PHENOTYPIC AND MOLECULAR CHARACTERISATION OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM PATIENTS IN SECONDARY AND TERTIARY HEALTHCARE FACILITIES IN ZARIA METROPOLIS, KADUNA STATE, NIGERIA

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

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ZARIA, NIGERIA

SEPTEMBER, 2019

DECLARATION

I declare that the work in this thesis entitled **"Phenotypic and Molecular Characterisation of *Staphylococcus aureus* isolated from patients in secondary and tertiary healthcare facilities in Zaria Metropolis, Kaduna State, Nigeria"** has been performed by me in the Department of Veterinary Public Health and Preventive Medicine under the supervision of Prof. J.K.P.Kwaga, Prof. Junaidu Kabir and Dr. F.J.Giwa. The assistance obtained from other individuals during the course of the study has been duly acknowledged and directly referred to in the text.

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

Istifanus Anekoson JOSHUA …… …….
Name of Student Signature Date

CERTIFICATION

This thesis titled **"Phenotypic and Molecular Characterisation of *Staphylococcus aureus* isolated from Patients in secondary and tertiary healthcare facilities in Zaria Metropolis, Kaduna State, Nigeria"** by Istifanus Anekoson Joshua meets the regulations governing the award of Doctor of Philosophy Degree of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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Dean, School of Post Graduate Studies	Signature	Date

DEDICATION

This research is dedicated to God Almighty and my beloved family- My beloved wife and children, namely: Mrs Wazi Istifanus Joshua, Ovye (Joshua Jnr), Ahogbresha (Joseph), Ashe-ulu (Joanne), Abesla (Jotham) and my beloved mother- Mrs Martina Joshua.

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Yammu Joshua and Abaka Joshua; and Mrs Cecilia Jatau (my mother-inlaw) and Naomi Jatau (my sister in-law); may God bless them.

ABSTRACT

Staphylococcus aureus is one of the most pathogenic and cosmopolitan pathogen in nature. It is associated with hospital (HA-MRSA), community (CA-MRSA) and livestock (LA-MRSA) infections which are of public health importance. As the epidemiology of MRSA disease changes, there is need for current information on the prevalence and dynamics of MRSA infections in Zaria metropolis, Nigeria. A cross sectional descriptive study was carried out to determine the phenotypic and molecular characteristics of *S. aureus* isolated from patients in secondary and tertiary healthcare facilities in Zaria metropolis. A total of 420 semi-structured, interviewer-administered questionnaires and 420 samples from patients in 5 healthcare facilities were used (Ahmad Bello University [ABU] Medical Centre, Major Ibrahim Bello Abubakar Hospital, St Luke's Hospital, Gambo Sawaba Hospital and ABU Teaching Hospital [ABUTH]). The results revealed majority of the patients (54.3%) were within the age bracket of 21-40 years, with mean age of 26.04 ± 12 years, 58.6% females, 58.3% married, 36% had secondary education and 32.1% were housewives. Approximately 70% had a history of antibiotic use prior to consultation in the hospital, which were self-prescribed, 91.2% were outpatients and 6.0% had history of surgery. The most commonly used antibiotics were ampiclox (19.5%) and cotrimoxazole (10.0%) with mean duration of use of 3.5 ± 1.3 days. The most common type of surgery the patients underwent was appendicectomy (28.0%), followed by caesarian section (24.0%). The detection rate for *S. aureus* was 10% (42/420) and 5.2% (22/420) for MRSA with ABUTH, Zaria having the highest detection rate (χ^2 =17.66, p < 0.819, df=4). The *S. aureus* isolates showed highest frequency of resistance against ampicllin 42 (100%), followed by penicillin G 39 (92.9%), 15 (35.7%) cefoxitin and least to gentamicin 5 (11.9%). The multiple antibiotic resistance (MAR) indices of *S. aureus* ranged from 0.2 - 0.9 for all the isolates. The overall detection rate for MRSA in the 5

hospitals was 5.2%, with ABUTH Zaria having the highest value of 2.1% (χ^2 =8.060, df-8, p< 0.427). The frequency of resistance for the MRSA were ampicillin 22 (100%), penicillin G 21(95.5%) and least gentamicin 2 (9.1%) and ciprofloxacin 5 (22.7%). Two isolates (4.7%) (297 and No 3) showed identical antibiogram (ampicillin, cefoxitin, penicillin); the remaining 40 isolates showed different multiple antibiotic resistance patterns. Thirty six (85.7%) of the isolates exhibited multiple drug resistance and 6 (14.3 %) extensive drug resistance. For the minimum inhibition concentration of the MRSA isolates, 22 (100%) had values greater than 256 μg /ml for vancomycin, while 18 (81.8%) had value greater than 256 μg/ml for oxacillin. Of the 60 isolates evaluated by multiplex polymerase chain reaction, the detection rates of the genes were *mec* A (15%), *pvl* (10%), *fem* B (10%) and *spa* (13.3%). The isolates positive for *fem* B, were found to belong to MLST 1, with similar allelic profiles at all seven loci and they belonged to the clonal complex (CC) 1. The *S. aureus* genes were relatively uniform with no variable nucleotide sites at the seven loci. All the isolates (23448_1#126, 23448_1#127 and 23448_1#130) were multidrug resistant. The phylogenetic relationship established based on a subset of core genes using the 16S rRNA sequences of the strains showed 100% identity with the available *S. aureus* (BX571857, BA000033, AP015012, CP017115 and CP01780) genome in the database. The 3 isolates (1B, 1237 and 3279) were 100% similar to each other with no divergence within the species level as observed by clustering tightly together. The ST1 (CC1) clones are known to be community acquired human biotypes. The detection rate of *S. aureus* and MRSA strains are of great public health concerns which requires strict preventive measures and control. Therefore, there is need for rational use of antibiotics (antimicrobial stewardship), periodic clinical auditing using molecular analysis, effective hospital infection control measures, surveillance and the practice of one-health.

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ABBREVIATIONS

agr	accessory gene regulator
ARC	Antimicrobial resistance classification
ATCC	American Type Cultue Collection
BHI	Brain Heart Infusion
bp	Base pair
BSAC	British Society for Antimicrobial Chemotherapy
BSI	Blood stream infection
CA-MRSA	Community Associated Methicillin Resistant *S. aureus*
CC	Clonal complex
ccr	Cassette chromosome recombinase
CDC	Centres for Disease Control and Prevention
clfA	Clumping factor A
CLSI	Clinical and Laboratory Standards Institute
CoNS	Coagulase Negative staphylococci
DNA	Deoxyribonucleic Acid
EARSS	European Antimicrobial Resistance Surveillance System
EFSA	European Food Safety Authority
ET	Exfoliative toxin
EUCAST	European Committee of Antimicrobial Susceptibility Testing
FBD	Food borne diseases
fem B	Factors essential for methicillin resistance B
FDA	Food and Drug Admiistration
FnBP	Fibronectin binding protein
HA-MRSA	Hospital Associated MRSA
HCL	Hydrochloric acid
HIV	Human Immunodeficiency Virus
HVR	Hyper variable region
Kb	Kilo-base pair
LA-MRSA	Livestock Associated Methicillin Resistant *S. aureus*

LGA	Local Government Area
MAR	Multiple antibiotic resistance indice
MHA	Mueller Hinton medium
min	minutes
MDR	Multi-drug resistance
MGE	Mobile Genetic Element
MIC	Minimum Inhibitory Concentration
MMWR	Mortality momthly and weekly report
µl	Microlitre
MLEE	Multilocus enzyme electrophoresis
M-PCR	Multiplex polymerase chain reaction
MLST	Multilocus sequence typing
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin sensitive *S. aureus*
MRSA	Methicillin resistant *S. aureus*
NAR	Number of antibiotics resistant to
NCCLS	National Committee for Clinical Laboratory Standards
OIE	Office International des Èpizooties
PBP	Penicillin binding protein
PBP2a	Altered penicillin binding protein
PCR	Polymerase chain reaction
PDR	Pan drug resistance
PFGE	Pulsed field gel electrophoresis
PVL	Panton Valentine Leukocidin
RAPDF	Random amplified polymorphic DNA fingerprinting
SAB	*Staphylococcus aureus* bacteraemia
SAIE	*Staphylococcus aureus infective* endocarditis
SCC*mec*	Staphyloccocal cassette chromosome *mec*
SE	Staphylococcal enterotoxin

SFM	Société Française de Microbiologie
SNP	Single nucleotide polymorphism
spa	Staphylococcal protein A
SRGA	Swedish Reference Group for antibiotics
SSA	Sub-saharan Africa
SSSS	Staphylococcal scalded skin syndrome
SSTI	Skin and soft tissue infection
ST	Sequence type
TSS	Toxic shock syndrome
Tsst	Toxic shock syndrome toxin
UK	United Kingdom
USA	United States of America
UPMGA	Unweighted pair group method with rrithmetic mean
VISA	Vancomycin-intermediate resistant *Staphylococcus aureus*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
VNTR	Variable number tandem repeat
VRE	Vancomycin resistant *Enterococcus faecalis*
WHO	World Health Organisation
XDR	Extensive drug resistance

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

The genus *Staphylococcus* contains both pathogenic and non-pathogenic organisms. They are Gram positive cocci, non-motile and non-spore forming that is highly resistant to drying, especially when associated with organic matter such as blood, pus and other tissue fluids (Kloss, 1997). The genomic size of the bacteria is in the range of 2000 to 3000kb (Prevost *et al*., 1992; George and Kloss, 1994), and most staphylococci are found routinely on the surface of the skin. Breaks in skin and mucous membranes allow entrance of these organisms into the body.

The genus *Staphylococcus* is currently composed of about 47 species and 21 subspecies (Prax *et al*., 2013). The three major species include *S. aureus, S. epidermidis* and *S. saprophyticus*, which can be distinguished from each other based on colony morphology, Gram stain reaction, cultural and biochemical characteristics, among others.The biochemical tests include catalase, coagulase, growth characteristics and fermentation of mannitol and resistance or susceptibility to antimicrobials. *Staphylococcus lugdunensis* is considered a significant pathogen in human infections (Argemi *et al*., 2017) and clinically, its virulence is most likely lower than that of *S. aureus* (Argemi *et al*., 2019). *S. lugdunensis* has been described as emerging zoonotic pathogen causing skin infections and invasive infections such as endocaditis, osteomylitis and sepsis in humans (Davis *et al*., 2013).

Staphylococcus aureus is Gram positive, spherical cocci with a diameter of 0.5-1.5um. It is the most pathogenic species of staphylococci, widely distributed and found almost everywhere, particularly on the skin of humans and animals (Mathanraj *et al*., 2009)

causing wide range of infections from mild to life threatening conditions (Lulitanond *et al*., 2013; Bazzi *et al*., 2015). Various disease conditions caused by this organism include: wound infection, skin pustules, impetigo, osteomyelitis, renal abscess, pneumonia, endocarditis, meningitis, gastroenteritis, and sometimes serious conditions in patients undergoing hemodialysis and diabetes mellitus (Jean, 1996; Lewis and Jorgensen, 2005). In addition, staphylococcal infections that arise exclusively from production of toxins include staphylococcal scalded skin syndrome (SSSS), toxic shock syndrome (TSS) and staphylococcal food poisoning (Lowy, 2013). *S. aureus* causes food poisoning through the production of heat-stable staphylococcal enterotoxins (SEs), namely- SEA, SEB, SEC, SED, SEG, SEH, SEI and SEJ; SEs K and Q which lack the cysteine loop structure have been identified (Orwin *et al*., 2002; Intrakamhaeang *et al*., 2012). The enterotoxin produced by the organism is heat-stable that can resist heating at 100^0C for 30-70 min and is thus responsible for food borne infections (Garcia-Alvarez, 2011). Typical foods associated with *S. aureus* outbreaks include high protein products that are not properly handled during processing or preparation, e.g. fermented meat, cheese, cooked poultry, sea products and dry pasta.

