4001 transposon and *dfrB* is considered to confer resistance in humans while *dfrG* is animal associated (Argudín *et al.,* 2011; Foster, 2017).

1.7 *S. aureus* virulence determinants and pathogenesis

Virulence is a series of processes that are usually coordinated and regulated to give an organism the ability to evade, adapt and become successful pathogens in a host. The virulence factors promote colonization, tissue damage and disease while evading host defenses and antimicrobials (Kaur & Chate, 2015).

S. aureus harbors various virulence mechanisms including enterotoxins, leucocidins, proteases, exfoliative toxins, hemolysins and immune-modulatory factors (Foster, 2005; Oogai *et al.*, 2011). Secreted toxins may include α- hemolysin, toxic shock syndrome toxin-1(TSST-1), Panton valentine leucocidin (PVL), phenol soluble modulins and multiple secreted tissue-damaging exoenzymes (Gordon *et al.*, 2013). These enterotoxins have been reported to cause food poisoning (Hennekinne *et al*., 2012; Jackson *et al.*, 2013). The *agr* gene has been reported to play a major role in the regulation of these virulence factors (Cheung *et al.*, 2004). It regulates a gene coding for RNA, named RNA Ⅲ. The regulation of these virulence factors occurs by RNA-RNA interaction in the post-transcriptional phase. This interaction is explained by the occurrence of transcription of genes encoding for secreted toxins and enzymes while the transcription of genes encoding for cell surface proteins is repressed. Apart from *agr,* there are other transcriptional regulators said to be responsible for virulence regulation inclusive of *arl, sae, sar, mgr* amongst others (Cheung *et a*l., 2004; Novick, 2003). The *arl* (autolysin regulated locus) is a negative regulator of autolytic process and downregulates RNA Ⅱ and RNA Ⅲ expression resulting to *agr* repression. It has a significant impact on extracellular proteolytic activity and clumping in the presence of fibrinogen. The *sae* drives the expression of several essential exoproteins including hemolysins, PVL, and exfoliative proteins amongst

others. Induction of exoproteins and repression of spa is facilitated by *sar*. It boosts the expression of the *agr* regulatory system. The *srrAB* facilitates resistance towards nitrosative stress. It also confers hydrogen peroxide resistance during high dioxygen-dependent respiration by activating hydrogen peroxide resistance genes. It is also required for defense against neutrophils. The *mgr* facilitates the repression of surface proteins and the induction of efflux pumps and capsule expression (Jenul & Horswill, 2018). All these virulence determinants facilitate adherence to cells and tissues, spread, successful evasion of host defenses and subsequently infection. (Gordon & Lowy, 2008).

S. aureus can form biofilm enabling it to persist by avoiding host defenses (phagocytosis) and antimicrobials (Foster, 2005). Furthermore, MRSA produces a variety of enterotoxins including SEA, SEB, SEC, and SED amongst many others. They are usually located in mobile genetic elements such as plasmids, prophages, transposons, *S. aureus* pathogenicity islands and enterotoxin gene clusters. They are considered non-specific T-cell stimulators that cause unregulated activation of immune responses resulting in cytokine activation with mild clinical symptoms such as fever and subsequently organ failure and mortality. Staphylococcal enterotoxins differ to other *S. aureus* toxins because of their tolerance to extreme changes such as low pH, heating, and proteolytic digestion (Fisher *et al.*, 2018).

Major factors that confer virulence in MRSA are mainly: (1) adherence, (2) antiphagocytosis, (3) production of exoenzymes, (4) immune invasions, (5) iron uptake, (6) plasmogen uptake, (7) harboring secretion system, (8) and toxin production (Otto, 2012).

1.7.1 Adherence

Adherence is facilitated by surface proteins called microbial surface components recognizing adhesive matrix molecules (MSCRAMM) made up of adhesin regulatory molecules that facilitate bacterial binding to host

tissue inclusive of collagen, fibrinogen and fibronectin. Adherence is the initial step of staphylococcal infection with various adherence factors such as collagen-binding proteins, clumping factors, extracellular adherence proteins, elastin binding proteins, fibronectin-binding proteins, intracellular adhesion proteins and serine-aspartate repeat protein (Ahangari *et al*., 2017).

Collagen binding protein is composed of an N-terminal signal peptide, a non-repetitive A region, four repeated subunits followed by a cell wall anchor region, transmembrane and a positively charged cytoplasmic tail. The A region attributes to collagen-binding activity. It is usually associated with osteomyelitis and septic arthritis (Foster *et al.*, 2013; Rhem *et a*l., 2000).

Clumping factors are a series of fibrinogen binding genes, clump A factor (ClfA) and clump B factor (ClfB). They bind to fibrinogen using a dock, lock and latch mechanism. ClfA and ClfB are structurally similar; however, they are distinct genes that are not closely linked (Foster et al., 2013). Extracellular adherence proteins function as a pro and anti-inflammatory proteins that bind to host glycoproteins. The similarity of this protein to the major histocompatibility complex (MHC) (which is a locus of DNA containing closely related polymorphic genes that encode cell surface proteins essential for adaptive immune response) peptide-binding groove allows the MHC complex to reduce lymphocyte proliferation. It redirects the response to Th_2 direction suppressing Th_1 responses that facilitate bacterial clearance during infection in animals (Bur *et al*. ,2013).

Elastin binding protein (Ebps) is located on the cell wall and binds to elastin. Ebps functions as an adhesin although bacterial adhesin is also said to be mediated by fibronectin-binding proteins. Moreover, it has been suggested that this protein may be involved in nutrient metabolism. Ebps can form secondary structures based on environmental conditions (Nakakido *et al*., 2014).

Fibronectin binding protein (FnBP) is a cell wall anchored protein with two distinct binding proteins, FnBPA and FnBPB. They bind on the N-terminal type Ⅰ module of fibronectin using their C-terminal by a tandem β-zipper mechanism. This allows fibronectin to become a bridge between the bacterial cell and the $\alpha_5\beta_1$ on host cells triggering endocytosis of the bacterial cell. Binding of FnBPA has been associated with cardiac infections implicating them in intravascular infections. Moreover, FnBPs can also bind to elastin, capture plasminogen and some MRSA strains form FnBP derived biofilm (Foster, 2016; Foster *et a*l., 2013; Geoghegan *et al*., 2013).

The synthesis of biofilm and micro-colonies requires intercellular aggregation of this bacteria. *S. aureus* can produce exopolysaccharides to achieve successful biofilm accumulation. This exopolysaccharide is called polysaccharide intercellular adhesion (PIA) or known as poly-N-acetyl-glucosamine (PNAG). PIA is a positively charged exopolysaccharide that enables intercellular interactions (Arciola *et al.*, 2015). It is synthesized by four major proteins, IcaA, IcaD, IcaB and IcaC that are encoded by the *ica* operon. IcaA and D act as an acetylglucosaminyltransferase synthesizing PIA oligomers. IcaC acts as a transporter functioning in the carriage of IcaAD oligomers across the cell membrane. IcaC also functions in the elongation of IcaAD oligomers. IcaB facilitates the deacetylation of PIA resulting in a positively charged polymer which assist in interacting with negatively charged surfaces. IcaADBC is regulated by SarA and σβ regulatory proteins as well as IcaR and TcaR (Cue *et al*., 2012). Serine-aspartate repeat protein is encoded by sdrC, sdrD and sdrE genes within the *sdr* locus. Different sdr genes perform different functions in *S. aureus* pathogenicity. Sdr proteins are constituted of B motifs, the R region the C-terminal that is associated with anchoring the protein onto a bacterial cell wall (Liu *et al*., 2015). SdrD facilitates the attachment of *S. aureus* on nasal epithelial cells and contributes to abscess formation (Askarian *et al*., 2016). SdrC promotes bacterial adherence to surfaces and biofilm synthesis through the formation of hydrophobic interactions with cell surfaces (Feuillie *et al*., 2017).

1.7.2 Antiphagocytosis

Antiphagocytosis is facilitated by cap 8 variants. *S. aureus* evades phagocytosis by expressing a capsular polysaccharide that disguises the bacterial surface and its proteins. The polysaccharide has two major types, CP5 and CP8. The anti-phagocytosis factor is conferred by capsule specific antibodies that function in opsonophagocytosis (Nanra *et al*., 2013). *S. aureus* harbors protein A that inhibits opsonophagocytosis by neutrophils. It functions by hindering antibody binding and allowing *Fcy* binding, a complement binding portion. Moreover, the creation of a fibrinogen shield by *Efb* protects the bacteria by surrounding it and binding C3b complement and fibrinogen (Kuipers *et al*., 2016).

1.7.3 Exoenzymes

S. aureus possesses the ability to survive within host cells by evading detection by phagocytes and thus has been termed a facultative intracellular pathogen (Fraunholz & Sinha, 2012). Exoenzymes are secreted enzymes that function to breakdown host and bacterial molecules for the acquisition of nutrients, bacterial survival and spread (Tam & Torres, 2019). *S. aureus* harbors a variety of these enzymes, namely: aureolysin, hyaluronate, lipases, staphopain, staphylocoagulase, von Willbrand factor-binding protein and V8 proteases.

Aureolysin is a metallo-protease that coordinates zinc (Zn^{2+}) and calcium (Ca^{2+}) ions for activation. It cleaves proteins on the N- terminal side of aliphatic hydrophobic residues promoting detachment and the spread of infection. It functions in catalysis for activation of serine protease zymogen (Laarman *et al*., 2011). Aureolysin promotes the cleavage of proteinase inhibitors, activates prothrombin in human plasma and activates the precursor of glutamyl endopeptidase (V8 protease) (Sabat *et al*., 2000).

Hyaluronate is an enzyme encoded by *hysA* gene. It cleaves the β-1,4 glycosidic bond of hyaluronic acid in *N*-acetyl glucosamine and D-glucuronic acid. Hyaluronic acid is found in the plasma membrane of mammalian

cell likely in skin, skeletal tissue, lungs, heart valves, brain and other tissues. It allows the spread of *S. aureus* by allowing dissemination of pathogen through host tissues during an infection (Ibberson *et al*., 2014).

Lipases are pre-pro-proteins with pre-regions similar to a signal peptide, hydrophilic propeptide and a mature peptide. The lipase pro-peptide is responsible for the secretion, stability and activity of lipase. The lipase pro-peptide acts as an intramolecular chaperone for lipase translocation. *S. aureus* harbors 2 lipase genes namely, geh/gehA and gehB which are responsible for the hydrolysis of short chain glycerides and long chain triglycerides respectively (Cadieux *et al*., 2014). The lipases enhance biofilm formation and cell invasion especially in skin colonization (Nguyen *et al*., 2017).

Staphopain is constituted of two major cysteine proteases, staphopain A (scpA) and staphopain B (sspB). ScpA promotes invasion by breaking down fribronectin binding MSCRAMM that facilitate adherence to components on the extracellular matrix of the host (Ghasemian *et al*., 2015). They are secreted as inactive precursors that require N-terminal propeptide cleavage for activation. ScpA and sspB allow the production and secretion of staphostatins which protect staphylococci from premature activation of staphopains during protein translocation (Nickerson *et al*., 2010). Animals are thought to harbor a third staphopain, C which enhances virulence (Kalinska *et al*., 2012).

S. aureus secretes two major factors for coagulation, i.e., coagulase and von Willebrand factor binding protein (vWbp). The N-terminal of coagulase binds with prosite C- terminal prothrombin which facilitates the cleavage of fibrinogen to fibrin resulting in clotting of blood (McAdow *et al.*, 2012; Peetermans *et al.*, 2015). vWbp is structurally similar to coagulase in the D_1-D_2 domains, however, has a unique binding site. It binds prothrombin and fibrinogen with a lower affinity than coagulase (McAdow *et al*., 2012).

