NASAL CARRIAGE AND MOLECULAR CHARACTERIZATION OF STAPHYLOCOCCUS AUREUS IN PIGS AND OCCUPATIONALLY EXPOSED HUMANS IN JOS METROPOLIS, PLATEAU STATE, NIGERIA

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A DEGREE OF DOCTOR OF PHILOSOPHY IN VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE

DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE, AHMADU BELLO UNIVERSITY, ZARIA

OCTOBER, 2015

DECLARATION

I declare that the work in the thesis entitled, **"Nasal Carriage and molecular characterization of *Staphylococcus aureus* in pigs and occupationally exposed humans In Jos Metropolis, Plateau State, Nigeria"**has been performed by me in the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, under the supervision of Prof. J.K.P. Kwaga, Dr. M. Bello and Prof. A.K.B. Sackey.

The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this thesis has previously presented for another degree or diploma at this or any other University

AsabeHalimat MOMOH	----------	----------
Name of Student	Signature	Date

CERTIFICATION

This thesisentitled **"NASAL CARRIAGE AND MOLECULAR CHARACTERIZATION OF STAPHYLOCOCCUS AUREUS IN PIGS AND OCCUPATIONALLY EXPOSED HUMANS IN JOS METROPOLIS, PLATEAU STATE, NIGERIA''** by AsabeHalimat MOMOH meets the regulations governing the award of Doctor of Philosophy Degree in Veterinary Public Health and Preventive Medicine of Ahmadu Bello University, Zaria and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This work is dedicated to my beloved mother MaimunatUmoruMomoh. I am indeed what I am today because you have always stood by me and believed in my dreams and aspirations.

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ABSTRACT

Most staphylococci species are commensals andpathogenic bacteria that cause a wide variety of diseases in humans and animals with high impact on public health and the livestock industry**.** The aim of this study was to investigate the nasal carriage and antimicrobial resistance pattern of staphylococci speciesand to molecularly characterize*S. aureus*in pigs and occupationally exposed humans from both pig farms and abattoir.A total of 300 pigs and 101 humans from both pig farms and abattoir in Jos metropolis were screened for nasal carriage ofcoagulase negative staphylococci species (CoNS) species, *S. hyicus, S. intermedius* and*S. aureus*. Risk factors associated with *S. aureus* carriage among occupationally exposed humans was also determined using a structured questionnaire. Fifty three (53/401) 13.2% out of the 401 isolates were CoNS, 4.9% (20/401) were *S. hyicus* and 4.2% (17/401) of the isolates were *S. intermedius* based on confirmatory test with microgen biochemical kit. Twenty nine of the isolates (29/401)7.2% were found to be *S. aureus*after confirmatory test with microgen biochemical kit and MALDI-TOF technique. Eight different CoNS species were identified and they include; *S. haemolyticus, S. simulans, S. chromogen, S. warneri, S. xylosus, S. epidermidis, S. schleiferi, S. hominis*. 48.9% (44/90) of the CoNS, *S. hyicus* and *S. intermedius* isolates showed marked resistance to 3 or more of the 10 antibiotics tested. Six (6) of the seven(7)*S. intermedius*isolates that were phenotypically resistant to vancomycin had MIC valuesthat were resistant to the vancomycin when tested with Etest. The 29*S. aureus* isolates were subjected to antibioticsusceptibility testing.Three different multiplex PCRs were used to detect enterotoxin genes and staphylococcal protein A, *mec* A, *scn*, and *pvl* genes.Staphylococcal protein A (*spa*) typing was undertaken and DNA microarray analysis was also used to

detect antibiotic resistance and virulence genes on the isolates. All the *S. aureus*isolates were resistant to at least one antibiotic and had multiple antibiotic resistance (M.A.R.I) value >0.2. The isolates were resistant to beta-lactams (cefoxitin, ceftarbiproleand ceftaroline), macrolides (erythromycin), aminoglycoside (gentamicin, kanamycin), mupirocin, linezolide. The microarray analysis of a subset of the methicillin susceptible *Staphylococcus aureus* (MSSA) isolates identified numerous virulence genes (*ica, agr, pvl,* enterotoxins, *cap, chp, scn, sak, fnb*) associated with pathogenesis which are commonly identified in isolates of human origin and sometimes in animal isolatesand antibiotic resistance genes(*bla*Z, *erm*A, *msrA, tet*K and cat-*pC221*). There was a positive correlation between the phenotypic and genotypic expression of antibiotic resistance. All the 29 *S. aureus*isolates carried *spa* A gene, 27 (93%) carried *scn* gene, 6 (21%) carried the *pvl* gene and other leukocidin genes (*lukF, lukX* and *lukY*) were also detected. The 29 *S. aureus* obtained from both pigs and in-contact humans belonged to five (5) clonal complexes (CCs); CC15, CC152, CC5, CC1 and CC8 and a cluster of *spa* type t5427, t5126 and t5576 could not be assigned to any CC-group. Among these genotypes, the genotype CC15 was the most prevalent genotype (44.8%). Three (3) accessory gene regulator (*agr*) (*agr* I, II, IV) allelic groups were identified among the *S. aureus* isolates. Among these, *agr* II was the most prevalent (87.5%).The capsule and biofilm associated genes detected include capsular polysaccharide synthesis enzymes (*cap*), surface protein involved in biofilm formation (*bap*) and intercellur adhesion protein (*icaA, icaC* and *icaD*). All the isolates either carried the capsular polysacharide gene; *cap* 5 or*cap* 8; 58% and 100% of the pig and human isolates carried *cap* 8 while 80% and 25% of the pig and human isolatescarried the *cap* 5 gene, *icaA, icaC, icaD* and the *bap* genes. Some of the virulence genes detected were haemolysin (*hlg*), staphylokinase (*sak*), and chemotaxis-inhibiting protein (*chp*) genes.

Carriage of *hla*and *hlb*was 100% and 75% for pigs and 25% each for humansrespectively while humans and pigs had 25%each and 75% each for the *sak*and *chp*gene, respectively. The genes encoding enterotoxins including classical enterotoxins were also observed as well as the genes for microbial surface components recognizing adhesive matrix molecules (MSCRAMM) genes. The results of the questionnaire showed that age, gender, occupation and work experience were not significantly ($p>0.05$) associated with carriage of *S. aureus.* However, medical related occupation of household members was a significant factor ($p<0.5$) for colonization with *S. aureus*. This study revealed the presence of MDRSA, MSSA and the absence of MRSA carriage among the study population, but revealed a diverse range of virulence and resistance determinants among the isolates.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figures **Titles** **Page**

LIST OF PLATES

LIST OF APPENDICES

Appendix Title Page

ABBREVIATIONS

<Less than

*agr*Accessory gene regulator

AIP Auto inducing peptide

CA-MRSA Community Associated Methicillin Resistant *Staphylococcus aureus*

cap Capsular polysaccharide

CC Clonal complex

ccrCassette chromosome recombinase

CHIP Chemotaxis inhibiting protein

*clf*AClumping factor A

CLSI Clinical and laboratory standards institute

*cna*Collagen binding protein

CONS Coagulase negative staphylococci

DNA Deoxyribonucleic acid

ET Exfoliative toxin

EUCAST European committee on antimicrobial susceptibility testing

FIBFibrinogen binding protein

Fnbp A Fibronectin binding protein A

HA Hospital Associated

*hlg*Haemolysin

IEC Immune evasion cluster

Kb Kilo-base pair

LA-MRSA Livestock associated Methicillin Resistant *Staphylococcus aureus*

MALDI-TOF MS Mass assisted laser desorption/ionization time-of-flight mass spectrometry

M.H.C Major histocompatibility complex

MDRSA Multi Drug Resistant *Staphylococcus aureus*

MGE Mobile genetic element

MIC Minimum inhibitory concentration

MLEE Multilocus enzyme electrophoresis

MLST Multilocus sequence typing

MSCRAMM Microbial surface components recognizing adhesive matrix molecules

MSSA Methicillin Susceptible *Staphylococcus aureus*

PBP2 Penicillin binding protein

PCR Polymerase Chain Reaction

PFGE Pulse field gel electrophoresis

PVL Panton Valentine Leukocidin

S. aureusStaphylococcus aureus

SAK staphylokinase

*sas*G*S. aureus* surface protein G

SCC*mec*Staphylococcal cassette chromosome *mec*

scn Staphylococcal complement inhibitor

SE Staphylococcal enterotoxin

*spa*Staphylococcal protein A

SSTI Soft skin tissue infection

ST Sequence type

TNFR 1 Tumor necrosis factor receptor 1

TsstToxic shock syndrome toxin

VNTR Variable number tandem repeat

vwbrvon Willebrand factor

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

The genus *Staphylococcus* is basically divided into coagulase positive or coagulase negative species based on their ability to coagulate plasma (Pyorala and Taponen, 2009). The coagulase negative species constitute the majority of the genus and are opportunistic pathogens that can be isolated from the skin or mucous membrane flora of humans and animals (He *et al*., 2013). They can act as reservoir for resistance genes and may promote the distribution of resistance genes into different staphylococcal species or even other bacteria genera (Shen *et al*., 2013). *Staphylococcus aureus* are mostly coagulase positive, nasal commensal Gram positive cocci which colonize 20-30% of the human population (van Belkum *et al*., 2009), as well as livestock and domestic animals (Lindsay *et al*., 2014; Peton and Le Loir, 2014) as well as among animal production workers (Eko *et al*., 2015).

In humans, it causes a wide range of infections ranging from mild to life threatening diseases (Lulitanond *et al*., 2013; Bazzi *et al*., 2015). Approximately 20-30% of humans are colonized with *S. aureus*, with the most common site for colonization being the anterior nares (Graham *et al*., 2006), and it becomes virulent once the immune system of the individual is compromised. Clinical syndromes associated with this pathogen include; pneumonia, bacteraemia, endocarditis, osteomyelitis, septic arthritis and deep abscess formation (Chambers, 2001). In the past, colonization by strains of *S. aureus* that were resistant to methicillin was less common in humans, but a report in the United States

estimates that 1.5% of the population (approximately 4.1 million persons) are colonized with Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Gorwitz *et al*., 2008).

Staphylococcus aureus is one of the species of the genus *Staphylococcus* which is able to elaborate numerous virulence factors (Lozano *et al*., 2014). In addition to being a human pathogen, *S. aureus* causes an array of infections in economically important livestock animals, particularly pigs (Chuang *et al*., 2015). The success of *S. aureus* as a pathogen is partly due to its ability to express a variety of virulence factors that mediate host colonization, tissue invasion and dissemination (Gordon and Lowy, 2008). Among the most known secreted virulence factors is a large family of exotoxins that include the staphylococcal enterotoxins which cause food poisoning (Portillo *et al*., 2013). Also, an important virulence factor produced by many *S. aureus* (both MRSA and MSSA) strains is the Panton Valentin Leukocidin (PVL), which is often associated with community associated MRSA (CA-MRSA) (Chambers, 2005). In addition to this, *S. aureus* has a unique ability to develop resistance to antimicrobial agents (Chambers and Deleo, 2009).

Over the last few decades, there have been an enormous increase and emergence of *S. aureus* strains resistant to the antibiotic methicillin (MRSA strain) through the acquisition of the *mec*A gene (Lulitanond *et al*., 2013). *S. aureus* also causes nosocomial and community associated infections worldwide (Stegger *et al*., 2010). It is one of the most important hospital-associated (HA) pathogen being responsible for serious infections such as pneumonia and sepsis (Pantosis and Venditti, 2009) and is commonly characterised by multi-drug resistant phenotype (Monaco *et al*., 2013). Since 2003, unique complex (CC398) of MRSA associated with livestock (e.g pigs) has been described as a new lineage infecting or colonizing humans in several countries worldwide (Camoez *et al*., 2013).

MRSA was first isolated from animals (cattle with mastitis) in 1972 and in pigs in the Netherlands (Voss *et al*., 2005). However, prior to 2004, animals were not considered to have any significant role in the epidemiology of MRSA in humans (Voss *et al*., 2005). MRSA colonizing pigs and more rarely other farm animals have been reported in Europe and other parts of the world (van Cleef *et al*., 2010a).

Several studies have shown that human exposure to livestock constitute a risk for carriage of livestock associated *S. aureus* (LA-*S.aureus*) and development of a possible infection (Voss *et al*., 2005) and carriage prevalence in livestock farming profession is high (Denis *et al*., 2009; Stegger *et al*., 2010) but some strains have been detected in people without risk factors (van Loo *et al*., 2007; Welinder-Olsson *et al*., 2008). In addition to livestock associated methicillin resistant *S. aureus* (LA-MRSA), mounting evidence suggests that livestock, particularly pigs, can also represent an important reservoir of CA-MRSA strains that can colonise and infect humans in close contact with them (Faccioli-Martins and Cunha, 2012).

The identification of bacterial clones with enhanced virulence or increased ability to spread is important. Currently, PCR-based techniques are commonly used for typing as they are easy and fast. Among such techniques, *Staphylococcus aureus* protein A gene-typing (*spa* typing) and Multilocus Sequence Typing (MLST) have been considered a very useful tool for epidemiological studies (Sabat *et al*., 2013). Also, DNA based micro-array analysis is a fast method for analyzing the gene content of a pathogen. An example is the simultaneous analysis of the antimicrobial resistance and virulence gene content of *S. aureus* (Sabat *et al*., 2013). Most livestock associated MRSA (LA-MRSA) strains belonged to CC398 as defined by multilocus sequence typing (MLST) (Wulf *et al*., 2008), although, other clones

have occurred (CC5, CC9, CC30, and CC97) (Köck *et al*., 2013). The emergence of livestock associated *S. aureus* in major food animal species has raised concerns about animals as reservoirs of MRSA (Leonard and Markey, 2008).

1.2 Statement of Research Problem

The ubiquitous nature of the genus *Staphylococcus* generally and CoNS specifically enhances opportunities for infection (Davis *et al*., 2013) and furthermore the transmission of antibiotic resistance genes. *S. aureus* infects a variety of animals including cattle, poultry, horses (Juhasz *et al*., 2007; Hansman *et al*., 2010; Weese, 2010), companion animals ((Baptiste *et al*., 2005; Loeffler *et al*., 2011), pigs (de Neeling *et al*., 2007; Khanna *et al*., 2008) and some wildlife, but of all livestock, pigs appear to most commonly harbour MRSA.

Dutch investigators have identified pigs as important reservoirs of a specific type of MRSA (ST398) which may be an emerging occupational hazard (Stegger *et al*., 2010). Additional studies on pigs have shown that isolates from pigs and pig personnel are frequently indistinguishable, suggesting transmission from the animals to humans and vice versa (Huijsdens *et al*., 2006). In the Netherlands, investigations have demonstrated that LA-MRSA (ST398) now accounts for 20% of all MRSA detected in that country, suggesting the importance of considering livestock and other animals when examining the epidemiology of MRSA (van Loo *et al*., 2007).

The widespread use of antibiotics has evolved the emergence of multidrug resistant strains, therefore, making eradication more difficult and as such the spread of multi-drug resistant (MDRSA) strains has become a tough challenge for human infection control and antibiotic

therapy (Fall *et al*., 2012). Direct contact with MRSA positive animals have been reported to be a risk factor for MRSA carriage (5.6% - 37.8% among farmers) with these LA-MRSA strains now becoming increasingly responsible for human infections (Otter and French, 2012) and have even entered the hospital setting (Graveland *et al.*, 2012).

However, except for the report of Fall *et al*. (2012) in Senegal, there are no relevant data available on MRSA/MSSA in pigs in Africa and Nigeria in particular. Knowledge of MRSA/MSSA prevalence among pigs and farmers, as well as the genetic background of methicillin-susceptible *Staphylococcus aureus* (MSSA) and MRSA strains and associated virulence factors is unknown in Nigeria.

1.3 Justification of the Study

Coagulase negative staphylococci (CoNS) species have shown the ability to develop resistance against a wide spectrum of antibiotics. *S. aureus* has become a pathogen of increasing importance in hospitals, the community, and in recent years also in livestock (Huber *et al*., 2009). *S. aureus* has the unique ability to develop resistance to antimicrobial agents (Chambers and Deleo, 2009). Because antibiotics have been the first line of defense in treating clinical infections in both man and animals, therefore, bacteria that are resistant to antibiotics are of public health concern. Bacterial resistance to antibiotics is usually the result of the indiscriminate use of antibiotics as growth enhancer in food animal production. Its cosmopolitan spread has become a serious challenge for human infection control and antibiotic therapy (Fall *et al*., 2012).

Among food animals, pigs have been implicated as major source of potential infections to humans, especially farmers, slaughterhouse workers, and Veterinarians who are in frequent

contact with MRSA colonized pigs (Voss *et al*., 2005: Wulf *et al*., 2008). It is an established fact that MRSA colonization of healthy pigs is prevalent in many countries like the Netherlands, Germany, U.S.A and Canada (Voss *et al*., 2005; Khanna *et al*., 2008). Also, pig farm workers and slaughter plant workers with live animal exposure are at elevated risk of colonization with LA-MRSA (van Cleef *et al.*, 2010b).

In Nigeria, the rate of MRSA carriage among healthy carriers working in a unit of a hospital was found to be 52% (Fadeyi *et al*., 2010). Other studies have shown MRSA to be common in the community (Kesah *et al*., 2003; Adesida *et al*., 2005; Taiwo *et al*., 2005; Azeez-Akande *et al*., 2008; Nwankwo and Nasiru, 2011). Udobi *et al*. (2013) also reported 23.8% and 37.8% from wounds of patients and the skin from the orthopaedic ward of Ahmadu Bello University, Zaria.

However, there is a general paucity of information on *S. aureus* in livestock especially pigs as well as in-contact humans in Nigeria. Therefore, there is need to obtain information on both MSSA and MRSA prevalence in the study population, as well as to determine the genetic background of the isolates in order to understand the dynamics of the emergence of livestock associated *S. aureus*, their adaptation to humans and their potential impact on human health.

1.4 Aim of the Study

The aim of this study was to determine the prevalence of *S. aureus* in pigs and workers in Jos abattoir and piggeries, characterise the isolates and to evaluate possible risk factors for

S. aureus carriage among occupationally exposed humans in Jos metropolis of Plateau state.

1.5 Objectives of the Study

1. To isolate and identify *S. aureus* and other staphylococcal spp in pigs and in-contact humans in the study location using conventional biochemical test, rapid kit (Microgen kit) and MALDI-TOF MS technique.
2. To determine antimicrobial susceptibility profiles of the isolates to commonly used antibiotics using disc diffusion and broth dilution method.
3. To determine the minimum inhibitory concentration (MIC) of Vancomycin using Etest.
4. To detect the carriage of *mec*A, *scn*, *pvl* and *spa* A gene among *S. aureus* isolates using multiplex PCR.
5. To carry out *spa* typing of the *S. aureus* isolates.
6. To detect the carriage of antibiotic resistance genes, virulence genes, and enterotoxin genes using DNA microarray analysis.
7. To determine risk factors associated with *S. aureus* colonization among in-contact humans.

1.6 Research Questions

1. Is *S. aureus* present in pigs on backyard pig farms and abattoir in Jos, Nigeria?

2. If present, are they susceptible to commonly used antimicrobial agents and do they carry antibiotic resistance genes?

3. What are the MIC values of the isolates to Vancomycin?

4. Do the *S. aureus* isolates carry *mec*A, *scn, pvl* and enterotoxin genes?

5. What are the *spa* types and CC-groups of the *S. aureus* strains in circulation?

6. Do the *S. aureus* isolates carry virulence and antibiotic resistance genes?

7. What are the risk factors associated with *S. aureus* colonization among workers in pig farms and abattoir in Jos metropolis, Nigeria?

CHAPTER TWO

LITERATURE REVIEW

2.1 General Characteristics

Staphylococci belong taxonomically to the family Staphylococcaceae. They are gram-positive that occur in grape-like clusters and catalase-positive (Forbes *et al*., 2002) with a GC content of 30%-35%. Presently, 47 species and 24 subspecies of the genus *Staphylococcus* have been described. Of these species, include coagulase negative and coagulase positive species and this is based on their ability to coagulate plasma. *S. aureus* is characteristically associated with acute pyogenic infections whereas CoNS cause infections in susceptible hosts with certain predisposing conditions. They are normally found on mucous membranes and skin of humans and animals, with the nasal cavity being the predominant site (Winn *et al*., 2006). Among the staphylococci, *S. aureus* is easily identified by its ability to produce coagulase and hence clot human plasma and it also differs from other staphylococcal species on the basis of colony morphology (smooth colonies of 6-8mm diameter) with golden pigmentation, deoxyribonuclease test, and fermentation of mannitol (Wilkerson *et al*., 1997).

S. aureus is a facultative anaerobe with an inherent ability to withstand harsh environmental conditions such as high temperatures (50ºC), high salt concentrations (7.5-10%), and dry environmental conditions (Somerville and Proctor, 2009; Beaume *et al*., 2010). They have cell walls made of peptidoglycan (linked polysaccharide subunits) and may have activity that is similar to endotoxin. Other important structural components of staphylococci include polysaccharide capsules and surface proteins (for example, Protein A) which forms the basis for the agglutination test used for *S. aureus* identification and enterotoxins that can cause food poisoning and shock (Jimenez *et al*., 2011). *S. aureus* has been commonly noted as a commensal organism; one that exists without apparent harmful effects on the skin or in

the nasal passage of healthy humans and animals. However, under stress and injury to skin surfaces, even a commensal organism may opportunistically be capable of causing infection.

S. aureus is without doubt the most virulent of the staphylococci due to the wide spectrum of diseases it causes ranging from localized and systemic infections to toxin mediated illness (Forbes *et al*., 2002).

2.2 Biology of *Staphylococcus* Species

Staphylococci are spherical gram-positive cocci arranged in irregular grape like clusters. They all produce catalase an important virulence factor which degrades the microbicidal H_2O_2 into O_2 and H_2O. *Staphylococcus aureus* is distinguished from other staphylococci by the production of coagulase an enzyme that clots plasma. *S. aureus* produces a carotenoid pigment that imparts a golden colour to its colonies. This pigment enhances the pathogenicity of the organism by inactivating the microbicidal effect of superoxides and other reactive oxygen species within neutrophils. *S. aureus* haemolyzes red blood cells and is also able to ferment mannitol (Lowy, 1998; Levinson, 2008).

2.2.1 *Staphylococcus aureus* cell wall

The cell wall of *S. aureus* is mainly peptidoglycan. The main function of peptidoglycan is to provide a rigid envelope for the cell content. Peptidoglycan consists of alternating

polysaccharide subunits of N-acetylglucosamine and N-acetylmuramic acid with 1,4-β linkages (Lowy, 1998). The peptidoglycan chains are cross-linked by tetrapeptide chains bound to Nacetylmuramic acid and by a pentaglycine bridge specific for *S. aureus* (Lowy, 1998). Peptidoglycan also has endotoxic properties, and has been reported to cause organ dysfunctions in experimental animals (Holtfreter and Broker, 2005). Most *S. aureus* strains produce a slimy, extracellular capsular polysaccharide. A total of eight capsular serotypes have been described, and serotypes 5 and 8 are predominant in isolates found in humans (O'Riordan and Lee, 2004). Microorganisms that cause invasive disease commonly produce extracellular capsular polysaccharides. Capsules enhance microbial virulence by rendering the bacterium resistant to phagocytosis resulting in bacterial persistence in the bloodstream of infected hosts. Animal studies suggest that it also promotes bacterial colonization and persistence on mucosal surfaces (O'Riordan and Lee, 2004).

2.2.2 *Staphylococcus aureus* genome

The first genome sequences of two related *S. aureus* strains (N315 and Mu50) were determined by shot-gun random sequencing in 2001 by Kuroda *et al*. (2001). The *S. aureus* genome consists of a singular circular chromosome of about 2.7 to 2.9 Mbp containing about 2600 genes composed of core and auxiliary (accessory) genes. It has a G+C content of 33%. The majority of genes comprising the core genome are those associated with central metabolism and other housekeeping functions. Supplementing these are genes that are associated with common species functions but that are not essential for growth and survival, including virulence genes. Also included are surface binding proteins, toxins, exoenzymes and the capsule biosynthetic cluster (Lindsay and Holden, 2004). The

accessory genome accounts for about 25% of any *S. aureus* genome, and mostly consists of mobile genetic elements that transfer horizontally between strains. These elements include bacteriophages, pathogenicity islands, chromosomal cassettes, genomic islands, plasmids and transposons. Accessory genes typically have a different G + C content than those in the core genome, often because they are obtained from other species of bacteria (Lindsay and Holden, 2004).