Staphylococcus aureus are mostly coagulase positive, nasal commensals that colonize 20-30 % of human population (van Belkum *et al*., 2009) as well as livestock and other domestic animals (Lindsay, 2014). 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 of America estimates that 1.5% of the population (approximately 4.1 million persons) are colonized with methicillin resistant *S. aureus* (MRSA) (Gorwitz *et al.*, 2018).

The most remarkable feature of *S. aureus* is its ability to elaborate numerous virulence factors (Lozano *et al*., 2014), that mediate host colonization, tissue invasion and dissemination (Gordon and Lowy, 2008). Some of these virulence factors include

staphylococcal enterotoxins which cause food poisoning (Portillo *et al*., 2013), the panton valentine leukocidin gene *(pvl)* often associated with community infections (Chambers, 2005) and *mec* A gene among others. *S. aureus* also has a unique ability to develop resistance to antimicrobial agents (Chambers and Deleo, 2009). Over the years, there have been enormous increase and emergence of *S. aureus* strains resistant to methicillin through the acquisition of the *mec* A gene and the strain is commonly characterized by multidrug resistant phenotype (Lulitanond *et al*., 2013). Over the past several decades, *S. aureus* has developed resistance to several important antibiotics and virulence genes, thereby making it more difficult to treat infections due to it (Harkins *et al*., 2017).

The spread of drug-resistant bacterial pathogens poses a major threat to global health. It is widely recognized that the widespread use of antibiotics has generated selective pressures that have driven the emergence of resistant strains. Methicillin resistant *S. aureus* was first observed in 1960, less than 1 year after the introduction of this 2nd generation beta-lactam antibiotic into clinical practice (Harkins *et al*., 2017).

Methicillin Resistant *Stapylococcus aureus* are bacteria that are resistant to penicillinase stable semi-synthetic penicillins such as methicillin, nafcillin, oxacillin and cloxacillin that are used for treatment of infections due to *S. aureus*. Hospital associated MRSA (HA-MRSA), Community acquired MRSA (CA-MRSA) and Livestock associated MRSA (LA-MRSA) are the three types of MRSA that have been reported. Hospital associated MRSA and Community acquired MRSA are bacteriologically, clinically and epidemiologically distinct from each other. Methicillin-resistant *S. aureus* has been found to colonize livestock including pigs, cattle and poultry. Since many of the MRSA clonal lineages identified in livestock were uncommon for MRSA isolates found then in human hosts, they are classified as livestock-associated MRSA (LA-MRSA) (Kock *et al.*, 2013).

Livestock associated MRSA isolates usually belong to clonal complex (CC) 398 and majority of them to multilocous sequence type 398 (ST 398). However, MRSA of other clonal lineages including CC5, CC9, CC30 and CC97 have also been detected in livestock in Germany (Kock *et al.,* 2013). LA-MRSA genotypically classified under CC398 has been detected in pigs and some farmers in Netherlands and other countries and is known to cause infections in humans and other animals (De cleoq *et al*., 2008). MRSA can spread from human to human (Lu *et al*., 2005), from animal to human (Huber *et al*., 2009) and most likely from human to animal. MRSA has been reported in animals and animal products in Nigeria (Otalu *et al*., 2011; Umaru *et al*., 2013; Kwoji *et al.,* 2018) with public health implications. This is a major concern in the emergence of resistant zoonotic bacterial pathogens which can be transmitted to humans via food chain.

The presence of *mec* A gene and either an oxacillin Minimum Inhibitory Concentration (MIC) of >2mg/L and methicillin MIC of >4mg/L or a cefoxitin MIC of >4mg/L and production of altered penicillin binding protein (PBP2a), are accepted criteria for methicillin resistance (NCCLS, 2000).

Prolonged hospital stay, prior exposure to broad spectrum antimicrobial therapy, nasal colonization, admission to intensive care unit, surgical wounds, use of intravenous catheter also appear to be important factors for MRSA colonization (Hardy *et al*., 2004). MRSA infections were first reported in the early 1960s and they continued to be significant cause of morbidity and mortality worldwide in both healthcare and community settings (Kock *et al*., 2010). The *mec* A gene encodes resistance, and expression of this gene results in production of altered penicillin binding protein.

Methicillin-resistant strains usually possess more than four genes encoding different resistant mechanisms; *mec* A is a 2,130 bp fragment of deoxyribonucleic acid (DNA) coding for penicillin binding protein and is part of a mobile genetic element the

staphylococcal cassette chromosome *mec* (SCC*mec*), which is incorporated in the chromosome (Katayama *et al*., 2000).

Initially, nine types of SCC*mec* (types I to VIII and VT) have been defined, which can be distinguished by the type of cassette chromosome recombinase (*ccr)* gene complex that mediates the site-specific excision and insertion of the SCC*mec* cassette out of or into the bacterial genome and the class of *mec* complex that they bear (Ito *et al*., 2004; O'Brien *et al.,* 2004).

Conventional and molecular methods are used for complete identification of *Staphylococcus* and MRSA and in general, a combination of two or more conventional typing techniques is used for strain identification. Currently, Polymerase Chain Reaction (PCR) based techniques are commonly used for typing as they are easy and fast. Among such techniques, *S. aureus* protein A gene typing (*spa* typing) and Multilocus Sequence Typing (MLST) have been considered very useful tools for epidemiological studies (Sabat *et al*., 2013). Molecular techniques for the detection of *mec* A are viewed as the 'gold standard' for determining resistance (Terry-Alli *et al*., 2007). Polymerase chain reaction amplification of staphylococcal *mec* A gene is thus very important.

Research on African isolates of *S. aureus* has been largely neglected in the past despite the cultural and geographical diversity in Africa which has a significant impact on the epidemiology of this pathogen (Schaumburg *et al*., 2014). However, studies in Zaria, Ilorin, Jos and Kano showed prevalence of MRSA to be 69.0%, 34.7%, 43.0% and 10.7% respectively (Onanuga *et al.,* 2005; Taiwo *et al*., 2005; Ikeh, 2003; Nwankwo and Nasiru, 2011). Studies in Sokoto, Ahmadu Bello University Teaching Hospital (ABUTH), Zaria, Southern Nigeria, Ghana and India reported MRSA prevalence of 25% (Olowo-Okere *et*

al., 2017), 12.3% (Abdulaziz and Olayinka, 2016), 28.7% (Anie *et al.*, 2017) 17% (Saba *et al*., 2017) and 47.5% (Sathish and Wadekar, 2017) respectively.

1.2 Statement of Research Problem

The wide spread distribution of the genus *Staphylococcus* and wide colonization by the organism enhances opportunities for infection (Davis *et al*., 2013) that could further enhance the transmission of antibiotic resistance genes. *S. aureus* has unique ability to develop resistance to antimicrobial agents (Chambers and Deleo, 2009), thereby making human infection control and antibiotic therapy a serious challenge (Fall *et al*., 2012). Also, the cosmopolitan spread of *S. aureus* has become a serious challenge for human infection control and antibiotic therapy (Fall *et al*., 2012).

The indiscriminate use of antibiotics without prescriptions in developing countries such as Nigeria where there are no effective regulatory policies in this respect has rendered the commonly used antibiotics completely ineffective in the treatment of *S. aureus* infections (Odugbemi, 1980; Shittu *et al.*, 2011). The wide use of antibiotics (in veterinary, human, animal husbandry and aquaculture farming) has evolved the emergence of multidrug resistant strains, therefore making eradication more difficult.

Methicillin-resistant *S. aureus* is a significant major public, clinical and epidemiological health problem (Appelbaum, 2006). Infections with MRSA are more serious than other MSSA and the available treatment options are limited, and the development of new antibiotic classes has slowed (Talbot *et al*., 2006). They are multidrug resistant (MDR), which can result in high cost of treatment, longer treatment time and higher rates of hospitalization and co-morbidities (Noskin *et al*., 2005; Cosgrove, 2006).

Resistance to non-beta lactam agent such as quinolones, tetracycline, aminoglycosides, macrolides and lincosamides are increasingly reported among MRSA strains, further diminishing the treatment options available for infected patients (Jensen and Lyon, 2009).

In addition, resistance of MRSA to vancomycin, a last choice therapeutic agent has been reported (Appelbaum, 2006; Graber *et al*., 2007; Howden *et al*., 2009). The increasing resistance to anti-staphylococcal antibiotics including vancomycin-resistant *S. aureus* (VRSA) represents a grave threat to public health ***(***Karmakar *et al*., 2016*).*

MRSA strains have in some cases, been associated with more severe clinical outcomes and a substantially increased cost of care for affected patients (Kuehnet *et al.,* 2005). MRSA infections have continued to be the significant cause of morbidity and mortality worldwide in both healthcare and community settings (Kock *et al.,* 2010). *S. aureus* causes nosocomial and community acquired infections worldwide (Stegger *et al., 2010*), and is an important pathogen being responsible for serious infections such as necrotizing pneumonia and sepsis (Pentosis and Vanditti, 2009).

The high MRSA prevalence of 69% and high level of multiple drug resistance observed among MRSA isolates from healthy women volunteers in Zaria is of great concern (Onanuga *et al*., 2005).

1.3 Justification of the Research

Staphylococcus aureus has a unique ability to develop resistance to antimicrobial agents (Chambers and Deleo, 2009). Because the organism has become a pathogen of increasing importance in hospitals, communities and in recent years in livestock (Huber *et al*., 2009), controlling MRSA remains a primary focus of most hospital infection control programmes.

The prevention of this public health problem is very important because of the dramatic increase in the incidence of infections caused by MRSA and the range of effective antibiotics is becoming limited, costly and potentially toxic (CDC, 2002).

There is paucity of information on this organism in Zaria, and the few studies that have been reported are of limited scope. There is need to determine the genetic background of the isolates in order to understand the dynamics of the emergence of MRSA among others.

As the epidemiology of MRSA disease changes, accurate information on the scope and magnitude of the burden of MRSA in Zaria is needed to set priorities for prevention and control.

In view of the importance of MRSA morbidity and mortality, and few detailed systematic studies in Nigeria, especially Zaria, there is need for further studies in order to shed more light on the phenotypes and molecular features of the clinical strains. This will be beneficial in terms of reducing morbidity and mortality caused by the organism, reduced duration of hospital stay and cost of treatment, and better patient outcome. It will also provide opportunity for effective hospital infection control strategy, training on antimicrobial stewardship and rational antimicrobial use. Further more, it will give better understanding of the relevance of veterinary control, as well as the one-health approach towards control of antimicrobial resistance.

1.4 Aim of the Study

The aim of the study was to determine the phenotypic and molecular characteristics of *S .aureus* isolated from patients in secondary and tertiary healthcare facilities in Zaria metropolis, Kaduna State, Nigeria.

1.5 Objectives of the Study

The objectives of the study were:

i) To describe the socio-demographic characteristics of patients from the selected healthcare facilities infected with *S. aureus* in Zaria metropolis.

ii) To isolate and identify *S. aureus* from patients from the selected healthcare facilities in Zaria metropolis using conventional and commercial test kit (Microbact staphylococcal 12S identification system).

iii) To determine the antimicrobial susceptibility profiles of the isolates to 14 commonly-used antibiotics using Kirby Bauer disk diffusion method and the minimum inhibition concentration (MIC) of vancomycin and oxacillin using E-test.

iv) To detect altered Penicillin Binding Protein (PBP2a) among the MRSA

v) To detect *mec* A, C, *fem* B genes encoding methicillin resistance and *spa* and *pvl* toxin gene by Polymerase Chain Reaction (PCR) among the isolates.

vi) To carry out multilocus sequence typing (MLST) and use data to determine the phylogenetic relationship of *S. aureus* isolated from hospital patients in Zaria metropolis.