V8 proteases are usually glutamyl endopeptidases that are synthesized as a pre-pro-enzyme which requires proteolytic activation by metallo-protease for activation. It is encoded by an Sspa gene expressed in an

operon with cysteine protease sspB. They function in the proteolytic activation of sspB, possess an autolytic activity and act as plasminogen activators by degrading the N-terminus of the fibrinogen β-chain at the same site as plasmin. V8 proteases have been reported to facilitate biofilm formation (Mootz *et al*., 2013).

1.7.4 Immune evasion

S. aureus has successfully acquired the ability to evade host immune systems by a series of virulence mechanisms that give them the ability to evade host defenses by enzyme and peptide synthesis and secretion. Virulence mechanisms *S. aureus* uses for immune evasion include Adenosine synthase A, chemotaxis inhibitory protein of Staphylococcal binder of immunoglobulin, staphylococcal complement inhibitor, Staphylococcus protein A (Zhang *et al*., 2017).

Adenosine synthase A is a cell wall anchored protein essential for the survival of *S. aureus* in the bloodstream. The ability of *S. aureus* to avoid host innate immune system can be attributed to the production of extracellular adenosine by 5'nucleotide of adenosine synthase A (AdsA). The synthesis of adenosine occurs in two metabolic steps (1) hydrolysis of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to adenosine monophosphate (AMP) by ecto- nucleoside triphosphate diphosphorylase (ecto-NTDPase), and, (2) hydrolysis of AMP to adenosine by 5'nucleotidases. Biosynthesis of adenosine results in the inhibition of T-cell proliferation and cytokine secretion (Thammavongsa *et al*., 2011). It can also confine macrophages from moving onto abscess areas by degrading neutrophil extracellular traps into deoxyadenosine monophosphate (dAMP) which is a purine involved in nucleotide binding then converts into dAdo by AdsA (Zhang *et al*., 2017).

Chemotaxis inhibitory protein of Staphylococcus (CHIPS) is a protein that inhibits neutrophil and monocyte degradation. It confers this evasion by binding onto C5a receptor and formylated peptide receptor resulting in inhibition of phagocyte recruitment. It is encoded by chp located on a mobile genetic element known as β-hemolysin converting bacteriophage (Guerra *et al*., 2017; McGuinness *et al*., 2016).

Staphylococcal binder of immunoglobulin (Sbi) is a protein associated with the bacterial envelope. Immune evasion is conferred by sbi manipulation of the molecular link between the innate and adaptive immune response creating conducible conditions for *S. aureus* infection and progression. It forms a tripartite complex with C3d and factor H which results in the consumption of C3 to inhibit opsonophagocytic clearance of the bacteria. It blocks antigen recognition of B-cells (Kim *et al*., 2012).

Staphylococcal complement inhibitor (SCIN) is an exoprotein that binds and inhibits C3 convertase inhibiting C3b generation. It also confers immune evasion by competing with factor H, which facilitates C3 convertase decay (Guerra *et al*., 2017). C3 deposition triggers C3b deposition, phagocytosis and C5a generation (Jongerius *et al*., 2010). De Jong and associates reported a SCIN variant called equine SCIN harbored by an equine specific prophage. They suggested that equine SCIN is animal adapted and has been isolated from horses, pigs and humans (de Jong *et al*., 2018).

Staphylococcus protein A (Spa) is a surface protein that binds *fcy* of immunoglobulin and forms cross-links of fab and V_{H3} B-cell receptors (IgM). Spa binding onto *fcy* protects *S. aureus* from opsono-phagocytosis. Formation of cross-links of V_{H3} results in apoptotic collapse of activated B-cells (Falugi *et al*., 2013; Kobayashi & DeLeo, 2013).

1.7.5 Iron uptake

Iron acquisition is a crucial step in a host-pathogen interaction (Hammer & Skaar, 2011). *S. aureus* has the ability to obtain iron from host cells even though it is usually bound by iron-binding proteins such as transferrin and lactoferrin. It achieves this by producing siderophores that have an extremely high affinity for iron and through competitive binding it dissociates the host iron-binding proteins. *S. aureus* produces two siderophores; namely, staphyloferrin A and staphyloferrin B. Biosynthesis is regulated by the fur gene. A third siderophore, auroechelin has been suggested but its function is not yet known. An alternate method for iron

uptake occurs through the lyses of erythrocytes that harbor hemoglobin containing heme. *S aureus* secretes hemolysins resulting in the liberation of hemoglobin; however, the host counters this mechanism by releasing haptoglobin, a protein with high hemoglobin binding affinity. Haptoglobin is contained in the serum and is plentiful in inflammation sites. *S. aureus* potentially damages erythrocytes releasing heme. The host cell counters this action by binding heme to hemopexin and translocates it to the liver. *S. aureus* is unable to use hemopexin-heme as an iron source (Hammer & Skaar, 2011; Sheldon & Heinrichs, 2015).

1.7.6 Plasminogen Activator

Staphylokinase (Sak) is a plasminogen activator protein that protects *S. aureus* by binding and inhibiting certain antimicrobial peptides. It forms complexes that cleave plasminogen to active plasmin targeting fibrin clots and allowing *S. aureus* to escape abscess areas and facilitates the spread of infection (Kwiecinski *et al*., 2016). Sak promotes bacterial entry and spread but decreases the severity of bloodstream infection. Subsequently, it has been used in cardiovascular medicine as a fibrinolytic agent (Marijke Peetermans *et al*., 2014).

1.7.7 Secretion System

Type Ⅶ secretion system

S. aureus harbors a broad spectrum of secretion enzymes inclusive of type Ⅶ secretion system, also known as T7SS. It was initially identified in Mycobacteria via the T7ss pathway but has since been reported in *S. aureus*. Like Mycobacteria, *S. aureus* harbors homologous genes encoding T7ss namely: EsxA, EsxB, EsxC and EsxD. EsxC results in persistent infection while EsxA and EsxB facilitates apoptosis and *S. aureus* release from epithelial cells (Dai *et al*., 2017). The T7ss comprises of integral membrane proteins, namely

EssA, EssB, EssC, however, only EssC is considered significant for protein secretion. EssB regulates ess activity at either the post-transcriptional or post-translational level (Warne *et al*., 2016).

1.7.8 Toxins

Toxins are described as poisonous substances that interfere directly with the host and are secreted to increase the pathogenicity of a microorganism (Otto, 2014). *S. aureus* synthesizes and secretes a variety of toxins inclusive of hemolysins, exfoliative toxins, enterotoxins amongst others. *Staphylococcu*s produces and secretes four hemolysins namely α- hemolysin, β- hemolysin, γ- hemolysin and δ- hemolysin (Oliveira *et al.,*2018).

Alpha-hemolysin is a pro-type of pore-forming cytotoxins that is encoded by the *hla* gene and regulated by *agr* and *sae* system. It functions by lysing red blood cells and leukocytes by binding on a disintergrin metalloproteinase (ADAM) 10 receptors which facilitate adhesion. In sepsis, binding on myeloid cells and platelets may be fatal. During pore formation on host cells, calcium influx and potassium efflux disrupts homeostasis resulting in necrotic cell death (Kong *et al*., 2016; Monecke *et a*l., 2013).

β-hemolysin is a sphingomyelinase that facilitates the hydrolysis of sphingomyelin, a plasma membrane lipid. It subsequently destabilizes the lipid bilayer causing irregular membrane fluidity and ceramide rich platforms. It plays a significant role in lysis of monocytes and erythrocytes at low temperatures (Kong *et a*l., 2016; Vandenesch *et al*., 2012).

γ-hemolysin is constituted of three protein components HlgA, HlgB, and HlgC. It requires the assembly of two peptides for function, a class S (HlgA or HlgC) and F (HlgB) component. Independently these components remain inactive. It is a pore-forming cytotoxin that forms HlgAB and HlgCB which share HlgB component (Alessandrini *et al*., 2013).

δ-hemolysin is encoded by the *hld* gene within RNAⅢ that functions in the regulation of transcription of alpha toxins, toxic shock syndrome toxin-1 and as a protein A repressor. It confers virulence by lysing erythrocytes, sub-cellular structures like protoplasts and other membrane-bound organelles (Burnside *et al*., 2011).

1.7.9 Exfoliative toxin

The exfoliative toxin is an epidermolytic toxin usually associated with skin and soft tissue infections like staphylococcal scalded skin syndrome and bullous impetigo. These toxins function by cleaving desmosomal cadherins of the skin layers resulting in severe skin diseases. The disruption of desmosomal attachment subsequently confers disruption of the skins epidermal layer promoting infection progression. These toxins have been suggested to act as super-antigens (Kong *et al*., 2016).

1.7.10 Panton-Valentine Leucocidin (PVL)

PVL is a significant virulence determinant among *S. aureus.* The genetic receptors for PVL are C5a. It consists of two exoproteins encoded by phage-encoded genes, viz., LukS-PV and LukF-PV (Bhatta *et al*., 2016). It is considered a genetic marker for CA-MRSA due to its prominence on this MRSA epidemiological class. It is a pore-forming toxin that lyses neutrophils, necrosis and apoptosis (Dahms *et al*., 2014). It has been associated with necrotizing pneumonia infections (Liu *et al*., 2011).

1.7.11 Staphylococcal enterotoxin

Staphylococcal enterotoxins (SE) are usually located on mobile genetic elements such as plasmids and bacteriophages. There are over 24 SEs differentiated based on antigenic heterogeneity(Fisher *et a*l., 2018) however; the most clinically significant are SEA and SEB. SEA is usually associated with food- poisoning. SEB, although a potential cause of food poisoning, has potential use as an inhaled bioweapon. SEF plays a

role in toxic shock syndrome. SEA and SEB are believed to carry a cavity that has been implicated in T-cell binding subsequently triggering T-cell activation, proliferation and eventually cell death due to apoptosis and toxic shock syndrome (Pinchuk *et al*., 2010).

Toxic shock syndrome toxin-1 (TSST-1) is a toxin that is encoded by *tst* on the staphylococcal pathogenicity island 1 (SaPI1). It stimulates the release of chemokines which trigger activation of immune cells, enhancing inflammation, the release of cytokines and T-cells subsequently allowing the interaction of T-cells with toxin resulting in toxic shock syndrome (Hema *et al*., 2018; Kong *et al*., 2016).

1.8 Prevalence of LA-MRSA in Pigs

The overuse and inappropriate use of antibiotics by humans for animal farming facilitates the emergence and transmission of antibiotic resistance bacteria, including multidrug-resistant organisms (Pantosti, 2012). MRSA is known to colonize various sites in pigs including the nares, pharynx, mouth, skin and rectum. The most common strain CC398 has been reported to infect both humans and animals and recently found in dust particles, especially where animals are in confined systems. Pigs have been reported to be important reservoirs of antibiotic resistant *S. aureus,* and their evolution of virulence factors poses a threat not only to healthcare but in veterinary medicine, food production and public health. Several studies have reported the occurrence of MRSA in livestock, slaughterhouses and food (meat) products (Beneke *et al*., 2011; Lassok et al., 2013; Sun et al., 2017).