Many of these genetic elements are known to carry genes associated with virulence, drug and metal resistance, substrate utilization and miscellaneous metabolism. Therefore, the distribution and horizontal spread of these elements can have important clinical implications. The identification and characterisation of these elements provides insights into how *S. aureus* cause disease, and their diversity (Shittu *et al*., 2009).

Bacteria obtain genetic information from other cells or the surrounding environment in three ways: (1) uptake of free DNA from the environment (transformation), (2) bacteriophage transduction, and (3) direct contact between bacterial cells (conjugation). Bacteriophages or bacterial viruses seem to have the greatest impact on staphylococcal diversity and evolution. They are known to transfer genes such as *luk*F-PV and *luk*S-PV that encode the Panton Valentine leukocidin (PVL) components which is strongly associated with severe forms of pneumonia (necrotic pneumonia) caused by community-acquired *S. aureus* strains; the staphylokinase (*sak*) gene, which is a potent plasminogen activator that could facilitate bacterial spreading through its fibrin-specific blood clotting activities and the enterotoxin genes (Shittu *et al*., 2009; Malachowa and DeLeo, 2010). *S. aureus* pathogenicity islands (SaPIs) often carry superantigen genes, such as toxic shock syndrome (tst) and enterotoxins B and C, implicated in toxic shock and

food poisoning. Three families of genomic islands have been discovered in sequenced strains of *S. aureus* strains. They carry exotoxin and lipoprotein genes (Shittu *et al*., 2009). All the sequenced *S. aureus* strains are known to carry one or more free or integrated plasmids. All types of *S. aureus* plasmids frequently carry antibiotic resistance genes. Some virulence genes are also known to be carried on plasmids, such as exfoliative toxin B and some superantigens (Lindsay and Holden, 2004). Other mobile genetic elements found in the *S. aureus* chromosome include Staphylococcal cassette chromosomes (SCCs). They encode antibiotic resistance and/or virulence determinants. SCCs encode the methicillin resistance gene (*mec*A) (Shittu *et al.,* 2009; Malachowa and DeLeo, 2010).

2.3 Carriage of *S. aureus*

S. aureus colonizes the skin, oral cavity, perineum, axillae, throat, but the main carriage site of this bacterium is the mucosa of the anterior nares (nose) (Weitheim *et al*., 2005; Andersen *et al*., 2013). Approximately 20-30% of humans are persistent carriers of a single or related strain type(s) while 60% are intermittently colonized with different strain types and 20% never carry *S. aureus* (von Eiff *et al*., 2001). Carriers of *S. aureus* have a higher risk of infection than non-carriers and may as well pose a risk for transmission to other individuals by direct contact or through contaminated environment (Deleo *et al*., 2010). Host and bacterial factors appear to co-determine the *S. aureus* carrier state in a process that is not fully understood (Wertheim *et al*., 2005; Mulcahy *et al*., 2012). Highlighting co-morbidities, hospitalization, exposure to animals, sharing household with carrier, young age, male sex, and ethnicity have been shown to be risk factors for colonization (von Eiff *et al*., 2001; Bogaert *et al*., 2004; Aubry-Damon *et al*., 2008; Gorwitz *et al*., 2008; Munckhof

et al., 2009; Herwaldt *et al*., 2013). Several studies have illustrated that *S.aureus* carriers (man and animal) can be decolonized by chlorohexidine baths and nasal application of mupirocin, contributing to reduce the risks of *S. aureus* infection (Kluytmans, 1998).

2.4 Transmission of *Staphylococcus aureus*

S. aureus can be acquired through several means for instance by man through contact with pus from infected wound, skin to skin contact with an infected person, or by contact with contaminated fomites, such as towels, clothing, or athletic equipment used by an infected person. For many years, *S. aureus* was considered primarily a human pathogen, but the report of a MRSA infected dairy cow in 1972 altered that perception (Devriese *et al.*, 1972). Investigators initially thought that the primary route of *S. aureus* transmission between humans and animals was solely from humans to animals. This was supported by the fact that a majority of MRSA infections found in cats, dogs, pet birds and horses were caused by human strains (Baptiste *et al*., 2005). In contrast, the most predominant strains in food animals (pigs, cattle) tended to be animal in origin.

However, a recent study demonstrated that *S. aureus* can be transmitted in both directions, from human to animal and animal to human (Loeffler *et al*., 2011). Once exposed to *S. aureus*, animals can become colonized, and may serve as reservoirs from which it can be transmitted to other animals and to their human handlers. A study by Sequin *et al*. (1999) found that animal caretakers and veterinary personnel that come in contact with *S. aureus*-infected animals may become colonized. These colonized humans subsequently may transmit the germ further to susceptible humans or animals (Lloyd, 2006). Also, two human cases of *S. aureus* bactereamia and wound infection respectively have been linked to a livestock reservoir on the patients' farms (Petersen *et al*., 2012). Factors involved in the

transmission of *S. aureus* include: crowding, compromised skin (scratches and abrasions), contaminated items (fomites) or surfaces, and poor hygiene. These factors apply to humans and animals alike. In particular, people who handle animals in their work or who have animals as pets in their homes have very close physical contact with these animals, which may facilitate transmission of MRSA. Transmission between humans and their animal contacts may be facilitated by their contaminated shared surroundings (Leonard and Markey, 2008).

2.5 Pathogenesis of *S. aureus*

S. aureus is associated with a wide range of infections, mainly skin and soft tissue infections (SSTI) (Gorwitz *et al*., 2008) and bactereamia, which is linked to considerable (20%) mortality rates. Prevailing host conditions and virulence of the infecting strain are known to influence the outcome of *S. aureus* infections (Foster, 2005; Wertheim *et al*., 2005; Winn *et al*., 2006). Strain pathogenicity is elicited by a repertoire of virulence factors comprising structural and secreted products (Lowdy, 1998; Gordon and Lowdy, 2008). The virulence factors described in *S.aureus* are involved in adherence to human tissue, immune evasion, toxin secretion and regulation of virulence gene expression (Foster, 2005; Winn *et al*., 2006). Surface proteins known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) such as fibronectin binding protein A (FnbpA), clumping factor A (ClfA) and staphylococcal protein A (Spa A) have been described (Foster and Hook, 1998; Winn *et al*., 2006). The MSCRAMMs facilitate adherence and favour infection by binding to fibrinogen, fibronectin and collagen in host tissue (Winn *et al*., 2006; Gordon and Lowdy, 2008). Different patterns of MSCRAMMs can be found in strains causing different kinds of infection (Lowy, 1998; Menzies, 2003; McCathy and

Lindsay, 2010). Host immune evasion by *S. aureus* is facilitated by several secreted proteins including staphylococci complement inhibition protein (SCIN) encoded by the *scn* gene, chemotaxis inhibitory protein (CHIPS, encoded by *chp* gene) and staphylokinase (SAK, encoded by the *sak* gene) (Foster, 2005; Winn *et al*., 2006). *S.aureus* can also subvert the host immune system and prevent opsonization by the binding of protein A and it is also known to activate tumor necrosis factor receptor 1 (TNFRI) (Bekeredjian *et al*., 2007), B cells and other ligands to promote inflammation (Gomez *et al*., 2004; David and Daum, 2010). Liu *et al*. (2005) demonstrated that staphyloxanthin (golden pigmentation) produced by *S. aureus* confers resistance to oxidative killing by host neutrophils.

Among the several toxins described in *S. aureus*, the most well characterised are toxic shock syndrome toxin-1 (encoded by *tst*-1 gene), food poison causing enterotoxins B and C, (encoded by *seb* and *sec*) (Dinges *et al*., 2000), and scalded skin syndrome exfoliative toxins A and B (encoded by *eta*, and *etb*). PVL (encoded by the *lukS-PV* and *lukF-PV* genes) has been associated with leucocyte destruction by pore formation in host cell membrane, skin and soft tissue infections (SSTI), abscesses, and severe necrotizing pneumonia (Lina *et al*., 1999; Gillet *et al*., 2002; Chambers and Deleo, 2009; Daskalaki *et al*., 2010; David and Daum, 2010). The role of *pvl* in pathogenesis is still controversial because no difference in virulence between isogenic strains differing for presence of *pvl* was observed *in vivo* using mouse infection models (Voyich *et al*., 2006; Wardenburg *et al*., 2008). In contrast, Loffler *et al*. (2010) reported that in the presence of *pvl*, neutrophils from monkeys or mice did not lyse, but those from rabbit and human were lysed, indicating the important role of *pvl* in pathogenesis in humans. David and Daum, (2010) showed that mouse models may probably not be a suitable way to measure the role of this virulence

factor in pathogenesis. *S. aureus* virulence factors are usually carried on mobile genetic elements (MGE) (Lindsay, 2010) such as bacteriophages and pathogenicity islands (Malachowa and Deleo, 2010). For example, the genes that code for SAK, SCIN, CHIP and PVL are carried on pathogenicity islands (Malachowa and Deleo, 2010). Expressions of *S. aureus* virulence factors are carefully modulated to prevent undue metabolic demand (Lowdy, 1998; Gordon and Lowdy, 2008). For example expression of MSCRAMMs usually occurs during logarithmic phase to facilitate initial adherence to host cells, unlike secreted toxins and enzymes, which are expressed at the stationary phase to facilitate the spread of *S. aureus* (Gordon and Lowdy, 2008).

2.6 Transition of *S. aureus* To MRSA

S. aureus is a major inhabitant or transient colonizer of both humans and primates. It is sometimes present on domestic animals, although these are usually colonized by other species of staphylococci (Pantosti *et al*., 2013), hence, it is of importance in both human and veterinary medicine. When *S. aureus* gains entrance into a host, it is capable of causing a range of infections from mild skin infection to life threatening invasive infections (Lulithanond *et al*., 2013; Pantosti *et al*., 2013). With the introduction of antimicrobial therapy, some clones of *S. aureus* have shown the ability to develop resistance easily against most classes of antibiotics to which they are exposed (Lowy *et al*., 2003; Pantosti *et al*., 2007; Malachowa and Deleo *et al*., 2010). Antibiotics have been the first line of defense in treating clinical infection in both man and animals. Prior to the introduction of penicillin for treatment of *S. aureus* infections, the mortality rate from *S. aureus* infections was over 80% (Skinner and Keefer, 1941). Penicillin became available for medical use around 1940. *S. aureus* being one of many organisms exhibiting resistance were becoming resistant to

antibiotics such as penicillin. The resistant bacteria produced penicillinase, an enzyme that breaks down penicillin, rendering it ineffective. Another antibiotic methicillin was then released in 1959, and it became the first antibiotic developed to resist penicillinase. However, the first methicillin-resistant *S. aureus* isolate was identified only two years later, and eventually about 80% of all *S. aureus* isolates were again resistant to this antibiotic (Deurenberg and Stobbering, 2008). By 1961 the first case of MRSA infection was reported in England (Jevon, 1961). Since that time, MRSA has become a worldwide problem in both human and veterinary medicine. Resistance to methicilin indicates resistance to all beta-lactam agents which is of great concern as it has contributed to a great burden of disease worldwide. Methicilin resistance is due to the acquisition of *mec*A gene, which encodes a new protein assigned *PBP2a*.

Since the carriage of *mec*A gene in methicilin susceptible *S. aureus* has resulted in MRSA (Enright *et al*., 2002), the source of *mec*A has long been investigated. Hence, series of sources have been linked to *mec*A gene. However, Couto *et al*. (1996) found a *mec*A homolog with 80% identity to the *S. aureus* gene in *S. sciuri*, a MSSA in rodents and other primates. Also, Schnellman *et al*. (2006) found 91% identity with *mec*A present in *S. vitulinus* isolated from horses. Recent data have shown *S. fleurettii*, belonging to *S. sciuri* group could be the origin of *mec*A as this species contains *mec*A and the chromosomal locus surrounding *mec*A that are almost identical to the corresponding sequence of staphylococcal cassette chromosome *mec* type (SCC*mec)* (Tsubakishita *et al*., 2010). These collective studies are an indication that animal staphylococci are the likely origin and reservoir of *mec*A.

MRSA was first recognized as a major cause of nosocomial infection also known as hospital associated MRSA (HA-MRSA) (Pantosis and Venditti, 2009). In 1990, MRSA epidemiology largely changed, due to the emergence of new MRSA lineages, responsible for infections occurring in the community among patients without known risk factors for the acquisition of MRSA and without previous hospital contacts (Deleo *et al*., 2010). These strains, defined as community acquired (CA)-MRSA (Hawkey, 2008) and in 2004, forms of MRSA associated with livestock contact emerged and have been described as livestock associated MRSA (LA-MRSA). These various types of MRSA is often associated with different sequence types (STs) and virulence factors (Chambers and Deleo, 2009; Fitzgerald, 2012). MSSA strains became resistant to methicillin by acquisition of *mec*A (Katayama *et al*., 2000; Ito *et al*., 2013). Morbidity, mortality rate and socio economic cost associated with MRSA infections seem to be higher compared to MSSA (Cosgrove *et al*., 2003; Grundmann *et al*., 2006) and this has been linked to patients underlying diagnosis, the limited therapeutic options available for treatment of MRSA infections and the high cost of patient management (Grundmann *et al*., 2006; Makgotlho *et al*., 2009). Resistance to non-beta lactam agents such as quinolones, tetracyclines, aminoglycosides, macrolides and lincosamides are increasingly reported among MRSA strains, further diminishing the treatment options available for infected patients (Jensen and Lyon, 2009). Additionally, resistance of MRSA to vancomycin, a last choice therapeutic agent, has been reported (Graber *et al*., 2007; Howden *et al*., 2009). Antimicrobial resistance genes in *S. aureus* are located on MGE such as plasmids, transposons and staphylococcal cassette chromosome *mec* (SCC*mec*), the genetic element associated with methicillin resistance (Sung *et al*., 2008).

2.7 Types of Methicillin Resistant *Staphylococcus aureus* (MRSA)

MRSA are classified as HA, CA and LA-MRSA, which are often associated with different sequence types (STs) and virulence factors (Chambers and Deleo, 2009; Fitzgerald, 2012).

2.7.1 Community associated methicillin resistant *S. aureus* (CA-MRSA)

This strain of MRSA emerged in the 1990s and since then it has become cosmopolitan (Hawkey, 2008) because it has an unusual virulence, capacity to spread and cause infections in young and healthy individuals (Deleo *et al*., 2010). CA-MRSA is not associated with short health-care exposures. It commonly causes SSTIs with different degrees of severity, from mild to serious infections and can rarely cause severe form of pneumonia associated with high mortality (Francis *et al*., 2005). CA-MRSA possess small, mobile genetic elements (SCC*mec* type IV, V or VI) which contains the *mec*A gene with or without additional antibiotic resistance genes and are more easily transferred to other strains of *S. aureus* than larger SCC*mec* (I, II and III) elements (O'Brien *et al.*, 2004), rare multi-resistance (Naimi *et al*., 2003) and variably resistant to clindamycin and fluoroquinolones. Large majority of CA-MRSA harbour the genes for Panton Valentine Leukocidin (*pvl*), a toxin that is absent in HA-MRSA. PVL has been considered the principal virulence determinant (Lina *et al*., 1999; Boyle-Vavra and Daum, 2007) or a simple classical marker of CA-MRSA strains (Voyich *et al*., 2006). Generally, before now, CA-MRSA infections were thought not to be associated with previous healthcare exposures. However, it is becoming difficult to differentiate between HA-MRSA from CA-MRSA (Song *et al.*, 2011; Stefanni *et al.*, 2012) because the CA-MRSA types have been increasingly incriminated as the cause of diseases in healthcare facilities and clones of HA-MRSA entering the community (Maree *et al*., 2007; Al-Rawahi *et al*., 2010; Companile *et*

al., 2012). It has even being assumed that CA-MRSA maybe adding to the overall number of infections in hospital populations rather than replacing HA-MRSA (Klein *et al*., 2009); alternatively, CA-MRSA strain may eventually become the dominant MRSA strain in the hospital settings (Webb *et al*., 2009). The emerging spread of CA-MRSA strains poses a significant threat to public health (Vandenesech *et al*., 2003; Lindsay and Holden, 2004).

2.7.2 Hospital associated methicillin resistant *S. aureus* (HA-MRSA)

It has long been considered the model of multi-drug resistant nosocomial pathogen in hospital and healthcare facilities (Grundmann *et al*., 2006). Typically, HA-MRSA are resistant to fluoroquinolones and clindamycin, they cause invasive infections, are associated with the carriage of SCC*mec* I, II, and III and definitely lack the Panton Valentine Leukocidine gene (*pvl* gene) (Grundmann *et al.*, 2006). HA-MRSA took more than 20years to become the epidemic that is known today (Hawkey, 2008) even after its emergence in the late 1950s as a result of the introduction of the antibiotic methicillin (Jevons, 1961). Generally, HA-MRSA infections are associated with prolonged hospitalization. The proportion of MRSA infections has increased greatly in the last 3 decades and strains defined as HA-MRSA have become endemic in industrialized countries as causes of serious infections such as septicemia, pneumonia, ventilator-associated pneumonia, and surgical sites infection (Diekema *et al*., 2001).

2.7.3 Livestock associated methicillin resistant *S. aureus* (LA-MRSA)

Recently, a unique MRSA clone was shown to be a frequent colonizer of food animals in Europe and emerging worldwide (Crombe *et al*., 2013). This colonization is possibly favoured by the indiscriminate and large use of antibiotics in animal production either as growth performance or for therapeutic use. This clone is quite different from the HA-

MRSA and CA-MRSA lineages. In food animals, mainly pigs, this new MRSA strain with the zoonotic potential of transmission of *S. aureus* between livestock, companion animals and humans (Lowder *et al.,* 2009; Loeffler *et al*., 2011). This new clone has been recognized and designated Livestock associated MRSA (LA-MRSA) with sequence type 398 (ST398) (Price *et al*., 2012) and other sequence types (STs) like ST9 has also been reported (Lulitanond *et al*., 2013). The first report of this novel change in the epidemiology of MRSA (LA-MRSA) was reported in 2005 among a family of pig farmers and their pigs in the Netherlands (Voss *et al*., 2005). In line with this, pig farming was observed to be a risk factor for *S.aureus* colonization in humans in France and ST398 was included among several lineages identified in pigs and pig farmers (Armand-Lefevre *et al*., 2005). The Agricultural environment has also been linked as probable reservoirs in LA-MRSA transmission however it has not been closely examined, although preliminary evidence shows that floor surfaces and feed from poultry facilities can be a source for MRSA. Since the discovery of LA-MRSA ST398, they have been steadily reported among livestock, especially pigs in a number of European countries (De Neeling *et al*., 2007; Witte *et al.*, 2007; Pomba *et al*., 2009; Lazano *et al*., 2011a; Lazano *et al*., 2011b), the USA (Smith *et al.*, 2009), Peru (Arriola *et al.*, 2011), Canada (Khanna *et al*., 2007) and Asia (Sergio *et al*., 2007; Cui *et al*., 2009; Lim *et al.,* 2012). However, in Asia, ST9 constitutes the predominant LA-MRSA clone among swine (Crombe *et al*., 2013).

Colonization rates at animal level vary widely across studies, ranging from 6-80% (Wesse *et al*., 2010; Crombe *et al*., 2013). However, care must be taken when comparing results due to differences in sampling and isolation procedures, sample size, number of pigs sampled and sample populations (finishing vs. breeding pigs, piglets vs. older pigs, open vs.

closed farms, pigs at the abattoir vs. pigs at the farm, etc.) (Broens *et al*., 2011). Even though LA-MRSA ST398 is more associated with colonization in farm animals, especially in pigs, it has been isolated as etiologic agent of infection in other animals (Sergio *et al*., 2007; Kadlec *et al*., 2009; Schwarz *et al*., 2008; Meemken *et al*., 2010; van der Wolf., 2012).

2.8 Mechanisms of Antibiotic Resistance

S. aureus has an exceptional ability to develop resistance to antimicrobial agents (Chamber and Deleo, 2009). MRSA strains have acquired and integrated into their genome a mobile genetic element, termed Staphylococcal Cassette Chromosome *mec* (SCC*mec*) which harbours the *mec*A and other resistance antibiotic determinant. Methicillin resistance in staphylococci is often mediated by the expression of the *mec*A gene that results in the production of a modified penicillin-binding protein (PBP*2a* or 2') which is an important component in building the bacterial cell wall (Chambers, 1997). This PBP*2a* has reduced affinity to beta-lactam antibiotics (Katayama *et al*., 2001; van Duijkeren *et al*., 2004) by producing enzymes that can destroy β-lactams, known as β-lactamases (Norris *et al*., 1994).

Methicillin-sensitive *Staphylococcus aureus* (MSSA) are susceptible to β-lactam antibiotics that bind their PBP and disrupt the cell wall; however, the altered PBP2' that are present in MRSA cannot be bound by β-lactam antibiotics. This enables MRSA to grow in the presence of most antibiotics (Deurenberg and Stobbering, 2008). However, heterogenous resistance to methicillin occurs among *S. aureus* isolates due to variations in the expression of the *mec*A gene, or alteration of constitutive PBPs (Chambers, 2001).

MRSA can be resistant to antimicrobials other than β-lactams since resistance is often acquired by the horizontal gene transfer from outside sources, however, chromosomal mutations from antibiotic selection have also been reported (Duerenberg and Stobberingh, 2008). For instance, additional drug resistance genes can become integrated into SCC*mec* as plasmids or transposons; examples include plasmid-mediated resistance to some aminoglycosides, tetracyclines, and heavy metals, and transposon-mediated resistance to macrolides, lincosamides, and streptogramin as well as cadmium (Deurenberg and Stobbering, 2008). Some MRSA are also resistant to fluoroquinolones because of a mutation in the DNA gyrase gene (Hawkey, 2008). In addition, resistance to the powerful antibiotic vancomycin has recently been observed (Appelbaum, 2006).

The causes of antimicrobial resistance are complex. Antimicrobial resistance in human forms of MRSA has been linked to the indiscriminate use of antimicrobials by man (Teale, 2002). There is significant controversy on the virulence of methicillin-susceptible *S. aureus* (MSSA) vs. MRSA isolates; both have enormous capacity for virulence and pathogenicity that enables them to reach high rates of infection (Gould 2006). However, they differ in their genotypes, geographical distribution and the infections they cause (Kim *et al*., 2006).

MRSA strains of human origin can occasionally colonize animals, including companion animals (dogs and cats) and pigs (Rich and Robert, 2004; Khanna *et al*., 2008; Cuny *et al*, 2010). Similarly, resistant bacteria in livestock are likely associated with selective pressures that result from the therapeutic and sub-therapeutic use of antimicrobials in livestock (Hunter *et al.,* 2010). Specifically, the use of tetracycline has been linked to livestock related forms of MRSA in pigs (de Neeling *et al*., 2007). The methods by which antimicrobial resistant organisms spread outside the farm have also been investigated.

Evidence suggests that antimicrobial resistant bacteria, including *S. aureus*, can be transmitted from pig farms to people in close contact with pigs (Monaco *et al*., 2013).

2.9 Antimicrobial Resistance

S. aureus has the ability to rapidly develop resistance to any novel antimicrobial agent (Lowdy, 2003; Makgotlho *et al*., 2009). As a result, multi drug resistant strains are frequently isolated worldwide (Lowdy, 2003). Penicillin was the drug of choice for treatment of *S. aureus* infections until the 1950s (Lowdy, 2003). Few years after the introduction of this drug in clinical practice, the prevalence of strains producing penicillinase (beta-lactamase encoded by the *bla*Z gene) increased, rendering penicillin ineffective in the treatment of *S. aureus* infections (Rammelkamp, 1942). The introduction of a semi-synthetic penicillin (methicillin), in 1959 (Espedido *et al*., 2012) offered a new opportunity to treat efficiently infections caused by penicillinase-producing strains. Unfortunately, in 1960, strains resistant to methicillin emerged in UK hospitals and then in Europe and the rest of the world (Jevon, 1961). These strains are termed methicillin resistant *S. aureus* (MRSA). These strains are resistant to practically all beta lactams and their derivatives due to the acquisition of a low affinity to penicillin-binding protein (PBP2a/PBP2') encoded by the *mec*A gene and MSSA strains became resistant to methicillin by acquisition of *mec*A (Katamaya *et al*., 2000; Ito *et al*., 2013). Antimicrobial resistance genes in *S. aureus* are located on MGE such as plasmids, transposons and staphylococcal cassette chromosome *mec* (SSC*mec*), the genetic element associated with methicillin resistance (Sung *et al*., 2008).