1.6 Research Questions

i) What are the socio-demographic characteristics of hospital patients infected with *S. aureus* in Zaria metropolis?

ii) Is *S. aureus* present in samples collected from patients in the selected healthcare facilities in Zaria metropolis?

iii) If present, are they susceptible to commonly used antibiotics? And what are the minimum inhibitory concentration values of the isolates to vancomycin and oxacillin?

iv) Do the *S. aureus* isolates elaborate PBP2a?

v) Do the *S. aureus* isolates carry *mec* A, C, *fem* B, *pvl* and *spa* genes?

vi) What are the sequence types (ST), clonal complex (CC) and the phylogenetic relationship of *S. aureus* isolated from hospital patients in Zaria metropolis?

1.7 Limitation of the Study

Firstly, the strike action embarked by the hospital staff during the data collection affected the total number of the in-patients.

Secondly, there was non-availability of some antimicrobial disks such as imipenem, carbapenem, linezolid and teicoplanin during the research, so they were not used in the antimicrobial susceptibility testing.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Classification of *Staphylococus* species

Table 2.1: Summary of the classification of *Staphylococcus*
(http://www.textbookofbacteriology.com*)*

Domain	**Bacteria**
Kingdom	**Eubacteria**
Phylum	**Firmicutes**
Class	**Bacilli**
Order	**Bacillales**
Family	***Staphylococcaceae***
Genus	***Staphylococcus***
Species (implicated in human disease)	***S. aureus***
	S. epidermidis
	S. saprophyticus
	S. haemolyticus
	S. lugdunensis

2.2 Morphology and Characteristics

Taxonomically, *Staphylococcus* belongs to the family *Staphylococcaceae* (Holcomb *et al*., 2008), and the genus *Staphylococcus*, is a group of facultative anaerobic, salt resistant, gram-positive that occur in grape-like clusters, include the major pathogen – *Staphylococcus aureus* (Forbes *et al*., 2002). Staphylococci are Gram-positive bacteria, with diameters of 0.5 – 1.5 μm and characterised by individual cocci, which divide in more than one plane to form grape-like clusters (Costa *et al*., 2013). *Staphylococcus* is usually either beta haemolytic or not haemolytic at all (called gamma hemolysis). Pathogenic staphylococci can produce a variety of virulence factors, including toxins, coagulase, leucocidins, and hydrolytic enzymes that can damage host tissues (Costa *et al*., 2013).

The staphylococci are non-motile, non-spore forming facultative anaerobes that grow by aerobic respiration or by fermentation (Harris *et al*., 2002). Most species have a relative complex nutritional requirement, however; in general they require an organic source of

nitrogen, supplied by 5 to 12 essential amino acids, e.g. arginine, valine, and B vitamins, including thiamine and nicotinamide (Wilkinson, 1997). Members of this genus are catalase-positive and oxidase-negative, distinguishing them from the genus *Streptococcus*, which are catalase-negative, and have a different cell wall composition to staphylococci (Wilkinson, 1997). Staphylococci are tolerant to high concentrations of salt (Wilkinson, 1997) and show resistance to heat. Pathogenic staphylococci are commonly identified by their ability to produce coagulase, and thus clot blood (Kloos and Musselwhite, 1975). This distinguishes the coagulase positive strains, *S. aureus* (a human pathogen), and *S. intermedius* and *S. hyicus* (two animal pathogens), from the other staphylococcal species such as *S. epidermidis*, that are coagulase-negative (CoNS). They all produce catalase, an important virulence factor which degrades the microbicidal H_2O_2 into O_2 and H_2O.

Macroscopically, *S. aureus* is a facultative anaerobic bacterium, which grows rapidly on blood agar and non-selective solid media including nutrient agar under both aerobic and anaerobic conditions (Yu and Washington, 1985). Colonies appear smooth, convex and sharply defined on blood agar plates when grown at room temperature (20^oC to 25^oC) (Lowy, 1998). The colonies are gold pigmented due to carotenoids, but this may not be apparent under certain conditions, such as anaerobic conditions or in liquid medium (Waldvogel, 2000). *Staphylococcus aureus* usually produce beta-haemolysis on horse, human or sheep blood agar plates, whereas *S. epidermidis* is non-haemolytic on blood agar plates when grown at 37^oC (Todar, 2005).

2.2.1 *Staphylococcus aureus* cell wall

The cell wall of *S. aureus* is a tough protective coat, which is relatively amorphous in appearance, about 20- 40 nm thick (Shockman and Barrett, 1983). Its main function is to provide a rigid envelope for the cell content. Underneath the cell wall is the cytoplasm

that is enclosed by the cytoplasmic membrane. Peptidoglycan is the basic component of the cell wall, and makes up 50% of the cell wall mass (Waldvogel, 1990). It is integral in the formation of the tight multi-layered cell wall network, capable of withstanding the high internal osmotic pressure of staphylococci (Wilkinson, 1997). Another cell wall constituent is a group of phosphate-containing polymers called teichoic acids, which contribute about 40% of cell wall mass (Knox and Wicken, 1973). There are two types of teichoic acids, cell wall teichoic acid and cell membrane associated lipoteichoic acid; bound covalently to the peptidoglycan or inserted in the lipid membrane of the bacteria. Teichoic acids contribute a negative charge to the staphylococcal cell surface and play a role in the acquisition and localization of metal ions, particularly divalent cations, and the activities of autolytic enzymes (Wilkinson, 1997). Peptidoglycan and teichoic acid together only account for about 90% of the weight of the cell wall, the rest is composed of surface proteins, exoproteins and peptidoglycan hydrolases (autolysins). Peptidoglycan has endotoxic properties, and has been reported to cause organ dysfunction in experimental animals.

Most *S aureus* produce a slimy, extracellular capsular polysaccharide, and a total of eight serotypes have been described; serotypes 5 and 8 are predominant in humans (O'Riordan and Lee, 2004). Capsules enhance microbial virulence by rendering the bacterium resistant to phagocytosis resulting in bacterial persistence in the blood stream of infected host.

2.2.2 *Staphylocoocus aureus* genome

Staphylocoocus aureus genome has a G+C content of 33% and it consists of a singular circular chromosome of about 2.7 to 2.9 Mbp containing about 2600 genes composed of core and auxillary (accessory) genes. The accessory genome accounts for about 25% of any *S. aureus* genome, and mostly consists of mobile genetic elements that transmit

horizontally between strains. These elements include bacteriophages, chromosomal cassettes, *pathogenicity islands*, *genomic islands*, plasmids and transposons. Many of genetic elements are known to carry genes associated with virulence, drug and metal resistance, substrate utilization and miscellaneous metabolism.

Bacteria obtain genetic information from other cells or the surrounding environment in three ways which are: uptake of free DNA from the environment (transformation), bacteriophage transduction and direct contact between bacterial cells (conjugation).

Staphylococcus aureus pathogenicity islands often carry superantigen genes such as toxic shock toxin and enterotoxins B and C, implicated in toxic shock and food poisoning. Staphylococcal cassette chromosome (SCC) is another mobile genetic element integrated into the *S. aureus* chromosome, within which the *mec* A gene encodes a specific methicillin-resistant transpeptidase known as penicilin-binding protein 2a (PBP2a) (Ito *et al*., 1999; Malachowa and DeLeo, 2010). The protein has a low affinity for beta-lactam antimicrobial drugs, thus bacteria expressing this protein are resisitant to all types of these drugs (Shittu *et al*., 2012). In recent times, there is resisitance to aminiglycosides and fluoroquinolone (Shittu *et al*., 2012).

2.3 Pathogenesis and Virulence of *S. aureus* infections

Staphylococcus aureus causes a broad range of infections. This variety is related to a number of virulence factors that allow it to adhere to surface, invade or avoid the immune system, and cause harmful toxic effects to the host. The virulence factors of *Staphylococcus aureus* include antigens, enzymes and toxins like Antigens, Capsule, Adhesins, enzymes: Coagulase, Lipase, Hyaluronidase, Staphylokinase, Nuclease, Toxins: α –Toxin, β –Toxin, δToxin, P-V Leukocidin, Enterotoxin, Exfoliative Toxin, Toxic Shock Syndrome Toxin (Costa *et al*., 2013).

It has been documented that there is probably no other bacterium that produces as many cellular components, enzymes, extracellular toxins and haemolysins as *S. aureus* (Todar, 2005). The cell wall of *S. aureus* is composed of a thick peptidoglycan layer, which contributes to the virulence of the bacterium (Lowy, 1998). The peptidoglycan stimulates the production of cytokines by macrophages resulting in complement system activation and platelet aggregation (Lowy, 1998).

The virulence factors 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 fibronectic binding protein A (FnbpA), clumping factor A (ClfA) and staphylococcal protein A (Spa) have been described (Winn *et al*., 2006).

Table: 2.2: Summary of molecules produced by *Staphylococcus aureus* and their effects

Gene(s)	Molecule or Protein	Activity
ahpC, ahpF	Alkyl hydroperoxide reductase subunits C and F AhpC and AhpF	Promotes resistance to reactive oxygen species (ROS)
aur	Zinc metalloproteinase aureuslysin (Aur)	Degrades LL 37
cap5, cap8	Capsular polysaccharide	Inhibits phagocytosis
katA	Catalase (katA)	Detoxifies hydrogen peroxide
Chp	Chemotaxis inhibitory protein of *Staphylococcus aureus*, chemotaxis inhibitory protein of *S. aureus*	Inhibits chemotaxis
clfA	Clumping factor A (ClfA)	Inhibits phagocytosis causes platelet activation
crtM, crtN	Carotenoid pigment, staphyloxanthin	Promotes resistance to ROS
dit operon	Dlt operon, dltABCD	Promotes resistance to cationic antimicrobial peptides (AMPs) and groups IIA phospholipase A2
Eap	Extracellular adherent protein (Eap)	Inhibits leukocyte adhesion
Ecb	Extracellular compliment binding protein (Ecb)	Inhibits C5a generation
Efb	Extracellular fibrinogen-binding protein (Efb)	Inhibits C5a generation
fnbA, fnbB	Fibronectin-binding proteins A and B (FnbA and FnbB)	Causes platelet activation
hla, hly	Alpha-haemolysin (α haemolysin), Hla	Causes host cell lysis
Hld	Delta-haemolysin (Hld)	Causes host cell lysis
hlgA, hlgB, hlgC	Gamma-haemolysin, subunits A,B and C (HlgA, HlgB, HlgC) Two-component leukocidin	Causes leukocyte and erythrocyte lysis
icaA, icaB, icaC, icaR	Polysaccharide intercellular adhesion (PIA)	Resistance to cationic AMPs

isdA, isdB	Iron-regulated surface determinants of *S. aureus* (isdA and isdB)	Resistance to AMPs, skin fatty acids and neutrophil ROS
lukS-PV, lukF-PV	Leukocidin S-PV and F-PV subunits, luk S/F-PV; OVL; two-component leukocidin	Causes phagocyte lysis
lukD, lukE	Leukocidin D and E; (lukD and lukE); two-component leukocidin	Causes leukocyte lysis
mprF	Multiple peptide resistance factor (MprF)	Promotes resistance to cationic AMPs
Psm	Phenol-soluble module like peptide (PSMs)	Causes leukocyte lysis
Sak	Staphylokinase (SaK)	Inhibits host α defensives
sbi	IgG-binding protein (Sbi)	Sequesters host IgG
Scn	Staphylococcal inhibitor of complement (SCIN)	Inhibits complements
sea, seb, sec, sed, see, seg, she, sej, sek, sel, sep, sel	Staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SHE, SEJ, SEK, SEL and SEP)	Activate T-cells
sodA, sodM	Superoxide dismutase (SodA, SodM)	Promotes resistance to ROS
Spa	Protein A	Sequesters host IgG, inhibits phagocytosis
ssL5	Staphylococcal superantigen-like 5, ssL5	Binds to CPSGL-1 and inhibits neutrophil
ssL7	Staphylococcal superantigen-like 7, SS7	Binds to C5a and IgA
Tst	Toxin shock syndrome toxin-1 TSST1	Activates T-cells

Source (adapted from): Todar, 2005

2.4 Diseases caused by *S. aureus*

Staphylococcus aureus is a pathogen that causes both human and animal infections and food intoxication (Hennekinne *et al*., 2012; Stryjewski and Corey, 2014). It causes simple infections, such as furuncle, boil, stye, impetigo, carbuncle, and keratitis, and serious infections, including septicemia, necrotizing pneumonia, endocarditis, osteomyelitis, and pericarditis (Taylor, 2013). The ability of *S. aureus* to cause a multitude of infections is probably due to the expression of a wide range of virulence factors, such as adhesion proteins, toxins and enzymes (Gebremariam and Zelelow, 2014). *Staphylococcus aureus* very commonly causes infections in humans: virtually every person will have one or more *Staphylococcus aureus* infections in his or her lifetime and most infections occur after an abrasion or cut of the skin. One of the reasons that *S. aureus* is a frequent cause of infections is that it can survive for months on any type of surface.