MRSA lineages are not restricted and can cause infections in humans and various animal species. A study was conducted by Cuny *et al*. (2009) to evaluate the potential transmission of MRSA from pigs to humans with or without occupational exposure. In this study, 47 pig farms were selected where nares of 113 farmworkers, 116 family members, 18 veterinarians with 44 of their family members and 462 school pupils from a dense pig farming area were swabbed. This study revealed that MRSA colonization was 86% and 4%

in the farmworkers and their family members, respectively. Veterinarians displayed 45% MRSA colonization with 9% of their family members similarly colonized. Only three school pupils presented with MRSA and they lived on pig farms. It was then concluded that MRSA dissemination was higher in farmworkers and less onpeople without occupational exposure/ family (Cuny *et al.*, 2010). In a study conducted in Belgium in 2009, 50 swine farms were selected to investigate MRSA carriage in pigs and farm workers. Swabs were taken from the nares, face and hands. Results showed that 48 persons (37.8%) out of 49 swine farmers carried the MRSAST398 clone and 1 (0.8%) had a concurrent skin infection. They concluded that risk factors for MRSA carriage included regular contact with pigs, companion animals. The use of protective clothing did not seem to reduce colonization and infection rate (Denis *et al.*, 2009). A study by Angen *et al.* (2017) elucidated the frequencyand duration of MRSA carriage in humans over a short-term period upon exposure to swine farms. In this study, 34 participants were exposed for 1hour on an MRSA positive swine farm for four sessions. Nasal swabs, throat swabs and air samples were collected and assessed at different time intervals based on the relevance of covariates. Ninety-four percent of volunteers acquired MRSA. However, two hours after non- exposure, MRSA nasal count had decreased by 95% of the samples, and after 48 hours, they all tested negative to MRSA. This led to a conclusion that increased human carriage with pig contact depends on the increased concentration of airborne MRSA and not directly on physical contact with pigs (Angen *et al*., 2017).A study was conducted in Sri Lanka (2019) on 100 pig farms where MRSA tested in 493 pigs, 228 humans and 119 dust samples. MRSA prevalence was reported as 1.2%, 2.2%, and 0.8% from pigs, humans and dust samples respectively. Low prevalence of MRSA (10%) was observed, and it was concluded that it wasdue to farm management, open-air and low dust (Kalupahana *et al.*, 2019).

The prevalence of LA-MRSA was influenced by farm size, farming systems, usage of disinfectants and infeed zinc (Cuny *et al*., 2015). It has been suggested that the use of infeed Zinc as an antidiarrheal agent has the ability to influence the emergence and spread of MRSA in swine through increased selection pressure to maintain SCC*mec* elements (Hau *et al*., 2017). In the Netherlands, a longitudinal study on MRSA

dissemination within pig herds was investigated. A total of 63 pigs and their piglets were sampled at various stages of production. MRSA prevalence in piglets remained >60% throughout the study and it increased from 33.3% before farrowing to 58,8% after farrowing and to 77,3% before weaning. The recurrent MRSA through the life cycle may be attributed to contamination and transmission influenced by the use of selective antimicrobials, and by the age of the pigs (Broens *et al*., 2011).

A comparative review on MRSA in Africa reported that LA-MRSA due to occupational exposure in South Africa is estimated at 12.5% and 1.3% in Senegal. The ST88 type III/IV was the most common strain within the continent similar to East Asia. Isolates were predominantly resistant to penicillin, tetracycline and co-trimoxazole. Additionally, a high number of methicillin-sensitive *S. aureus* (MSSA) isolates harbored PVL. It was concluded that *S. aureus* active surveillance programs were imperative (Schaumburg *et al*., 2014). In Germany, a study was conducted to investigate the occurrence of MRSA in farm workers and livestock. In this study, 78 workers were sampled from pig farms, cattle farms and poultry farms. Samples on each farm were pooled based on source as either from animal, dust, or human. MRSA was detected only in pig farms. Six of 17 pooled dust samples were also MRSA positive with resistance prevalent to tetracycline and isolates belonging to the CC398 strains. This study emphasized the importance of monitoring MRSA on pig farm workers in rural settings (Dahms *et al*., 2014).

S. aureus has also been shown to survive cleaning on steel areas, hence supporting survival in abattoir equipment (de Boer *et al*., 2009; Pu *et al*., 2009). In a study in Georgia (2013) the prevalence of MRSA from retail meat and humans was investigated. One hundred retail pork and retail beef samples were collected from 14 retail stores; human isolates were collected from a local hospital for comparison. Results showed that 45% of pork and 63% of beef was contaminated with *S. aureus*, and 50% of human isolates were MRSA positive. Three retail beef isolates had a similar PFGE pattern with human MRSA isolates suggesting contamination during processing. It was concluded that contamination during processing of meat may present

a risk of MRSA transmission to consumers or anyone who handles raw meat (Jackson et al., 2013). MRSA has been regarded as a zoonotic agent with pigs as one of the leading reservoirs for this pathogen. This has suggested the existence of the bacterium in food processing facilities, including workers in those production plants. However, the source of carriage remains unclear. The occurrence of MRSA in the food production chain has been documented in countries like North America, Belgium, and some European countries (Molla *et al*., 2012). A study conducted in 2015 by Ivbule *et al*. to determine the presence of MRSAin a slaughterhouse environment from workers, pigs and carcasses concluded that the higher the capacity ofslaughter, the higher the contamination of pig carcasses and related pork products (Ivbule *et al*., 2017). In this study, a total of 100 pigs, 105 carcasses, 19 workers, 24 environmental samples were screened for MRSA. Overall, 78 isolates were MRSA positive. Prevalence of MRSA in slaughtered pigs ranged between 8-88,6% depending on slaughterhouse and slaughter capacity. Of the workers, 21,1% were colonized, and 6,7% carcasses were contaminated (Ivbule et al., 2017). The question of whether contaminated meat can bea source of human infection remains debatable (Molla *et al*., 2012).

1.9 Antibiotic use and resistance in pigs

The emergence of resistant bacteria and the discovery of gene transfer in animals play a significant role in the global challenge of antimicrobial resistance. According to the World Health Organization (WHO), the World organization for Animal Health (OIE)and the Food and Agriculture Organization (FAO) this is a cause for concern, and they have thus started implementing action plans to reduce antimicrobial use in animals (Maron *et a*l., 2013; WHO., 2015). In pigs, antibiotics are used as growth promoters, for prophylaxis, metaphylaxis or disease treatments. Growth promotion involves the use of antibiotics in low concentrations by in-feed medication. Antibiotic growth promoters create the ideal environment for a selection of antibiotic-resistant bacteria and the proliferation ofresistance genes between enteric bacteria in the pig's intestinal tract.

Prophylactic (disease prevention) and metaphylactic (disease control to prevent spread) pre-emptive use of antibiotics also involves in-feed addition of antibiotics. However, the concentration of antibiotics usually is much higher and typically equivalent to therapeutic concentrations. This again favors the selection of antibiotic-resistant bacteria as these medicatedfeeds can be used over an extended period (Barton, 2014). The overuse of antibiotic has made pigs a reservoir for MRSA and has facilitated the emergence of multiple drug-resistant strains, thus making their eradication difficult. Resistance patterns may differ based on geographical location and types of antibiotics used within the farm for promotional growth and/or disease treatment.

A study was conducted in the Eastern Cape (2015-2016) to determine the resistance profiles of *S. aureus* isolated from meat carcasses (pork, beef, sheep) and bovine milk in abattoirs and dairy milk farms. Samples were obtained from meat carcasses and milk samples from a total of 1000 meat swabs and 200 milk samples. The carcass samples were collected from cows, pigs and sheep. Overall, 14% of pork samples were contaminated with *S. aureus* with resistance ranging from 20 to 100% to oxacillin, penicillin-G and tetracycline. It was concluded that antibiotic resistance was high within this region especially to antibiotics that are widely used in both veterinary medicines for animal husbandry and human medicine therefore prudent use was recommended (Pekana & Green, 2018).

Another study in India in 2018 was conducted to evaluate the prevalence, antimicrobial susceptibility pattern and virulence genes of *S. aureus* in pork on retail outlets. In this study, 120 retail pork samples were screened for *S. aureus* contamination. Prevalence of *S. aureus* was reported as 67% in retail pork meat. All isolates were 100% resistant to both ampicillin and tetracycline. Variable susceptibility was observed to ciprofloxacin, chloramphenicol, vancomycin and gentamycin. Prevalence of enterotoxigenic genes was 82.6%. It was concluded that the prevalence of MDR *S. aureus* and its virulence was a clear indication of *S. aureus* as a potential cause of food poisoning to consumers (Savariraj *et al*., 2019). A study conducted in Western

Switzerland on the assessment of antibiotic-resistant *S. aureus* acquired by farmers from pigs reported that isolates were commonly resistant to tetracyclines and macrolides. In this study, 48 isolates from farmers/veterinarians and 130 isolates from pigs were investigated for possible animal-human transmission. Resistance prevalence to tetracycline and macrolides were comparative between isolates from farmers and/veterinarians (52% and 21%) and pigs with resistance levels of 39% and 23% respectively. The study concluded an evidently that transmission of MDR isolates were possible (Oppliger *et a*l., 2012).

Antibiotic resistance is a global threat and a major concern. However, MRSA carriage from farm to fork remains poorly documented in several countries, including Africa. In South Africa, studies documenting the incidence, prevalence and possible transmission of MRSA from farm to fork at a molecular level are scarce/absent. We thus conducted a study on determining the molecular epidemiology of antibiotic-resistant *Staphylococcus aureus* in an intensive pig production system in KwaZulu-Natal, South Africa.

1.10 Aim

To characterize the molecular epidemiology of antibiotic-resistant *Staphylococcus aureus* from farm-to-fork in an intensive pig production system in uMgungundlovu district KwaZulu Natal, South Africa.

1.11 Objectives

i. To identify and determine the prevalence of *Staphylococcus aureus* from different stages of the pigproduction chain, viz.:
    - on the farm from faeces, slurry water and litter samples of pigs and the hands and nares of farm workers,
    - holding areas from trucks and/or crate swabs,
    - post-slaughter at the abattoir from caeca, carcass swabs and carcass rinsate,

- retail meat

using selective media, biochemical tests and conventional polymerase chain reaction (PCR) methods of the *nucA* gene.

ii. To delineate antibiotic susceptibility profiles of isolates to the following antibiotics by Kirby Bauer disc diffusion method according to CLSI/EUCAST guidelines to ampicillin, penicillin-G, ciprofloxacin, moxifloxacin, levofloxacin, linezolid, cefoxitin, amikacin, gentamicin, tigecycline, tetracycline, doxycycline, erythromycin, clindamycin, rifampicin, sulfamethoxazole-trimethoprim, nitrofurantoin, chloramphenicol, teicoplanin, vancomycin.

iii. To ascertain the prevalence of methicillin-resistant Staphylococcus aureus (MRSA) using disc diffusion method for the detection of cefoxitin resistance and polymerase chain reaction (PCR) for the detection of *mec*A gene.

iv. To genotypically identify the presence of selected resistance genes based on the antibiotic susceptibility profile by PCR, viz: *tetM*, *tetK*, *blaZ*, *ermC, msrA*, *aac*(6')-aph(2''), *vanA, vanB*, and *mecA*.

v. Identify the presence of putative virulence genes, viz: *hla, hld, eta, etb, tst, luk/PVL sea, seb and sed.*

vi. To determine the molecular relatedness of selected MRSA isolates by strain typing using repetitive element sequence-based PCR (REP PCR).

1.12 Summary of Methodology

Ethical approval

Ethical approval for the study was obtained from the Biomedical Research Ethics Committee (Ref. BCA444/16) (Appendix I) and Animal Research Ethics Committee (Ref. AREC/073/016PD) (Appendix II) of the University of KwaZulu-Natal (UKZN). Further approval was obtained from the Department of Agriculture, Forestry and Fisheries (Ref: 12/11/1/5 (879)) (Appendix III). Human samples were obtained from participants 18 years and older upon voluntarily informed consent (Appendix IV). All the information obtained from the farm (noted as Farm A) was kept confidential as part of the memorandum of understanding (MOU) between the Antimicrobial Research Unit and the farm.