2.10 Diagnosis

2.10.1 Direct detection method

The primary method use to identify staphylococci is colonial morphology using Gram staining while speciation of isolates is essential to distinguish *S. aureus* from coagulase-negative staphylococci (CoNS). Various tests can be used to identify *S. aureus*, they include; coagulase test, sugar fermentation test, DNase test, heat-stable nuclease test and commercial biochemical kits. Latex agglutination kit is also one of the tests that can be use too. It detects protein A and/or clumping factor (Spanu *et al*., 2004).

2.10.2 Molecular detection method

Staphylococci may be typed, based on their susceptibility to bacteriophages. Most molecular methods for identification of *S. aureus* have been PCR based. Early tests required the Southern blotting of amplified products to confirm their identity, 36 but a range of primers designed to amplify species-specific targets have now been developed. Such targets include the nuclease (*nuc*), coagulase (*coa*), protein A (*spa*), *fem*A and *fem*B, 16S rRNA and surface-associated fibrinogen-binding protein genes (Grisold *et al*., 2002; Mason *et al*., 2001). For further molecular investigation, Microarray analysis, single nucleotide polymorphism (SNPs) – these are looking to replace PFGE and MLVA) and even whole-genome sequencing (WGS) may be options, as new technology. However, some of these methods remain accessible to reference laboratories only and are difficult to implement for routine bacterial identification in a clinical laboratory. These tools support infection control strategies to limit bacterial spread.

2.11 Antimicrobial Susceptibilty Testing

Two main methods are used in determining antimicrobial susceptibility of *S. aureus*: the disk diffusion (Kirby Bauer) and the broth dilution methods (Bauer *et al*., 1966). The outcome of these methods is used by practitioners in the selection of treatment options for patient management. In disk diffusion method, *S. aureus* is cultured on Mueller-Hinton (MH) agar in the presence of selected antimicrobial impregnated filter paper disks or tablets. Zone sizes around the filter paper disk/tablet which indicate activity of the antimicrobial agent, are measured and interpreted according to clinical breakpoints established by international organizations such as the European Committee on Antimicrobial Sensitivity Testing (EUCAST) (www. Eucast.org) or Clinical and Laboratory Standard Institute (formerly NCCLS) (CLSI) (www.clsi.org).

The broth dilution method is a quantitative antimicrobial susceptibility testing methods used to determine the minimum inhibitory concentration (MIC), the lowest concentration (mg/L) of an antimicrobial agent that under a defined *in-vitro* condition completely inhibits growth of the microorganism tested within a defined time period (Matuscheck *et al*., 2014). It is the gold standard for susceptibility testing (EUCAST, 2003). MICs are recorded and interpreted according to a standard guidelines provided by EUCAST or CLSI.

Following EUCAST guidelines, strains interpreted as resistant by disk diffusion using 30ug cefoxitin disk/tablets (< 22mm zone diameter) or by broth microdilution (cefoxitin MIC >4 mg/L) are reported as MRSA (www.Eucast.org). However, phenotypic detection of MRSA can be complicated by the heterogenous nature of MRSA populations; presumptive MRSA should therefore be confirmed by molecular detection of *mec*A, which is considered to be the gold standard (Chambers, 1997).

2.12 Molecular Typing

S. aureus is highly clonal organism. Molecular typing methods are therefore used to assess strain diversity, clonal relationship and evolutionary changes among *S. aureus* isolates, for example as part of surveillance and outbreak investigations in hospitals, communities and livestock (Sabat *et al.*, 2013). Ideally, typing methods should be fast, less expensive, reliable (able to distinguish related from unrelated), reproducible, and the results should be easy to interpret and communicate (van Belkum *et al*., 2007). The choice of a suitable method(s) depends on the epidemiological question being addressed (Sabat *et al*., 2013). For example, MLST measures variations that accumulates slowly in long term evolution (Enright *et al*., 2002), whereas PFGE indexes short-term variations and can be applied for outbreak investigations (Sabat *et al*., 2013). Other methods used to assess genetic diversity of *S. aureus* isolates include *spa* typing, which has gradually replaced PFGE in routine typing, SCC*mec* typing and DNA microarray.

2.12.1 Typing of the polymorphic region of *spa* gene (*spa* typing)

Staphylococcal protein A (*spa*) typing is a sequence based method that targets the polymorphic variable-number tandem repeats (VNTR) of the *spa* gene encoding protein A (Koreen *et al*., 2004; Strommenger *et al*., 2006). The *spa* gene region is polymorphic as a result of spontaneous mutations and loss or gain of repeat (Harmsen *et al*., 2003). Each repeat consist of 24bp; variation in the sequences is used to assign repeats numbers. Based on the variation defined by the order of repeats succession within the *spa* gene for a given *S.aureus* strain, *spa* types are assigned (Harmsen *et al*., 2003) from a central database (http://spaserver.ridom.de) hosted in Munster, Germany. It has been observed that this region provides information not only about short-term epidemiology but also about long-

term phylogeny and contains a reliable signal that could be utilized for the determination of clonal relatedness among individual strains (Mellmann *et al*, 2006). The method of *spa* typing is impressive for its ease of interpretation and suitability for international comparison, with results that can give useful epidemiological information for local investigations (Harmsen *et al*., 2003; Hallin *et al*., 2007). *spa* typing is able to detect both rapidly and slowly accumulated molecular variations, it can be used to investigate outbreaks in epidemiological studies as well as molecular evolution in studies of population structure (Shopsin *et al*., 1999; Tang *et al*., 2000; Koreen *et al*., 2004; Strommenger *et al*., 2006). This method has advantages regarding speed, inter-laboratory comparison and interpretation compared to PFGE. Although *spa* typing is a single locus typing method, it generates data compared to the more expensive MLST and generally allows strain assignment to specific STs and CCs based on associations between *spa* types and STs/CCs obtained by MLST (Shopsin *et al*., 1999; Oliveira *et al*., 2001; Koreen *et al*., 2004). To date, 657 diverse repeats and more than 14,000 *spa* types have been described (http://spaserver.ridom.de).

2.12.2 DNA based micro-array analysis

DNA based micro-array analysis is a fast method to analyze the gene content of a pathogen. An example is the simultaneous analysis of the antimicrobial resistance and virulence gene content of *S. aureus* (Sabat *et al*., 2013). The test DNA obtained from monoclonal *S. aureus* cultures following an approximately 40-fold amplification and biotin-labelled of the test DNA, (www.clondiag.com) are hybridized to the microarray (targeting 336 genes) and visualized by adding horseradish peroxidase. Based on the resulting hybridization profiles, affiliation of isolates to specific lineages are done by software (Monecke *et al.*, 2008; Sabat

et al., 2013). The major disadvantage of this method is that unusual alleles not included in the array cannot be identified (Sabat *et al*., 2013).

2.12.3 Staphylococcal cassette chromosome *mec* typing (SCC*mec*)

This is a mobile genetic element that carries *mec*A or the recently described *mec*C homologue encoding methicillin resistance (IWG-SCC, 2009). This element also carries a complete or part of the regulatory genes (*mec*R1-*mec*I) and the *ccr* genes responsible for the movement of the cassette (IWG-SCC, 2009; Ito *et al*., 2012). SCC*mec* elements integrate specifically into the 3' end of orFX, an open reading frame of unknown function and may carry other resistance genes on transposons or integrated small plasmids in the junkyard regions (J1-J3). The SCC*mec* typing is a PCR method (Kondo *et al*., 2007) employed in addition to other typing methods to characterise MRSA strains. The origin of these SCC*mec* elements is unknown but it has been hypothesized that they may have originated from coagulase negative staphylococci (CoNS) (Garza-Gonzalez *et al*., 2010). It is characterised by the presence of terminal inverted and direct repeats, two essential parts; the *mec* gene complex (*mec*) and the cassette chromosome recombinase gene complex (*ccr*) (Ito *et al*., 2003), and the junkyard (J) regions (Ma *et al*., 2002; Ito *et al*., 2004). The *mecA* gene which confers methicillin-resistance and the cassette chromosome recombinase (*ccr*) genes which facilitate inclusion and excision into the *S. aureus* genome are both located in the SCC*mec*.

Although the origin of SCC*mec* is not definitively known, it is generally thought that the *mecA* gene arose from transfer between two staphylococcal species (Wielders *et al*., 2001). However, two theories exist regarding the evolution of MRSA. Kreiswirth *et al*. (1993) suggested that all MSRA clones have a common MSSA ancestor which acquired SCC*mec*

only once. They later introduced multi-clone theory suggests that the SCC*mec* element was introduced into different *S. aureus* lineages multiple times (Gomes *et al*., 2006). Currently, the multiclone theory seems to be gaining support.

Currently, eleven major types (http://www.sccmec.org/) of SCC*mec* elements (I to XI) have been identified based on the organization of the *mec* gene complex, *ccr* gene complex, and integrated plasmids. To date, there are three classes (A, B and C) of *mec* complex and four allotypes (types 1, 2, 3 and 5) of ccr complex (Zhang *et al*., 2005). Different combinations of these complex classes and allotypes generate various SCC*mec* types. SCC*mec* elements are currently classified into types 1, II, III, IV and V based on the nature of the *mec* gene complex and *ccr* allotypes (Ito *et al*., 2001; Ito *et al*., 2004; IWG-SCC, 2009; Turlej *et al*., 2011). It's however, an important molecular tool and very useful in understanding the epidemiology and clonal strain relatedness of MRSA (Zhang *et al*., 2005).

2.12.4 Mass assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique

The mass assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is both a typing and identification tool. In this study it was used for species identification. It is a rapid and reliable species identification tool. It is an alternative to various other identification tools which could serve as hindrance to quick and successful treatment and management of diseases (Panda *et al*., 2013).

This technique is based upon the detection of highly abundant proteins in a mass range between 2 and 20 kDa by computing their mass (m) to charge (z), m/z values. Thus, a typical fingerprint is generated for each microorganism which is used for comparison with the stored reference spectra and thereby providing identification for the sample. The

striking advantage of mass spectral approach over phenotypic and genotypic procedures is a simple straight forward sample preparation procedure and the short time required for analysis (Panda *et al*., 2013).

2.13 Virulence Components of *S. aureus*

S. aureus is one of the most important opportunistic pathogens for mammalian species. The success of *S. aureus* as a pathogen is partly due to its ability to express a variety of virulence factors both structural and secreted, that mediate host colonization, tissue invasion and dissemination (Gordon and Lowy, 2008). It is the most invasive and an etiological agent of diverse human and animal illnesses, including skin infection, abscesses, food poisoning, toxic shock syndrome septicemia, endocarditis and pneumonia. Almost all strains secrete a group of enzymes which include coagulase, hyaluronidase, catalase, thermonuclease and certain toxins (Iandolo, 1989). Also, cell wall adhesion components of the bacteria are also involved in virulence. The expression of these virulence factors help the bacteria adapt to hostile environments, facilitating their survival and promoting infection due to cell wall invasion, the degradation of the immune system cells and tissues, facilitate the multiplication of the bacteria and are involved in the onset of clinical symptoms (Gordon and Lowy, 2008). The toxins described in *S. aureus* include; cytotoxins (leukocidins, haemolysin), exfoliative, toxic shock syndrome and enterotoxins (Chambers, 2005).

2.13.1 Leukocidins

Panton Valentine Leukocidin (a pore forming toxin) is one of the most potent toxins encoded by the genes *LukS-PV* and *LukF-PV*. This cytotoxin is joined to the host defense cells, in particular white blood cells, monocytes and macrophages inducing the formation of pores, altering the permeability and thus destroying the cell. PVL produce leukocyte destruction causing necrotizing pneumonia- an aggressive condition that often kill patients with skin and soft tissue infections (SSTIs) (Gillet *et al*., 2002; McClure *et al*., 2006), polymorphonuclear lysis and epidemiologically linked to community associated MRSA (CA-MRSA) (Boyle-Vavra and Daum, 2007), but its contribution to disease has been subject of debate (Bubeck *et al*., 2007; Labandeira *et al*., 2007). Isolates of *S. aureus* harbouring *pvl* genes have been epidemiologically linked to specific human *S. aureus* manifestations: not only to primary skin and soft tissue disease but also to severe necrotizing pneumonia and severe bone and joint infections. It is also often associated with community-acquired MRSA infections (Lina *et al*., 1999). *pvl* positive MSSA strains have been linked to outbreaks of skin infections in Swiss schools (Boubaker *et al*., 2004) in a German town (Weise-Poselt *et al*., 2007), in French soldiers in the Ivory Coast (Strauss *et al*., 2007) and in a group of pupils in Lebanon (Beyrouthy *et al*., 2013).

However, there are a number of un-described genes in the LA-MRSA stains which could encodes virulence factors associated with infections in animals. The world-wide emergence of CA-MRSA has been linked to the carriage of the PVL genes (DeLeo *et al*., 2010). Based on a gene knockout screen, the PVL genes have been identified as contributing transiently to CA-MRSA pathogenesis in a rabbit bacteremia model (Diep *et al.*, 2008). In addition there is a strong association between PVL and severe skin infections in humans (Lina *et al.*, 1999). By comparative genomics the PVL genes have been identified in some human-

associated MSSA ST398 isolates (Price *et al*., 2012) which could contribute to increased virulence in these strains.

Only 2-3% of *S. aureus* strains produce this toxin. The *LukE* and *LukD* (*lukE/D* genes) produce dermonecrosis in rabbits but have a weak leukotoxic activity and hemolytic activity. Ruminant polymorphonuclear leukocytes are highly sensitive to the leukotoxin *LukM*/*LukF-PV* (encoded by *lukM*) and its presence has been associated with cases of bovine mastitis (Kaneco and Kamio, 2004).

2.13.2 Haemolysins

Five haemolysins have been described up to date; alpha (α-haemolysin, encoded by *hla*), beta (β-haemolysin, encoded by *hlb*), delta (δ-haemolysin, *hld*), gamma (γ-haemolysin, *hlg*) and gamma variant (*hlgv* or *hlg-2*). The majority of *S. aureus* cells produce any of these haemolysin. Its broad distribution is due in part, to their location in very stable regions of the chromosomal DNA (Kaneco *et al*., 2004).

In addition, this gene also help the bacteria to survive in the human host via evasion of the innate immune system has been described (Van Wamel *et al.*, 2006). It is named Immune Evasion Complex (IEC) and compiles the modulators staphylokinase (SAK, *sak* gene), Staphylococcal Complement Inhibitor (SCIN, *scin* gene) and Chemotaxis inhibitor (CHIPS, *chp* gene) which cluster on the conserved 3' end of β-haemolysin (*hlb*)-converting bacteriophages (Van Wamel *et al*., 2006). These human-specific innate immune modulators SCIN, CHIPS, and SAK from IEC that are easily transferred among *S. aureus* strains by a diverse group of β-haemolysin-converting bacteriophages. The IEC is gaining interest in the last years due to its specificity for human-adapted *S. aureus* strains and it has been

suggested as a good predictor of the human or animal origin of a given isolate (McCarthy *et al*., 2012; Price *et al*., 2012; Vincze *et al*., 2014).

2.13.3 Enterotoxins

Enterotoxins are small peptides of about 26-29kDa in size and they have a great deal of similarity at the amino acid level (Marrack and Kappler, 1990). Eighteen Staphylococcal enterotoxins (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER, SEU) have been described in addition to some variants of SEC, SEG, SEH, SEI. They are the main causes of food poisoning and intensive intestinal peristalsis. However, SEA and SED are the most frequently implicated in food poisoning outbreaks (De Buyser *et al*., 2001) possibly because they can produce in a wide range of growth conditions (Gundogan *et al*., 2005). However, in the last few years, new types of SEs have been discovered and staphylococcal-like (s*el*) proteins have also been described (Hennekinne *et al*., 2012).

These toxins are heat stable and resistant to digestive enzymes being responsible for food poisoning caused by *S. aureus*. In *S. aureus*, a putative nursery of enterotoxin genes, named *egc*, has been detected and observed to be highly prevalent (Jarraud *et al*., 2001).

2.13.4 Adhesins

Staphylococcus aureus possesses various secreted and surface-anchored proteins by which it binds to host extracellular matrix and plasma components. Microbial surface components recognizing adhesive matrix molecules (MSCRAMM) adhesins are generally covalently anchored to cell wall peptidoglycan. MSCRAMMs recognize collagen (collagen -binding

protein (CNA), fibronectin (fibronectin-binding proteins (FnB*P*), such as FnBP*A* and FnBP*B*), and fibrinogen (clumping factors (*clf*), such as *clfA* and *clfB*) (Foster and Hook, 1998). MSCRAMMs play a major role in adhesion to host tissues. Staphylococcal protein A (SPA) belongs to the MSCRAMM family and it binds to the von Willebrand factor (VWBR), a large glycoprotein that mediates platelet adhesin at site of endothelial damage. *spa* displays several additional properties: it interferes with immunoglobulin-mediated opsonization; it mimics a B-cell superantigen; and it binds to tumor necrosis factor (TNF)-R1, a TNF-α receptor (Cheung *et al*., 2002; Silverman and Goodyear, 2002).

2.13.5 Accessory gene regulator (*agr*)

The accessory gene regulator (*agr*) quorum-sensing system was first described in *S. aureus* but is a global regulatory system of the genus *Staphylococcus* and has been found in the majority of staphylococcal species examined (Dufour *et al.,* 2004; Bannoerh *et al*., 2009). It is an operon that includes a system of two-signal transduction components responsible for controlling the expression of many virulence factors and pathogenicity. This operon is self-induced by a peptide named AIP (Auto inducing peptide), located in the same locus. AIP diffuses into the target cell and acts as a receptor of the signal when the cell density is sufficient. This is a population density sensor or quorum sensing and is the only quorum-sensing system in the staphylococcal genome (Traber *et al*., 2008).

Expression of most virulence factors in *S. aureus* is controlled by the accessory gene regulator (*agr*) locus, which encodes a two-component signaling pathway whose activating ligand is a bacterial-density sensing peptide (auto-inducing peptide) also encoded by *agr* (Lina *et al*., 2003). A polymorphism in the amino acid sequence of the autoinducing peptide and of its corresponding receptor (*agr*C) has been described. *S.*

aureus strains can be divided into four major groups on this basis: within a given group, each strain produces a peptide that can activate the *agr* response in the other member strains, whereas the autoinducing peptides produced by the different groups are usually mutually inhibitory (Ji *et al*., 1997). Links between a peculiar *agr* type and a specific staphylococcal syndrome have been shown for toxic shock syndrome (TSS) and staphylococcal scalded skin syndrome (SSSS). TSST-1-producing isolates belong to *agr* specificity group III and mostly belong to a single clone, as shown by multilocus enzyme electrophoresis (MLEE) (Ji *et al*., 1997) and pulsed-field gel electrophoresis (PFGE). Most exfoliatin-producing strains responsible for TSS belong to *agr* group IV, but the clonality of these strains has not been investigated (Jarraud *et al*., 2002). The accessory gene regulator (*agr*) locus influences the expression of many virulence genes such as exotoxins and capsular polysaccharides type 5 and 8 during *S. aureus* infections (Luong *et al*., 2002). Interference in virulence gene expression caused by different *agr* groups has been suggested to be a mechanism for isolating bacterial populations and a fundamental basis for subdividing the species (Robinson *et al*., 2005). The capsular polysaccharide is a cell wall bacterial component that protects bacterium from phagocytic uptake and enhances microbial virulence (Verdier *et al*., 2007). Numerous serotyping studies based on capsular type of *S. aureus* strains from diverse human sources have revealed that serotype 5 and 8 strains account for about 25% and 50%, respectively, of clinical isolates (Watts *et al*., 2005). These two serotypes are clinically relevant in that they are predominant among clinical isolates of varied geographic origin. Because of their prevalence, *cap*5 and *cap*8 have been used as targets for vaccine development, and specific antibodies against CAP 5 and CAP 8 have been shown to be protective against *S. aureus* infections (Luong *et al.*, 2002).

2.14 MRSA in Food Animal Products

Both animal and human MRSA have been detected in meat products. The contamination of food products by animal MRSA is a big threat, as it has a potential for wide dissemination in the general population (Kluytmans, 2010). A study in the Netherlands found that 11% of raw meat samples including pork, beef, veal, lamb and chicken from the retail market were contaminated by MRSA, represented mainly by MRSA ST398 (de Boer *et al*., 2009). A study in South Italy reported the presence of MRSA *spa* types corresponding to ST398 in mozzarella cheese products. In a study carried out in Belgium 2006, 12% of the isolates obtained from healthy chickens were MRSA that belonged to *spa* types associated with ST398 (Nemati *et al.*, 2008). S. aureus is commonly associated to mastitis leading to contamination of milk and milk products (Jablonsky and Bohach, 1997). A study in Plateau state, Nigeria reported the prevalence of 30.9% of *S. aureus* in mastitic milk (Suleiman *et al*., 2013).

2.15 LA-MRSA as an Occupational Hazard

Before now, close contact with pigs was not considered a risk for MRSA colonization. However, pigs have now been associated with an important reservoir of LA-MRSA which may be an emerging occupational hazard (Witte *et al*., 2007). Several reports have shown that people with occupational exposure to live animals especially farmers, veterinarians, abattoir workers and their family members constitute a very high carriage prevalence of LA-MRSA compared to the general populace (Wulf *et al*., 2008; de Jonge *et al*., 2010; van Cleef *et al*., 2010a; 2010b; Camoez *et al*., 2013). However, people without risk factors have also been shown to be colonized or even infected with other strains of MRSA (Welinder-Olsson, 2009). A skin and soft tissue infection (SSTIs) is most frequently associated with

LA-MRSA (Lozano *et al*., 2012). Also, several studies have reported that severe infections can occur and such have been reported in Europe, Asia and United States (Yu *et al*., 2008; Smith *et al*., 2009 and van Cleef *et al*., 2011). Voss *et al*. (2005) also reported MRSA infections among the daughter of a pig farmer, the son of a veterinarian and another pig farmer in the Netherlands. The type of MRSA found in these people was an unknown type and also similar clone was found by the same researchers in the pigs of both owners (Voss *et al*., 2005).

2.16 *Staphylococcus aureus* in Africa

2.16.1 Prevalence of human *S. aureus* nasal carriage

In Africa, very few studies have investigated the prevalence of nasal carriage in patients and healthy people and there are few or no reports on occupationally exposed humans with pigs. However, in recent years, a growing interest in the epidemiology of *S. aureus* has been observed in the African continent (Aiken *et al*., 2014; Schaumburg *et al.*, 2014). The few reports available on *S. aureus* carriage indicate that carriage rate vary significantly between countries and often between study population within the same country. In Nigeria, carriage rastes have been reported among HIV patients (33%), healthy carriers (21%) and among hospital patients (Olalekan *et al*., 2012). However, only the report by Fall *et al*. (2012) on carriage rate of *S. aureus* among pig workers in Senegal is available online to my knowledge.

2.16.2 Prevalence of antimicrobial resistance

The available data on prevalence of antimicrobial resistance in *S. aureus* in Africa mainly originate from North, South, Central and West Africa (Breurec *et al*., 2011; Fall *et al*., 2012; Falagas *et al*., 2013; Schaumburg *et al.,* 2014). Resistance to penicillin (74-100%) (Ramdani-Bouguessa *et al*., 2006; Shittu *et al*., 2011; Kolawale *et al*., 2013), tetracycline (22-92%) (Shittu *et al.,* 2011; Djoudi *et al*., 2013; Conceicao *et al*., 2014) and sulphamethoxazole/trimethoprim (15-89%) are relatively common compared to other antimicrobial agents (Shittu *et al*., 2011; Mariem *et al*., 2013; Seni *et al*., 2013). Nasal isolates from a remote community in Gabon were however, often susceptible to tetracycline (94%), sulphamethoxazole/trimethoprim (88%) and penicillin (65%) (Schaumburg *et al*., 2011), indicating that prevalence of antimicrobial resistance is lower in remote populations with limited access to hospitals and antimicrobials. The hypothesis is indirectly supported by the different frequencies of penicillin (94.2%), tetracycline (37.5%) and sulphamethoxazole/trimethoprim (7.8%) resistance among clinical isolates from urban areas in the same region (Alabi *et al*., 2013). Differences in antimicrobial resistance patterns seem to exist between Africa regions. For example, fusidic acid resistance appears to be more frequent in North Africa (13-62%) than in sub-Saharan Africa (0-2%) (Ramdani-Bouguessa *et al*., 2006; Shittu *et al*., 2011; Elazhari *et al*., 2012; Conceicao *et al*., 2014). These variations might be due to differences in availability and usage of antimicrobial susceptibility testing (Falagas *et al*., 2013). A systematic review on antimicrobial resistance in *S. aureus* in five (5) countries in Central Africa (DR Congo, Cameroon, Congo Brazzaville, Gabon and Central African Republic) indicate that most of the published studies did not conform to standard procedures for antimicrobial testing (Vleighe *et al*., 2009). This might explain some of the striking differences between the antimicrobial resistance patterns reported by different studies.