Diseases that arise from exclusively staphylococcal toxins include staphylococcal scalded skin syndrome, staphylococcal food poisoning and toxic shock syndrome (Murray *et al*, 2005). Other staphylococcal diseases include suppurative infections, wound infections and catheter related infections (Murray *et al*., 2005).

2.4.1 Bacteraemia

Staphylococcus aureus is a major cause of bacteremia, and *S. aureus* bacteremia is associated with higher morbidity and mortality, compared with bacteraemia caused by other pathogens. The burden of *S. aureus* bacteraemia, particularly methicillin-resistant *S. aureus* bacteraemia, in terms of cost and resource use is high (Naber, 2009). An episode of staphylococcal bacteremia was defined as a single positive blood culture specimen for *Staphylococcus* spp. in a patient with signs consistent with infection (Gupta and Chaudhary, 2018).

The incidence of *S. aureus* bacteraemia (SAB), particularly bacteraemia caused by methicillin-resistant *S. aureus* (MRSA) strains, has increased dramatically in recent years in the United States and in some European countries (Wisplinghoff *et al*., 2004; Shorr and Lodise, 2006).

Staphylococcus aureus bacteremia is an urgent medical problem due to its growing frequency and its poor associated outcome. The incidence of SAB has increased significantly during the past few decades and *S. aureus* has become a leading cause of blood stream infections (BSI) in most of the industrialized world (EARSS, 2007).
This burden is increased by the high likelihood that life-threatening complications of SAB will occur, including infective endocarditis (IE) and metastatic infections (Fowler *et al*., 2005; Troidle *et al*., 2007). *S. aureus* infective endocarditis (SAIE) is associated with significantly higher mortality, compared with IE caused by other bacteria (Miro *et al*., 2005).

Increased antibiotic resistance, in addition to the increased frequency of invasive surgery, increased use of intravascular devices, and increased number of patients with immunocompromised status because of HIV infection or immunosuppression after transplantation or cancer treatment, has led to sharp increases in the incidence of SAB and SAIE over the past 30 years (Fowler *et al*., 2005). It is estimated that more than 50% of *S. aureus* associated bacteraemia are acquired in the hospital after surgical operation or resulting from constant use of contaminated intravascular catheters (Mylotte and Tayara, 2000). The increased risk in staphylococcal bacteraemia is mostly attributed to catheterisation and patients with a high nasal carriage (85%) of *S. aureus* in hospital settings (Morin and Hadler, 1998).

2.4.2 Endocarditis

Staphylococcus aureus is the leading cause of infectious endocarditis and its mortality has remained high despite better diagnostic and therapeutic procedures over time (Fernandez *et al*., 2009). *Staphylococcus aureus* related endocarditis has accounted for 25% to 35% of cases worldwide between 1985 and 1993 (Sandre and Shafran, 1996).

Infective endocarditis is a complication often arising from *S. aureus* associated bacteraemia with a 12% incidence in infants and children in North Carolina, USA, between 1998 and 2001 (Valente *et al*., 2005). The mortality rate for hospital-infective endocarditis between 1972 and 1992 in Spain was 40% to 56% and it has been demonstrated that the mortality is even higher in patients when the isolated bacteria was *S. aureus* (Fernandez- Guerrero *et al*., 1995).

The infection is abundant in elderly patients, children (Valente *et al*., 2005), prosthetic valve patients, intravenous drug users and hospitalized patients. In the US it is standard care for IE/SAB patients to repeat the blood cultures until they are negative to document resolution of bacteremia whereas blood cultures only are repeated if complications arise in many European countries (Mermel *et al.*, 2009).

For these reasons it is very important that in patients with SAB the treating physician takes all of the clinical, microbiological, biochemical and echocardiographic findings into consideration when developing a management plan for an individual patient.

Prognosis of *S. aureus* related endocarditis is worsened in patients with HIV infection, as it usually presents as an advanced infective endocarditis (Fernandez-Guerrero *et al*., 1995).

2.4.3 Toxic shock syndrome

Toxic shock syndrome (TSS), caused by toxic shock syndrome toxin-1 (TSST-1) and other enterotoxins and the disease is characterised by diarrhoea, erythroderma, high fever, hypotension, mental confusion and renal failure. Toxic shock syndrome was initially described in 1978 (Todd *et al*., 1978); the disease came to public attention in 1980 with the occurrence of a series of menstrual-associated cases (Davis *et al*., 1980). The majority of clinically reported cases of TSS have been due to methicillin-susceptible *S. aureus* (MSSA). Cases of TSS due to MRSA have also emerged as rates of infection due to MRSA have increased (Durand *et al*., 2006).

By 1980, young menstruating women using high absorbency tampons were identified as a high risk group, with cases also observed in men and non-menstruating women. As the pathogenesis was better understood, it became clear that *S. aureus* toxins called superantigens in conjunction with host susceptibility from the absence of anti-superantigen antibodies were risk factors for the development of TSS (Spaulding *et al*., 2013).

The estimated incidence of TSS in 1980 among young menstruating women was 13.7 per 100,000 persons (Davis *et al*., 1980). By 1986 the rates of menstrual and non-menstrual TSS cases were 1 and 0.3 per 100,000, respectively (Durand *et al*., 2006), following multiple public health interventions including removal of highly absorbent tampons and messages regarding proper use of tampons, the number of cases declined sharply.

Many strains of *S. aureus* are known to carry genes for superantigens including toxic shock syndrome toxin–1 (TSST 1), the causative superantigen in most TSS cases (Smit *et al*., 2013). Prevalent strains of *S. aureus* are in constant flux. Over the past 15 years, community-associated methicillin-resistant *S. aureus* (CAMRSA) strains, most notably

USA300 and USA400, have emerged as predominant causes of skin and soft tissue infection in many geographic regions of the United States (Smit *et al*., 2013). Skin and soft tissue infections are a common primary site of non-menstrual TSST 1 (Smit *et al*., 2013).

Initial symptoms include diarrhoea, fever, myalgias and vomiting (Waldvogel, 2000). Hypovolemic shock develops due to loss of colloids and fluids (Chuang *et al*., 2005). Diagnosis and treatment of TSST 1 includes identification of the *S. aureus* strain and resistance profiling of the identified strain (White *et al*., 2005). Electrolytes and fluid replacement should be given to the patient as part of the overall therapy (White *et al*., 2005). An adjuvant treatment approach included agents that can block TSS superantigens, such as intravenous immunoglobulin that contains superantigen neutralizing antibodies (Chuang *et al*., 2005).

2.4.4 Food poisoning

Food-borne diseases (FBD) are defined by the World Health Organization (WHO) as "diseases of infectious or toxic nature caused by, or thought to be caused by the consumption of food or water (WHO, 2017). To date, around 250 different food-borne diseases have been described, and bacteria are the causative agents of two thirds of food-borne disease outbreaks (Le Loir *et al*., 2003). Among the predominant bacteria involved in these diseases, *Staphylococcus aureus* is a leading cause of gastroenteritis resulting from the consumption of contaminated food. Staphylococcal food poisoning is due to the absorption of staphylococcal enterotoxins preformed in the food (Le Loir *et al*., 2003).

The onset of *S. aureus* food poisoning is rapid, ranging from 30 min to 8 hr after ingestion, with spontaneous remission after 24 hr and the symptoms ranges from abdominal pain to nausea, vomiting and sometimes diarrhoea but never diarrhoea alone (Wieneke *et al*., 1993). Some *S. aureus* strains are able to produce staphylococcal

enterotoxins and are the causative agents of staphylococcal food poisonings. *Staphylococcus aureus* enterotoxins involved in food poisoning are highly stable and resistant to neutralisation by proteolytic enzymes, such as pepsin or trypsin (Bergdoll, 1989).

The main sources of contamination of these foods are food-handlers by manual contact, coughing or sneezing since up to 50%-70% of the human populations are *S. aureus* carriers (Solberg, 2000; Le Loir *et al*., 2003). *Staphylococcus aureus* is indeed found in the nostrils, and on the skin and hair of warm-blooded animals. Other sources involve contamination from animal origins either by animal carriage or zoonosis (Le Loir *et al*., 2003).

The foods that are involved in *S. aureus* food poisoning differ from one country to another (Wieneke *et al*., 1993), and various high sugar, protein and salt content foods are involved with *S. aureus* food poisoning including milk and milk products (cheese and ice cream), sausage, canned meat, salads (potato salads) and sandwich fillings (Bergdoll, 1989). Considerable research effort is still required for better understanding of the interactions between *S. aureus* and the food matrix, and of the mechanisms of SE production in foodstuffs (Le Loir *et al*., 2003).

2.4.5 Staphylococcal scalded skin syndrome

Staphylococcal scalded skin syndrome (SSSS) was first described in 1878 by Ritter von Rittershain as a disease manifested by a bullous exfoliative dermatitis in infants less than 1 month old (Rogolsky, 1979). Staphylococcal scalded-skin syndrome has been shown to be due to exfoliative toxins (Yamasaki *et al*., 2005). The exfoliative toxin genes, *eta* and *etb*, have been detected in 30% and 19% of SSSS presenting patients by polymerase chain reaction (Yamasaki *et al*., 2005). The disease presents occasionally with an onset of general localized erythema and spreads to the entire body in less than two days

(Rogolsky, 1979). Others include systemic manifestation involving the liver and other internal organs, osteomyelitis and pneumonia.

2.5 Antimicrobial Resistance

2.5.1 Bacterial Resistance strategies

Antimicrobial resistance is the ability of a microorganism to survive and multiply in the presence of an antimicrobial agent that would normally inhibit or kill this particular kind of organism. It is just one of the many adaptive traits that resilient bacterial subpopulations may possess or acquire, enabling them to out-compete and out-survive their microbial neighbors and overcome host strategies aimed against them.

There are four major bacterial resistance strategies namely: by prevention of the antimicrobial agent from reaching its target by reducing its ability to penetrate into the cell, by expulsion of the antimicrobial agents from the cell via general or specific efflux pumps, by inactivation of antimicrobial agents via modification or degradation and by modification of the antimicrobial target within the bacteria (Giguere *et al*., 2006). As in other parts of the world, antimicrobial resistance is emerging in *S. aureus* in Africa, particularly methicillin resistance (Schaumburg *et al*., 2014).