General methodology

Samples were obtained from different stages of an intensive pig production chain (feaces, litter, slurry water, transport, retail meat) and evaluated for the presence of *Staphylococcus aureus*. Selective media and biochemical tests methods were used for isolate identification and confirmation was done by using PCR and targeting the *nuc* gene. MRSA was determined by targeting the *mec*A gene using PCR and gel electrophoresis to assess amplicon sizes and banding patterns. Antibiotic susceptibility was determined by the Kirby-Bauer disk diffusion method and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints. The Clinical and Laboratory Standards Institute (CLSI) guidelines were used for those antibiotic breakpoints that were absent from the EUCAST 2017 guideline. Antibiotic resistance and virulence genes were investigated using PCR and gel electrophoresis. Genetic relatedness between isolates throughout the production chain was determined by using REP-PCR.

1.13 Dissertation Structure

This study elucidates the molecular epidemiology of antibiotic-resistant *Staphylococcus aureus* in an intensive pig production system in uMgungundlovu district KwaZulu-Natal, South Africa.

Chapter 1 provides the background of this study as well as a literature review.

Chapter 2 of this dissertation provides a concise methodology, results and discussion in a manuscript format intended for submission to the Microorganism (MDPI).

Chapter 3 is the conclusion, limitation and recommendations of this study.

1.14 References

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CHAPTER TWO - MANUSCRIPT

MOLECULAR CHARACTERIZATION OF ANTIBIOTIC-RESISTANT *STAPHYLOCOCCUS AUREUS* FROM AN INTENSIVE PIG PRODUCTION SYSTEM IN KWAZULU-NATAL, SOUTH AFRICA

AUTHOR CONTRIBUTIONS

Ncomeke Sineke, as the principal investigator, co-conceptualized the study, undertook the laboratory work and data collection, analysed the data and drafted the manuscript.

Jonathan Asante, aided in the laboratory work, vetted the results and reviewed of the manuscript.

Daniel G. Amoako, as principal supervisor, co-conceptualized the study, guided the literature review, supervised the laboratory work, facilitated data collection and analysis, vetted the results and undertook critical revision of the manuscript.

Akebe L. K. Abia, as co-supervisor, co-conceptualized the study, supervised the laboratory work, facilitated data collection and analysis, vetted the results and undertook critical revision of the manuscript.

Keith Perrett, aided in the sampling, facilitated data collection, vetted the results and undertook critical revision of the manuscript.

Linda A. Bester, as the co-supervisor, co-conceptualized the study, supervised the laboratory work and undertook a critical revision of the manuscript.

Sabiha Y. Essack, as co-supervisor, co-conceptualized the study, guided the literature review and ethical clearance application, facilitated data collection and analysis and undertook critical revision of the manuscript.

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Article

Staphylococcus aureus in Intensive Pig Production in South Africa: Antibiotic Resistance, Virulence Determinants, and Clonality

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Abstract: Although *Staphylococcus aureus* is a major threat to the veterinary, agricultural, and public health sectors because of its zoonotic potential, studies on its molecular characterisation in intensive animal production are rare. We phenotypically and genotypically characterised antibiotic-resistant *S. aureus* in intensive pig production in South Africa, using the farm-to-fork approach. Samples ($n = 461$) were collected from the farm, transport vehicles, and the abattoir using the World Health Organisation on Integrated Surveillance of Antimicrobial Resistance (WHO-AGISAR) sampling protocol. Bacteria were isolated using selective media and identified using biochemical tests and polymerase chain reaction (PCR). Phenotypic resistance was determined using the disk diffusion method. Selected resistance and virulence genes were investigated using PCR. Clonality among the isolates was determined using the repetitive element sequence-PCR. In all, 333 presumptive staphylococcal isolates were obtained, with 141/333 (42.3%) identified as staphylococci biochemically. Ninety-seven (97; 68.8%) were confirmed as *S. aureus* using PCR, 52.6% of which were identified as methicillin-resistant *S. aureus* (MRSA) through the *mec*A gene. All the 97 *S. aureus* isolates (100%) were resistant to at least one of the antibiotics tested, with the highest resistance observed against erythromycin and clindamycin (84.50% each), and the lowest observed against amikacin (2.10%); 82.47% (80/97) were multidrug-resistant with an average multiple antibiotic resistance index of 0.50. Most of the phenotypically resistant isolates carried at least one of the corresponding resistance genes tested, *erm*C being the most detected. *hla* was the most detected virulence gene (38.14%) and *etb* was the least (1.03%). Genetic fingerprinting revealed diverse MRSA isolates along the farm-to-fork continuum, the major REP types consisting of isolates from different sources suggesting a potential transmission along the continuum. Resistance to antibiotics used as growth promoters was evidenced by the high prevalence of MDR isolates with elevated multiple antibiotic resistance indices >0.2, specifically at the farm, indicating exposure to high antibiotic use environments, necessitating antibiotic stewardship and proper infection control measures in pig husbandry and intensive pig production.

Keywords: *Staphylococcus aureus*; antibiotic resistance; foodborne pathogens; multidrug resistance; MRSA; pig production chain; South Africa; genetic diversity; virulence determinants; molecular epidemiology

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1. Introduction

According to recent World Health Organisation estimates, food contamination affects over 600 million people worldwide, with over 420,000 dying every year [1]. This condition is further exacerbated by the presence of antibiotic-resistant bacteria in contaminated foods. Today, antibiotic resistance is a global public health concern [2,3] that poses a severe threat to human and animal health [4,5]. The escalating antibiotic resistance rate may be attributed to the excessive and inappropriate antibiotics use in humans and animals, including in animal husbandry [2,4].

Staphylococcus aureus is a bacterium that exists as either a commensal or pathogen in humans and animals [6]. Its success as a pathogen may be attributed to the production of many virulence factors, including enterotoxins, leucocidins, exfoliative toxins, haemolysins, and immune-modulatory factors [7–9] that promote colonisation, tissue damage, and infection while facilitating the evasion of host defence mechanisms. Moreover, its ability to resist a wide range of antibiotics has led to limited therapeutic options for treating its infections [10]. *S. aureus* has shown resistance to most β-lactam antibiotics, linezolid, daptomycin, and vancomycin, which are the last-resort antibiotics for Gram-positive bacteria [11]. Its resistance mechanisms encompass the enzymatic inactivation of antibiotics, alteration of the target penicillin-binding proteins that decrease the antibiotic's binding affinity, and efflux pumps that remove antibiotics from the bacteria's cytoplasm. Resistance is acquired through mutations and horizontal gene transfer of resistance genes on various mobile genetic elements such as plasmids, bacteriophages, and transposons [10,12]. Methicillin resistance in *S. aureus* is mediated by the *mec*A gene that is harboured by a mobile genetic element, the staphylococcal cassette chromosome mec (*SCCmec*) [13].

Epidemiologically, methicillin-resistant *S. aureus* (MRSA) is divided into three classes, hospital-acquired MRSA (HA-MRSA), community-associated MRSA (CA-MRSA), and livestock-associated MRSA (LA-MRSA) [14]. Pigs were identified as important reservoirs for LA-MRSA as early as 2004, and LA-MRSA lineages have been currently reported in humans, suggesting a possible transmission from animals to humans, blurring the epidemiology of MRSA [15].

Antibiotic overuse is the primary driving force of resistance in pig production. Antibiotics are used as growth promoters for metaphylaxis and prophylaxis to improve health, produce high-quality products, and increase overall production yield [16]. In 2016, it was estimated that South Africa consumes 200,000 tons of pork, which is the second most consumed source of meat after chicken [17]. Due to high demand, different antibiotics are extensively used during food animal production [16,18]. The high demand for pork also imposes the need to adopt intensive production approaches, requiring the farming of many animals within limited and confined spaces. However, this approach has the downside of promoting stress and increasing disease transmission within the animal farm [19], thus requiring extensive antibiotics use to treat sick animals. The use of these antibiotics, including the critically important and clinically relevant ones in food animals, can create a selective environment for the emergence of multidrug-resistant pathogenic strains.

Despite the safety issues associated with drug-resistant bacterial contaminants in food, there are limited studies on antibiotic-resistant *S. aureus* in intensive pig production or the possibility of transmission to humans in South Africa. Furthermore, no study has investigated this along the pig farm-to-fork continuum in Africa. Therefore, we elucidated the molecular epidemiology of antibiotic-resistant *S. aureus* in an intensive pig production chain in uMgungundlovu District KwaZulu-Natal, South Africa, using the farm-to-fork approach. This study would provide the foundations for implementing measures to curb antibiotic use in food animals and identify areas along the continuum that may be prioritised in such interventions.

2. Results

2.1. *Staphylococcus Aureus* Detection Rate in the Pig Production Chain

A total of 333 presumptive staphylococcal isolates were obtained throughout the pig production chain based on culture characteristics. However, the biochemical analysis yielded 141 (42.3%) *Staphylococcus* isolates, of which 97 (68.8%) were confirmed as *S. aureus* through PCR. The least number of *S. aureus* isolates was obtained on Week 4 from litter and faecal samples. Most *S. aureus* isolates were obtained on Week 7, while no isolates were obtained on Week 9. In addition, no isolates were recovered from caecal samples (Table 1).

Table 1. Distribution of presumptive staphylococcal isolates obtained throughout the study.

Week	Production Stage	Source	No. Collected
Weeks 1–17	Farm (n = 293)	Faeces	117
		Slurry	119
		Human (Nasal)	57
Week 18	Transport (n = 4)	Before Loading	4
		After Loading	4
Week 18	Abattoir (n = 32)	Carcass Rinsate	12
		Caecal contents	0
		Retail Meat (Whole Carcass)	4
		Retail Meat (Head)	8
		Retail Meat (Thigh)	8
	Total		333

Furthermore, 51 (52.6%) of the 97 *S. aureus* isolates were positive for the *mec*A gene, confirming them as MSRA.

2.2. *Antibiotic Susceptibility Profiles*

The antimicrobial susceptibility was only performed on PCR-confirmed *S. aureus* isolates. These isolates displayed varying percentages of resistance to the various antibiotics tested (Figure 1). The highest resistance was observed against clindamycin (84.50%) and erythromycin (84.50%), while the lowest resistance was against amikacin (2.10%). Overall, all the isolates (100%) were resistant to at least one of the 20 antibiotics tested. There was substantial resistance to the glycopeptide antibiotics, vancomycin (69.10%), and teicoplanin (51.50%).

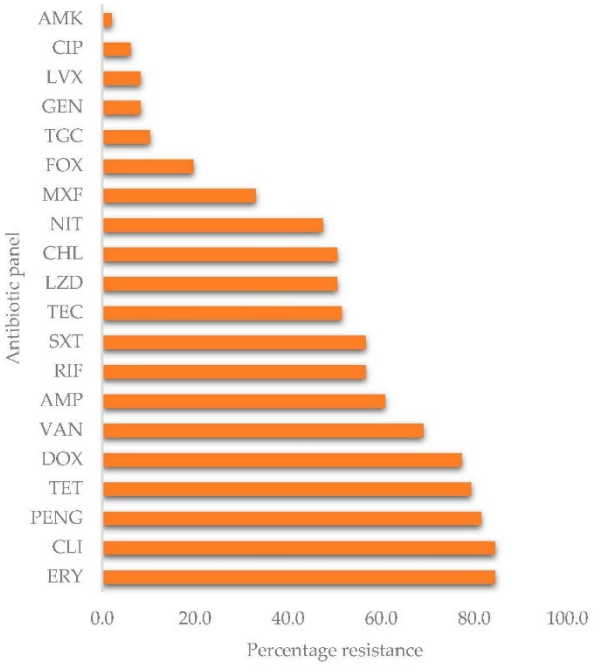

Figure 1. Overall percentage resistance of *S. aureus*. AMP = ampicillin, PENG = penicillin-G, CIP = ciprofloxacin, MXF = moxifloxacin, LVX = levofloxacin, LZD = linezolid, FOX = cefoxitin, AMK = amikacin, GEN = gentamicin, TGC = tigecycline, TET = tetracycline, DOX = doxycycline, ERY = erythromycin, CLI = clindamycin, RIF = rifampicin, SXT = sulfamethoxazole-trimethoprim, NIT = nitrofurantoin, CHL = chloramphenicol, TEC = teicoplanin, VAN = vancomycin.