2.17 Population Structure of *S. aureus* in Africa

Although extensive information on *S. aureus* population structure is available in developed countries of the western world, *S. aureus* diversity has been poorly explored on the African continent. Based upon the few African studies that investigated the clonal structure of *S. aureus* isolates by *spa* typing, t311 (ST5), t064 (ST8), t084 (ST15), t314 (ST121) and t355 (ST 152) appear to be the most common lineages (Chua *et al*., 2008; Breurec *et al*., 2011; Shittu *et al*., 2011; Schaumburg *et al*., 2011; Ateba *et al*., 2012; Moremi *et al*., 2012; Kolawale *et al*., 2013; Djoudi *et al*., 2013; Seni *et al*. 2013; Conceicao *et al*., 2014) often associated with MSSA (Biber *et al.,* 2012; Conceicao *et al*., 2014). PVL-positive ST152 (t355) *S. aureus* is a common clone in several African countries (Ruimy *et al*., 2008; Bruerec *et al*., 2011; Elazhari *et al.,* 2012; Kolawale *et al*., 2013). This clone is rare in non-African countries and mainly associated to PVL-positive MRSA as described from the Balkan region (Monecke *et al*., 2007; Francois *et al*., 2008). Among the MRSA clones, ST88-IV has been reported frequently in several African countries (Chua *et al*., 2008; Alabi *et al*., 2013; Moremi *et al*., 2013; Schaumburg *et al*., 2014) ST80-IV is a common MRSA clone in North Africa (Breurec *et al*., 2011; Schaumburg *et al*., 2011; Tokajian, 2014) and ST612-IV in South Africa (Moodley *et al*., 2010; Oosthuysen *et al.,* 2013). Other frequent MRSA clones found in Africa include ST5, ST8, and ST239/241 (Breurec *et al*., 2011; Moremi *et al*., 2012; Scahumburg *et al*., 2014).

2.18 The Perspective of *S. aureus* from the Pig Industry in Nigeria

In Nigeria, small scale pig production often referred to as backyard piggeries is a very lucrative business with the income for family support. *S. aureus* is not a significant

pathogen in pig production and in line with the fact that only a single report is available on the occurrence of *S. aureus* in pigs and pig workers in Africa (Fall et al., 2012). This maybe indication of little or no interest in the study of the bacterium in pigs in this region. In Europe and the US, human infections have been caused by this germ, however, in Africa and Nigeria at large there are no reports on LA- *S. aureus* in both humans and animals even though some of other MRSAs have been incriminated in hospitalized patients (Fadeyi *et al*., 2010) and this could simply be because they are not routinely screened for or have not been intensely researched.

2.19 Prevention and Control

Spread of *S. aureus* (including MRSA) generally is through human-to-human, animal-human contact and vice versa (Sing *et al*., 2008). Emphasis on basic hand washing techniques are, therefore, effective in preventing its transmission. The use of disposable aprons and gloves by staff reduces skin-to-skin contact and, therefore, further reduces the risk of transmission. Transmission of the pathogen is facilitated in settings where worker hygiene is insufficient. *S. aureus* is an incredibly hardy bacterium, as was shown in a study where it survived on polyester for just under three months (Neely and Maley, 2000) polyester is the main material used in hospital privacy curtains.

The bacteria are transported on the hands of human and animal healthcare workers, who may pick them up from a seemingly healthy patient carrying a benign or commensal strain of *S. aureus*, and then pass it on to the next being treated. Introduction of the bacteria into the bloodstream can lead to various complications, including, but not limited to endocarditis, meningitis, and sepsis.

Pig workers who are found to carry resistant strains of *S. aureus* may be required to undergo "eradication therapy", which may include antiseptic washes and shampoos (such as chlorhexidine) and application of topical antibiotic ointments (such as mupirocin or neomycin) to the anterior nares of the nose. *S. aureus* is killed in 1 minute at 78°C and 10 minutes at 64°C (Shafiei *et al*., 2011). In summary, active surveillance and decolonization are the two main targeted control measures for reducing the transmission of both MSSA and MRSA (Robotham *et al*., 2011). The use of protective clothing when working with livestock in the farm and routine use of disinfectant and proper cleaning of the environment cannot be overemphasized. Controlled use of antibiotics for either therapeutic or as growth promoters and public education of livestock workers and the general public on the transmission of infectious agents is also a very important aspect of controlling the spread of microorganism.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Major equipment

Incubator, weighing balance, centrifuge, binocular microscope, autoclave, deep freezer, hot air oven, refridgerator, thermocycler (PCR) machine, heating block, water bath, electrophoresis machine, computer, photographic machine, votex machine and eppendorf centrifuge.

3.1.2 Minor equipment

Bijou bottles, glass slides, petri dishes, test tubes, measuring cylinder, conical flasks, pipettes, Bunsen burner.

3.2 Methods

3.2.1 Study area

The study was conducted in Plateau state. Piggeries located in Jos North and Jos South local government areas of Plateau state were selected for the study and also, the main abattoir in Jos metropolis (fig 3.1). Plateau state is a tropical area with cool and windy climate and the mean ambient temperature ranges between a minimum of 18.7°F and 51.7°F. The coldest wheather is between the months of December and February. The warmest temperatures usually occur in the dry season months of March and April. The state is endowed with natural resources and livestock with majority of the people engaged in farming and keeping of livestock such as pigs (Qadeer, 2008).

3.2.2 Sampling technique and sampling location

Based on convenience sampling, two local government areas in Plateau state, namely Jos North and Jos South were selected for this study. Five communities were identified in the two local government areas based on their pig production activities. For Jos South LGA,

the selected three communities are Zawan, Vom and Gyel and for Jos North LGA, Kabong and Tudunwada.

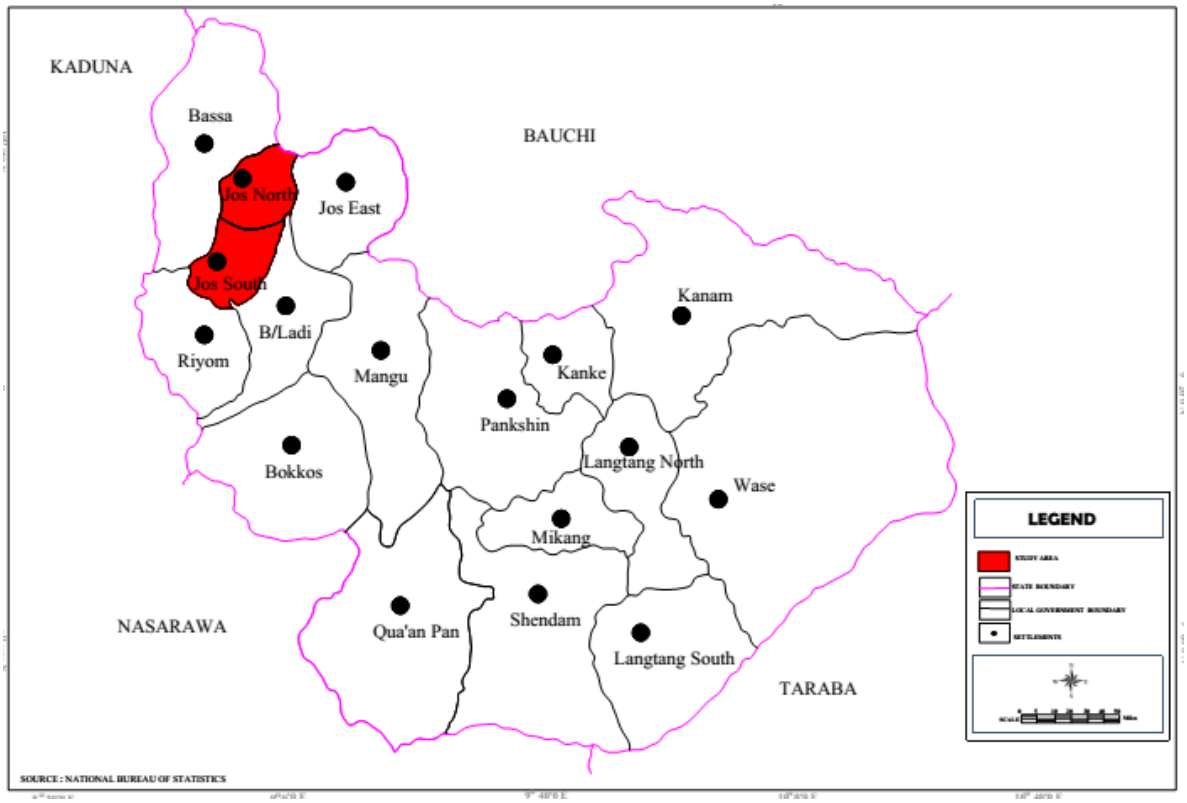

Fig 3.1: Map of Plateau State showing Jos North and South Local Government areas.

Source: Department of Geography, Ahmadu Bello University, Zaria.

3.2.3 Study design and sample collection

A cross-sectional study was conducted based on convenience sampling of 30 selected piggeries in the study areas and from the Jos main abattoir. Sterile swab were used to collect nasal swabs from pigs and humans by rotating the swab stick. Using simple random sampling technique, twenty percent (20%) of pigs in every farm visited was sampled while

for abattoir samples, every third pig to be slaughtered was sampled. Human samples from farms and abattoir workers were collected based on consent and availability from each of the sampled locations on a weekly basis. For each animal and human sampled, the following information were recorded; date, age and sex. Nasal swabs were collected from humans and pigs from six different locations namely; Tunduwada (T/wada), from Vom, Kabong, Gyel, Zawan and Abattoir as shown on Table 3.1.

A structured questionnaire (Appendix I) regarding risk factors for *S. aureus* colonization was administered to participating pig farmers and persons who work in the pig section of the abattoir. Obtained samples were properly labeled, stored on an ice pack and transported to the Bacterial Zoonoses Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria for processing.

Table 3.1: No of samples collected from humans and pigs at six Locations

S/No	Location	Humans	Pigs
1	T/Wada	5	42
2	Vom	13	32
3	Kabong	21	38
4	Gyel	14	36
5	Zawan	11	46
6	Abattoir	37	107
Total		101	300

3.2.4 Sample size determination

The sample size was calculated using the formula of Thrusfield (2005).

Prevalence for pigs**;** $n = \frac{Z^2pq}{d^2}$

Where n = sample size of the study

q= 1-p

p= expected prevalence (12.5%) in Senegal (Fall *et al*., 2012).)

Z = standard normal deviation for the desired confidence= 1.96

d= degree of precision 5%= 0.05.

$n=\frac{1.96^2 \times 0.125 \times (1-0.125)}{(0.05)^2}$= 168 pigs from backyard farm

For pigs at slaughter in the abattoir, 7% prevalence (Bayleyegn *et al.*, 2012) was used to calculate the sample size and a total of 100 was arrived at, while occupationally exposed (pig farmers and abattoir workers) human samples were collected based on availability and consent, however, 101 occupationally exposed human samples were taken for this study.

3.2.5 Criteria used for sample collection

3.2.5.1 Inclusion criteria

a. Pigs owners consent
b. Oral consent from farm and abattoir workers
c. Pigs that were adequately restrained for samples collection
d. Farms with minimum of 5 pigs.
e. Only occupationally exposed individuals (pig farmers and Abattoir workers) were sampled

3.2.5.2 Exclusion criteria

a. Pigs owners disapproval
b. Workers who do not give consent.
c. Pigs that cannot be restrained for samples to be collected
d. Farms with less than 5 pigs.

3.2.6 Ethical Clearance for Human Samples

Ethical clearance (Appendix II) for the collection of human samples was obtained from the Ministry of Health, Jos, Plateau State, Nigeria.

3.2.7 Isolation and characterization of *Staphylococcus* species using conventional methods.

3.2.7.1 Enrichment

Swab samples were inoculated into 5ml Brain-heart infusion broth containing 6.5% NaCl for enrichment and incubated for 24 h at 37°C.

3.2.7.2 Conventional culture

A loopful of broth was inoculated onto Baird Parker agar and incubated for 48 h at 37ºC, following which suspected colonies were subcultured onto tryptic Soy agar and incubated at 37ºC for 24 h so as to obtain pure colonies.

3.2.7.3 Morphological identification and Gram staining

Characteristic growths on each medium were subjected to bacteriological identification; the size, shape, colour were noted. On examination microscopically, isolates that appear as violet cocci, predominantly clusters were selected for further identication procedures.

3.2.7.4 Haemolysis on blood agar

Haemolysis was carried out by plating the isolates onto 5% sheep blood agar and incubated for 24 h at 37ºC. Complete clearing of the blood was indicative of beta haemolysis, partial clearing was alpha haemolysis while no clearing was indicative of no haemolysis.

3.2.7.5 Biochemical characterization

Each isolate was stained using Gram staining and positive isolates were subjected to sugar fermentation (glucose, xylose and mannitol), catalase test, the tube coagulase test and Dnase. The tube method for coagulase test was carried out as follows: 0.5ml of rabbit serum was diluted at 1:9ml of distilled water. It was then mixed with the test isolates in a tube and incubated at 37°C for 4-24 h, clotted plasma indicated positive result. For catalase test, a small smear of the isolate was made on a glass slide and a drop of hydrogen peroxide was placed on the test isolate, presence of effervescence indicated catalase positive.

3.2.7.6 Deoxyribonuclease (Dnase) test

Dnase test was carried out by culturing the isolates on a Dnase agar medium and incubated at 37ºC for 24 h and a zone of clearing around the streaked areas after adding a drop of 1N HCl was indicative of Dnase activity.

3.2.7.7 Storage of pure isolates

All isolates positive for catalase, coagulase, Gram stain, glucose, mannitol, Dnase were then stored on nutrient agar for further analysis. Also isolates that were coagulase negative were also stored.

3.2.8 Confirmation of *Staphylococcus* isolates

3.2.8.1 Microgen 1DStaph confirmation

The test was carried out and interpreted based on the instructions of the manufacturer. An homogenous suspension was prepared by mixing 2-5 isolated colonies in 3ml staphylococci suspending medium provided in the kit (Microgen Bioproducts, U.K), then four drops of the bacterial suspension were added to each well with a Pasteur pipette; well no 7 indicated by a black circle on the test strip coated with arginine was overlaid with two drops of mineral oil. The inoculated test strips were incubated at 35°C ± 2ºC for 24hr. After incubation, two drops of fast blue reagent was added to well no 12 indicated by a green circle on the test strip (Beta galactosidase). A colour change within 5-10seconds to plum purple was observed for positive test. All results were recorded on the organism ID report forms provided in the kit and interpreted using the microgen identification package.

3.2.8.2 Confirmatory test using MALDI-TOF MS (mass assisted laser desorption ionization time-of-flight ms) technique

All S. aureus isolates were confirmed using MALDI-TOF technique. Sample preparation for MALDI-TOF MS: Ethanol/ formic acid extraction procedure was followed according to the manufacturer's instruction (Bruker Daltonik GmbH, Bremen, Germany). Briefly, 2-3 isolated colonies were transferred into the 1.5 ml screw cap extraction tube containing 300 µl of double distilled water with an inoculation loop and mixed thoroughly. Absolute ethanol (0.9 ml) was added, the contents of the tube were carefully mixed, and the tubes were then centrifuged at 13,000 × g for 2 min; the supernatant was centrifuged at 13,000 × g for 2 min, then 1 µl of the supernatant was placed onto a ground steel MALDI target plate and allowed to dry at room temperature. Subsequently, each sample was overlaid with 1 µl of matrix solution, which consisted of a saturated solution of α-cyano-4 hydroxy-cinnamic acid (HCCA) in 50 per cent acetonitrile and 2.5 percent trifluoroacetic acid (final concentration: 10 mg

HCCA/ml) and air dried at room temperature. The MALDI-TOF MS target was subsequently introduced into the MALDI-TOF MS mass spectrometer (Brucker Daltonik GmbH, Bremen, Germany) for automated measurement and data interpretation using the MALDI Biotyper software package with the reference database v.2.0 by AHL.

3.2.9 Antibiotic susceptibility testing

To demonstrate phenotypic properties, strains were tested for their antibiotic susceptibility patterns using the disc diffusion method for 10 antibiotics by single disc method (Bauer *et al*., 1966). The following antibiotics were used: ampicillin (10µg), cefoxitin (30µg), ceftriazole (30µg), erythromycin (15µg), gentamicin (10µg), oxacillin (1µg), penicillin (10units), sulphamethoxazole/trimethoprim (25µg), tetracycline (30µg), and vancomycin (30µg). Procedure: one or two colonies of the isolates was emulsified in 5ml of sterile normal saline to prepare an equivalent of 0.5 McFarland standard and with the aid of a sterile swab the inoculum was spread evenly onto prepared Mueller-Hinton agar plate. With the aid of a disk dispenser, 5 antibiotics each were dispensed onto the Mueller-Hinton agar plate. The plates were incubated at $37^{o}C \pm 2^{o}C$ for 24 h. After incubation, zones of inhibition around each antibiotic were measured to the nearest millimeter and recorded as susceptible (S), intermediate (I) and resistant (R) based on the Clinical and Laboratory Standards Institute guideline (CLSI, 2011), (Appendix III).

3.2.10 Minimum inhibitory concentrations (M.I.Cs)

MICs of vancomycin was determined using the Episolmeter strip (Etest strip) (Oxoid). This was carried out as described by the manufacturer and interpreted using the CLSI guidelines (CLSI, 2011). Procedure: 0.5 McFarland standard bacterial suspension was

made from an over night culture plated on Mueller Hinton agar on which the Etest strips were placed with a forcep one on each plate. The plates were incubated for 24 h at 37ºC. After the period of incubation, an eclipse corresponds to the antimicrobial concentration no longer inhibitory to the growth of the organism. The corresponding concentration of antibiotic at the point of intersection on the strip were read as the MIC in µg/ml.

If the growth intersects on the line between two sections, the MIC was read as the value in the lower section. If there was growth along the entire length of the strip, the MIC was read as greater than the highest value on the M.I.C.E scale. MIC values were interpreted as susceptible (S), intermediate (I) and resistant (R).

The *S. aureus* isolates were further subjected to antibiotic susceptibility at the Statens Serum Institut (SSI). The antibiotic susceptibility of the isolates to penicillin, ceftaroline, ceftobiprole, clindamycin, gentamicin, daptomycin, oxacillin, teicoplanin, vancomycin, gentamicin, tetracycline, ciprofloxacin, moxifloxacin, trimethoprim/sulfamethoxazole, fusidic acid, erythromycin, rifampicin, mupirocin, norfloxacin, linezolid and tigecycline was determined by the broth microdilution method using sensititre custom plates (Thermo Fisher). *S. aureus* ATCC 29213 was the control strain and resistance was determined following European Committee on Antimcrobial Sensitivity Testing (EUCAST, 2013) guideline.

3.2.11 Molecular characterization of *S. aureus* isolates

The molecular studies were carried out in the Reference Laboratory for Staphylococci and Antimicrobial Resistance in Statens Serum Institut, Copenhagen, Denmark.

3.2.11.1 DNA extraction

Crude DNA of the isolates was used for the PCR. Template DNA was prepared by simple boiling. Briefly, portions of individual bacterial colonies were suspended in 200µl of lysis buffer containing 1% Triton X-100, 0.5% Tween 20, 10mM Tris-HCl (pH 8.0), and 1mM EDTA and incubated in a screw cap reaction tube for 10 minutes in a boiling water bath. After centrifugation for 2minutes at 10,000 x g to sediment the debris, a 2µl aliquot of the clear supernatant was then used for the various PCR reactions.

3.2.11.2 Multiplex polymerase chain reaction (PCR)

The detection of *mec*A, *pvl*, *scn* and *spa* A genes was carried out using a multiplex polymerase chain reactions (PCR) as described by Larsen *et al*. (2008). Briefly, DNA templates was prepared according to Kumari *et al*. (1997). Each PCR reaction mixture contained 0.45 µM *mec*A primers, 0.18 µM *spa* primer, 1 µM *pvl* primers, 0.25 µM *scn* primers, 1 X multiplex PCR mastermix (Qiagen, Valencia, CA, USA) and 1 µl of DNA template preparation. Pre-staining of the PCR product was done using Ethidium bromide. Amplification was performed in a DNA Engine DYAD (Bio-Rad, Hercules, CA, USA) with initial denaturation for 15min at 94^{o}C, followed by 30cycles of 30s at 94^{o}C, 1min at 59^{o}C and 1min at 72^{0}C, with a final 10 min at 72^{0}C. The resultant PCR products were visualized on electrophoresis gels 2% w/v (Invitrogen, Carlsbad, CA, USA). The expected amplicon size and primer sets are shown on Table 3.1.

Table 3.2: Primer sets used for detection of *mec*A, *spa*, *pvl* and *scn* genes

Target gene	Primers	Amplicon size (bp)	Reference
*mec*A P4	5'-TCCAGATTACAACTTCACCAG-3'	162	Larsen *et al*., (2008)
P7	5'-CCACTTCATATCTTGTAACG-3'		
spa FP	5'-TAAAGACGATCCTTCGGTGAGC-3'	Variable	Larsen *et al*., (2008)

	RP	5’-CAGCAGTAGTAGTGCCGTTTGCTT-3’	(165-550)	
pvl	FP	5’-GCTGGACAAAACTTCTTGGAATAT-3’		
	RP	5’-GATAGGACACCAATAAATTCTGGATTG-3’	80	Larsen *et al*., (2008)
scn	FP	5’-TACTTGCGCGAACTTTAGCAA-3’	130	
	RP	5’-AATTCATTAGCTAACTTTTCGTTTTGA-3’		

The detection of staphylococcal enterotoxin (SE) genes was carried out using 2 separate multiplex PCRs. Each PCR reaction contained 0.25 µM each of primers *sea-see* and *seh-sej* and 12.5 µl PCR master mix (Jena Bioscience Co. Jena, Germany) and 1µL of DNA template.The PCR products were pre-stained using Ethidiun bromide and amplification was performed in a DNA Engine DYAD (Bio-Rad, Hercules, CA, USA) with initial denaturation for 5min at 94^{o}C, followed by 30cycles of 30s at 94^{o}C, 1min at 59^{o}C and 2min

at 72^0C, with a final 5 min at 72^0C. Then PCR products were visualized on electrophoresis gels 2% w/v (Invitrogen, Carlsbad, CA, USA. The expected amplicon size and primer sets are shown on Table 3.2.

Table 3.2 Primer sets for *sea-see* and *seh-sej*

Target gene	Primers	Amplicon size (bp)	Reference
M1- *sea-see*			
sea	SEA-3 CCTTTGGAAACGGTTAAAACG SEA-4 TCTGAACCTTCCCATCAAAAAC	127	Larsen *et al*., 2008
seb	SEB-1 TCGCATCAAACTGACAAACG SEB-4 GCAGGTACTCTATAAGTGCCTGC	477	

sec	SEC-3 CTCAAGAACTAGACATAAAAGCTAGG	271		
	SEC-4 TCAAAATCGGGATTAACATTATCC			
sed	SED-3 CTAGTTTGGTAATATCTCCTTTAAACG	319		
	SED-4 TTAATGCTATAGATAAAGTTAAAACAAGC			
see	SEE-3 CAGTACCTATAGATAAAGTTAAAACAAGC	178		
	SEE-2 TAACTTACCGTGGACCCTTC			
M2-*seh-sej*				
seh	SEH-1 TTAGAAATCAAGGTGATAGGGTGGC	235	Larsen *et al*., 2008	
	SEH-2 TTTTGAATACCATCTACCCAAAC			
sei	SEI-1 GCCACTTTATCAGGACAATACTT	330		
	SEI-2 AAAACTTACAGGCAGTCCATCTC			
sej	SEJ-1 CTCCCTGACGTTAACACTACTAATAA	641		
	SEJ-2 TTGTCTGGATATTGACCTATAACATT			

3.2.11.3 spa A gene typing of S. aureus.

The variable region of the *Staphylococcus* protein A (*spa*) gene was amplified as described previously (Harmsen *et al*., 2003) and sequenced. Isolates were assigned to *spa* type using Ridom StaphType software (Ridom GmbH, Germany). Associations of the assigned *spa* type with multilocus sequence type (MLST) were determined using the Ridom spa server database (www.spaserver.ridom.de/).