2.5.2 Molecular mechanisms of bacterial resistance

2.5.2.1 Intrinsic Resistance

The abilities of bacterial organisms to utilize the various strategies to resist antimicrobial compounds are genetically encoded. Intrinsic resistance is that type of resistance which is naturally coded and expressed by all (or almost all) strains of that particular bacterial species. Intrinsic resistance is the innate ability of a bacterial species to resist activity of a particular antimicrobial agent through its inherent structural or functional characteristics, which allow tolerance of a particular drug or antimicrobial class. This can also be called ‘insensitivity’ since it occurs in organisms that have never been susceptible to that

particular drug. Such natural insensitivity can be due to: lack of affinity of the drug for the bacterial target, inaccessibility of the drug into the bacterial cell, extrusion of the drug by chromosomally encoded active exporters and innate production of enzymes that inactivate the drug (Giguere *et al*., 2006).

2.5.2.2 Acquired Resistance

Acquired resistance is said to occur when a particular microorganism acquires the ability to resist the activity of a particular antimicrobial agent to which it was previously susceptible. This can result from the mutation of genes involved in normal physiological processes and cellular structures, from the acquisition of foreign resistance genes or from a combination of these two mechanisms. Acquired resistance results from successful gene change and/or exchange that may involve: mutation or horizontal gene transfer via transformation, transduction or conjugation (Alekshun and Levy, 2007).

A mutation is a spontaneous change in the DNA sequence within the gene that may lead to a change in the trait which it codes for. Horizontal gene transfer or the process of swapping genetic material between neighbouring contemporary bacteria is another means by which resistance can be acquired. Many of the antibiotic resistance genes are carried on plasmids, transposons or integrons that can act as vectors that transfer these genes to other members of the same bacterial species, as well to bacteria in another genus or species. Horizontal gene transfer may occur via three main mechanisms: transformation, transduction or conjugation. Transformation involves uptake of short fragments of naked DNA by naturally transformable bacteria. Transduction involves transfer of DNA from one bacterium into another via bacteriophages, while conjugation involves transfer of DNA material via sexual pilus and requires cell to cell contact (Alekshun and Levy, 2007).

Table 2.3: Antibiotic modes of action and mechanisms of resistance by bacteria

Antibiotic classes	Antibiotic mode of action	Bacterial mechanism of resistance
Beta-lactam e.g. penincillin, ampicillin, mezlocillin, peperacillin, cefazolin, cefotaxime, ceftazidime, aztreonam, imipenem	Target and bind to penicillin binding proteins (PBP), inhibiting bacterial cell wall synthesis	Enzymatic destruction of beta-lactam rings Target (PBP) modification Reduced intracellular accumulation
Glycopeptides e.g. vancomycin	Inhibit the last stages of cell wall assembly by preventing cross-linking reaction	Target modification Production of false target
Quinolones e.g. ciprofloxacin, levofloxacin, norfloxacin, lomefloxacin	Targets DNA gyrase and topoisomerase IV of the bacteria and inhibit the necessary step of supercoiling	Target modification Reduced intracellular accumulation of the drug
Aminoglycosides e.g. gentamicin, tobramycin, amikacin, netimicin, streptomycin, kanamycin	Target and bind to the 30S ribosomal subunit to cause misreading of the genetic code which results in inhibition of protein synthesis	Antibiotic (structural) modification Target modification Reduced uptake
Macrolides	Target and bind to 50S ribosomal subunit to inhibit translocation and transpeptidation process, resulting in inhibition of protein synthesis	Reduced intracellular uptake Target modification
Tetracyclines	Target and bind to 30S ribosomal subunit to prevent aminoacyl-tRNA to attach RNA ribosome complex, inhibiting protein synthesis	Reduced intracellular accumulation Target modification
Rifampicins	Interacts with the beta subunit of the bacterial RNA polymerase to block RNA synthesis	Target modification
Sulfonamides	Targets dihydropteroate	Target modification

	synthase (DHPS) and prevents addition of para-aminobenzoic acid (PABA), inhibiting folic acid synthesis

Source: Forbes *et al*., 1998; Berger-Bachi, 2002.

2.6 Mechanisms of Action for specific Antimicrobial Resistance

2.6.1 Resistance to beta lactams

The introduction of penicillin in the early 1940s dramatically improved the prognosis of patients with staphylococcal infection (Franklin, 2003). However, as early as 1942, penicillin-resistant staphylococci were recognized, first in hospitals and subsequently in the community (Rammelkamp and Maxon, 1942).

By the late 1960s, more than 80% of both community and hospital-acquired staphylococcal isolates were resistant to penicillin. This pattern of resistance, first emerging in hospitals and then spreading to the community, is now a well-established pattern that recurs with each new wave of antimicrobial resistance (Chambers, 2001). Kirby first demonstrated that penicillin was inactivated by penicillin-resistant strains of *S. aureus* (Kirby, 1944). More than 90% of staphylococcal isolates now produce penicillinase, regardless of the clinical setting.

The gene for β-lactamase is part of a transposable element located on a large plasmid, often with additional antimicrobial resistance genes (e.g. gentamicin and erythromycin) and spread of penicillin resistance primarily occurs by spread of resistant strains.

Penicillin is inactivated by penicillinase, a beta-lactamase that hydrolyses the beta-lactam ring of penicillin. Staphylococcal resistance to penicillin is mediated by *blaZ*, the gene that encodes β-lactamase. Four types of *blaZ* genes, A, B, C and D, have been distinguished by serotyping and differences in hydrolysis of beta-lactam substrates (Olsen *et al*., 2006). The *blaZ* gene is a transposable gene located on a plasmid, *pBW15* and transposon, *Tn4002* (Gillespie *et al*., 1988; Olsen *et al*., 2006). The *pBW15* is a 17.2-kb beta-lactamase plasmid that is present in 96% of *S. aureus* strains (McMurray *et al*., 1990). The transposon, *Tn4002* is 6.7 kb in size (Gillespie *et al*., 1988).

This predominantly extracellular enzyme synthesized when staphylococci are exposed to β-lactam antibiotics, hydrolyzes the β-lactam ring, rendering the β-lactam inactive. *blaZ* is under the control of two adjacent regulatory genes, the antirepressor *blaR1* and the repressor *blaI* (Kernodle, 2000). The signaling pathway responsible for β-lactamase synthesis requires sequential cleavage of the regulatory proteins BlaR1 and BlaI. Following exposure to β-lactams, BlaR1, a transmembrane sensor-transducer, cleaves itself (Zhang *et al*., 2001). Zhang *et al*. (2001) hypothesized that the cleaved protein functions as a protease that cleaves the repressor BlaI, directly or indirectly (an additional protein, BlaR2, may be involved in this pathway) and allows *blaZ* to synthesize enzyme.

2.6.2 Resistance to Methicillin

Methicillin, introduced in 1961, was the first of the semi-synthetic penicillinase resistant penicillins (Franklins, 2003). Its introduction was rapidly followed by reports of methicillin-resistant isolates (Jevons, 1961). For clinicians, the spread of these methicillin-resistant strains has been a critical one.

The *mec A* gene present in MRSA strains encodes the altered protein (PBP2a), which is not inactivated by methicillin (Gaze *et al*., 2008). The *mec A* gene resides on the staphylococcal cassette chromosome *mec* (SCC*mec*) and is expressed by the regulator genes *mecR1* and *mecI* (Lowy, 1998).

The regulator gene *mecR1* is activated by beta-lactam antibiotics and serves as a signal transducer that inactivates the *mecI* repressor gene product (Lowy, 1998). To date, atleast eleven different SCCmec types (I-XI) have been defined (Ito *et al*., 2016). However, only SCCmec type I-V are globally distributed while others are uncommon and may exist as local strains in their original countries (Ghaznavi-Rad *et al*., 2010).

Some SCC*mec* types contain genetic elements for other antibiotic resistance, such as *Tn554*, a transposon responsible for resistance to macrolides, clindamycin and streptogramin B, while the *pT181* plasmid accounts for resistance to tetracyclines (Oliveira and De Lencastre, 2002).

Methicillin resistance requires the presence of the chromosomally localized *mecA* gene. *Mec A* is responsible for synthesis of penicillin-binding protein 2a (PBP2a; also called PBP2′) a 78-kDa protein (Song *et al*., 1987). PBPs are membrane-bound enzymes that catalyze the transpeptidation reaction that is necessary for cross-linkage of peptidoglycan chains (Ghuysen, 1994).

2.6.3 Resistance to Chloramphenicol

Evidence accumulated suggests that strains of *Staphylococcus aureus* which are resistant to chloramphenicol (chl-r) owe their resistance to inactivation of the antibiotic (Miyamura, 1964) and further studies indicate that acetyl coenzyme A (CoA) is required for the inactivation of the drug by resistant *S. aureus* (Suzuki *et al*., 1966).

Chloramphenicol resistance in gram positive bacteria is mainly due to enzymatic inactivation. Efflux system which confers either only chloramphenicol resistance or combined resistance to chloramphenicol has also been described. Chloramphenicol-resistant strains of *Staphylococcus aureus* contain an inducible enzyme which inactivates chloramphenicol by acetylation in the presence of acetyl coenzyme A (Shaw and Brodsky, 1967).

2.6.4 Resistance to Aminoglycosides

Resistance to aminoglycoside is mainly based on enzymatic inactivation by aminoglycoside – modifying enzymes (Shows *et al*., 1993). Moreover, decreased uptake of aminoglycoside and chromosomal mutation conferring high-level resistance to streptomycin has been described. Enzymatic inactivation of aminoglycosides is conferred

by N-acetyltransferases, o-adenyltransferases or o-phosphotransferases (Show *et al*., 1993). Most of these enzymes exhibit specific substrate spectrums. However, several other enzymes have been identified, some of which are part of integrons/gene cassettes. Four classes of N-acetyltransferase are known which acetylate the amino acid groups at positions 1-, 3-, 2^1- and 6^1 (Show *et al*., 1993).

2.6.5 Resistance to Sulphamethoxazole and trimethoprim

Sulphamethoxazole and trimethoprim block different enzymatic steps in tetrahydrofolate biosynthesis. Sulfonamides are structural analogues of p-aminobenzoic acid and competitively inhibit the enzyme dihydropteroic acid synthetase (DHPS) while trimethoprim competitively inhibits the enzymes dihydrofolate reductase (DHFR). While some bacteria are intrinsically resistant, acquired resistance may be due to chromosomal mutations or to plasmid–ended DHPS or DHFR enzymes which are resistant to sulphamethoxazole and Trimethoprim respectively (Quintilian *et al*., 1999).

2.6.6 Resistance to Tetracycline

Several different mechanisms of tetracycline resistance have been described, among which active efflux and ribosome protection are the most prevalent mechanisms among gram-positive and negative pathogens. The energy dependent efflux of tetracycline is mediated by at least two types of membrane proteins, both of which exchange a proton for tetracycline cation complex. At least fourteen different classes can be differentiated on the basis of hybridization experiments (Levy *et al*., 1999), and the most intensively studied classes are A, B, C, D, H, K and L. The genes *tet* (k) and *tet* (L) are mainly present in gram- positive bacteria and code for a protein which consists of 14 transmenbrane regions and these genes are frequently found on small plasmids which on rare cases may be integrated into other plasmids or into the chromosomal DNA, but may also undergo interplasmic recombination with other resistance plasmids. Their Tc-

inducible expression is regulated by a mechanism known as 'translational attenuation'. Tc-inducible expression of these *tet* genes based on the binding of tetracycline Mg2+ complex to the *tet* repressor protein which in the absence of tetracycline, blocks transcription of *tet* structural gene.