Furthermore, when stratified by sampling source, isolates obtained from transport samples were 100% resistant to erythromycin, clindamycin, and tetracycline, while faecal samples showed 100% resistance to penicillin-G (Figure 2).

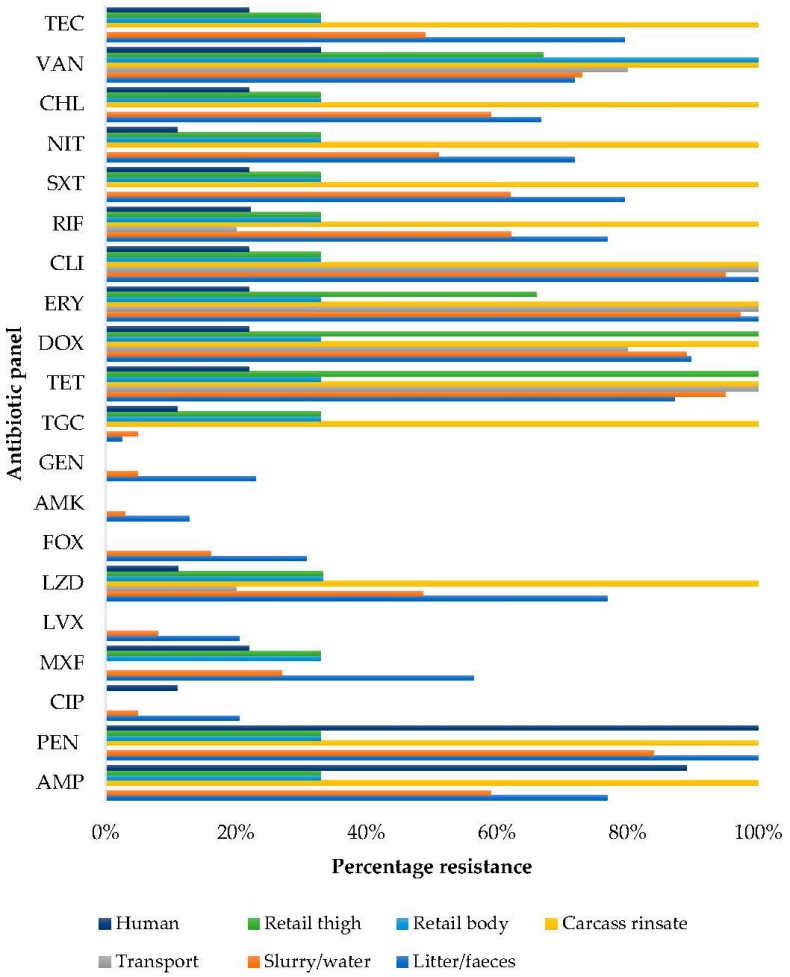

Figure 2. Percentage resistance of *S. aureus* isolates stratified by source. AMP = ampicillin, PENG = penicillin-G, CIP = ciprofloxacin, MXF = moxifloxacin, LVX = levofloxacin, LZD = linezolid, FOX = cefoxitin, AMK = amikacin, GEN = gentamicin, TGC = tigecycline, TET = tetracycline, DOX = doxycycline, ERY = erythromycin, CLI = clindamycin, RIF = rifampicin, SXT = sulfamethoxazole-trimethoprim, NIT = nitrofurantoin, CHL = chloramphenicol, TEC: teicoplanin, VAN = vancomycin.

2.3. *Multidrug Resistance and Risk Assessment Parameters*

Out of 97 *S. aureus* isolates, multidrug resistance (MDR) was recorded in 82.47% (80/97) of the isolates, which were mostly from the farm (faeces, slurry, and litter) and the least being from humans (Figure 3).

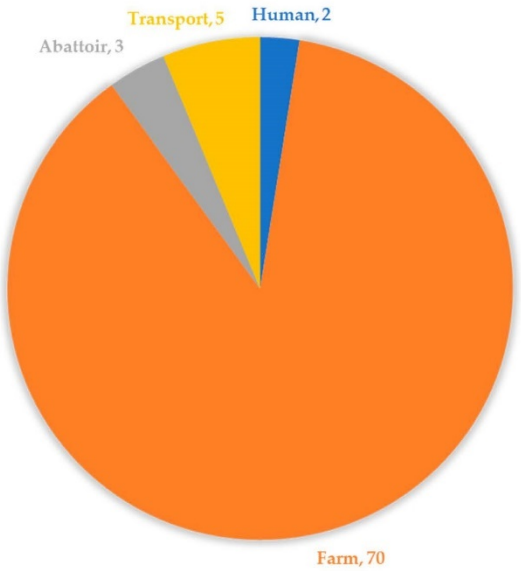

Figure 3. The overall distribution of multidrug-resistant *S. aureus* isolates along the pig production chain.

The isolates displayed varying resistance patterns that were grouped into 56 different antibiograms, LZD-RIF-ERY-CLI-AMP-PEN-SXT-MXF-TET-DOX-NIT-CHL-VAN-TEC being the most common pattern (Table S1). Most MDR isolates (66; 82.50%) were resistant to six or more tested antibiotics (Table S1). No isolate was pan-drug resistant (i.e., showing resistance to all antibiotics tested in this study).

The overall MARI for all the isolates in the current study ranged between 0.02 and 0.95. However, most isolates recorded an MAR (multiple antibiotic resistance) index of 0.80 throughout the production chain. The highest MARI (0.95) was recorded on the farm; this isolate was resistant to 19 of the 20 antibiotics tested (Table S1). On the farm, the MARI ranged between 0.20 and 0.95 (mean = 0.50). The transport system ranged between 0.20 and 0.30 (mean = 0.25), while at the abattoir, isolates recorded MARIs of 0.75 and 0.80. The two MDR human isolates had MARIs of 0.50 (hands) and 0.85 (nasal).

2.4. Detection of Antibiotic Resistance and Virulence Genes

The tested resistance genes were detected at varying percentages in the isolates that showed phenotypic resistance to the corresponding antibiotics or antibiotic classes (Table 2). For example, the *erm*C gene was detected in 97.56% (80/82) of the isolates that were phenotypically resistant to erythromycin. The vancomycin resistance genes, *van*A and *van*B, were not detected, although phenotypic resistance was observed. There was no correlation between the antimicrobial resistance genes (ARGs) from the different sampling points and sources.

Table 2. Prevalence of antibiotic resistance and virulence genes in *S. aureus* isolates.

Resistance Gene *	Prevalence	Virulence Genes **	Prevalence
*tet*M	27 (35.05%)	*hla*	37 (38.14%)
*tet*K	56 (72.73%)	*hld*	21 (21.65%)
blaZ	71 (88.75%)	*sea*	0 (0.00%)
*mec*A	51 (63.75%)	*seb*	3 (3.09%)
*erm*C	80 (97.56%)	*sed*	2 (2.06%)
*msr*A	15 (18.29%)	*eta*	0 (0.00%)
aac (6′)-aph (2″)	5 (62.50%)	*etb*	1 (1.03%)
*van*A	0 (0.00%)	*lukS/F-PVL*	29 (29.90%)
*van*B	0 (0.00%)	*tst*	11 (11.34%)

* The following genes confer resistance to the corresponding antibiotics and were tested in isolates that displayed phenotypic resistance to these antibiotics: *tet*M and *tet*K (tetracycline; $n = 77$), *blaZ* (penicillins; $n = 80$) *ermC*, *msr*A (erythromycin; $n = 82$), *aac*(6′)-*aph*(2″) (gentamicin; $n = 8$), *van*A and *van*B (vancomycin; $n = 67$), and *mec*A (methicillin/cefoxitin/β-lactams; $n = 80$). ** The following virulence genes encode the corresponding protein: *hla and hld* (α and δ hemolysins), *eta and etb* (exfoliative toxins), *sea, seb and sed* (staphylococcal enterotoxins), *lukS/F-PVL* (leucocidin), *tst* (toxic-shock syndrome/exotoxin).

The distribution of virulence genes was *hla* (39%), *hld* (23%), *seb* (3%), *sed* (2%), *etb* (1%), *LukS/F-PV* (30%), and *tst* (11%). The most common virulence factor was the α-hemolysin cytotoxin encoded by the *hla* gene (Table 2). Low prevalence was recorded for exfoliating toxins encoded by *etb* and staphylococcal enterotoxin genes *seb* and *sed*. Virulence genes *eta* and sea were not detected.

2.5. Repetitive Element Palindromic PCR (REP-PCR)

Twenty-eight (28) clusters were identified from A-AC (Figure 4). Amongst these, 38% constituted five major rep types, namely P ($n = 5$), J ($n = 4$), F ($n = 3$), I ($n = 3$), and X ($n = 3$). The largest clonal cluster was P ($n = 5$), with isolates originating from the farm (faeces, slurry, and human samples). J ($n = 4$) contained Week 4 isolates from faeces and litter. Repetitive element palindromic (REP)-F ($n = 3$) consisted of Week 5 faecal and slurry isolates. REP-type X ($n = 3$) consisted of Week 7 isolates obtained from human swabs, and REP-type I ($n = 3$) contained Week 3 human and slurry isolates and a Week 4 litter isolate. REP-I was the only major REP-type with isolates originating from different but consecutive sampling weeks.

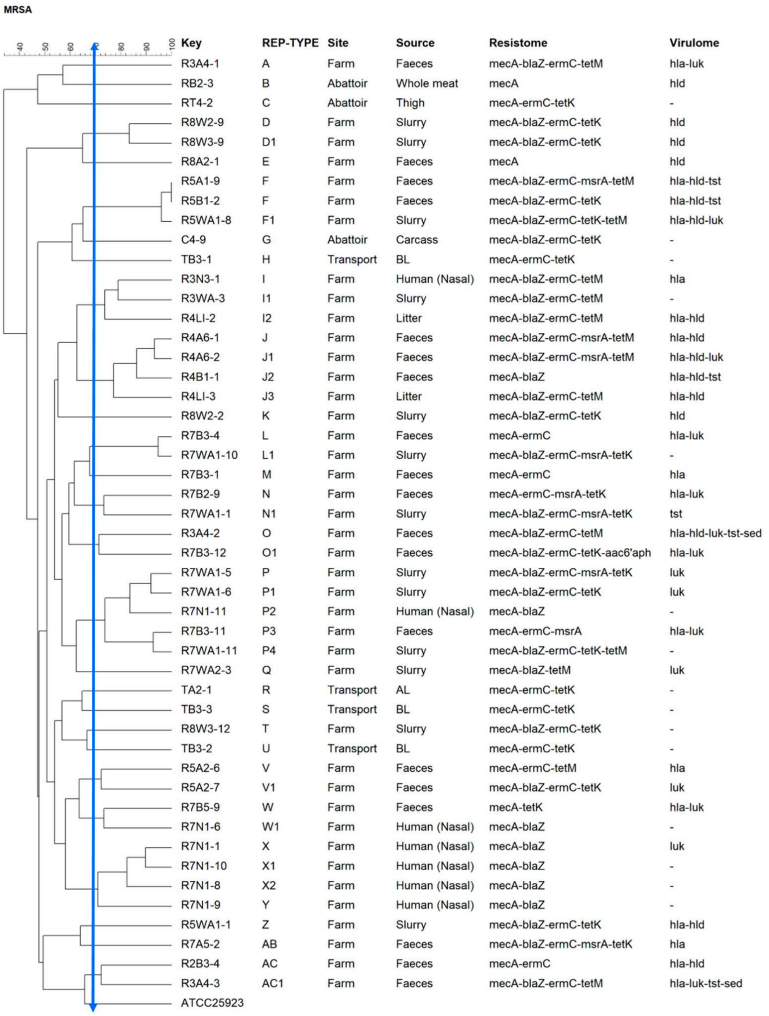

Figure 4. Dendrogram showing repetitive element palindromic (REP)-type groups of *S. aureus* isolates, based on the 70% similarity index, recovered along the farm-to-fork continuum. The solid blue line indicates the REP-type cut-off.