3.2.11.4 DNA based microarray analysis

DNA based microarray typing was used to analyze the gene content of the isolates (genes encoding resistance and virulence associated genes). The Alere StaphType based microarray was used in this study. According to manufacturer's instruction, DNA was extracted and quantified following manufacturer's instruction. DNA probes are bound to a solid surface (microwell) to detect the presence of complementary sequences of the strain tested in an already prepared Arraystrips. Extraction of DNA was performed as follows: a loopful of an overnight culture was added to 10µl of proteinase K and 100µl of the lysis buffer and then vortexed briefly. The mixture was then incubated for 30-60 min at 56ºC and 550 rpm in the thermoshaker. Then 200µl of ethanol was added to the mixture and vortex and centrifuged. The content was then transferred into a spin column that was placed in a 2 mL collection tube. It was centrifuge at room temperature and all collected liquids were washed and incubated at room temperature for 5 min to elute DNA. DNA concentration was then measured using a spectrophotometer. DNA targets obtained were amplified, biotin labelled and hybridized to the DNA microarray targeting 336 genes.

Amplification and Internal Biotin-Labelling was carried out as follows: a Master Mix was prepared by combining 4.9 µl of labelling reagent and 0.1 µl of labelling enzyme. Then 5 µl of DNA was added to a 5 µl aliquot of the Master Mix. Then amplification was performed in a DNA thermocycler with initial denaturation for 60 sec at 96ºC, followed by 55 cycles, 20 sec at 50ºC, with a final 40 sec at 72ºC. It was then allowed to cool down to 4ºC

Hybridisation was performed by the following procedure. All 30 µl of labeled PCR product was baked in a thermoshaker for about 2 h at 55°C until dry and then diluted in 200 ul of hybridisation buffer. The mixture was then incubated at 55°C for 60 min and 550 rpm in a water bath. After incubation, the array strip was washed sequentially in a wash solution

four times, 100 µl of conjugated buffer solution (horseradish Peroxidase Substrate) was used to fill the wells and incubated for 15 min and washing was repeated again then the strip was dried under a gentle airstream before it was scanned. Thereafter, the microwell plates were run on the Arraymate reader for 20 minutes and affiliation to a specific lineage was done by Iconoclust software based on the hybridisation profile.

3.2.12 Questionnaire survey

Structured questionnaire were administered to pig farmers and abattoir workers regarding possible risk of *S. aureus* colonization. One questionnaire was administered to each of the farm (30) visited while only 20 out of the total questionnaire administered to abattoir workers who also had pig farms were retrieved. In total, fifty (50) questionnaire were analyzed for the study. Risk factors included medical history and contact with pigs.

3.2.13 Data analyses

Data were analyzed with the aid of the Graphpad prism version 5.0 for windows. Chi-square and Odds ratio were used to analyze for association between variables from in-contact humans and pigs and carriage of *S. aureus*. Values of $p < 0.05$ were considered significant.

CHAPTER FOUR

RESULTS

4.1 Occurrence of Coagulase Negative Staphylococcus species, *S. hyicus* and *S. intermedius* and Antibiotic Resistance Pattern

4.1.1 Coagulase negative *Staphylococcus* species (CoNS), *S. hyicus* and *S. intermedius*

4.1.1.1 Prevalence of coagulase negative Staphylococcus species (CoNS), S. hyicus and S. intermedius identified in both humans and pigs

Out of the 401 samples collected, 53 of them were identified as coagulase negative *Staphylococcus* species while 20 and 17 were *S. hyicus* and *S. intermedius*, respectively. The following eight (8) different CoNS species were identified; *S. haemolyticus* 18.9% (10/53), *S. xylosus* 22.6% (12/53), *S. chromogen* 16.9% (9/53), *S. simulans, S. warneri, S.*

schleferi and *S. epidermidis* all had 59.4% (5/53), respectively and *S. hominis* showed the least percentage of occurrence 3.8% (2/53) (Table 4.1).

4.1.1.2 Antibiotic Resistance Pattern exhibited by the different CoNS species, S. hyicus and S. intermedius

Table 4.2 shows that most of the CoNS species were resistant to penicillin, tetracycline and trimethoprim while a few were resistant to phenicol (chloramphenicol), cephalosporin (cefoxitin and oxacillin) and aminoglycoside (gentamicin and vancomycin). The in-vitro antimicrobial resistance profile of all the CoNS isolates tested and by each species are summarised in (Figure 4.1 and Table 4.2).

Table 4.1 Distribution of Coagulase Negative *Staphylococcus* (CoNS), *S. hyicus* and *S. intermedius* among humans and pigs

CoNS/CoPS species	Humans (%)	Pigs (%)
S. haemolyticus	8 (80)	2 (20)
S. hycuis	0 (0)	20 (100)
S. intermedius	6 (35.3)	11 (64.7)
S. simulans	2 (40)	3 (60)
S. warneri	3 (60)	2 (40)
S. schleiferi	2 (40)	3 (60)

S. xylosus	4 (44.4)	8 (66.7)
S. chromogen	1 (11.1)	8 (88.9)
S. epidermidis	5 (100)	0 (0)
S. hominis	2 (100)	0 (0)
Total	33	57

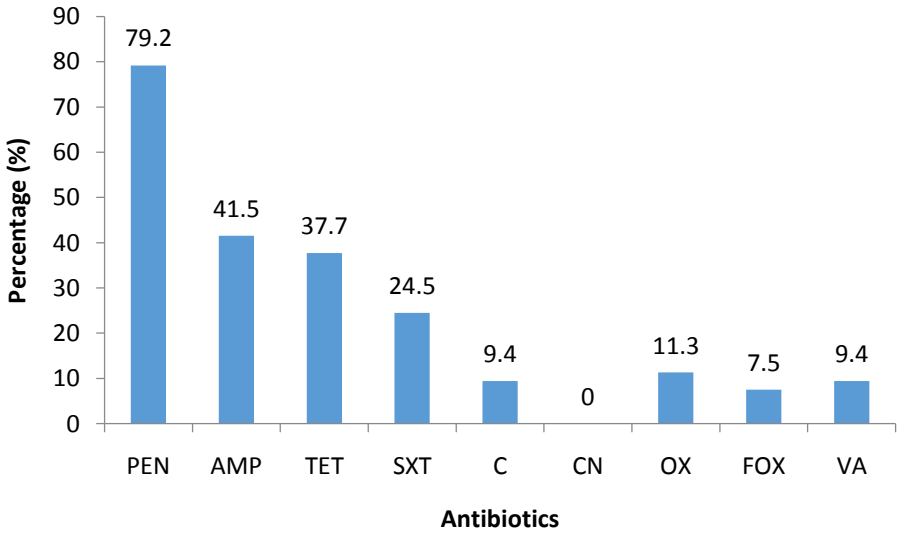

Figure 4.1 shows the percentages and antibiotic resistance profile of all CoNS, *S.hyicus* and *S. intermedius* isolates tested.

Keys; PEN = Penicillin, AMP = Ampicillin, TET = Tetracycline, SXT = Trimethoprim/sulphamethoxazole, C = Chloramphenicol, CN = Gentamicin, OX = Oxacillin, FOX = Cefoxitin, VA = Vancomycin.

Table 4.2. Prevalence of antimicrobial resistance by species of coagulase negative staphylococci isolates from humans and pigs

Antibiotics	No of Resistant staphylococci species (%)									
	S. haemolyticus (n = 10)	*S. simulas* (n = 5)	*S. warneri* (n = 5)	*S. schleiferi* (n = 5)	*S. xylosus* (n =12)	*S. chromogens* (n = 9)	*S. hominis* (n = 2)	*S. epidermidis*(n = 5)	*S. hyicus* (n = 20)	*S. intermedius* (n = 17)
PEN	8 (80)	3 (60)	4 (80)	2 (40)	6 (50)	9 (100)	2 (100)	4 (80)	14 (70)	7 (41)
AMP	6 (60)	0 (0)	2 (40)	2 (40)	4 (33)	5 (56)	1 (50)	2 (40)	4 (20)	2 (12)
TET	5 (50)	2 (40)	1 (20)	2 (40)	3 (25)	5 (56)	2 (100)	0 (0)	6 (30)	3 (18)
SXT	4 (40)	0 (0)	1 (20)	1 (20)	2 (16.5)	4 (44)	1 (50)	0 (0)	4 (20)	2 (12)
C	1 (10)	0 (0)	0 (0)	1 (20)	2 (16.5)	1 (11)	0 (0)	0 (0)	2 (10)	1 (6)
CN	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (6)
OX	0 (0)	0 (0)	0 (0)	1 (20)	3 (25)	2 (22)	0 (0)	0 (0)	1 (5)	1 (6)
FOX	0 (0)	0 (0)	0 (0)	0 (0)	3 (25)	1 (11)	0 (0)	0 (0)	1 (5)	1 (6)
VA	0 (0)	1 (20)	0 (0)	1 (20)	2 (16.5)	1 (11)	0 (0)	0 (0)	1 (5)	1 (6)

Keys; PEN = Penicillin (10 units), AMP = Ampicillin (10 µg), TET = Tetracycline (30 µg), SXT = Trimethoprim/sulphamethoxazole (25 µg), C = Chloramphenicol (30 µg), CN = Gentamicin (10 µg), OX = Oxacillin (1 µg), FOX = Cefoxitin (30 µg), VA = Vancomycin (30 µg).

4.1.1.3 Most occurring patterns of multidrug resistance by CoNS, S. hyicus and S. intermedius isolatates

Table 4.3 summarises the number of isolates resistant to 3 or more antibiotic agents tested. Of the 90 isolates tested, 48.9% (44/90) were resistant to 3 or more of the 10 antimicrobial agents tested. All the isolates showed almost similar resistance pattern. S. *hyicus* had the most occurring resistance pattern type (5) closely followed by S. *intermedius* (4). Generally, 3 pattern types were most occurring; (PEN), (PEN, AMP, TET) and (PEN, AMP, TET, SXT).

4.1.1.4 Vancomycin MIC values for S. hyicus and S. intermedius

The values of the 2 *S. hyicus* isolates that were resistant to vancomycin had MIC values of 2 and 0.12 µg/ml respectively while the 5 *S. intermedius* isolates had MIC values >256 µg/ml (Table 4.4).

4.1.2 Coagulase positive *Staphylococcus* species (CoPS)

4.1.2.1 Prevalence of S. aureus based on different techniques

Out of the four hundred and one samples collected from both humans (101) and pigs (300) in this study, 120 (30%) of the total samples were positive for *S. aureus* based on conventional biochemical testing; 63 (53%) were human samples while 57 (47%) were pig samples. After subjecting the 120 isolates to further test using Microgen kit, 50 (42%) were positive; 17 (34%) were humans while 33 (66%) were pigs. The 50 isolates were further confirmed by MALDI-TOF and 29 (58%) were *S. aureus*; 13 (44%) were human isolates and 16 (55%) were isolated from pigs (Table 4.5). Therefore, an overall prevalence of 7.2% (29/401) *S. aureus* was observed from this study with pigs showing 16/300 (5.3%) and humans 13/101 (12.8%) respectively (Table 4.5).

Table 4.3: No of CoNS isolates resistant to 3 or more antibiotics and the resistance patterns most frequently observed among the isolates

CoNS species (n = 8)	No of isolates tested	No of isolates resistant to 3 or more antibiotics (%)	No of resistance pattern types	Pattern types	No of isolates (%)
S. haemolyticus	10	5 (50)	7	PEN	2 (20)
				PEN, AMP, TET	2 (20)
				PEN, AMP, TET, SXT	2 (20)
S. simulans	5	1 (20)	3	PEN, TET	2 (40)
S. warneri	5	1 (20)	4	PEN	2 (40)
S. schleiferi	5	2 (40)	3	PEN, AMP, TET	2 (40)
S. xylosus	12	5 (42)	7	PEN, AMP, TET	2 (17)
				PEN, TET, SXT	2 (17)
S. chromogen	10	6 (60)	7	PEN, TET, SXT,	2 (20)
S. hominis	2	1 (50)	2	NONE	
S. epidermidis	5	0 (0)	2	PEN	2 (40)
				PEN, AMP	2 (40)

PEN = Penicillin, AMP = Ampicillin, TET = Tetracycline, SXT = Trimethoprim/sulphamethoxazole

Table 4.4 Values of Vancomycin Minimum Inhibitory Concentration for *S. hyicus* and *S. intermedius* isolates from Humans and Pigs using Etest

Staphylococcus species	No Resistant to vancomycin	MIC (µg/ml)	Interpretation
S. hyicus	2	2	S
		0.12	S
S. intermedius	5	>256	R
		>256	R
		>256	R
		>256	R
		>256	R

Key: S= Susceptible, R=Resistant

Table 4.5: Detection of *S. aureus* in pig and human samples based on three different methods

Methods	Source		No positive (%)
	Humans (%)	Pigs (%)	
Conventional	63 (53)	57 (47)	120 (30)
Microgen	17 (34)	33 (66)	50 (42)
MALDI-TOF	13 (44)	16 (55)	29 (58)
Overall Prevalence	12.8 (13/101)	5.3 (16/300)	7.2 (29/401)

4.1.2.2 Prevalence and Association of S. aureus nasal carriage in pigs and humans based on sex, age and source of samples

The piglets had 2% (2/102) prevalence for *S. aureus* while the adult pigs had 7% (14/198). The male pigs had a prevalence of 5% (6/130) and the female 6% (10/170). The samples from abattoir gave a prevalence of 6.5% (7/107) while those from backyard farms were 5% (10/193). There were no significant ($p > 0.05$) association between the prevalence of *S. aureus* and the age, sex and source of pigs sampled (Table 4.6).

For the human samples, 25% of the children were *S. aureus* positive while 9% of the adults were positive. The females had a higher prevalence (23%) than males (7%), while samples from farms had 14% prevalence and those from abattoirs, 10.8% (Table 4.6). There was no significant association ($p> 0.05$) between the prevalence of *S. aureus* and the age and source of human samples. There was however, a significant ($p< 0.05$) association between the prevalence of *S. aureus* and the sex of the humans sampled (Table 4.6).

4.1.2.3 Antibiotic resistance profile frequencies of S. aureus isolates from pigs and humans

The twenty nine (29) *S. aureus* isolates obtained in this study were analysed for antibiotic resistance and were resistant to at least one or more antibiotic used (Table 4.7). They include beta-lactams (cefoxitin, cetabiprole, cetaroline, penicillin and oxacillin), macrolides (erythromycin, clindamycin), lipopeptide (daptomycin), aminoglycosides (gentamicin, kanamycin), floroquinolone (norfloxacin), sulphonamides (trimethoprim/sulphamethoxazole), tetracycline, rifampin, moxil, linezolide and mupirocin. For the beta-lactams, 6.9% of the isolates were resistant to cefoxitin, ceftobiprole, oxacillin and ceftaroline, 6.9% of the isolates were resistant to rifampicin while 10.3% of the isolates were resistant to moxil, gentamicin, kanamycin, and clindamycin and 13.8% to norfloxacin. Ninety seven percent (97%) of the isolates were resistant to penicillin, 62% to tetracycline, and 52% to trimethoprim. The antibiotic resistance phenotype of the *S. aureus* (MSSA)

isolates and multi-drug resistance pattern is shown in (Table 4.8). The multidrug resistance in this study is defined as resistance to two or more antibiotics. Antibiograms were used to calculate the multiple antibiotic resistance (MAR) indices. All the *S. aureus* isolates had MAR values greater than 0.2 (Figure 4.2).

Plate 4.1 shows that all the 29 isolates carried the *spa* A gene, 27 out of the 29 isolates carried the s*cn* gene while 7 (5 pig and 2 human) isolates carried the *pvl* gene. None of the isolates was found to carry the *mec* A gene.

4.2 Molecular Characterisation of *Staphylococcus* species

4.2.1 Molecular diversity of *S. aureus* isolated from pigs and humans using *spa* typing

Twenty nine *spa* types were obtained in this study with t084 (9) being the most commonly occurring followed by t355 (5) then t2216 (3). Six (6) clonal complexes (CC) were identified based on the *spa* types of which one of the CC-groups was not defined (N.D). The CC- groups identified include; CC15, CC152, CC5, CC1 and CC8. The *spa* types were clustered into each of the CC-groups. *spa* type t084, t2216, t5691 were clustered under CC15, t355 was under CC152, t311, t002 and t442 were under CC5, t1931 and t127 were grouped into CC1, only t304 was under CC8, while the CC groups for these *spa* types (t5427, t5126 and t5576) were not defined. Table 4.9 shows the molecular *spa* characteristics of *S. aureus* from pigs and humans. Twenty seven (27) out of the 29 *S. aureus* isolates recovered from both pigs and humans carried the *scn* gene encoding staphylococcal complement inhibitory gene (SCIN) with each of the CC-group harbouring at least one *scn* gene. Altogether 27 (93%) of pig and human strains of *S. aureus* carried the *scn* gene while two (2) *spa* types (t5691 and t002) both from pigs did not carry the *scn*

gene. A total of 7/29 (24%) of the strains grouped into the following CC-group; CC15, CC152 and CC5 carried the *pvl* gene (Tables 4.9a and 4.9b, Plate 4.1).

4.2.2 Association of virulence determinants in *S. aureus* strains from pigs and human using microarray analysis

The following species markers were identified in all the isolates; catalase A (*katA*), coagulase (*coA*), thermostable extracellular nuclease (*nuc1*) and IgG-binding protein (*sbi*) genes. The analysis indicated that the isolates tested (n = 16 both pig and human isolates) possessed certain genes responsible for antibiotic resistance and virulence. Fourteen of the isolates carried the (*bla*Z, *bla*I, *bla*R) genes which are responsible for resistance to penicillin. Two isolates carried the *erm*A and *msr*A gene responsible for resistance to erythromycin and inducible clindamycin or constitutive clindamycin resistance respectively. Two isolates were also shown to carry the *msrA* gene which confers resistance to gentamicin. *cat-pC*221 gene which confers resistance to chloramphenicol was detected in one isolate while fourteen isolates were observed to carry the *fosB* gene which confers resistance to fosfomycin. Ten of the isolates carried the *tetK* gene which is responsible for resistance to tetracycline. Staphylococcal enterotoxin genes were detected in some of the isolates. Two isolates were found to carry *sea* and *seh* while *seg, sei, sem, sen, seo,* and *seu* were detected in 3 isolates. Amongst these enterotoxin genes detected, the following; *seg, sei, sem* and *sen*, were detected in the isolates from CC5, while *sea* gene was detected in CC15. All of the isolates were observed to harbour the accessory regulatory genes, *agr* I, II and IV with the following percentages 13%, 88% and 13%, respectively. All the strains carrying *agr* gene were from pigs. All the virulence factors except for the presence of *agr* II did not show any significant association ($p > 0.05$) between virulence determinants and carriage of *S. aureus*. (Tables 4.10).

4.2.3 Antimicrobial susceptibility patterns, *spa* type and presence of *agr* in *S. aureus* strains

Seven (7) *spa* types were associated with *agr* II, one *spa* type to *agr* II and IV and two *spa* types to *agr* III and IV. Two antibiotypes were associated with t084 *spa* type; Penicillin and Tetracycline (P^R, TET^R) and Penicillin, Tetracycline and Trimethoprin/sulphamethazole (P^R, TET^R and SXT^R). *spa* type t2216 strains were resistant to 2 different antibiograms; Penicillin and Tetracycline (P^R and TET^R), Penicillin, Tetracycline and Trimethoprim/Sulphamethazole (P^R, TET^R and SXT^R). All t355 *spa* types were resistant to five antibiotypes (Table 4.11).

4.2.4 Correlation between phenotypic and genotypic resistance pattern of the *S. aureus* strains

Out of the 15 isolates that were resistant to penicillin, 93.7% correlation was observed between the presence of the *bla*Z gene and resistance to the antibiotic. Seventy five percent (75%) correlation for the *tet* K gene, 100% for chloramphenicol gene (*cat-pC221*) and 18.8% for *erm* A (erythromycin) and *msr* A (clindamycin) were observed (Table 4.12).

Table 4.6: Prevalence of *S. aureus* nasal carriage in pigs and humans based on demographic variable (age, sex and location)

	Pigs					Human			
Variable	Total	No. Positive (%)	p value	OR	Variable	Total	No. Negative (%)	p value	OR
Age									
Piglet	102	2 (2)	0.100	0.262	Children	16	4 (25)	0.095	3.21
Adult	198	14 (7)			Adult	85	8 (9)		
Sex									
Male	130	6 (5)	0.797	0.774	Male	70	5 (7)	0.043*	0.264
Female	170	10 (6)			Female	31	7 (23)		
Source									
Farm	193	10 (5)	0.612	0.781	Farm	64	9 (14)	1.000	1.18
Abattoir	107	6 (6.5)			Abattoir	3	4 (10.8)		

*Denotes significant association ($p<0.05$)

Table 4.7: Antibiotic resistance frequencies of the human and pig *S. aureus* isolates

Antibiotics	No and percentages of isolates from humans and pigs resistant to:		
	Pigs (n = 16)	Humans (n = 13)	No. of isolates (n =29)
Cefoxitin	1 (6)	1 (7.7)	2 (6.9)
Ceftaroline	1 (6)	1 (7.7)	2 (6.9)
Cetobiprole	1 (6)	1 (7.7)	2 (6.9)
Clindamycin	2 (12)	1 (7.7)	3 (6.9)
Daptomycin	1 (6)	1 (7.7)	2 (6.9)
Erythromicin	3 (18)	1 (7.7)	4 (14)
Gentamicin	2 (12)	1 (7.7)	3 (10.3)
Kanamycin	2 (12)	1 (7.7)	3 (10.3)
Oxacillin	1 (6)	1 (7.7)	2 (6.9)
Penicillin	16 (100)	12 (92.3)	28 (97)
Rifampicin	1 (6)	1 (7.7)	2 (6.9)
Tetracycline	12 (35)	6 (46.2)	18 (62)
SXT	9 (53)	6 (46.2)	15 (52)
Linezolid	0 (0)	2 (15.4)	2 (6.9)
Moxilfloxacin	2 (12)	1 (7.7)	3 (10.3)
Norfloxacin	3 (18)	1 (7.7)	4 (13.8)
Mupirocin	0 (0)	1 (7.7)	1 (3.4)

SXT = Trimethoprim/sulphamethoxazole

Table 4.8: Antibiotic resistance phenotype and multi-drug resistance pattern of *S. aureus* (MSSA) from pigs and human carriage

Resistance Phenotype	Number of isolates	
	Humans	Pigs
P	3	2
P, TET	2	4
P, SXT	2	0
P, ERY	1	0
P, TET, SXT	3	5
P, SXT, NORFLO	0	1
P, TET, ERY, SXT	0	1
P, ERY, LINEZOLID, SXT, NORFLO, MOXIL	0	1
P, ERY, CN, NORFLO, MOXIL, CLIND, KAN	0	1
P, FOX, CEFTA, CEFTOB, DAP, ERY, CN, KAN, OX, RIFAMP, TET, SXT	0	1
P, FOX, CEFTA, CEFTOB, DAP, ERY, CN, KAN, OX, RIFAMP, TET, SXT, LINEZOLID, MUPIROCIN, CLIND	1	0

P = Penicillin, TET = Tetracycline, SXT = Trimethoprim/sulphamethoxazole,

FOX = Cefoxitin, NORFLO = Norfloxacin, ERY = Erythromycin,

MOXIL = Moxilfloxacin, OX = Oxacillin, CN = Gentamicin, KAN = Kanamycin,

DAP = Daptomycin, CLIND = Clindamycin, RIFAMP = Rifampicin, CEFTA = Ceftaroline

CETOB = Ceftobiprole

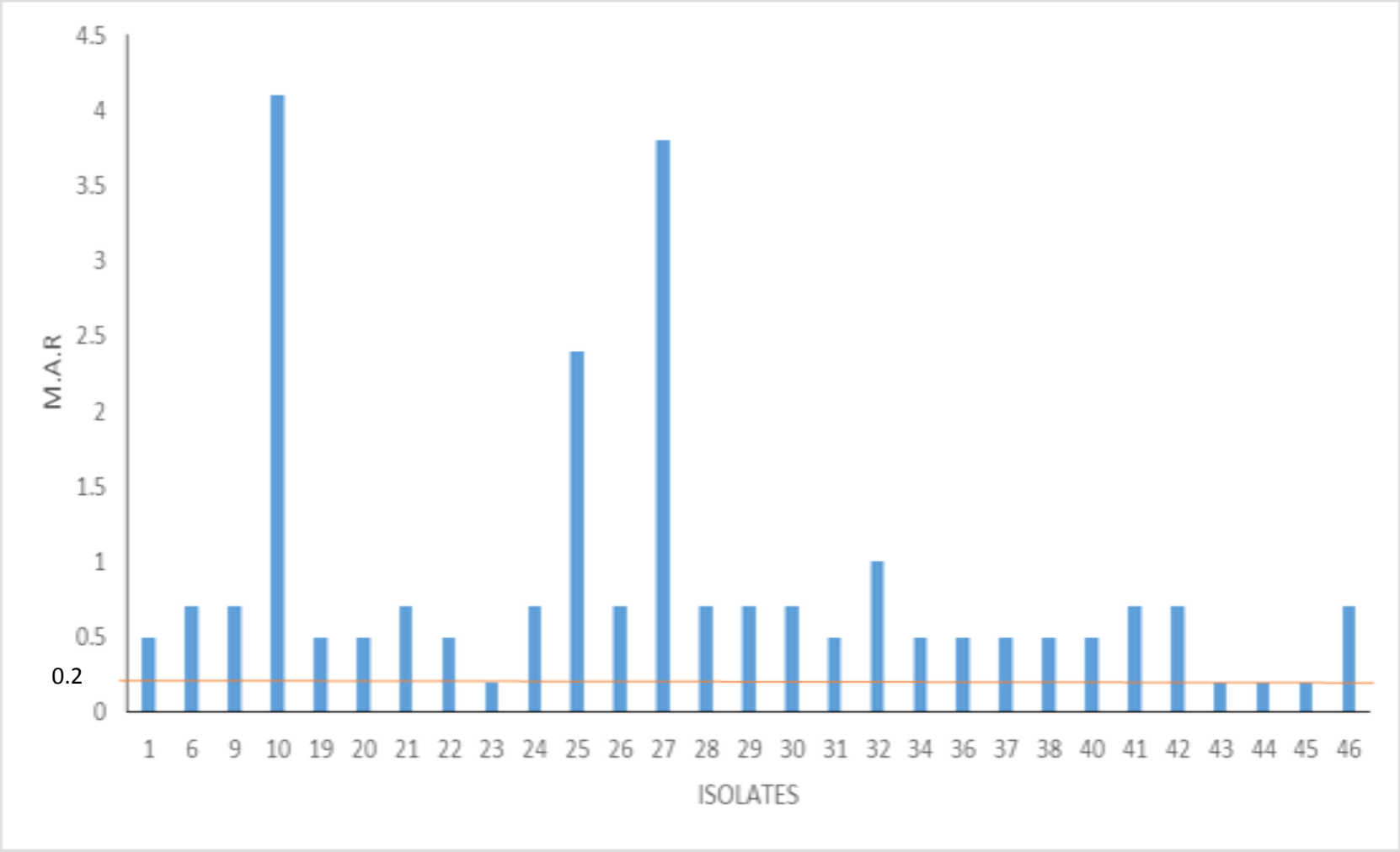

Figure 4.2 showing the M.A.R index of *S. aureus* isolates from pigs and in-contact humans.