2.6.7 Resistance to Macrolides, Lincosamides and Streptogramins

Resistance to erythromycin in staphylococci is usually associated with resistance to other macrolides, to the lincosamides, and to type B streptogramin (MLS). The most important mechanism conferring high-level resistance is structural changes in ribosomal RNA (rRNA) that prevent macrolide binding. Different bacterial species are able to synthesise an enzyme that methylates rRNA encoded by a series of structurally related erythromycin-resistant (*erm*) methylase genes (Pechère, 2001). Production of methylase results in the *N*6-dimethylation of an adenine residue at position 2058 of 23S rRNA. The conformational changes that occur in the P site of rRNA 23S prevent macrolide binding; therefore the inhibitory effect of the macrolide on protein synthesis is overcome. The erythromycin genes that have been identified in staphylococci are *ermA*, *ermB*, *ermC* and *ermM*, with *ermA* being the predominant gene (Sekiguchi *et al*. 2003). Another mechanism of inducible resistance to erythromycin is conferred by the gene *msrA*, which encodes an ATP- dependent efflux pump.

2.6.8 Resistance to vancomycin

The emergence of *S. aureus* resistant to vancomycin has caused considerable concern (Daum *et al* 1992; Ploy *et al* 1998) and staphylococcal resistance to vancomycin in a clinical isolate was first reported in a strain of *Staphylococcus haemolyticus*. In 1996, the first MRSA to acquire vancomycin intermediate-resistant *S. aureus* (VISA) was isolated in Japan, and additional cases were subsequently reported from other countries such as USA, France, Scotland, South Africa and Brazil (Hiramatsu, 2001). In 2002, the first

vancomycin resistant *S. aureus* (VRSA) infection was documented in a patient in the United States. This strain was shown to carry a *van* gene, suggesting that the resistance determinant might have been acquired through the genetic exchange of material between vancomycin resistant enterococci (VRE) and *S. aureus.* Unusually, a thickened cell wall is responsible for the vancomycin resistance in clinical VRSA strains (Walsh and Bowe, 2002). The thickened cell wall not only traps a greater number of vancomycin molecules, but also significantly impedes other glycopeptide molecules from reaching the sites of cell wall biosynthesis at the plasma membrane (Sieradzki *et al.*, 1999).

Showsh *et al*. (2001) reported that enterococcal plasmid containing *vanA* encodes a sex pheromone that is synthesized by *S. aureus*, suggesting a potential facilitator of conjugal transfer of the *van* A operons from a vancomycin-resistant *E. faecalis (*Franklin, 2003*).*

Two forms of vancomycin resistance have been demonstrated (Walsh and Howe, 2002).The first form involves the VISA strains with MIC of 8-16 μg/ml (Walsh and Howe, 2002), and the reduced susceptibility to vancomycin postulated to be due to changes in the peptidoglycan synthesis (Walsh and Howe, 2002). The second form of resistance involves vancomycin resistant *S. aureus* with a MIC of ≥128 μg/ml (Walsh and Howe, 2002). The mechanism is hypothesised to be due to conjugation with vancomycin resistant *Enterococcus faecalis* (VRE). The process of conjugation results in the transfer of the *vanA* operon of the *E. faecalis* bacterium to the MRSA strain (Showsh *et al*., 2001).

2.6.9 Resistance to Quinolones

Fluoroquinolones were initially introduced for the treatment of Gram-negative bacterial infections in the 1980s. However, because of their Gram-positive bacterial spectrum, they have also been used to treat bacterial infections caused by pneumococci and staphylococci (Franklin, 2003). Quinolone resistance among *S. aureus* emerged quickly, more prominently among the methicillin-resistant strains. Fluoroquinolone resistance

develops as a result of spontaneous chromosomal mutations in the target of the antibiotic, topoisomerase IV or DNA gyrase, or by the induction of a multidrug efflux pump. When quinolones are used to treat infections caused by other bacterial pathogens, subjects colonized with *S. aureus* (e.g., on their skin or mucosal surfaces) are likely exposed to subtherapeutic antibiotic concentrations and are therefore at risk of becoming colonized with resistant mutants (Hooper, 2002).

Resistance to quinolones results from the stepwise acquisition of chromosomal mutations. The confluence of high bacterial density, the likely preexistence of resistant subpopulations, and the sometimes limited quinolone concentrations achieved at sites of staphylococcal infections creates an environment that fosters selection of resistant mutants (Hooper, 2002). The quinolones act on DNA gyrase, which relieves DNA supercoiling, and topoisomerase IV, which separates concatenated DNA strands. Amino acid changes in critical regions of the enzyme- DNA complex (quinolone resistance–determining region (QRDR) reduce quinolone affinity for both of its targets. The ParC subunit (GrlA in *S. aureus*) of topoisomerase IV and the GyrA subunit in gyrase are the most common sites of resistance mutations; topoisomerase IV mutations are the most critical, since they are the primary drug targets in staphylococci (Hooper, 2002).

An additional mechanism of resistance in *S. aureus* is induction of the NorA multidrug resistance efflux pump. Increased expression of this pump in *S. aureus* can result in low-level quinolone resistance (Ng *et al*., 1994). In an interesting linkage of virulence to antimicrobial resistance, a recent study showed that exposure of a quinolone-resistant isolate to a quinolone increased the organism's expression of fibronectin-binding protein, a surface protein that mediates adherence to tissue surfaces (Bisognano *et al*., 2000).

2.7 Transmission of *Staphylococcus aureus*

Staphylococcus aureus may occur commonly in the environment and transmitted through droplets or aerosol. When an infected person coughs or sneezes, he or she releases numerous small droplets of saliva that remain suspended in air. These contain the bacteria and can infect others. Another common method of transmission is through direct contact with objects that are contaminated by the bacteria or by bites from infected persons or animals. Approximately 20-30% of healthy persons are persistent carriers of *S. aureus* and 60% are intermittent carriers with high colonization rates among risk groups including hospital patients, children and jail inmates (Tekalign *et al.,* 2013). Although it is usually harmless at these sites, it may occasionally get into the body (e.g. through breaks in the skin such as abrasions, cuts, wounds, surgical incisions or indwelling catheters) and cause infections.

Staphylococcus aureus can be readily transmitted from one species to another. This includes transmission between humans and animals and vice verse (Loeffler *et al.,* 2011). 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). Report showed that majority of MRSA infections found in cats, dogs, pet birds and horses were caused by human strains (Baptiste *et al*., 2005).

Transmission between humans and their animal contacts may be facilitated by their contaminated shared surroundings (Leonard and Markey, 2008). Five factors or Cs have been implicated in MRSA outbreaks include- contact, lack of cleanliness, compromised skin integrity, contaminated items or surfaces and crowded living conditions (Deleo *et al*., 2011).

2.8 Emergence of Methicillin-Resistant *Staphylococcus aureus*

The introduction of methicillin in 1960 as an alternative antibiotic for the treatment of beta-lactamase (penicillinase) producing *S. aureus* was greeted with resistance almost immediately (Jevons, 1961). MRSA is any strain of *S. aureus* that has developed resistance to β-lactam antibiotics, which include the penicillins; methicillin, dicloxacillin, nafcillin, oxacillin and the cephalosporins (Seyed *et al.,* 2016). Strains unable to resist these antibiotics are classified as methicillin-sensitive *S. aureus* (MSSA) (Tenover and Goering, 2009). Methicillin-susceptible *S. aureus* (MSSA) become MRSA through the acquisition and insertion into their genomes of a large DNA fragment known as staphylococcal chromosome cassette *mec* (SCC*mec*), which contains the methicillin resistance determinant, *mec* A gene (Hiramatsu *et al*., 2001).

MRSA has become a major public health problem worldwide and recent reports have indicated that the prevalence of hospital-associated MRSA (based on the detection of the *mec* A gene) in health care institutions in Nigeria may vary from 1.5% to 20% (Okon *et al*., 2009; Ghebremedhin *et al*., 2009).

The importance of methicillin resistant *S. aureus* to public health is due to the ability of the organism to acquire resistance and virulence genes, thus leading to the emergence of new and highly virulent clones posing great difficulty in antibiotics chemotherapy thereby prolonging hospital admission stay (Liu, 2009). Different antimicrobial substances are used extensively at sub-therapeutic or therapeutic doses as growth promoters, routine prevention and treatment of bacterial diseases in food-animals (Waters *et al*., 2011). This practice is usually common in developing countries such as Nigeria, where there are poor legislations regulating the use of antimicrobials in food-producing animals (Bitrus *et al*., 2016: Ugwu *et al*., 2015). Global transmission of MRSA linked to international travel has been the subject of many studies (Harastani *et al*., 2014).

Methicillin resistance in *S. aureus* involves an altered target site due to an acquired penicillin-binding protein (PBP2a) with decreased affinity to b-lactams (Hamid *et al.*, 2017). The *mec A* gene encodes this protein and is located on a mobile SCC*mec* (Chibuike *et al.*, 2014).

To date, there are 12 SCC*mec* types described, varying greatly in size (from approximately 21- 67kb) and most commonly HA-MRSA strains carry SCC*mec* I,II and III, while CA-MRSA strains have SCC*mec* types IV and V (Wu *et al*., 2015).

SCC*mec* elements are highly diversed and eleven types have been recognized to date by some reserachers (Ito *et al*., 2012). Despite their diversity in size (from approximately 21-67kb) and gene content; they all share important defining characteristics. The smaller subtypes (I, IV, and V) encode only recombinase genes and the structural and regulatory genes for resistance to methicillin; they do not carry transposable elements and genes encoding resistance to non– beta-lactam antibiotics (Deresinki, 2005). It is noteworthy, however, that some SCC*mec* types carry various additional genetic elements (*Tn554*, which encodes resistance to macrolides, clindamycin, and streptogramin B; and *pT181*, which encodes resistance to tetracyclines) that can confer resistance to additional antibiotic classes; these genetic elements are especially common in HA-MRSA (Deresinki, 2005).

2.9 Types of Methicillin Resistant *Staphylococcus aureus*

These are classified as hospital- associated MRSA (HA-MRSA), Community associated MRSA (CA-MRSA) and Livestock associated MRSA (LA-MRSA) (Fitzgerald, 2012). The latter is bacteriologically, clinically and epidemiologically, distinct from the former, and continues to be prominently involved in nosocomial infections. Infections caused by CA-MRSA in particular, have become a major public health threat (Martin and Henry, 2008). On the basis of the differences in SCC*mec* genomic islands, it is possible to define

differences between CA-MRSA and hospital-acquired MRSA (Rimoldi *et al*., 2018). Staphylococcal cassette chromosome *SCCmec* typing, is used as epidemiological marker of isolates into either of noscomial or community-associated infections (Ma *et al*., 2000); SCC*mec* types IV or V is a signature for CA-MRSA (Sowash and Uhlemann, 2014).

2.9.1 Health care associated MRSA

Hospital-associated (HA) methicillin-resistant *Staphylococcus aureus* (MRSA) has been recognized as a primary cause of nosocomial infections that acquired multiple drug resistance, associated with its global spread since the 1960s (Chambers and Deleo, 2009).

The major reservoir of staphylococci in hospitals are colonized infected in-patients and colonized hospital workers, with carriers at risk for developing endogenous infection or transmitting infection to healthcare workers and patients. The major mechanism for patient to patient transmission has been through transient carriage of the organism on the hands of healthcare workers. Approximately 30% of healthy individuals are colonized with *Staphylococcus aureus* asymptomatically (Gorwitz *et al*., 2008).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is currently the most prevalent antibiotic-resistant pathogen causing nosocomial infections in hospitals in many parts of the world with increasing prevalence in various community populations (Abdulreesh, 2010).