3. Discussion

3.1. Prevalence of S. aureus in the Pig Production System

Many different bacteria have been implicated in foodborne disease outbreaks around the world. In the current study, staphylococcal species were isolated from different sources throughout the production chain. Overall, 69% (n = 97) of the total number of isolates were identified as *S. aureus*, of which 52.6% were confirmed as MRSA using molecular techniques. This prevalence was considerably higher than the 12% reported in another South African study conducted in 2017 assessing MRSA prevalence in commercial pig herds in the Western Cape, KwaZulu Natal, and Gauteng [20]. A lower prevalence was also reported in another South Africa study assessing the formal (30%) and informal (50%) meat sectors with isolates obtained from meats samples in abattoirs and slaughtering points [21].

These differences in prevalence may reflect the sampling framework used. Unlike the current study, most studies have been performed at single points along the continuum [22]. The differences in percentages could also be due to the methods used. Unlike our study that used PCR to detect the mecA gene, the previous studies only relied on culture using selective media and biochemical tests to confirm MRSA in their isolates. While being valuable in their respects, such narrow sampling could give an incomplete picture of the epidemiology of *S. aureus* in the food production chain. Thus, the farm-to-fork sampling approach used in the current study, as recommended by the WHO, provides a better understanding of the microbial pathogens' distribution in intensive production systems. This could also highlight the potential transmission along the continuum and identify hotspots needing prompt attention. The MRSA prevalence rate may also differ according to geographic location and herd size [23]. Globally, high prevalence rates (>50%) have been reported in the USA, Germany, Italy, and Sri Lanka [6,24–26]. Nevertheless, the scarcity of information in South Africa, as in many developing countries, on the current situation or possible dissemination of these bacterial pathogens through the food production chain remains a concern [27].

3.2. Antimicrobial Resistance Profile of S. aureus Isolated from the Pig Production Chain

Pig production is one of the leading sources of meat protein in South Africa, after poultry. However, the intensive conditions under which pigs are housed during production are a risk factor for spreading disease, resulting in high antibiotic use to control and treat infections [28]. The routine use of antibiotics as growth promoters for prophylaxis, metaphylaxis, and treatment exerts selective pressure for developing and escalating antibiotic resistance [26]. This creates large reservoirs of antibiotic-resistant bacteria, including MRSA, colonising the nares, skin, and rectum of the pigs and occupationally exposed workers. For example, significantly high resistance percentages, including high MDR rates, have been reported to some antibiotics frequently used in veterinary medicine for animal husbandry, such as tetracycline, penicillin, erythromycin, and sulphonamides [29]. The transmission of resistant bacteria between animals and humans has also been reported in many studies [30–32]. For example, a report on antimicrobial use and resistance in Africa indicated a 100% prevalence of MDR *E. coli* in South Africa with isolates highly resistant to sulphonamides, tetracycline, and penicillin [33]. The high percentage of resistance to these antibiotics may be because they are widely used, favoured by their low cost and availability [32]. In veterinary medicine, penicillins are commonly used for prophylaxis and treatment of urinary tract infection and have been frequently detected in foodborne *S. aureus* [34,35].

According to a document published by the WHO on critically important antibiotics for human medicine in 2018 [36], some classes of antibiotics used in food animals are also used to treat human infections. Hence, their indiscriminate use in animal production may cause resistance, compromising their efficacy in human infections [32]. Although the European Union has banned some of these antibiotics as growth promoters, they are still used in South Africa [29,37]. The >50% resistance observed for erythromycin, clindamycin, penicillin-G, tetracycline, and doxycycline may be attributed to the use of these antibiotics to promote growth, prevent, and treat infections. Similar resistance patterns reported in China and Portugal in pig production were correlated with overuse [38,39].

The resistance profiles observed in the current study suggest cross-resistance to frequently used antibiotic analogues for growth promotion. For example, resistance to erythromycin and clindamycin may be attributed to the use of tylosin and kitasamycin in the feed [22,40]. In addition, tetracycline and doxycycline analogues are commonly used for the treatment of respiratory infections. These antibiotics may be administered through drinking water or feed over a prolonged period [32]. The Stock Remedies Act No.36 of 1947 has made antibiotics available over the counter for growth promotion and prophylaxis. This laxity could increase antibiotic resistance in foodborne pathogens in the food

production chain in South Africa. However, as recommended by the WHO, a ban on such use is already effective in the European Union for the last decade [41]

Furthermore, the current study revealed that over 82% of the isolates were MDR, with 56 antibiograms, indicating diverse resistance patterns. This observation intimates the mobilisation and easy exchange of antibiotic resistance genes between isolates across the farm-to-fork continuum. The diversity of resistance patterns and the high MDR rate highlights the need for antibiotic stewardship to ensure prudent antibiotic use for animal production, as it may have grave consequences for human and environmental health [42]. The overall large number of isolates with MARI > 0.2 (average = 0.47) further illustrates the selection pressure of excessive antibiotic use, indicating that these isolates were from environments of high antibiotic exposure, as would be expected if antibiotics are used for growth promotion, metaphylaxis, or prophylaxis [43,44]. Comparatively, an average MAR index > 0.3 was reported in India in pork [45].

The possible dissemination of MDR strains along the production chain due to the handling and contamination emphasises the need to monitor and enforce infection prevention and control measures at each stage in the food production chain. For example, the two MDR human isolates had MARIs of 0.5 (hands) and 0.85 (nasal). While the number of isolates was small, the high MARI values indicate a potential health hazard for the farmworkers. However, it cannot be concluded that the isolates identified in the humans originated from the farm, as human samples were not collected before the workers entered the farm. Similarly, isolates in the abattoir recorded MARI values between 0.75 and 0.80. Although these isolates likely came from the farm, it may not be concluded that they were from the same batch of pigs, since the abattoir serves many other farms within the district. This could be further supported because *S. aureus* was also isolated from the truck before our animals were loaded. These observations indicate that the transmission of microorganisms along the farm-to-fork continuum, especially antibiotic-resistant ones, exhibits a complex dynamic that requires further investigation using advanced molecular tools such as whole-genome sequencing.

3.3. Antibiotic Resistance Mechanisms

Although there was a general agreement between phenotypic and genotypic resistance, there were a few discrepancies. The most common resistance gene detected in this study was the *erm*C gene in over 90% of the isolates resistant to erythromycin, while another macrolide resistance gene, *msr*A, was detected at much lower levels (Table 2). The *erm*C gene facilitates the methylation of the 23S rRNA ribosome's active site, triggering conformational changes, resulting in drug binding inhibition [7,46], while *msrA* encodes for an ATP-dependent efflux pump. Resistance to erythromycin reportedly co-selects resistance to other antibiotics such as the type B streptogramin (MLS_B) and lincosamides. The frequent use of antibiotics such as streptogramin, virginiamycin, or tylosin to promote growth in farm animals through feeds has accounted for increasingly high numbers of isolates carrying the macrolide resistance genes [10].

The use of virginiamycin (streptogramin associated with resistance to quinupristin-dalfopristin), amongst others, for growth enhancement, was endorsed by the Pig Veterinary Society of the South African Veterinary Association in its policy document on "guidelines for the use of antimicrobials in the South African pig industry" [47]. Additionally, a survey by Eager et al. on the animal use of antimicrobials in South Africa reported high tylosin sales as a registered growth promoter [29]. This raises concerns because tylosin was banned alongside virginiamycin, spiramycin, and bacitracin in the EU based on WHO recommendations due to their chemical and structural homologies to antibiotics used in humans [48].

The prevalence of the *bla*Z gene was reported in 88.75% of penicillin-resistant isolates. In *S. aureus*, this gene is found on transposon Tn522 located in plasmid pI524. *bla*Z produces β-lactamase, which inactivates penicillin by hydrolysing its β-lactam ring [10]. Zehra et al. earlier found the *bla*Z as the most prevalent resistance gene in *S. aureus* in

bovine and swine from Punjab, India [49]. Furthermore, tetracycline resistance is conferred by two mechanisms: the active efflux of drugs, facilitated by *tet*K and *tet*L, and ribosomal protection due to the acquisition of *tet*M and *tet*O [50]. Our study's isolates displayed a higher prevalence of *tet*K than *tet*M (Table 2), accounting for the tetracycline resistance observed in the phenotypically resistant isolates. This finding was similar to that of Sieber et al. in a Danish study on LA-MRSA in pigs and humans [51]. However, other studies have reported a comparatively higher prevalence of *tet*M than *tet*K [52,53]. It has been established that most MRSA harbour both *tet*K and *tet*M, which confer resistance to all tetracycline antibiotics [54].

Most isolates that were phenotypically resistant to gentamicin harboured the *aac(6′)-aph(2″)* gene. However, the absence of this in two isolates could suggest that other aminoglycoside resistance mechanisms that were not investigated in this study might have conferred resistance. Nevertheless, the low prevalence of resistance (phenotypic and genotypic) may imply that aminoglycosides can still be used to treat clinical staphylococcal infections successfully; hence, its prudent use is advised in food animal production.

It has been reported that using avoparcin to promote growth in agriculture has facilitated the emergence of glycopeptide-resistant enterococci [55]. The resistance genes involved have been disseminated into other Gram-positive bacteria, including MRSA. The emergence of vancomycin-resistant MRSA is a cause for concern, considering that vancomycin is a drug of choice for resistant hospital-acquired infections [48]. Although the current study reported phenotypic resistance to vancomycin, the targeted *van*A and *van*B genes were not detected. This was in line with another South African study on *Staphylococcus* in farm animals, which revealed that 12% of the phenotypically vancomycin-resistant MRSA did not harbour the *van*A and *van*B resistance genes [56]. This could be attributable to other plasmid-mediated vancomycin genes that were not investigated in the current study, such as *van*C, *van*D, *van*E, *van*F, and *van*G [57]. More so, vancomycin resistance may also be caused by decreased permeability by thickening the cell wall, thus inhibiting/decreasing vancomycin availability to intracellular target molecules [58]. High percentage resistance to teicoplanin was also observed in the study; however, this was not peculiar, as cross-resistance between glycopeptides has been reported [52,53].

Further studies involving whole-genome sequencing (WGS) to detect unknown or novel mechanisms would be useful to delineate the genetic basis of resistance [14]. Lastly, isolates showed over 50% *mec*A gene prevalence, which is not surprising, as the MDR rate was also high. Isolates phenotypically resistant to cefoxitin but lacking the *mecA* gene could be due to alternative mechanisms of cefoxitin resistance such as *mec*C [54].