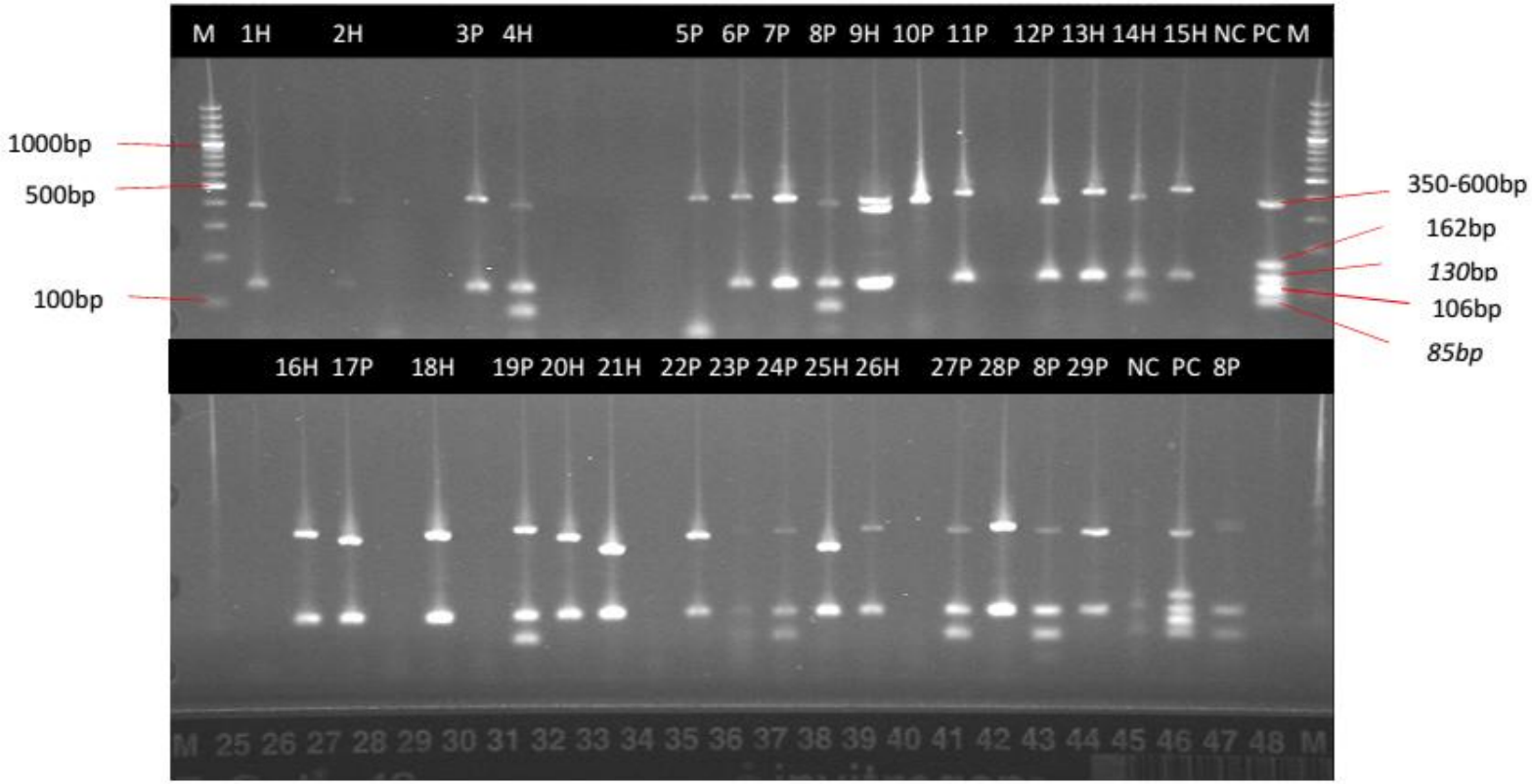

Plate 4.1 Multiplex PCR for *spa* A (350-600bp), *mec* A (162bp), *scn* (130bp), CC398 (106bp) and *pvl* (85bp) genes of *S. aureus* isolates. Molecular marker (M), NC = Negative control, PC = Positive control, H = Human strain and P = Pig strain.

Table 4.9a: Distribution of 29 *spa* types among *S. aureus* strains from occupationally exposed humans

Clonal complex (CC)	*spa* types	Humans	Pigs	No. of isolates
CC1	t1931	2	0	2
	t127	1	0	1
CC5	t311	1	1	2
	t002	0	1	1
	t442	0	1	1
CC8	t304	0	1	1
CC15	t084	2	7	9
	t2216	3	0	3
	t5691	0	1	1
CC152	t355	1	4	5
CC-N.D	t5427	1	0	1
	t5126	1	0	1
	t5576	1	0	1
Total		13	16	29

TABLE 4.9b: Clonal complexes and the *spa* types of *S. aureus* isolates from pigs and humans

Clonal complex (CC)	*spa* type and frequency	Source/No	*scn*	*pvl*
CC15	t084 (9), t2216 (3), t5691 (1)	Pig (8)	7	1
		Human (5)	5	1
CC152	t355 (5)	Pig (4)	4	4
		Human (1)	1	0
CC5	t311 (2), t002 (1), t442 (1)	Pig (3)	2	0
		Human (1)	1	1
CC1	t1931 (2), t127 (1)	Human (3)	3	0
CC8	t304 (1)	Pig (1)	1	0
N.D	t5427 (1), t5126 (1), t5576 (1)	Human (3)	3	0
Total			27 (93%)	7 (24%)

Table 4.10: Distribution of virulence genes in *S. aureus* isolates from pigs and humans.

Virulence genes	No. positive (%)	Humans	Pigs	p value
Species markers				
cat, coA, nuc1	29 (100)	29 (100)	29 (100)	
sbi	29 (100)	29 (100)	29 (100)	
cap 5 (n = 16)	5 (31)	1 (20)	4 (80)	0.3
cap 8	11 (69)	4 (36)	7(64)	
Enterotoxin genes (n = 29)				
sea	2 (7)	1 (50)	1 (50)	1.000
seg	3 (10)	1 (33)	2 (67)	1.000
sei	3 (10)	1 (33)	2 (67)	1.000
seh	1 (3.5)	1 (100)	0 (0)	0.414
sea + seh	1(3.5)	1 (100)	0 (0)	0.414
sem	3 (10)	1 (33)	2 (67)	1.000
sen	3 (10)	1 (33)	2 (67)	1.000
seo	3 (10)	1 (33)	2 (67)	1.000
seu	3 (10)	1 (33)	2 (67)	1.000
agr (n = 16)				
I	2 (13)	0 (0)	2 (100)	0.498
II	14 (88)	0 (0)	14 (100)	0.0001□
IV	2 (13)	0 (0)	2 (100)	0.498

□ denotes significant association ($p < 0.05$)

Table 4.11: Relationship of the occurrence of *spa* type, antibiogram and *agr* among 29 *S. aureus* strains isolated from pigs and humans

spa type	Antibiotic resistance phenotype	*agr*
t002	P, CLIND, ERY, CN, KAN, MOXIL, NORFLO, TET	
t084 (2)	P, TET	II
t084 (7)	P, TET, SXT	II & IV
t127	P, SXT	
t304	P, TET	
t442	P, FOX, CEFT, CEFTOB, CLIND, ERY, CN, KAN, OX, RIFAMP, TET, SXT	II
t311	P, SXT	II
t311	P, ERY, TET, SXT	II
t355	P, TET	II & IV
t355	SXT, NORFLO, MOXIL	II & IV
t355	P, SXT, NORFLO	II
/t355	P	
t355	P, FOX, CEFTAB, CEFTOB, CLIND, DAPT, CN, KAN, LINEZO, MOXIL, MUPIRO, NORFLO, OX, RIFAMP, TET, SXT	II
t2216 (2)	P	II
t2216	P, TET, SXT	II
t1931 (2)	P, TET	
t5427	P	
t5576	P, ERY, LINEZO	
t5691	P, TET	
t5126	P, SXT	

Table 4.12: Correlation between some antimicrobial resistance gene detection by micro array profiling and phenotypic detection of resistance by disc diffusion

Genes	Conferring Resistance to:	No of isolates with gene profile	Antimicrobial tested (susceptibility profile Obtained)	Correlation between genotype and phenotype		
				No of isolates Resistant	No of discrepancies	% correlation
Beta-Lactams (Excluding methicillin) Resistance gene, *bla*Z	Beta-Lactams	14	Penicillin susceptible (n=1)	15	1[e]	93.8 (15/16)
Tetracycline resistance Gene, *tet* (K)	Tetracycline	7	Tetracycline susceptible (n=4)	12	5[e]	75 (12/16)
Chloramphenicol Resistance gene, *cat-pC221*	Chloramphenicol	1	Chloramphenicol susceptible (n=15)	1	0	100 (16/16)
Erythromycin Resistance gene (*erm* A)	Erythromycin	2	Erythromycin susceptible (n = 13)	3	1[e]	18.8 (3/16)
Clindamycin Resistance gene (*msr* A)	Clindamycin	2	Clindamycin susceptible (n = 13)	3	1[e]	18.8 (3/16)

4.2.5 Presence of typing markers, antimicrobial resistance genes, virulence associated and other genes

The following virulence associated, MSCRAMM, adhesion and biofilm genes among the strains were detected; *sea, sei, seg, sem, sen* and *chp, scn, sak* and *bla*Z genes were detected in isolates from CC5. Typing marker *agr* II and *cap* 5 and 8, Sea, *chp, scn* and *bla*Z and *tet* K genes were common to isolates from CC15 while *sak, scn* and *bla*Z and *tet* K genes were the only genes associated with CC152. (Table 4. 13)

Plate 4.2 shows that only 5 out of the 29 isolates carried staphylococcal enterotoxin genes in lane 13, 15, 25, 27 and 28. Four (4) of the isolates carried *sea* gene while 1 carried *sed* gene. Plate 4.3 shows 9 isolates carrying enterotoxin (*seh. sei* and *sej*) genes in lane 1, 2, 11, 13, 17, 18, 19, 25 and 29. Six (6) out of the isolates carried *sei* while three (3) of the isolates carried *seh* genes.

4.2.6 Association between risk factors and presence of *S. aureus* among pig farmers and abattoir workers

The results revealed that adult respondents 8 (66.7%) and females 7(58.3%) had higher carriage rate than young 4 (33.3%) and male 5 (41.6%) respondents. Pig farmers had higher carriage rate 8 (66.7%) than abattoir workers 4 (33.3%). Respondents who had 5-10yrs experience had the highest carriage rate 6 (50.0%) closely followed by respondents who had spent less than 5years in the job, 4 (33.3%) carriage rate, while those with greater than 10yrs experience had 2 (16.9%). The age of the respondents, gender, occupation and years of working of the respondents did not show any significant association ($p > 0.05$) to *S.*

aureus carriage; however, medical related occupation of households showed a significant association ($p < 0.05$) between carriage and risk factors (Table 4.14).

The overall presence of the *pvl*, *cap* 5 and *cap* 8 genes among the isolates recovered from both pigs and pig workers were 21%, 31% and 69%, respectively (Figure 4.3) while the distribution of the *pvl* gene among humans and pigs were (33%) and (67%), respectively (Figure 4.4).

TABLE 4.13: *spa* types, *agr*, capsule, antibiotic resistance, virulence-associated, MSCRAMM, biofilm formation genes identified using DNA Microarray among isolates from pigs and humans

Clonal complex	*spa* type	*Agr*	*cap*	Antimicrobial Resistance genes	Virulence associated genes	MSCRAMMS, Adhesion, and Biofilm genes
CC5	t311 (2) t002 (1) t442 (1)	II	5, 8	*blaZ, fos* B	*sea, sei, seg, sem, sen, chp, scn and sak*	*bbp, clf* A, *ica* A, *ica* C, *ica* D, *ebh, ebp* S, *eno, fib, fnb* A, *fnb* B, *map, sas* G, *sdr* C, *sdr* D, *vwb* ,
CC15	t084 (9) t2216 (3) t5691 (1)	II	5, 8	*blaZ, tet* K, *fos* B	*sea,chp, scn*	*bbp, clf* A, *ica* A, *ica* C, *ica* D, *ebh, ebp* S, *eno, fib, fnb*A, *fnb* B, *map, sas* G, *sdr,* C, *sdr* D, *vwb*
CC152	t355 (5)	IV	5, 8	*blaZ, tet* K	*sak, scn*	*bbp, clf* A, *ica* A, *ica* C, *ica* D, *ebh, ebp* S, *eno, fnb* A, *fnb* B, *map, sdr* D

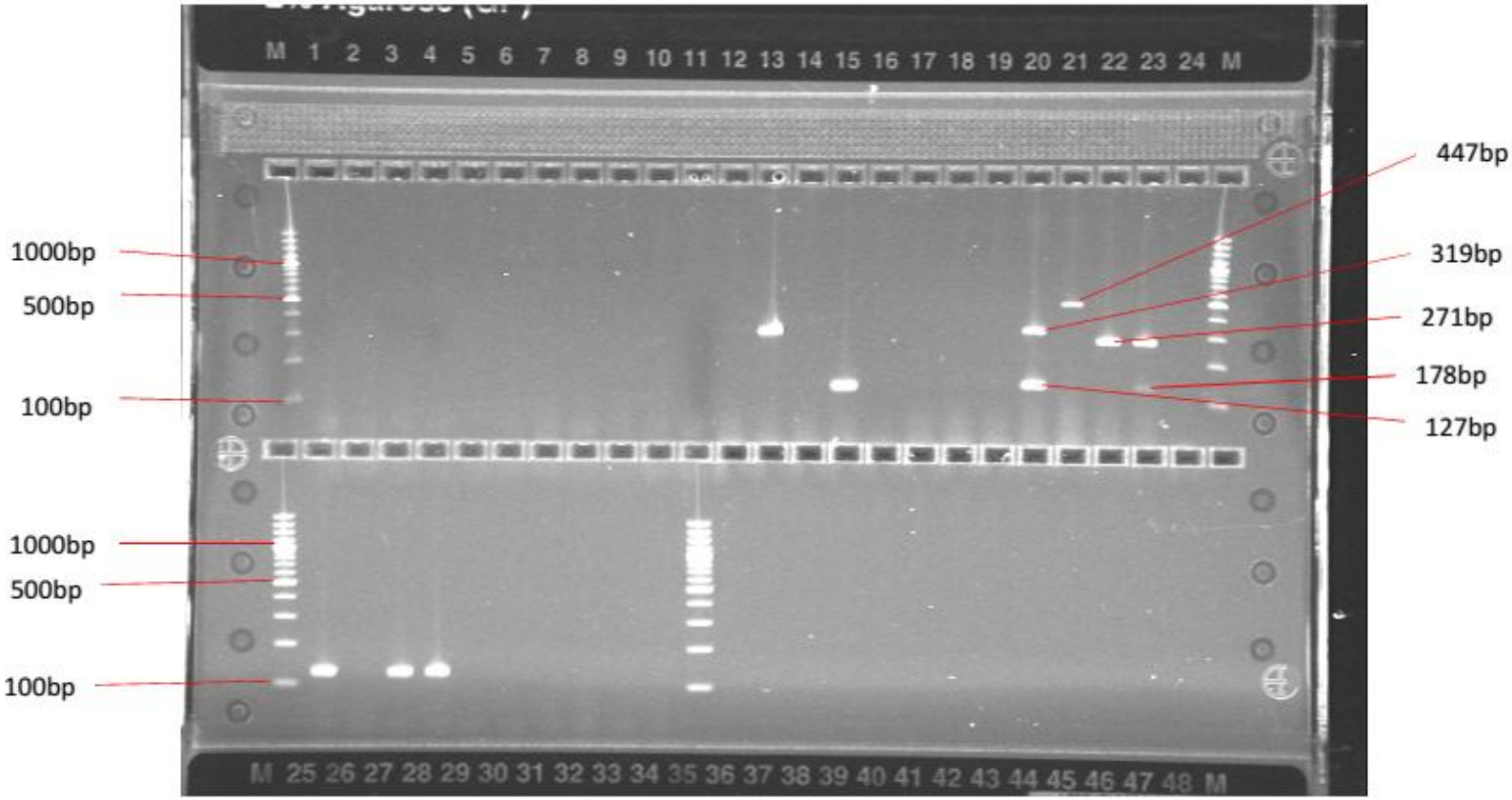

Plate 4.2 Multiplex PCR for enterotoxin A-E genes carried by the *S. aureus* isolates from pig and human. M = Molecular marker, lane 20-23 (positive controls), lane 24 (negative control). Lane 13 is *sed* positive, lane 15, 25, 27 and 28 are all *sea* positive (*sea*=127bp, *seb*=477bp, *sec*=271, *sed*=319, *see*=178).

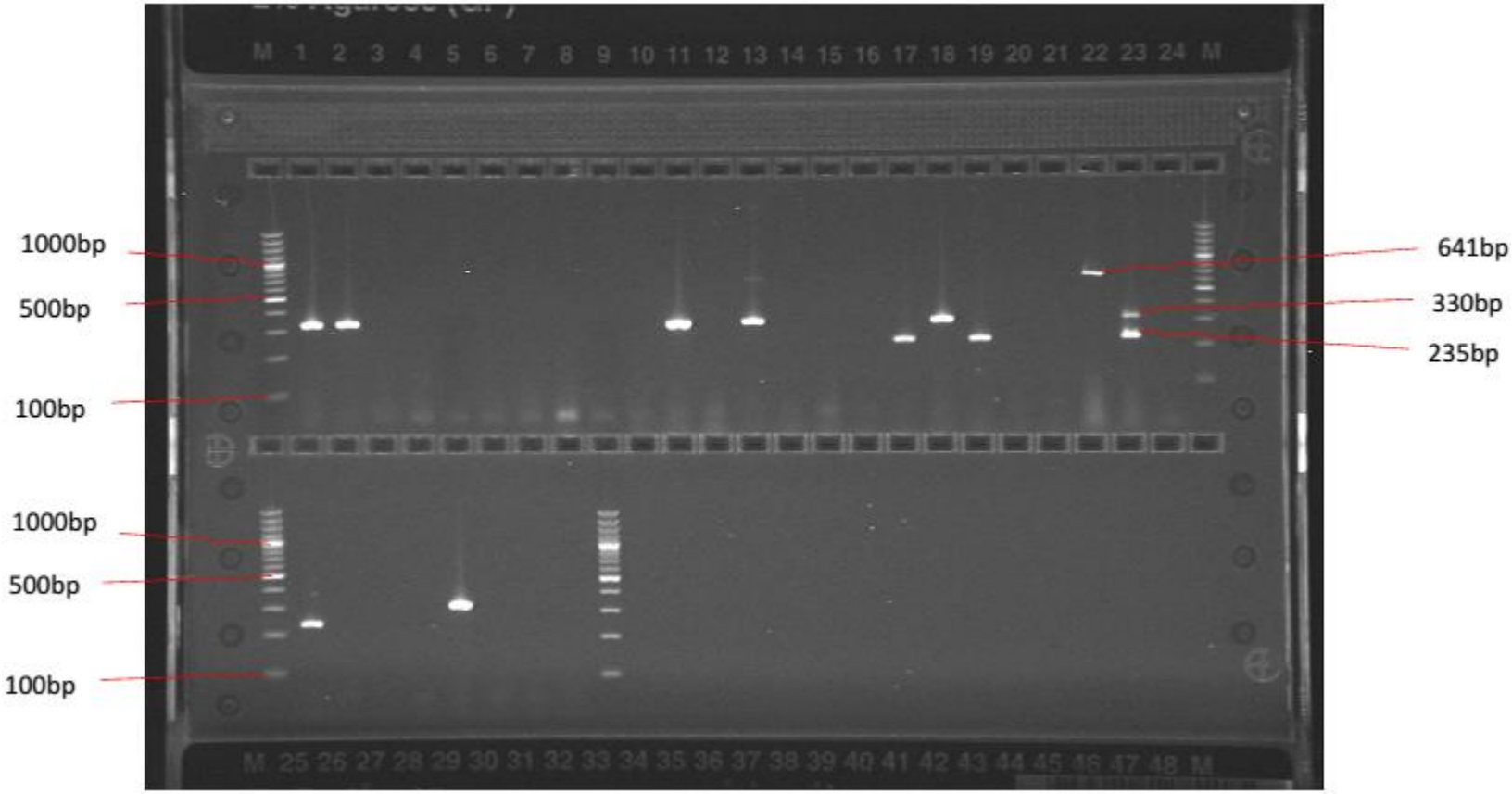

Plate 4.3 Multiplex PCR for enterotoxin SEH-SEJ genes carried by the *S. aureus* isolates from humans and pigs. M = Molecular marker, Lane 22-23 (Positive control), Lane 24 (Negative control). (*seh* = 235bp, *sej* = 641bp and *sei* = 330bp

Table 4.14 *S. aureus* prevalence among pig farmers and abattoir workers based on demographic and risk factor

Characteristics	No of subjects No positive (%) (n = 12)	No negative (%) (n = 89)	OR	C.I	p value
Age					
Young (>18yrs)	4 (33.3)	12 (13.5)	3.2210	0.8351-12.33	0.0950
Adult	8 (66.7)	77 (86.5)	Ref.		
Gender					
Male	5 (41.6)	65(73.0)	0.4615	0.1374-1.550	0.2288
Female	7 (58.3)	24 (26.9)	Ref.		
Occupation					
Pig farmer	8 (66.7)	56 (62.9)	1.1790	0.3292-4.219	1.000
Abattoir worker	4 (33.3)	33 (37.1)	Ref.		
Seniority					
<5yrs	4 (33.3)	19 (21.3)	3.895	0.6531-23.23	0.1828
5-10yrs	6 (50.0)	33 (37.1)	3.364	0.6344-17.83	0.2626
>10yrs	2 (16.7)	37 (41.6)	Ref.		
Medical related occupation of household	7 (58.3)	18 (20.2)	5.522	1.568-19.45	0.0087□
	5 (41.6)	71 (79.8)	Ref.		