Methicillin resistance of *Staphylococcus* is characterized by the presence of a transmissible genome element, staphylococcal cassette chromosome *mec* (SCC*mec*), which is inserted in the chromosome of bacterial cell. SCC*mec* in MRSA has been differentiated into at least 11 genetic types (I–XI) (IWG-SCC, 2009; Shore *et al.*, 2011) among which types I to III are commonly found in HA-MRSA, while type IV and V were reported to be frequently found in CA-MRSA (David and Daum, 2010). However, in the present circumstances, CA-MRSA with the dominant SCC*mec* types have been brought

to healthcare settings (Popovich *et al*., 2008), which makes distinction between HA- and CA-MRSA more difficult in terms of SCC*mec* type.

2.9.2 Community associated MRSA

Community associated (CA) MRSA have also emerged as cause of infections in individuals who have no healthcare-associated risk (David and Daum, 2010), posing a public health concern worldwide. The initially identified CA-MRSA strains were characterized by production of Panton-Valentine leukocidin, a two-component leukolytic toxin (Vandenesch *et al*., 2003), which is associated with severe symptoms in a wide spectrum of infections (Francis *et al*., 2005), including skin, soft tissue infection (SSTI) and necrotizing pneumonia. Prevalence of CA-MRSA harbouring *pvl* genes has been increasing recently in hospitalized patients as well as healthy individuals in the community (Hetem *et al*., 2012). Panton- Valentine leukocidin is believed to cause tissue necrosis and leukocyte destruction, and it is an important virulence factor that is mainly found in CA-MRSA, but it is also associated with CA-methicillin-susceptible *S. aureus* (MSSA) (Chiu *et al*., 2012). The increased virulence potential of CA-MRSA strains remains controversial and different virulence factors, such as pvl, phenol-soluble modulins, alpha toxin, arginine catabolic mobile element and protein A, contribute to the severity, persistence and increased transmission of the bacterium (Kale and Dhawan, 2016).

Although CA-MRSA is generally more susceptible to antibiotics than strains originating from the healthcare system, its resistance profile in certain populations, such as North American homosexuals, has broadened considerably. CA-MRSA most often harbour the staphylococcal chromosome cassette (SCC) *mec* type IV which contains the *mec A* gene as the sole resistance determinant.

2.9.3 Livestock associated MRSA

Livestock associated methicillin-resistant *Staphylococcus aureus* (MRSA) was first associated with human disease in 2003, when a MRSA clone associated with a reservoir in pigs and cattle was isolated from a human (Brigitte *et al*., 2011). Animals have also been identified as a reservoir and source for the emergence of novel resistance elements such as the novel bovine *mec*A gene homologue, *mec*A (LGA251), now designated *mec*C (Petersen *et al*., 2013).

Study showed MRSA clones to be frequent colonizers of food animals in Europe and emerging worldwide (Crombe *et al*., 2013). This clone is quite different from HA-MRSA and CA-MRSA lineages. The colonization by the organism is possibly favoured by the indiscriminate and large use of antibiotics in animal production either for therapeutic use or to improve growth performance of the animals (Gilchrist *et al*., 2007; Waters *et al*., 2011).

The detection of MRSA in livestock since 2006 especially pigs, but also in calves, chickens, horses, turkey and dairy cattle, has shown that livestock constitute a reservoir of MRSA belonging to sequence type (ST) 398, which can be transmitted to humans (Loeffler *et al*., 2011). MRSA of other sequence types like ST5, ST9, 97 have also been reported in livestock (Lulitanond *et al*., 2013; Kock *et al*., 2013). Multilocus sequence type 398 has been reported globally in horses, cattle and poultry (Butaye *et al*., 2016).

MRSA ST398 carriage have been high (25%–35%) for persons in the Netherlands who have frequent contact with pigs and veal calves, but associated illness is rare (Broek *et al*., 2008*)*. However, in Netherlands, Asia, and the United States, invasive infections and a hospital outbreak of MRSA ST398 have been reported (Wulf *et al.*, 2008).

2.10 Epidemiology of *S. aureus*

2.10.1 Prevalence of *S. aureus* colonization and infection

Research on African *S. aureus* has been largely neglected in the past, despite the cultural and geographical diversity in Africa, which has a significant impact on the epidemiology of this pathogen (Schaumburg *et al.,* 2014). However, in recent years, a growing interest in the epidemiology of *S. aureus* has been observed on the African continent (Aiken *et al*., 2014; Schaumburg *et al*., 2014).

HIV/AIDS, malaria, malnutrition, crowded living conditions, high temperatures and humidity increase the risk of other bacterial infections (Wang *et al*., 2013). Beside this, prolonged hospitalization, use of invasive medical devices, contact with healthcare workers, suppressed immune system, prolonged use of antimicrobials, living in crowded or unsanitary conditions are some risk factors for MRSA infection (Goyal *et al.,* 2013). Nasal colonization with MRSA is a significant risk factor for hospital acquired infections (Sachin-Sharma *et al.,* 2011). *S. aureus* colonization is a risk factor for subsequent infection caused by the colonizing clone (von Eiff, 2001). Confirmed risk factors for *S. aureus* colonization in Africa are HIV infection (Olalekan *et al*., 2012; Kinabo *et al*., 2013), living in rural areas, and being hospitalized on surgery wards. In Nigeria, colonization rates have been reported among HIV patients (33%), healthy carriers (21%) and among hospital patients (Olalekan *et al*., 2012). As in other parts of the world, *S. aureus* also colonizes animals, such as pets (e.g. dogs), livestock (e.g. donkeys, pigs, and sheep) or wild animals (e.g. monkeys, chimpanzees, gorillas, and bats) (Youn *et al*., 2014). So far, only MRSA colonization in pigs (12.5% in South Africa and 1.3% in Senegal) might point towards future problems (Schaumburg *et al*., 2014).

The spectrum of *S. aureus*-related infections differs from one part of the world to the other. Some studies have suggested a higher proportion of *S. aureus* in urinary tract

infections: 6.3– 13.9% of urinary tract infections are caused by *S. aureus* in Senegal (Dromigny *et al*., 2002), Ghana (Adjei and Opoku, 2004), and Nigeria (Otajevwo, 2013), as compared with 1.1% in Europe and Brazil (Naber *et al*., 2008). In general, *S. aureus* is a major pathogen in bloodstream infections (9.5–39.0%), skin and soft tissue infections (62.8–90.0%), ear, nose and throat infections (16.7–29.0%), and surgical site infections (20.4–32.0%) (Alabi *et al*., 2013). A few prospective studies have revealed a higher incidence of *S. aureus* infection in Africa than in industrialized countries (Landrum *et al*., 2012).

Research on African isolates of *S. aureus* has been largely neglected in the past despite the cultural and geographical diversity in Africa which has a significant impact on the epidemiology of this pathogen (Schaumburg *et al*., 2014). However, studies in Zaria, Ilorin, Jos and Kano showed prevalence of MRSA to be 69.0%, 34.7%, 43.0% and 10.7% respectively (Onanuga *et al.*, 2005; Taiwo *et al*., 2005; Ikeh, 2003; Nwankwo and Nasiru, 2011). Studies in Sokoto, ABUTH Zaria, South South Nigeria, Ghana and India reported MRSA prevalence of 25% (Olowo-Okere *et al*., 2017), 12.3% (Abdulaziz and Olayinka, 2016), 28.7% (Anie *et al.*, 2017) 17% (Saba *et al*., 2017) and 47.5% (Sathish and Wadekar, 2017) respectively.

Detailed molecular characterization of clinical *S. aureus* isolates from Africa has been largely neglected in the past (Schaumburg, *et al*., 2014). Only cases of *S. aureus*-related infections in travellers returning from Africa have suggested that African *S. aureus* strains might have a different genetic background and might be more virulent than isolates from Europe (Schaumburg *et al*., 2014). Field studies performed in Africa in the last two decades have showed PVL-positive *S. aureus* infection in travellers: Africa is now considered to be a pvl-endemic region with high rates of pvl-positive isolates, mainly MSSA, ranging from 17% to 74% (Breurec *et al*., 2011).

This is in stark contrast to Europe, where the prevalence of pvl-positive isolates is low (0.9–1.4%) (von Eiff *et al*., 2004). Studies from Gabon and South Africa support the association of PVL with abscesses and skin and soft tissue infection (Schaumburg *et al*., 2011). The reasons for the high prevalence of pvl are unknown, but might be related to the host (i.e. altered C5a receptors, which have been identified as pvl targets), so far unidentified virulence factors of *S. aureus* that facilitate dissemination and the humid environment of tropical Africa (Li *et al*., 2012; Spaan *et al*., 2013).

2.10.2 Prevalence of Antimicrobial Resistance

Antimicrobial resistance of *S. aureus* is emerging in Africa, particularly methicillin resistance (Schaumburg *et al*., 2014). Available data on prevalence of antimicrobial resistance in *S. aureus* in Afica mainly originated from north, south, central and west Africa. (Breurec *et al*., 2011; Fall *et al*., 2012; Falagas *et al*., 2013; Schaumburg *et al*., 2014). One striking feature of African MSSA in urban areas is the high level of resistance to penicillin (73.7–100%) (Shittu *et al*., 2011;Kolawole *et al*., 2013) and sulphamethoxazole-trimethoprim (15–89.1%) (Mariem *et al*., 2013; Seni *et al*., 2013). However, isolates from a remote community in Gabon were often susceptible to tetracycline (94%), and sulphamethoxazole-trimethoprim (88%) and penicillin (65%) (Schaumburg *et al*., 2011). Differences also exist in the antimicrobial resistance/ susceptibility patterns between African regions; fusidic acid resistance appears to be more frequent in North Africa (13-65%) than in sub-saharan Africa (0-2%) (Ramdani.-Bouguess *et al*., 2006; Shittu *et al*., 2011; Elazhari *et al*., 2012; Conceic~ao *et al*., 2014). This could be as a result of difficulties in availability, usage of antimicrobial agents and antimicrobial susceptibility testing (Falagas *et al.,* 2013). In addition, not conforming to standard procedures for antimicrobial testing in some of the studies may be responsible (Vieighe *et al*., 2009).

2.11 Diagnosis of *S. aureus* infections

2.11.1 Phenotypic detection method

The identification of *S. aureus* infection and MRSA has been based on phenotypic and molecular studies. The various tests include Gram staining, coagulase, catalase, Dnase, sugar fermentation and culture on mannitol salt agar, Baird Parker or blood agar (Waldvogel, 2000) and commercial biochemical kits. *S. aureus* is Gram positive cocci, catalase positive, coagulase and Dnase positive; the organism grows on mannitol salt agar, blood agar and Baird Parker agar at 37^0C for 18 to 24hrs. The colonies appear yellow on mannitol salt agar and creamy white on blood agar (Brown *et al*., 2005).

2.11.2 Molecular detection method

Molecular characterization of *S. aureus* is vital for the rapid identification of prevalent strains and will contribute to the control and prevention of *S. aureus* spread around healthcare settings if results are provided in real time (O'Hara *et al*., 2016). The molecular methods include phage typing, staphylococcal protein A (*spa*) typing, staphylococcal cassette chromosome mec (SCC*mec)* typing, multilocus sequence typing (MLST), pulse field gel electrophoresis, capsular typing, among others. Phage typing was gradually replaced by pulsed field gel electrophoresis (PFGE), the most recent gold standard method for the typing of *S. aureus* isolates (Bosch *et al*., 2015).