3.4. Virulence Determinants

S. aureus harbours various virulence determinants that contribute to its pathogenicity. Therefore, food animals may be a source of transmission of pathogenic strains in production facilities to humans and the environment [58]. Isolates predominantly harboured the *hla* gene (Table 2), an α-haemolysin cytotoxin, which contributes to biofilm formation in epithelial tissues, promoting infections and slowing down wound healing [59]. Staphylococcal enterotoxins (*sea, seb*, and *sed*) were recovered at lower rates, which agreed with other animal studies [60,61]. For example, Dweba et al. reported a prevalence of 6.4% (*sea*) and 6% (*sea*) for the gene amongst different animal species in South Africa [61]. Staphylococcal enterotoxins are usually associated with food poisoning, with *seb* considered a potential inhaled bioweapon [62,63]. Moreover, *S. aureus* may produce Panton-Valentine leucocidin (PVL), a pore-forming toxin encoded by phage-encoded genes [64]. PVL is also considered a genetic marker for CA-MRSA due to its prominence in this epidemiological class [64]. A substantial number of isolates carried the *PVL* gene, corroborating a study conducted on *S. aureus* isolated from backyard-raised pigs and pig workers in Nigeria, with 27% of isolates harbouring *PVL* [65]. However, although *PVL* has been associated with necrotising pneumonia and joint infection in humans, its role in pigs is not thoroughly investigated;

hence, the current findings should be interpreted with caution [66], as its presence does not necessarily imply diseased animals.

3.5. Clonal Relatedness of Isolates

Vancomysin has been regarded as the drug of choice to treat infections caused by MRSA; however, the increased resistance of these bacteria to vancomycin warrants rapid typing methods to characterise MRSA, as they have also been isolated from meat and meat products [67]. Although pulse-field gel electrophoresis (PFGE) has been regarded as the gold standard for typing MRSA, REP-PCR is more practical, time-efficient, and cost effective than other typing methods [68]. In addition, REP-PCR yields comparative results to PFGE [69] while outperforming more recent methods such as multilocus sequence typing (MLST) and PFGE in some instances [70].

Thus, using REP-PCR in the current study, the 48 MRSA isolates selected for typing yielded 28 REP types (A-AC) based on a 70% similarity index, with the majority concentrated within the farm environment. Five major REP types were identified on the farm, with isolates sourced from faeces, slurry, human swabs, and litter samples, usually at the same time points. Clonal relatedness was evident in isolates from pig faeces, human swabs, and the environment (slurry/litter), belonging to six REP types (F, I, J, L, N, and P). Although this was not surprising, due to the proximity between these sample sources, it further strengthens the knowledge of potential transmission of microbial species between humans, animals, and the environment within animal farms. Some isolates belonging to the same clones carried some similar resistance and virulence genes, although there was extensive diversity in the resistance, virulence, and clonal profiles. Such a high diversity could also be due to the small number of isolates included in the experiment. Selecting a few isolates from each sampling point may introduce bias that could allocate phenotypically similar isolates to different clonal groups. However, such selection could not be avoided, as the number of isolates obtained depended on the number of positive samples. Therefore, studies involving a larger number of isolates could provide a better picture of the clonality along the farm-to-fork continuum. A similar trend was observed in a study by Neyaz et al. on the characterisation of *S. aureus* from various meat products where a high prevalence of tetracycline resistance was reported in two different clones [65]. A 2019 study in Italy using other typing methods, including WGS, reported 94.1% of human MRSA isolates belonging to the same epidemiological group as swine MRSA isolates [26]. Of note, although REP-PCR has a shorter turnaround time, it is less discriminatory; therefore, further studies involving more resolute typing approaches such as WGS are recommended [71]. Nevertheless, it should also be noted that the diversity reported in the current study was based on a 70% similarity cut-off value and that changing this index could alter the number of REP-types in any given experiment.

4. Materials and Methods

4.1. Study Site and Sample Collection

The study was conducted in the uMgungundlovu District Municipality in KwaZulu-Natal (KZN), South Africa. This district is one of the largest districts in the KZN Province and contains all the major intensive food animal farms in the region.

Four hundred sixty-one (461) samples were collected from Farm P, its occupationally exposed farmworkers, farm environments, and associated abattoir over 18 weeks (September 2018–January 2019). The samples were collected across the farm-to-fork continuum (animal faeces on the farm, transport, and post-slaughter) as per the World Health Organisation on Integrated Surveillance of Antimicrobial Resistance (WHO-AGISAR) protocol [72]. The farm, transport, and abattoir samples were collected as previously described [73]. Block sampling was used to make sure the entire herd was well represented. Faecal and slurry samples were collected bi-weekly from day 0 to 126 (slaughter).

Additionally, hand and nasal swabs were obtained from farm employees. On the 18th week, the same herd was followed to the abattoir. Samples were collected from the

transport (truck, before and after loading the pigs) and at different stages from slaughter to packaging for human consumption from the same herd sampled on the farm [73]. All collected samples were immediately stored in a cooler box containing ice packs and transported to the laboratory for processing within 4 h of sampling.

4.2. Isolation and Identification of Staphylococcus aureus

4.2.1. Isolation of *S. aureus*

The samples were inoculated into tryptone soya broth (TSB) (Basingstoke, Hampshire, England) and incubated at 37 °C for two hours while shaking (100 rpm). Then, these samples were streaked on HiCrome Aureus Agar Base (Himedia Laboratories, Mumbai, India) and incubated overnight at 37 °C under aerobic conditions. After incubation, colonies showing a unique brown-black colour with a clear zone were streaked onto mannitol salt agar (Himedia Laboratories, Mumbai, India) for further screening. Presumptive *S. aureus* colonies were examined for coagulase-activity by the tube plasma test and DNAse tests [74]. The presumptive *S. aureus* colonies were maintained at −80 °C in 10% glycerol stocks for further analysis.

4.2.2. Molecular Confirmation of *Staphylococcus aureus* and Identification of Methicillin-Resistant *S. aureus* (MRSA)

DNA was extracted using the GeneJet Genomic DNA purification kit according to the manufacturer's instructions (ThermoFischer Scientific, Waltham, MA, USA). The concentration and purity of the DNA were determined spectrophotometrically using the Nanodrop ND-1000 Spectrometer (ThermoFisher Scientific, Waltham, MA, USA). The extracted DNA was used as the template in the PCR. Molecular confirmation was performed using *S. aureus* species-specific primers for the *nuc*A gene, which codes for a thermostable nuclease [75]. The primer sequences used were *nuc*AF 5′-GCGATTGATGGTGATACGGTT-3′ and *nuc*AR 5′-AGCCAAGCCTTGACGAACTAAAGC-3′ (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa), generating a 270-base pair fragment [75]. PCR was performed in a 20 µL reaction volume with 3 µL DNA template, 10 µL Luna® Universal qPCR master mix (Biolabs, New England Ipswich, MA, USA), 0.5 µL from each forward and reverse *nuc*A primers (20 µM), and 6 µL of nuclease-free water (Thermo Scientific, Waltham, MA, USA). The PCR protocol included activation for 5 min at 94 °C; 35 cycles of 30 s at 94 °C (denaturation), 45 s at 62 °C (annealing), and 45 s at 72 °C (elongation), and a final extension step of 10 min at 72 °C. All reactions were carried out in a T100™ thermal cycler (BioRad, Hercules, CA, USA). The PCR products were subjected to electrophoresis on a 1.5% agarose gel stained with ethidium bromide in 0.5 Tris-acetate-EDTA (TAE) buffer (HiMedia, Mumbai, India) at 120V for 1 h. Gels were visualised in a Gel Doc™ XR + imaging system (Bio-Rad, Hercules, CA, USA). The confirmed isolates were coded according to their collection site. Then, isolates that were positive for the *nuc*A gene were tested for the presence of the *mec*A gene to identify MRSA isolates using primers and PCR conditions described in Table S2.

S. aureus ATCC 25,923 and *S. epidermidis* ATCC 12,228 were used as the positive and negative controls for the presumptive phenotypic and genotypic identification experiments.

4.3. Antimicrobial Susceptibility Testing (AST)

The isolates' antibiotic susceptibility profiles were determined using the disk diffusion method on Mueller–Hinton Agar (Merck (PTY) Ltd., Modderfontein, South Africa) and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [76]. The Clinical and Laboratory Standards Institute (CLSI) guidelines [77] were used for antibiotic breakpoints absent from the EUCAST 2017 guidelines. Care was taken to ensure that the recommended ≈25 mL of agar was poured in each 90 mm plate, as the agar depth/volume could affect the antimicrobial susceptibility testing (AST) results. Antibiotics were selected based on the WHO-AGISAR 2017 protocol, their availability, and frequency of use in veterinary and human medicine in the country. The following

20 antibiotics were used: penicillin G (PEN 10 µg), ampicillin (AMP 10 µg), tigecycline (TGC 15 µg), nitrofurantoin (NIT 300µg), cefoxitin (FOX 30 µg) (interpreted using EUCAST breakpoints), amikacin (AMK 30 µg), gentamicin (GEN 10 µg), ciprofloxacin (CIP 5 µg), moxifloxacin (MXF 5 µg), levofloxacin (LVX 5 µg), tetracycline (TET 30 µg), doxycycline (DOX 30 µg), erythromycin (ERY 15 µg), clindamycin (CLI 2 µg), teicoplanin (TEC 30 µg), trimethoprim-sulfamethoxazole (SXT 1.25/23.75µg), chloramphenicol (CHL 30 µg), linezolid (LZD 30 µg) and rifampicin (RIF 5 µg) (interpreted using CLSI breakpoints) (Oxoid, Basingstoke, UK). The diameters of the zone of inhibition around the disks were measured to the nearest millimetre (mm) using a ruler. The minimum inhibitory concentrations (MICs) for vancomycin (VAN) were determined through the broth microdilution method using the CLSI guidelines [77]. A methicillin-sensitive strain, *S. aureus* ATCC 29213, was used as a positive control.

4.4. Risk Assessment Parameters of S. aureus Isolates

Multidrug resistance is defined as resistance to one or more agents in three or more distinct antibiotic classes, and it was determined from the AST results [78]. The multiple antibiotic resistance index (MARI) was calculated as (a/b), where "a" is the number of antibiotics to which the isolates were resistant, and "b" is the total number of antibiotics to which the isolate was tested [44]. Bacteria having a MARI > 0.2 originate from a high antibiotic exposure environment, while values < 0.2 show bacteria from lower antibiotic use sources. A completely resistant isolate has a MARI of 1.0.

4.5. Genotypic Characterisation of Isolates' Resistance and Virulence Potentials

Resistance and virulence genes were determined by PCR using primers (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa) and PCR conditions listed in Table S2. PCR was performed in a 20 µL reaction mixture consisting of 10 µL One Taq Master Mix (x2) (Biolabs, New England Ipswich, MA, USA), 6 µL of nuclease-free water, 0.5 µL of each primer pair (final concentration of 0.5 µM), and 3 µL of template DNA. All reactions were carried out in a T100™ Thermal Cycler (Bio-Rad, Hercules, USA). Each PCR assay included a positive control and a No Template Control (NTC) consisting of the PCR mix with template DNA replaced by nuclease-free water.

4.6. Determination of Genetic Relatedness Using Repetitive Element Palindromic PCR (REP-PCR)

The REP-PCR was only conducted on the MRSA isolates. Each PCR reaction was carried out in a 25 µL reaction mixture containing 12.5 µL of Dream Taq (Thermo Fischer Scientific, Vilnius, Lithuania), 10.5 µL of nuclease-free water, 1 µL of GTG_5 primer, and 1 µL of template DNA. The cycling conditions were as previously reported [72]. PCR products were subjected to electrophoresis in a 1% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer containing 5 µL of ethidium bromide and run at 75 V for 3 h. The gels were visualised, and the images were captured with a Gel Doc ™ XR imaging system (Bio-Rad, Hercules, California, USA). A 1 Kb DNA ladder (Biolabs, New England, Hertfordshire, UK) was used as a reference molecular weight marker. The resultant electrophoretic patterns were analysed using the BioNumerics software version 6.6 (Applied Maths NV, Belgium) using the Dice coefficient. Clustering was done through the unweighted pair group with arithmetic averages (UPGMA) using 1% tolerance and 0.5% optimisation. Clusters were identified based on a similarity of $\geq$ 70% [50].