□ Denotes Significant Association

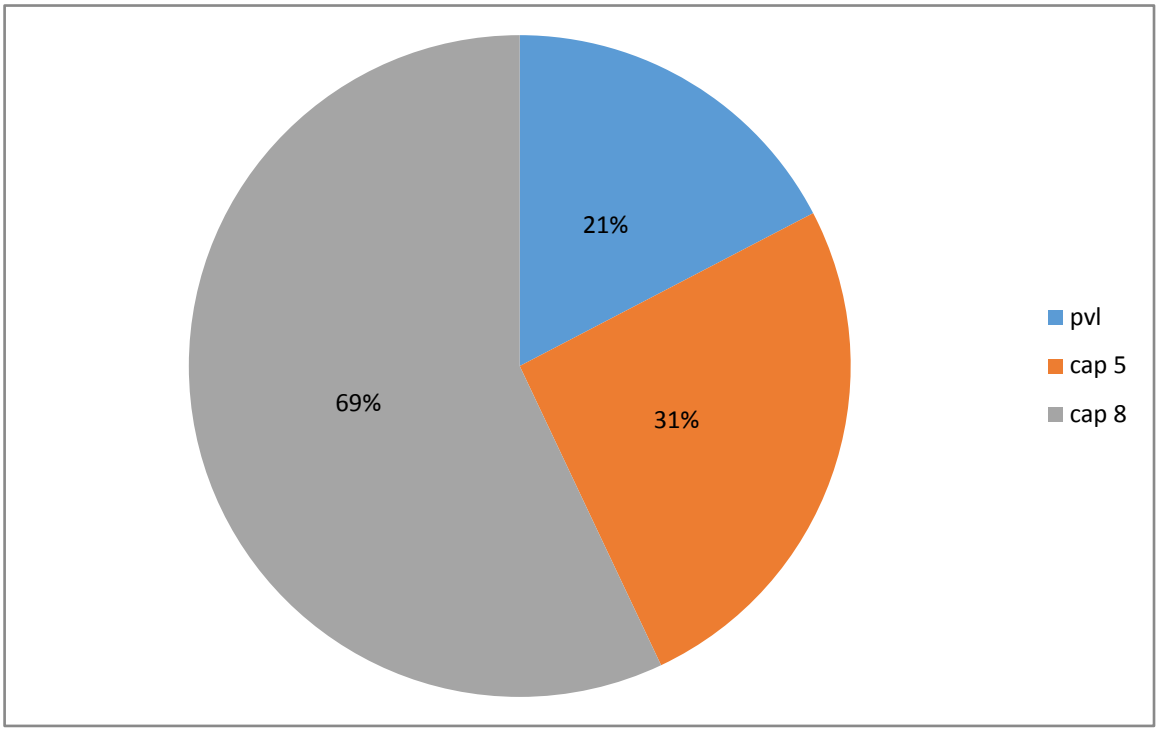

Figure 4.3: Overall percentages of *pvl*, *cap* 5 and *cap* 8 genes among 29 *S. aureus* isolates from humans and pigs

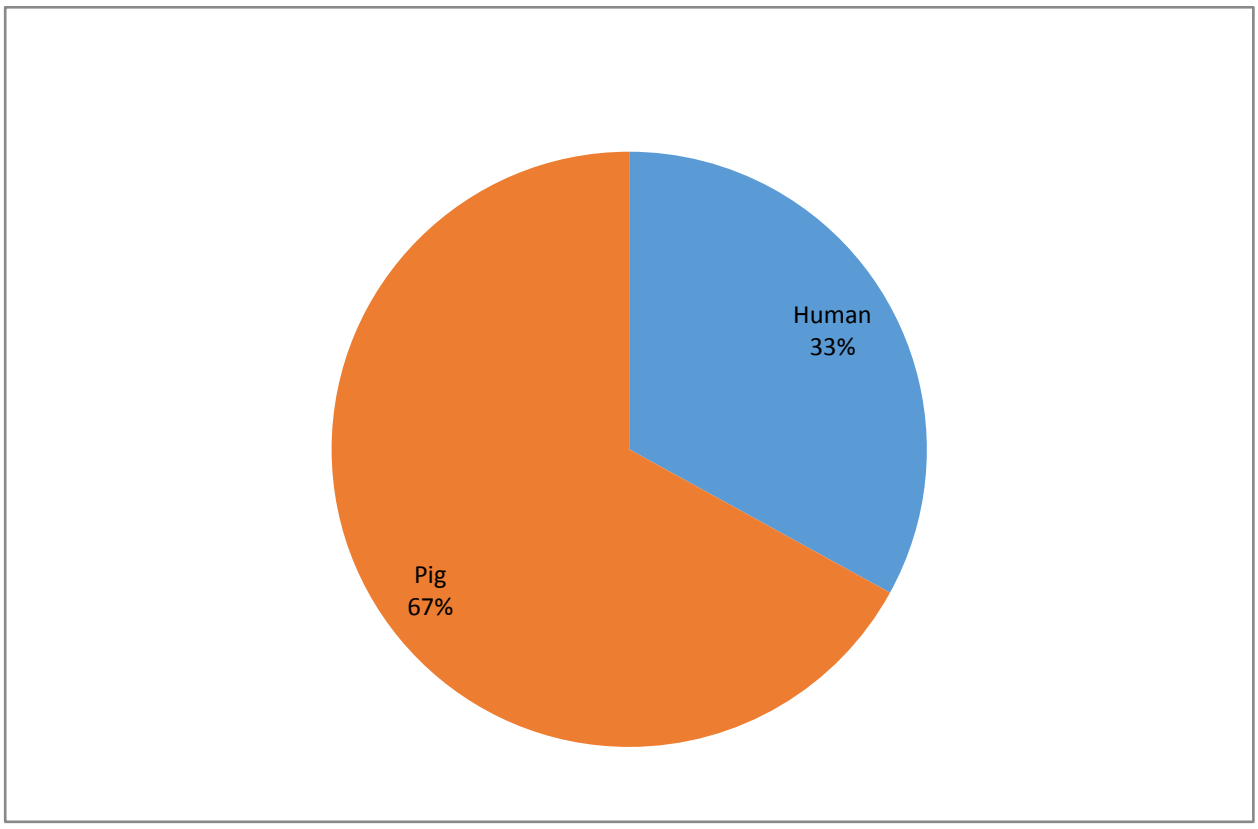

Figure 4.4: Distribution of *pvl* positive gene among pig and human isolates of *S. aureus*

CHAPTER FIVE

5.0 DISCUSSION

This study presents a phenotypic analysis of 53 coagulase negative staphylococci (CoNS) species, 20 *S. hyicus* and 17 *S. intermedius* and a comparative analysis of genotype and virulence profiles of 29 *S. aureus* isolates representing two major phenotypic groups; nasal carriage by pigs and those of humans who have close contact with pigs.

Out of the 401 samples collected, CoNS, *S. hyicus* and *S. intermedius* isolates constituted a prevalence of 22.4% (90/401). This prevalence is higher than the 11% prevalence reported by Ulrika *et al*. (2014) among canine with UTI and lower than the 47.4% prevalence reported by Penna *et al*. (2009) also among canine in Brazil. The differences in these prevalences could be associated with the fact that the isolates from this study erer from apparently healthy subjects. The ten (10) different species identified showed a marked multidrug resistance to the antibiotic agents 61.1% (55/90). All the CoNS, *S. hyicus,* and *S. intermedius* isolates showed high resistance to the beta-lactams (with oxacillin and cefoxitin inclusive though at a very low frequency) and this result is in agreement with the report of Detwiller *et al*. (2013) who reported that CoNS are often resistant to beta-lactams. Apart from the beta-lactams, most of the isolates were also resistant to 3 or more antimicrobial agents. This could be associated with the fact that CoNS are often reported as methicillin resistant with co-resistance to different classes of antibiotics (Huber *et al*., 2010). *S. hyicus* is part of the commensal flora of various animals and is primarily known as the causative agent of exudative epidermitis in pigs (Fudaba *et al*., 2008). The high level of resistance (60%) to betalactams (penicillin) by *S. hyicus* and the 20% and 15% resistance to oxacillin and cefoxitin is of immense concern. From a swine health standpoint, it is of importance because penicillin is a commonly used antibiotic agent for the treatment of

staphylococci infections. Also, resistance to oxacillin and cefoxitin raises questions whether phenotypic methicillin resistant *S. hyicus* (MRSH) could ultimately be a source of MRSA clones on pig farms (Park *et al*., 2014) since they can coexist. The 2 *S. hyicus* isolates in this study were vancomycin susceptible (MICs were 0.12µg/ml and 2µg/ml) even though they were resistant to the agent using disc diffusion. This could be as a result of the differences in concentration. Thus while *S. hyicus* is of little zoonotic relevance, the public health relevance cannot be completely dismissed. *S. intermedius* also showed high level of multidrug resistane (64.7%). It is a major opportunistic pathogen in many Veterinary infections (Youn *et al*., 2011). Human infections with *S. intermedius* have also been identified (Chuang *et al*., 2010) and direct transmission between animals and humans has also been demonstrated (Kempker *et al*., 2009). Such characteristics emphasize their potential as zoonotic pathogen. Furthermore, increasing evidence has identified the emergence of spread of antibiotic resistance *S. intermedius* isolates (Weese and Duijkeren, 2010) thus transmission seems very likely in animal-human interface. The 5 *S. intermedius* isolates that were resistant to vancomycin using disc diffusion were also resistant when Etest was used for their MICs (MIC >256 µg/ml). *S. schleiferi* which is one of the species isolated in this study is of public health concern because it has been reported to be an emerging potential zoonotic pathogen (Davis *et al*., 2013). Typically they have been associated with skin infections in companion animals (otitis or pyodermal in dogs) and cats and has recently been described as a human pathogen (Kumar *et al.*, 2007; Tzamalis *et al*., 2013). In this study, 3 out of the 5 isolates were from pigs while the other 2 from humans. Also, asymptomatic nasal or skin carriage in people with veterinary occupational contact has been demonstrated with prevalence ranging between ≤2% to 50% (Vanni *et al*., 2009; Ishihara *et al*., 2010; Morris *et al*., 2013; Cain *et al*., 2011a, b; Penna *et al*., 2013). These

species also showed multidrug resistance and it is in agreement with the reports of Starlander *et al*. 2011; Cain, 2013; Penna *et al*. 2013 who also reported multidrug resistance of this species. Generally, most of the isolates showed high resistance to penicillin, tetracycline, trimethoprim, chloramphenicol and a few of them to oxacillin and vancomycin. The reason for this resistance could be associated with the fact that most of the antibiotics are commonly used for treatment of infection and as growth performance and since more than one species of microorganism can be present within a host there is therefore the possibility of acquiring resistance. Also they could have environmentally acquired the resistance through horizontal transfer. The importance of most of these species is not fully understood most especially in pigs and this can be as a result of the fact that most reports have focused on surgical site infections in humans and little is known in animals especially pig. However, most of these CoNS species have been shown to be frequently associated with mastitis in animals (El Jakee *et al*., 2013). Hence, even though some of these isolated species have limited zoonotic potential they should be carefully monitored so as not to reduce therapeutic options for treatment of infections in both humans and animals.

A total prevalence of 5.3% and 12.9% *S. aureus* carriage was found in pigs and humans, respectively in this study. Nasal carriage of *S. aureus* may be considered as a situation in which bacterium in pigs are rarely associated with clinical diseases. However, the organism has been associated with conditions such as arthritis or polyarthritis and in systemic diseases like septicemia and abortion in pigs (van der Wolf *et al*., 2012). In humans, colonisation of a person does not result in any harm to the host, but serves as a risk for developing subsequent symptomatic infections (Graham *et al*., 2006; Fritz *et al*., 2009).

These colonized healthy persons who are often grouped as persistent or intermittent carriers could serve as sources of transmission of *S. aureus* to susceptible persons (Kadariya *et al.*, 2014) and even to animals that they may come in contact with. All the *S. aureus* strains isolated from this study were of MSSA phenotype. The prevalence rates observed in pigs and humans (12.9% and 5.3%), respectively are lower than the 12.3% (57/464) for pigs and 30.8% (16/52) for humans reported by Fall *et al*. (2012) in Senegal. The factors responsible for the different prevalence rates are unknown. In this study, single nasal swab was collected from the study participants and the sampled pigs were all from backyard piggeries. This could also reflect the lesser use of antibiotics for animal growth and therapy in the study area as well as the absence of evidence of the ST398 clone common in pigs in developed countries (Espionosa *et al*., 2012; Morcillo *et al*., 2012; van der Wolf *et al*., 2012). To the best of our knowledge, there are no published data available on the prevalence of MRSA or MSSA carriage among pigs and pig workers in the study area and in Nigeria as a whole. This observation show that MSSA strains remain an important source of infection, suggesting that MRSA has not yet replaced MSSA strains, thereby indicating the need for susceptible strains to be continually monitored and controlled, as resistant strains are due to their high pathogenic potential (Jimenez *et al*., 2011).

This study also showed that adult pigs had higher prevalence (7%) than piglets (2%). This agrees with the report that piglets born to *S. aureus* positive sow may in some way confer protection against *S. aureus* carriage at later production stages (Burns *et al*., 2014). However, there was no significant association between age and presence of *S. aureus*. There were more female carriers than males, which may be attributed to many factors in addition to sow colonisation status. These factors include selective pressure exerted by

antimicrobial medication post weaning which are likely to be important influences on carrier status particularly in older pigs (Burns *et al*., 2014). The prevalence (7%) of MSSA was higher in pigs sampled at abattoirs than those from farms (5%). This result is in contrast with the report of De Neeling *et al*. (2007) and Broens *et al*. (2011) who reported that pigs sampled at the point of stunning in the abattoir became MRSA positive due to MRSA contamination of the Lorries that were used to transport the pigs from farms to the abattoir. However, in this study, there were no conventional means of transportation of the pigs to abattoir. The sampled pigs were transported to the abattoir using motorcycles and commercial motor vehicles.

For human subjects, young persons had higher prevalence (25%) than adults (9%). This could be attributed to the fact that the young people easily exhibit behaviours such as poor hygiene and sanitary level which could serve as risk factors to enhance the transmission of *S. aureus*. More females (23%) than males (7%) were carriers and the result was statistically significant indicating that women working in contact with pigs are more prone to be carriers than their male counterparts. This can be supported with the fact that women do most of the cleaning and feeding of these pigs and as such in the process of cleaning they could be exposed to this bacterium generated from dust in piggeries. This result is partly in line with the report of Fang *et al*. (2014) who reported that being a female was a risk factor for the carriage of MRSA among pig workers. *S. aureus* has been shown to be present in dust in pig farms (EFSA, 2009). Pig farmers had relatively higher carriage rate (13%) than abattoir workers (11%). This can also be associated with the nature of work carried out by pig farmers which include cleaning, feeding and other forms of contact. Also, contact with pigs is a risk factor not only for MRSA carriage but also for carriage of

S. aureus strains sensitive to methicillin but resistant to tetracycline and often macrolides and lincosamides (Oppliger *et al*., 2012) as observed in this study.

The marked resistance to penicillin, tetracycline and trimethoprim/sulphathamexole is not surprising considering the fact that these drugs are relatively cheap and readily available over the counter for purchase even without prescription. In pigs, tetracycline and trimethoprim are commonly used antibiotics in pig medicine and they are both first choice antibiotics for many indications in pigs hence the abusive usage in both human and veterinary medicine. Similar reports of resistance to these antibiotics have been previously reported in both humans and animals around the world (Shittu *et al*., 2012; Egyir *et al*., 2013; Nemeghaire *et al*., 2014). There was a low frequency of resistance by isolates to gentamicin, rifampicin, daptomycin, mupirocin, clindamycin and linezolid by the MSSA strains in this study. This result is in contrast to that of Shittu *et al*. (2011) who reported that all MSSA strains were susceptible to these antibiotics. The difference in prevalence could be attributed to the fact that the isolates must have become resistance to these antibiotics via horizontal transfer from the environment. The prevalence observed in this study is however worrisome knowing that these antibiotics are not routinely used in both human and animal healthcare settings. It is therefore worthy of note that drugs to which susceptibilities are still high should be cautiously administered to prevent resistance to such drugs. The low level resistance (8%) to mupirocin in this study is quite surprising given the fact that it is a topical antibacterial agent that is clinically used for MRSA decolonization/decontamination in both human and veterinary medicine (Mai-siyama *et al*., 2014; Mustak, *et al*., 2014; Wendlandt *et al*., 2015). It is however partly in agreement with the report of Shittu *et al*. (2009) who reported a low prevalence of resistance of MRSA in

human isolates from Nigeria and South Africa to Mupirocin. Also, resistance to linezolide (LZD) was observed. Linezolide is the sole oxazolidinone in clinical use today (Oksuz and Gurler, 2013). Resistance to linezolide remains rare in clinical settings (Gabriel *et al*., 2015) but reports of its emergence are increasingly being reported (Billal *et al*., 2011; Gu *et al*., 2012; Mendes *et al*., 2012; Cui *et al*., 2013; Barros *et al*., 2014; De Almeria *et al*., 2014; Flamm *et al*., 2014; Tewhey *et al*., 2014). The 7% resistance to linezolide in this study was found among human isolates. The reason for this low resistance is however unknown but it however still remains one of the few options currently available for the treatment of MRSA among other infections (Gabriel *et al*., 2012). Therefore, in an effort to preserve LZD as a valuable therapeutic, its judicious use coupled with sensitivity testing prior to its prescription and the enforcement of infection control procedures should be implemented (Gabriel *et al*., 2015). Fourteen percent (14%) of the isolates were resistant to erythromycin and carried the *erm* A gene, which is responsible for inducible macrolide, lincosamide and streptogramin (MLS) resistance (Ito *et al*., 2001; 2003). Macrolides (erythromycin) and lincosamides (clindamycin) belong to different classes of antimicrobials but act through the same mechanism that is by inhibition of protein synthesis (Christine *et al*., 2005). Clindamycin has long been an option for treating both MSSA and MRSA infections (Lall and Sahni, 2014). Ten percent (10%) of the *S. aureus* isolates in this study were resistant to clindamycin, but the reason for this is not clear. However, expression of inducible resistance to clindamycin which was also observed among 7% and 10% isolates in this study could limit the effectiveness of clindamycin (Mukesh *et al*., 2006). Thus it was not surprising that some of the isolates in this study were resistant to erythromycin and clindamycin. The three isolates from humans (10%) that were found to be resistant to clindamycin showed no associated resistant gene on the micro array analysis.

The possible explanations include the presence of an allelic variant of a gene that was not detected by the micro array analysis or possibly, the presence of a novel gene(s) that remain to be described (Burns *et al*., 2014). Fourteen percent (14%) of the isolates were resistant to a member of the fluoroquinolones i.e norfloxacin. Resistance to fluoroquinolones occurs by point mutation in chromosomal genes that encode the subunits of DNA gyrase and topoisomerase IV. This class of antibiotic also selects for methicillin resistance in staphylococci (Dalhoff, 2012). In this study, all isolates were MSSAs and this further supports the long evolutionary history of MSSA to MRSA relationship. There was however a good correlation between resistance phenotype and the resistance genes detected for penicillin, tetracycline and chloramphenicol. All the *S. aureus* isolates had Multiple Antibiotic Resistance Index (MARI) greater than 0.2 which means they are indicative of being isolated from environments where antibiotics are abused or frequently used. This implies that the burden of antibiotics present in the study population is high and this may directly or indirectly increase resistance of *S. aureus* and other bacterial in the study population. The resistance patterns varied between various classes and within the different classes of antibiotics.

Successive acquisition of resistance to most classes of antimicrobial agents, such as penicillins, macrolides, aminoglycosides, chloramphenicol, and tetracycline has made treatment and control of staphylococcal infections increasingly difficult. Resistance to penicillin in *S. aureus* is mediated by production of penicillinase encoded by *bla*Z gene. Majority of the isolates in this study harboured at least one antimicrobial resistance gene including those encoding beta-lactams (*bla*Z), *msr*A, *erm*A, tetracycline (*tet*K) and chloramphenicol (*Cat-pC221*). All of the *S. aureus* strains (100%) in this study harboured

the *bla*Z gene. This is interesting because erroneous report of penicillin susceptibility could result in potentially inadequate therapy of *S. aureus* infection (Kaase *et al*., 2008). However, isolates that were resistant to trimethoprim/sulphamethaxazole were shown to be *dfr*A negative. This suggests that the predominant mechanism of trimethoprim resistance in *S. aureus* is via mutation of the dihydrofolate reductase (DHFR) which is selected even when trimethoprim is used in combination with sulphamethaxazole could have been responsible for the observed resistance as reported by Dale *et al*. (1997) and Shittu *et al*. (2011). This finding is in agreement with the report of Shittu *et al*. (2011). The *cat* gene encodes for chloramphenicol acetyltransferase and the first and still predominant mechanism of bacterial resistance to chloramphenicol is enzymatic inactivation of the drug (Mingoia *et al*., 2015). In this study, this gene was detected in only one isolate of pig origin. Even though chloramphenicol is not often used in swine medicine, it is frequently used in poultry and poultry droppings are usually fed to pigs most especially among those that are backyard reared. Based on this practice, the presence of the *cat* gene could be attributed to this reason. The *msr*A and *erm*A genes detected in this study were however dependent on erythromycin and clindamycin resistance phenotype. This finding is partly in agreement with the report of Lawung *et al*. (2014) who reported resistance to erythromycin and clindamycin in association with the presence of *erm*A gene, though all the resistant isolates were MRSAs. The *msr*A gene is involved in the active efflux of macrolides while *erm*A resistance is by methylation of rRNA. The *erm* and *msr* genes are two related genotypes (Hung *et al.*, 2008).

Genotyping analysis of the isolates in this study revealed that clinical (human) isolates were widespread even among pig strains. Among the six (6) clonal complexes (CC) identified in

this study, CC15, CC5, and CC152 accounted for 76% of the total isolates examined. Interestingly, these 3 clonal complexes have been reported to be prevailing among clinical isolates associated with bacteremia in Africa especially Ghana and Nigeria (Egyir *et al*., 2013; Shittu *et al*., 2012) and in China (He *et al.,* 2013). This report is partly similar to the report of Fall *et al*. (2012) who reported that the presence of CC15 and CC152 among pigs and pig farmers have already been described as major MSSA lineages responsible for human infections in Dakar. Interestingly, CC5 was observed to be one of the predominant clonal complexes of the MSSA observed among pigs and it is consistent with reports in studies of MRSA in pigs (Monecke *et al*., 2011; He *et al*., 2013). Since the initial detection of (ST) 398 MRSA in pigs in Europe (Voss *et al*., 2005), research in other regions has revealed considerable genetic diversity among MRSA found in pigs. In particular, CC5 appear to be common in North America (Khanna *et al*., 2008; Molla *et al*., 2012; Frana *et al*., 2013; Smith *et al*., 2013). The occurrence of CC5 and associated *spa* types among MSSA in this study suggest that CC15, CC152 and CC5 lineages of *S. aureus* are likely to have a long evolutionary relationship with pigs. Aside from CC398 and CC9, other strains that are human associated are carried by animals (Pantosti *et al.,* 2012). For example, *S. aureus* CC5 is a successful human associated strain and it has been isolated from poultry across the world (Lowder *et al*., 2009). In agreement with this report, four *spa* types were assigned to CC5 which amounted to 14% of *S. aureus* isolates. However, three out of these *spa* types were isolated from pigs of which all except t002 did not carry the *scn* gene. Clonal Complex 5 (CC5) has also been associated with bacteremia (He *et al*., 2013) and detected in raw and processed food in Shanghai (Song *et al*., 2015). This result is suggestive of the fact that humans could be a potential source of maintaining *S. aureus* in the pig population in this study area. CC5 has also been reported to be predominantly

isolated from poultry (Ho *et al*., 2012). It is also a common and widespread clonal complex, some of which have attained pandemic spread and it comprises a large number of both CA-MRSA and HA-MRSA (Monecke *et al*., 2011). Hence, it is therefore suggested that the term LA-MRSA should not be restricted to ST398 lineage, but to include othe sequence types like ST9 and ST5. Among the MSSA isolates, *spa* types of MLST CC15 (*spa* types t084 and t2216) and CC152 (t355) were most predominant. Similar results have been reported by Breurec *et al*. (2011) and Shittu *et al*. (2012) from other studies on the molecular structure of African MSSA.

Pigs in this study were found to be colonized by *S. aureus spa* types from lineages mostly found in humans (t084, t355 and t311) indicating transmission from humans to pigs. *spa* type t084 was not only the most frequently occurring genotype, but also displayed highest level of antimicrobial resistance. These t084 and t355 have been reported in other parts of Nigeria (Shittu *et al*., 2011; Kolawale *et al*., 2013), Mali (Ruimy *et al*., 2008), Uganda (Seni *et al*., 2013) Sao Tome and Principe (Conceicao *et al*., 2014) demonstrating that these *spa* types are widespread human epidemic clone established in African countries. *spa* type t084 has been reported among carriage and clinical isolates in Norway (Sangvik *et al*., 2011), Denmark (Annual reports on *S. aureus* cases in Denmark, 2011), and the United Kingdom (Feil *et al*., 2006) suggesting the global spread of this clone (Feil *et al*., 2003; Lindsay *et al*., 2006). But t355 which is not common in non-African countries where they are usually detected as MRSA especially in the Balkan areas (Monecke *et al*., 2007; Ruimy *et al*., 2008; Francois *et al*., 2008).

The recovery of *spa* type t127/CC1 in human isolate is quite intriguing because this *spa* type has fairly recently been isolated from pigs and humans in Italy, and the presence of a

pig reservoir was hypothesized (Franco *et al*., 2011). It can therefore, be deduced that humans must have been infected by pigs in this study area. This finding is partly in line with that of Monaco *et al*. (2013) who also reported this *spa* type in MRSA clone in human isolates. The *spa* type was reported in Ovine samples by Carfora *et al*. (2015) though identified as MRSA and not MSSA unlike in this study. *spa* type t084 and t311 have been reported from other studies on the molecular structure of African MSSA (Breurec *et al*., 2011; Shittu *et al*., 2012). Also, *spa* type t084 was reported in a pig farmer in the US (Smith *et al*., 2014).

spa types t002 and t127 do not belong to CC398 but have been previously reported to be found in humans, pigs and horses (Cuny *et al*., 2008; Khanna *et al*., 2008; Broens *et al*., 2011).

There was no direct association between *S. aureus* genotype and toxin gene profile among the isolates examined. However, in this study, CC5 was found to carry more toxin genes (>6) than other CC groups. This finding is in tune with the report of Varshney *et al*. (2009) where they reported a high content (>5) of toxin genes in CC5 among other CC-groups. In this study, CC5 was identified mainly among the pig isolates.

In this study a prevalence of 24% of *lukS-PV* and *lukF-PV* genes of the *pvl* operon in MSSA isolates was observed. The reason for this high prevalence is however, presently unknown, though, *pvl* positive strains are generally reported to be virulent whether they are MSSAs or MRSAs (Ghebremedhin *et al*., 2009; Shambat *et al*., 2012). In other parts of the world, the presence of *luk PV* genes encoding *pvl* is potentially of particular concern as the presence of this toxin has been associated with deep abscesses, severe necrotizing

pneumonia and severe bone and joint infections in human epidemiological studies (Badiou *et al*., 2010; Breurec *et al*., 2011). The high prevalence of *pvl* gene carriage is however consistent with several reports from other African countries (Cameroon, Madagascar, Morocco, Niger, Senegal) including Nigeria where the prevalence of *pvl* ranged between 17% and 74% (Okon *et al*., 2009; Breurec *et al*., 2011). This high prevalence indicate Africa has a far higher prevalence of *pvl* positive *S. aureus* than industrialized countries which record 0-2% carriage among human and swine isolates (O'Hara *et al*., 2008). This means that most of the African region may act as a potential reservoir for the *pvl* virulence factor with considerable impact on regional as well as global health care system (Kolawale *et al*., 2013) of both human and veterinary medicine. The presence of this *pvl* gene was higher in pig isolates and it is quite alarming in regards to the fact that the pig *pvl* positive strains were MSSA and no study has investigated *pvl* positive *S. aureus* in pigs in Nigeria. This result is in agreement with the report of Song *et al*. (2015) who reported a notably high prevalence of *pvl* gene in MSSA strains in Shanghai. The presence of *pvl* gene in pig could be associated with the fact that *pvl* encoding genes are carried on prophages as mobile genetic elements, which enable easy incorporation or spread into *S. aureus* lineages of host species (Chini *et al*., 2006). Also, in this study, *pvl* positive CC/ST152 was the most predominant clone. This finding is in consonance with the report of Okon *et al*. (2009) who observed that this clone was the most predominant in North-Eastern Nigeria and while Ruimy *et al*. (2008) observed it as the second most prevalent clone in a carriage study in Mali. The high prevalence of *pvl* positive MSSA ST/CC152 emerging in the community and in the hospital settings in West Africa has also been described by Breurec *et al*. (2010) but this is the first report to the best of our knowledge on the carriage of *pvl* gene among pig *S. aureus* clone in West Africa and in Nigeria in particular. In contrast to many other

clonal complexes that display worldwide occurrence, ST/CC 152 has been shown to be widespread in Africa, whereas it is comparatively rare elsewhere (Ruimy *et al*., 2009; Masiuk *et al*., 2010). Generally, co-occurrence of multiple genotypes of *S. aureus* in humans has been reported, but co-occurrence of MSSA and MRSA strains is not mentioned (Bloemendaeel *et al*., 2009; Mongkolrattanothai *et al*., 2011). Conversely, it is suggested that colonization with MSSA protects against co-colonization with MRSA (Dall'Antonia *et al*., 2005; Huang *et al*., 2011).

The capsular genotyping of *S. aureus* strains in this study showed that all the strains carried either *cap*5 locus (31%) or *cap*8 locus (69%). Of the human isolates 20% and 36% possessed *cap*5 and *cap*8, respectively, while 80% and 64% of pig isolates carried *cap*5 and *cap*8 loci respectively. A previous report had shown the predominance of the type 5 seroptype among MRSA isolates (Na'was *et al*., 1998). Also, an earlier study has demonstrated that a *S. aureus* strain expressing *cap*5 was more virulent than an isogenic *cap* 8 producing strain in a mouse model of bacteremia and abscess formation (Hanses *et al*., 2014).

The study showed that genes encoding newly identified types of staphylococcal enterotoxins (*seh, sel, seln, sem, seg, sei* and classical *sea*) were more frequently identified than the other classical SEs (*seb, sec, sed, see*) genes, indicating emergence of these "new" toxigenic strains in the study area. This is in agreement with report of Song *et al*. (2015) in Shanghai and other parts of the world (Bania *et al*., 2006; Aydin *et al*., 2011). A recent report by Omoe *et al*. (2013) showed that some of the new SEs such as *sek, sel, sem, sen, seq, sep and seo* could induce emetic reactions in monkeys implying a role of these new SEs in staphylococcal food poisoning. *seh* was found to be the most frequently occurring

SE, Kolawale *et al*. (2013) reported that SEH can induce emetic disease and that it was found to be associated with ST1 (CC1). Also, Bae *et al*., (2013) reported that it is associated with persistent *S. aureus* bacteremia, indicating that the strains reported in this study have a high potential to cause bacteremia as earlier reported by Japoni-Nejat *et al*. (2013) and could therefore also be considered a potential causative agent for food poisoning. Also, Franco *et al*. (2011) and Hummerjohann *et al*. (2014) reported that *seh* is a common finding in isolates of human and animal origins. The fact that some of these toxins were detected among isolates from pigs also is of public health concern because pigs are food-producing animals; with inherent concerns about contamination of food as reported by Neela *et al*. (2009). The higher prevalence of SE genes in MSSA isolates from pigs than human gives a signal that even though no MRSA was found in this study, the toxigenic nature of the observed genes could pose a greater risk to humans through consumption of contaminated food that was prepared under poor environmental conditions.

It is well known that most virulence factors in *S. aureus* are under the control of the quorum-sensing accessory gene regulator (*agr)* system (Painter *et al*., 2015; Song *et al*., 2015). In this study, it was revealed that isolates belonging to *agr* II and IV groups had a higher incidence of toxin genes than those belonging to *agr* I, suggesting that *agr* profiles may be associated with the virulence potential of *S. aureus* (Sugiyama *et al*., 2009; Cheung *et al*., 2011). It agrees with the report of Song *et al*. (2015) and partly with that of report Montanaro *et al*. (2010) that different *agr* groups exhibited distinct patterns of virulence genes. Majority of the isolates in this study were observed to carry *ica*A, *ica*D and *ica*C genes which encode polysaccharide intercellular adhesion (PIA). These genes mediate

biofilm formation in *S. aureus*. Though, a clear role for *ica* locus of *S. aureus* is not fully understood (Izano *et al*., 2008).

The result also revealed the presence of the immune evasion cluster (*scn, chp* and *sak* gene) which is rare in animal isolates and usually associated with *S. aureus* of human origin and involved in human host immune evasion (Sung *et al.,* 2008; Cuny *et al*., 2010) were detected in pig isolates. All of the isolates contained the *scn* gene except for two isolates from pig with *spa* type t002 and t5691. Absence of the *scn* gene has been described as an indicator for LA-MRSA (Cromwell, 2001). The reason for the high carriage of the *scn* gene by the isolates in this study may be attributed to the fact that majority of these *spa* types are human-adapted which may have possibly been shared with the associated pigs in this study.

Other important virulence genes observed in this study include the fibronectin-binding protein A (*fnb*A) and clumping factor A and B (*clf*A and *clf*B). They mediate staphylococcal adherence to epithelial cells. *Clf*A also plays a role in *S. aureus* binding to platelets (Hanses *et al*., 2014) an interaction that is critical to the induction of staphylococcal endocarditis. Also, *clf*A together with *agr*B has also been linked to stress response and biofilm formation. On the other hand, *clf*B is linked to nasal colonization and a combination of *Clf*A and *Clf*B genes allow the pathogen to sustain and survive in the anterior nares (Sivaraman *et al*., 2009). *fnb*A is another gene of importance in colonization which is present in almost all strains of *S. aureus* isolated from the human epithelium generally (Nashev *et al*., 2004) but it is also present in invasive strains. Also, the *sdr*C gene observed in this study is one gene that might have a role in host attachment in general and for nasal carriage. This gene encodes a host attachment domain (MSCRAMM) and is highly prevalent in almost all strains of *S. aureus*. Overall, Sabat *et al*. (2006) reported that

the gene was present in about 90% of tested bacterial strains in various countries. Generally, in humans there is abundant information about the host factors that play a role in colonization with *S. aureus* (Peacook *et al*., 2001; Ruimy *et al*., 2010; Mulcahy *et al*., 2012) while for animals, there is no information available about individual susceptibility for *S. aureus* colonization (Kroen *et al.,* 2014). Also, there is significant controversy on the virulence of MSSA versus MRSA isolates; both have enormous capacity for virulence and pathogenicity that enables them to reach high rates of infection (Gould, 2006). However, they differ in their genotypes, geographical distribution and the infections they cause (Kim *et al*., 2006). The *fnb*A, *fnb*B, *clf*A, *clf*B, *sdr*C genes are MSCRAMM domain genes and they augment the pathogens interaction with the host (Navarre and Schneedwind, 1999). However, molecular typing based surveillance of *S. aureus* should not only be based on MRSA but also on MSSA so as to follow the emergence of strains with capacity to cause invasive infections (Cuny *et al*., 2013). Carriage of haemolysin genes (*hl*, *hla*, *hld*) and leukocidin genes (*luk*F, *luk*X and *luk*Y) was identified in 94% of the isolates. The *scn* gene was present in almost all the isolates (27 out of 29) investigated. This gene is associated with virulence of *S. aureus* in humans (Bokarewa *et al*., 2006) and hence its presence could suggest that pigs have become colonized with these isolates following exposure to humans.

The result of the questionnaire survey showed no significant association between demographic and length of time working with pigs ($p< 0.05$) and colonization with *S. aureus*. However, hospital linkage of household members had a significant association with the presence of *S. aureus* among study subject. Several studies have shown that being medical personnel could serve as a risk factor for *S. aureus* carriage. This could suggest that people who are medical personnel and are in contact with pigs could easily transmit *S.*

aureus to pigs which may further corroborate the fact that most of the *spa* types recovered from pigs have been reported to be hospital-related *spa* types.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

This study provides insights into the carriage of coagulase negative staphylococci (CoNS) species, *S. hyicus*, *S. intermedius* and *S. aureus* among pigs, pig farmers and abattoir workers, highlights antimicrobial susceptibilities, virulence factors and molecular diversity of this opportunistic pathogen in Jos, Plateau State Nigeria. The data generated from this

study will serve as a baseline for future studies on *S. aureus* epidemiology among the study population. Conclusions made from this study are outlined below;

1. There is high carriage of CoNS species, *S. hyicus* and *S. intermedius* 22.4% (90/401) and 61.1% were multidrug resistant.

2. There is relatively low prevalence of 5.6% and 11.9% of MSSA among pigs and occupationally exposed humans in the study area and the absence of MRSA strains.

3. The isolation of multi drug resistant MSSA (MDR MSSA) in human strains, coupled with the absence of routine identification and susceptibility testing of *S. aureus* in clinical settings, could impede effective management of patients

4. The occurrence of MSSA which are MDR and harbouring some virulence genes with human adaptive genes in pigs should be investigated to understand their epidemiology in the pig population.

5. CC152 and CC15 seem to be the well-established clones in the study area as indicated by the widespread occurrence of *spa* types t084 and t355, respectively.

6. The remarkably high prevalence of *pvl* (24%) among the isolates detected in this study corroborates the endemicity of this virulence factor among *S. aureus* on the African continent.

7. The high prevalence of non-classic SE encoding genes may indicate their widespread distribution among *S. aureus* strains in the study area.

8. Despite the current lack of information on LA-MRSA in Nigeria (which maybe due to a lack of systematic national surveillance), there is still public health concern about potential broad dissemination of drug-resistant *S. aureus* to the general public.

9. The MALDI-TOF MS was more sensitive in the detection of *S. aureus* isolates in the study

10. DNA microarray analysis provides a valuable insight to the virulence genes carried by *S. aureus* isolates in pigs and in-contact humans in the study area.

6.2 Recommendations

Based on the findings of this study, the following recommendations are made:

1. A more sensitive confirmatory test like the use of MALDI-TOF MS should be used for correct and quick identification of *S. aureus* in research and in clinical settings in Nigeria because it is a sensitive and quick technique.

2. Control strains should be used when carrying out antibiotic susceptibility testing for quality control.

3. For effective surveillance and continuous improvement of antimicrobial policies both in the human and animal populations, it would be useful to conduct follow-up studies annually or biannually including other geographical areas not included in this study to determine local differences, monitor changes in antimicrobial resistance over time and to detect the emergence and spread of resistant clones in Nigeria .

4. Coagulase negative and positive isolates of public health importance should be subjected to molecular typing to detect presence of virulence and resistance genes.

5. Routine decontamination of animals should be carried out to control *S. aureus* carriage

6. Pig workers should always ensure to wear protective clothing when working with pigs to reduce human sources of pig colonization

7. Molecular typing methods for *S. aureus* should be established in research laboratories in Nigeria for epidemiological studies of this bacterium through an extended collaboration with the Statens Serum Institut, Denmark.

8. The use of probiotics for farm animal fattening should be encouraged rather than antibiotics.

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APPENDICES

APPENDIX I: Structured Questionnaire on Risk Factors for Carriage of *S. aureus*

Questionnaire

This questionnaire is intended to gather information on your current and past contact with swine and other potential risk factors for infection with methicillin-resistant *Staphylococcus aureus* (MRSA), a bacterium that can cause a variety of diseases in humans and animals. It will only take a few minutes to complete. Please check the appropriate boxes and answer the questions to the best of your knowledge. All responses will remain confidential.

Thank you

DEMOGRAPHICS

1. Today's date _____/_____/_____

2. Your age ___ (years)
3. Sex Male ( ) Female ( )

MEDICAL HISTORY AND EXPOSURES
4. Have you taken antibiotics in the past three months? Yes ( ) No ( )

4a. List all antibiotics taken in the past three months
_____
_____
_____

5. Have you participated in any social-gathering, team and/or contact sports in the last 3 months?
Yes ( ) No ( )

6. Have you been hospitalized in the previous 12 months?
Yes ( ) No ( )

7. Have you or any family members visited a patient in the hospital in the previous 12 months?
Yes ( ) No ( )

8. Do you or any immediate family members work in a hospital? Yes ( ) No ( )

9. Have you had boil or been diagnosed with a skin or soft tissue infection in the previous 12 months? Yes ( ) No ( )

OCCUPATIONAL AND ANIMAL EXPOSURE

11. Do you work in a pig production unit (pig farm or pig slaughter house/abattoir)?
Yes--pig farm *(continue with question 12)*
Yes—slaughter house/abattoir *(continue with question 12)*

12. How long have you been working with pigs?
(Please state total of all years in current and previous jobs.)
_____ (years)

13. What type of work do you currently perform in the farm? Tick all that apply.
- Breeding
- Slaughtering and/or butchering pig
- Transporting live pigs or carcass
- Swine waste disposal
- Examining and treating pigs

- Cleaning and disinfecting equipment and areas exposed to pigs, pig products, or pig waste.

14. What is the average number of pigs you are exposed to in a typical day?
_____ Pigs

15. Please write in one of the following spaces how long it has been since your last contact with pigs.
_____ (days) _____ (weeks) _____ (months)

16. On average, how many days per week do you work in direct contact with pigs?
_____ (days/week)

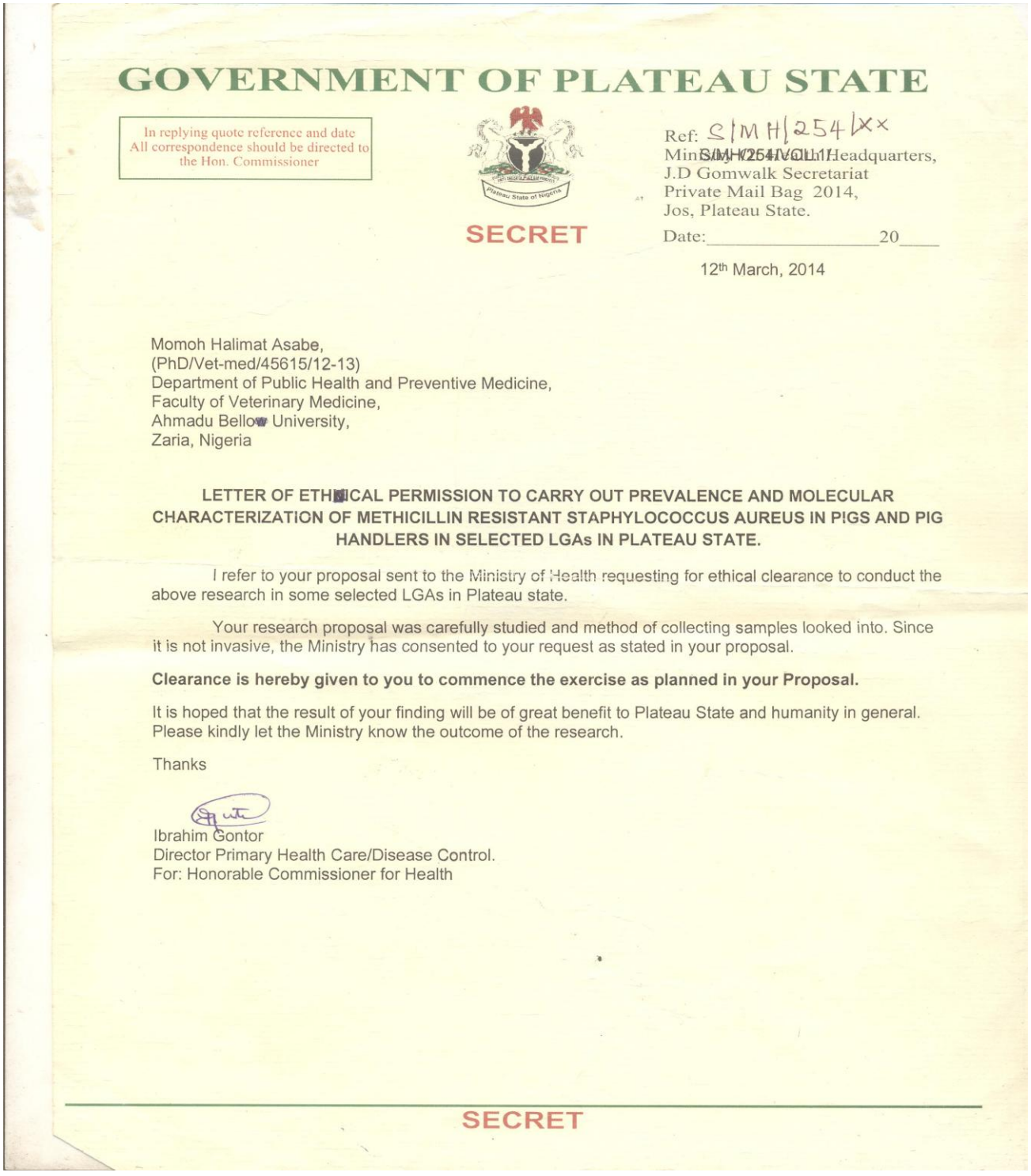
17. On days that you work with pigs, how many hours (on average) do you work in direct contact with them? _____ (hours/day)

18. In the past 12 months have you worked with/been in contact with any of the following types of live animals? (Tick *yes* or *no* for each)
Chickens yes ( ) no ( ) Cattle yes ( ) no ( ) Horses yes ( ) no ( )
Goats yes ( ) no ( ) Sheep yes ( ) no ( )
Other type of livestock, please specify _____

19. Do you have any of the following animals in or within your Household?
Chickens yes ( ) no ( ) Swine yes ( ) no( )
Cats yes ( ) no ( ) Dogs yes ( ) no ( )
Other type of animal, please specify _____

20. How often do you handle pork and pork products?
Less than once per week ( ) Approximately once per week ( )
2-3 times per week ( ) more than 4 times per week ( )

APPENDIX II: Ethical Clearance



GOVERNMENT OF PLATEAU STATE

In replying quote reference and date
All correspondence should be directed to
the Hon. Commissioner

SECRET

Ref: S/MH/254/xx
Ministry Headquarters,
J.D Gomwalk Secretariat
Private Mail Bag 2014,
Jos, Plateau State.

Date:_____20____

12th March, 2014

Momoh Halimat Asabe,
(PhD/Vet-med/45615/12-13)
Department of Public Health and Preventive Medicine,
Faculty of Veterinary Medicine,
Ahmadu Bello University,
Zaria, Nigeria

LETTER OF ETHICAL PERMISSION TO CARRY OUT PREVALENCE AND MOLECULAR CHARACTERIZATION OF METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS IN PIGS AND PIG HANDLERS IN SELECTED LGAs IN PLATEAU STATE.

I refer to your proposal sent to the Ministry of Health requesting for ethical clearance to conduct the above research in some selected LGAs in Plateau state.

Your research proposal was carefully studied and method of collecting samples looked into. Since it is not invasive, the Ministry has consented to your request as stated in your proposal.

Clearance is hereby given to you to commence the exercise as planned in your Proposal.

It is hoped that the result of your finding will be of great benefit to Plateau State and humanity in general. Please kindly let the Ministry know the outcome of the research.

Thanks

Ibrahim Gontor
Director Primary Health Care/Disease Control.
For: Honorable Commissioner for Health

SECRET

Appendix III: CLSI guideline (2011) interpretations of antibiotic zone of diameter for *S. aureus* and CoNS

Antibiotic	Disc Content	Zone diameter Break Points nearest whole mm S I R	MIC Interpretative standard (µg/ML) S I R	Species
Penicillin	10 units	≥29 - ≤28		*S. aureus*
Oxacillin	1µg	≥13 11-12 ≤10		
Cefoxitin	30µg	≥22 - ≤21		
Ampicillin	10µg	≥29 - ≤28		
Vancomycin	30µg		≤2 4-8 ≥16	
			≤4 8-16 ≥32	CoNS
Gentamicin	10µg	≥15 13-14 ≤12		
Tetracycline	30 µg	≥19 15-18 ≤14		
Chloramphenicol	1.25/23.75 µg	≥16 11-15 ≤10		
Trimethoprim/sulphamethoxazole	30 µg	≥18 13-17 ≤12		

Key: S = Susceptible

I = Intermediate

R = Resistant

M.I.C = Minimum Inhibitory Concentration