4.7. Statistical Analysis

Descriptive statistics were used to describe the prevalence *of S. aureus* isolates, phenotypic resistance profiles, and genotypic profiles from different sources. The association between MAR index, resistance, and virulence genes was determined by performing a Chi-square test using SPSS (Statistical Package for the Social Sciences) v 20 (IBM, Armonk, USA). Results were considered statistically significant at $\alpha = 0.05$.

5. Conclusions

This study confirmed that pigs serve as important reservoirs for MDR *S. aureus*, including MRSA, with significant zoonotic implications and transmission potentials to humans through occupational exposure. The resistance to a range of antibiotics used as growth promoters, high MDR prevalence, and MARI values suggest a transmission risk between animals and humans. This poses a challenge to food safety and human and veterinary medicine, necessitating proper surveillance, stewardship, and biosecurity programmes in intensive food animal production. However, it should be noted that although clonalilty was observed among the isolates in the current study, all major REP types were found on the farm with no transmission evidence across the farm-to-fork continuum. Therefore, while being crucial for understanding the molecular epidemiology of *S. aureus* in intensive pig farming, the results of the current study should not be over-generalised. The clonality was only based on MRSA isolates, and other staphylococci and microbial pathogens carrying resistance genes could still be transmitted across the continuum.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-0817/10/3/317/s1, Table S1: Multidrug resistance profiles of *S. aureus* isolates, Table S2: List of primers used to detect antibiotic resistance and virulence genes.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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CHAPTER THREE - CONCLUSION

The current study delineated the prevalence, antibiotic resistance profiles, selected antibiotic resistance and virulence genes and the clonal relatedness of *Staphylococcus aureus* isolates obtained from an intensive pig production chain from farm-to-fork in the uMgungundlovu district, KwaZulu Natal, South Africa.

3.1. Conclusions

The following were the main findings from the study according to the study objectives:

- There was a high prevalence of *S. aureus* isolates (69%) detected throughout the production chain.
- A majority of the isolates (82%) were multi-drug resistant with 56 antibiograms observed.
- The resistance patterns >50% resistance to clindamycin, erythromycin, penicillin-G, tetracycline and doxycycline with 53% of the isolates confirmed MRSA by the detection of *mec A.*
- Resistance genes were identified and detected based on antibiotic resistance petterns. The prevalence of the resistance genes detected were; *ermC (82%), blaZ (73%), tetK (58%), tetM (28%), mecA (53%), msrA (15%) and aac (6')-aph (2") (5%)*.
- The *hla, LukS/F-PV, hld, tst, seb, sed, etb, eta* and *sea* virulence genes were detected.
- The MAR indices > 0.2 indicate that the isolates were exposed to environments with high antibiotic use, i.e. the use of antibiotics during animal husbandry as drivers of antibiotic resistant *S. aureus*.
- Five clones were observed. There was no evidence of transmission across the production chain with the five clones restricted to the farm, however, a possible transmission was observed between isolates from pigs, humans and slurry sharing similar resistance and virulence factors.
- The diversity and complexity of the resistance and virulence profiles of *S. aureus* with its zoonotic potential poses a potential threat to public health. Resistance to a range of antibiotics used for growth

promotion with high prevalence of MDR isolates and a high MAR index with a 0.47 average poses a significant challenge to food safety and veterinary medicine.

3.2. Limitations

- The current study was limited to one farm, disallowing extrapolation to intensive pig production in South Africa more generally. .
- A limited number of human participants from the farm gave consent to participate in the study therefore, the result was unable to give us a clear picture of the pig-to-human and/or vice versa MRSA transmission.

3.3 Recommendations

- MRSA identification was limited to *mecA* detection although *mecC* has also been implicated in methicillin resistance therefore further studies should incorporate the detection of *mecC*.
- Virulence and resistance determinants investigated were limited due to costs. Therefore, further investigations of other resistance and virulence conferring genes should be investigated e.g. phenotypic vancomycin resistance was evident but *vanA* and *vanB* resistance were absent suggesting other resistance genes could potentially be responsible for the conferred resistance. Resistance genes conferring resistance to other antibiotics should also be investigated.
- Whole-genome sequencing of isolates should be considered as this will provide an in-depth understanding of the genomic profiles of isolates.
- Several other farms within KZN could be added as study areas inclusive of extensive and backyard farms or antibiotic- free farms to provide a holistic picture.

- Proper monitoring of *S. aureus* as a foodborne pathogen, surveillance of antibiotic use and resistance in food production, stewardship programmes of stringent infection prevention and control measures should be implemented to deter the rise of antimicrobial-resistant *S. aureus* in food production chain.

3.4 Appendices

Appendix 1: Biomedical Research Ethics Committee (BREC) approval letter

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
RESEARCH OFFICE
Biomedical Research Ethics Administration
Westville Campus, Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

17 May 2019

Prof SY Essack
Department of Pharmaceutical Sciences
School of Health Sciences
essacks@ukzn.ac.za

Dear Prof Essack

Title: One Health approach to the containment of antibiotic resistance.
Degree: Non-degree
BREC Ref No: BCA444/16

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	17 March 2019
Expiration of Ethical Approval:	16 March 2020

I wish to advise you that your application for Recertification received on 29 April 2019 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 11 June 2019.

Yours sincerely

[redacted]

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

Appendix 2: Animal Research Ethics Committee (AREC) approval letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

09 February 2018

Professor Sabiha Yusuf Essack (3951)
School of Life Sciences
Westville Campus

Dear Professor Essack,

Protocol reference number: AREC/007/018
Project title: Antibiotic Resistance & One Health

Full Approval – Field Research Application

With regards to your application received on 06 February 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following conditions:

CONDITIONS

1. Samples will only be collected from Baynesfield Estate for this study.
2. Necessary approval must be obtained from other appropriate Ethics Committees for the human component part.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 09 February 2019.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

.....

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Academic Leader Research: Dr P Naidoo
Cc NSPCA: Ms Anita Engelbrecht
Cc Registrar: Mr Simon Mokoena
Cc Joseph Baynes Estate (Pty) Ltd

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8350 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

Appendix 3: Department of Agriculture, Forestry and Fisheries (DAFF) record

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/5

Prof Sabiha Yusuf Essack
Antimicrobial Research Unit
College of Health Sciences
University of KwaZulu-Natal
Private Bac x54001
Durban
4000
essacks@ukzn.ac.za

Dear Prof Essack,

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your request for permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project/study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. Only bacterial cultures emanating from this study may be stored in microbanks in the biofreezer in the access-controlled Antimicrobial Research Unit at UKZN. All other potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes.
3. A dispensation application must be made to the Director Animal Health in the event that any of the samples/bacterial cultures are to be used for any further studies or distributed outside of the Antimicrobial Resistance Unit of UKZN;

– 1 –

4. The study is approved as per the application form dated 28 August 2018 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
5. Pig faecal and litter samples may only be collected from Baynesfield Farm for which a state veterinary letter has been provided;
6. Samples from pig holding pens may only be collected from Cato Ridge abattoir for which a state veterinary letter has been provided;
7. Caecal, carcass rinsates and carcass swabs may only be collected from Cato Ridge abattoir for which a state veterinary letter has been provided;
8. Removal of samples from the abattoir is subject to compliance with the provisions of the Meat Safety Act, 2000 (Act 40 of 2000), as well as written permission from the abattoir owner;
9. The study may only be performed in the Antimicrobial Resistance Unit laboratories at UKZN;
10. Should any aspect of the study change, please contact the Directorate Animal Health to enquire regarding the need for Section 20 permission;
11. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: Antibiotic Resistance and One Health
Researcher (s): Prof Sabiha Yusuf Essack
Institution: Antimicrobial Research Unit, College of Health Sciences, University of KwaZulu-Natal
Your Ref./ Project Number: AREC/007/018, BCA444/16
Our ref Number: 12/11/1/5
Expiry date: 2019-04

Kind regards,

[redacted]

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2018 -09- 17

Appendix 4: Farm personnel consent form

Appendix 1
Participant Information Leaflet – Farm/Abattoir Personnel

You are being asked to volunteer to participate in a research study entitled "One Health Approach to the Containment of Antibiotic Resistance" conducted under the auspices of the South African Research Chair in Antibiotic Resistance and One Health, funded by the Department of Science and Technology via the National Research Foundation (NRF) over a 5-year period 2016-2020.

Your participation is completely voluntary. Please read the following information about the project. If there is anything in this Consent Document that you do not understand, be sure to ask study personnel to explain that portion of the study. If you voluntarily agree to participate, please sign in the appropriate box below.

This study is being conducted at University of KwaZulu-Natal. The overall purpose of this study is to investigate antibiotic resistance in bacteria isolated from pigs and poultry production, including the personnel that work in the production system from farms to abattoirs to butchers and supermarkets. This is called the "farm-to-form" protocol.

Male and female employees in the profession-related to pigs 18 years and older are eligible to participate. We are asking you to take part in this study because you are member of this category of person. The study involves taking a sample/swab of your nares and hands to undertake bacteriological investigations.

Participation in this study is completely voluntary. If you decide not to participate there will not be any negative consequences. Please be aware that if you decide to participate, you may stop participating at any time and you may decide not to provide any samples.

The investigators believe that the risks or discomforts to you and your animals are minimal.
You will not receive any payment for your participation in this study. Your participation will provide information to improve practices in the pig and poultry food production systems.

Please do not hesitate to contact the following person should you have any queries or concerns related to your voluntary participation:

Professor Sabiha Essack
B. Pharm., M. Pharm., PhD
South African Research Chair in Antibiotic Resistance & One Health
Professor: Pharmaceutical Sciences
Director: Antimicrobial Research Unit
College of Health Sciences
University of KwaZulu-Natal
Private Bag X54001
Durban
4000
South Africa
Telephone: +27(0)31 2607785
Telefax: +27(0)31 2607792
Email: essacks@ukzn.ac.za

We thank you for your invaluable time and your assistance.

Appendix 2
Participant Consent Form

I the undersigned…….. certify that I have been invited to participate in a research study entitled "One Health Approach to the Containment of Antibiotic Resistance" conducted under the auspices of the South African Research Chair in Antibiotic Resistance and One Health, funded by the Department of Science and Technology via the National Research Foundation (NRF) over a 5-year period 2016-2020 conducted by Professor Sabiha Essack from the School of Health Sciences, College of Health Sciences of the University of KwaZulu-Natal in Durban-South Africa as Principal Investigator.

- I confirm that I have read and understood the contents of the information sheet.
- I have well understood the aim and objectives of the research as well as the potential risks and benefits.
- I confirm that the occasion has been given to me to ask any questions and I certify that I have received proper answers to any of my questions.
- I understand that my personal information will be strictly confidential with limited access and that I have the right to withdraw from the study at any time, for any reason, without any consequence, and without any influence to my legal rights.

I understand that I am free to contact the researcher the address below:

Professor Sabiha Essack
B. Pharm., M. Pharm., PhD
South African Research Chair in Antibiotic Resistance & One Health
Professor: Pharmaceutical Sciences
Director: Antimicrobial Research Unit
College of Health Sciences
University of KwaZulu-Natal
Private Bag X54001
Durban
4000
South Africa
Telephone: +27(0)31 2607785
Telefax: +27(0)31 2607792
Email: essacks@ukzn.ac.za
-

I also understand that for concerns related to my rights as participant or to the researcher, I can freely contact the Biomedical Research Ethics Committee at the address below:

BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
Research Office, Westville Campus
Govan Mbeki Building
University of KwaZulu-Natal
Private Bag X 54001, Durban, 4000
KwaZulu-Natal, SOUTH AFRICA
Tel: +27(0) 31 2602486
Fax: +27 (0) 31 2604609

_____
Signature

Signed this _____ day of _____ 2016 at _____

Witness 1: _____

Witness 2: _____