However, due to its laborious character and difficulties in exchanging data between laboratories, and the requirement for inter-laboratory standardization, PFGE was replaced by multi-locus sequence typing (MLST) and staphylococcal protein A (*spa*) typing (Harmsen *et al*., 2003). MLST is a great tool for evolutionary investigations and differentiates isolates according to nucleotide variations in 7 house-keeping genes. It is a method that relies on sequencing of internal fragments of specific housekeeping genes, i.e. seven gene loci are compared in *S. aureus*—carbamate kinase (*arcC*), shikimate

dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyl transferase (*pta*), triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyl transferase (*yqiL*). Sequence differences in each gene are considered alleles and the seven gene loci create an allelic profile by which the sequence type is determined (Stefani *et al*., 2012).

The MRSA isolates are genetically characterized by multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), SCC*mec* typing (I-VIII), accessory gene regulator (*agr*) typing and staphylococcal protein A (*spa*) typing (Chambers and Deleo, 2009; Kennedy and Deleo, 2009). The MLST classifies *S. aureus* isolates on the basis of allelic variation in seven housekeeping genes - clones consist of isolates with identical sequences at all the seven loci and are assigned a unique sequence type (ST); clonal complexes (CCs) comprise closely-related STs differing by single nucleotide polymorphisms (SNPs) at lesser than three loci (Chambers and Deleo, 2009).

Accurate and rapid detection of methicillin resistance in *S. aureus* is imperative for the use of appropriate antimicrobial therapy and for the control of nosocomial spread of MRSA strains. Early diagnosis and appropriate treatment can significantly limit the duration and outcome of infection (Salem-Bekhit, 2014).

The treatment of severe infections caused by some epidemic strains of MRSA is much more difficult and expensive than with MSSA because most of MRSA strains are resistant to multiple antibiotics (Johnson, 2011). Accurate and rapid identification of MRSA and their antimicrobial susceptibility profiles is essential for the selection of appropriate therapy (Mohanasoundaram and Lalitha, 2008). Therefore, it is clinically crucial to determine quickly whether *S. aureus* isolates are methicillin resistant or not, as this is very significant for both treatment and requires extensive hygienic precautions to limit the spread of such strains.

2.12 Antimicrobial Susceptibility Testing

Laboratory detection methods can determine resistance or susceptibility of an isolate against an array of possible therapeutic candidates, antimicrobial susceptibility testing results can be a useful clinical guideline in selecting the best antibiotic treatment option for each particular patient. Antimicrobial susceptibility testing methods are *in vitro* procedures used to detect antimicrobial resistance in individual bacterial isolates; and they can also be used for monitoring the emergence and spread of resistant microorganisms in the population (Watts and Lindeman, 2006).

Clinical Breakpoints are threshold values established for each pathogen-antibiotic combination indicating at what level of antibiotic the isolate should be considered to be sensitive, intermediate or resistant. The interpretative criteria for these are based on extensive studies that correlate laboratory resistance data with serum achievable levels for each antimicrobial agent and a history of successful and unsuccessful therapeutic outcomes. There are organizations responsible for harmonizing the criteria for human and veterinary standards (Watts and Lindeman, 2006).

Standard conditions for these assays have been established based on extensive batteries of laboratory testing. Guidelines and recommendations for these are continuously updated by certain organizations worldwide, such as Clinical and Laboratory Standards Institute (CLSI), European Committee of Antimicrobial Susceptibility Testing (EUCAST), Office International des Èpizooties (OIE), British Society for Antimicrobial Chemotherapy (BSAC), Société Française de Microbiologie (SFM), Swedish Reference Group for antibiotics (SRGA) and CDS disk diffusion method (CDS) (Watts and Lindeman, 2006). In vitro procedures are only approximations of *in vivo* conditions, which can be very different depending on the nature of the drug, the nature of the host and the conditions surrounding the interaction between the antibiotic and the target pathogen. Therefore,

following standardized procedures that will generate reproducible results is very important (i.e. quality control). These aspects of quality control include- standardized bacterial inoculum size, culture conditions (growth medium, pH, cation concentration, blood and serum supplements and thymidine content), incubation conditions (atmosphere, temperature, duration), concentration of antimicrobials for testing among others. Culturing of bacteria before conducting the antimicrobial susceptibility testing usually takes several days which is not ideal particularly in critical clinical cases demanding urgency. Often, practitioners may utilize locally established antibiograms as guideline for therapy. An antibiogram is a compiled susceptibility report or table of commonly isolated organisms in a particular hospital, farm, or geographic area, which can serve as a useful guideline in therapy before actual culture and susceptibility data becomes available for reference (Turnidge and Peterson, 2007).

There are several antimicrobial susceptibility testing methods available today, and each one has their respective advantages and disadvantages. They all have one and the same goal, which is to provide a reliable prediction of whether an infection caused by a bacterial isolate will respond therapeutically to a particular antibiotic treatment or not.

Some examples of antibiotic sensitivity tesing methods are: dilution method (broth and agar dilution method), disk-diffusion method, E-test, automated methods, mechanism-specific tests such as beta-lactamase detection test and chromogenic cephalosporin test, genotypic methods such as PCR and DNA hybridization methods.

The selection of the appropriate method will depend on the intended degree of accuracy, convenience, urgency, availability of resources, availability of technical expertise and cost (Watts and Lindeman, 2007), and the two most commonly used methods in

veterinary laboratories are the agar disk-diffusion method and the broth microdilution method *(*Turnidge and Peterson*, 2007;* Watts and Lindeman*, 2007).*

In the disk diffusion method (Bauer *et al*., 1966), which is a qualitative method, *S. aureus* is cultured on Mueller Hinton (MH) agar in the presence of selected panel of antimicrobial impregnated filter paper disks or tablets. The zones of inhibition (zone around the filter paper disk/tablet) which indicates activity of the antimicrobial agents are measured and interpreted according to clinical breakpoints established by international organisations such as NCCLS, EUCAST, OIE, BSAC, SRGA, among others. This method is qualitative in nature.

The broth dilution method involves subjecting the isolate to a series of concentrations of antimicrobial agents in a broth environment. It is a quantitative antimicrobial susceptibility testing method used to determine the minimum inhibitory concentration. Macrodilution testing uses broth volumes at about 1.0 ml in standard test tubes. For both of these broth dilution methods, the lowest concentration at which the isolate is completely inhibited (as evidenced by the absence of visible bacterial growth) is recorded as the minimal inhibitory concentration or MIC (mg/L) (Watts and Lindeman, 2007). The MICs are recorded and interpreted according to a standard guidelines provided by CLSI or EUCAST. The test is only valid if the positive control shows growth and the negative control shows no growth. It is the gold standard for susceptibility testing.

The E-test (AB Biodisk, Solna, Sweden) is a commercially available test that utilizes a plastic test strip impregnated with a gradually decreasing concentration of a particular antibiotic. The strip also displays a numerical scale that corresponds to the antibiotic concentration contained therein. This method provides for a convenient quantitative test of antibiotic resistance of a clinical isolate. However, a separate strip is needed for each antibiotic, and therefore the cost of this method can be high.

2.13 Treatment of *Staphylococcus aureus* infections

The MRSA has markedly influenced the empirical therapy for suspected staphylococcal infections. The treatment of serious MRSA infections presents a great challenge to clinicians, particularly bacteremias and infective endocarditis, for which bactericidal therapy is essential to maximize successful clinical outcomes. Vancomycin has been the preferred antimicrobial agent to treat such MRSA infections. However, the clinical efficacy of this glycopeptide has become more limited (Sader *et al*., 2007).

Cutaneous abscesses need surgical incision and drainage irrespective of the antibiotic susceptibility pattern of the causative organism (Pallab *et al*., 2011). Limited options are available for the therapeutic management of MRSA infections. The CA-MRSA-associated skin and soft-tissue infections are treated with oral antibiotics including doxycycline, minocycline, clindamycin, sulfamethoxazole- trimethoprim, rifampicin and fusidic acid. Severe CA-MRSA infections and HA-MRSA necessitate intravenous vancomycin therapy. Asymptomatic carriers represent an important MRSA reservoir. The transmission of MRSA infections may be limited by universal infection-control measures, patient education, screening and decolonization of asymptomatic MRSA carriers in both health-care and community settings (Pallab *et al.*, 2011).

Fluoroquinolones are usually not recommended for MRSA treatment. Therapy with these agents frequently results in selection of resistant mutants, and consequent relapse and treatment failure (Gorwitz *et al*., 2011). Vancomycin remains the first-line intravenous drug for severe CA-MRSA and HA-MRSA infections (Chambers and Deleo, 2009). However, high rates of microbiological and clinical failure, nephrotoxicity and emergence of nonsusceptible strains have limited the effectiveness of this drug (Chambers and Deleo, 2009).

Linezolid exhibits an excellent antistaphylococcal activity, comparable to that of vancomycin, and can also be administered orally (Chambers and Deleo, 2009). Resistance to this drug has been rarely reported. Nonetheless, owing to the expense and potential toxicity, linezolid has been approved by the Food and Drug Administration (FDA), United States of America, for the treatment of serious MRSA infections only (Chambers and Deleo, 2009).

In addition, daptomycin and tigecycline have been approved by the FDA for MRSA management (Chambers and Deleo, 2009). Many glycopeptide derivatives including telavancin, dalbavancin and oritavancin, and two cephalosporins (ceftobiprole and ceftaroline) are also effective against MRSA, both *in vitro* and in animal models (Deleo *et al*., 2010). Telavancin has now been approved by the FDA for treatment of complicated skin and soft-tissue infections.

Asymptomatic carriers, both patients and health-care workers, constitute important MRSA reservoirs (Johnston and Bryce, 2009). The use of hand hygiene, environmental cleaning, patient isolation as well as barrier precautions such as gloves, gowns and masks plays an important role in preventing MRSA transmission in hospital settings (Johnston and Bryce, 2009). Routine screening of health-care workers and patients is also effective in preventing the MRSA spread and is cost effective in the long run (Johnston and Bryce, 2009).

2.14 Prevention and Control

S. aureus is both a commensal organism and a pathogen. The anterior nares are the main ecological niche for *S. aureus*. However, numerous other sites may be colonized, including the axillae, groin, and gastrointestinal tract (Gordon and Lowy, 2008). The MRSA is usually transmitted by direct skin to- skin contact with a colonized or infected individual and occasionally via fomites (Deleo *et al.*, 2010; Otter and French, 2010). Five

factors or “Cs” have been implicated in MRSA outbreaks - contact; lack of cleanliness; compromised skin integrity; contaminated objects; and crowded living conditions (Deleo *et al.,* 2010). Emphasis on basic hand hygiene is crucial and effective in preventing the transmission of the organism. Also because of the spread of *S. aureus* through human-to-human, animal-human and vice versa (Sing *et al*., 2008, Umaru *et al*., 2013, Kwoji *et al*., 2018), the use of disposable aprons and gloves and other personal protective equipment by staff will further reduce the risk of transmission. The use of antiseptic washes and shampoo and the application of topical antibiotic ointment such as mupirocin or neomycin for decolonization have been documented (Shafiel *et al*., 2011). Mupirocin nasal ointment is currently the treatment of choice for eradicating *S. aureus* from the nose. Mupirocin is well tolerated and, when used appropriately (application to the nose twice daily for 5 days) development of resistance is minimal. Other important control measures include education of the general populace on rational use of antibiotics, controlled use of antibiotics for therapeutic and growth promotion in livestock, proper farm management practices by farm workers, periodic clinical and laboratory auditing in hospitals to determine antibiotic resistance profiles for quality control among others.

Centres for Disease Control and Prevention have developed 4 core actions to prevent antibiotic resistance. These are preventing infections that will prevent the spread of resistance, tracking resistance patterns, improving use of antibiotics (antibiotic stewardship) and developing new antibiotics and diagnostic tests for resistant bacteria (CDC, 2013).

2.14.1 Antimicrobial Stewardship

The recognition that antimicrobial resistance is caused in part by excessive antibiotic prescribing has prompted calls for reform (Schwartz, 1999). Antimicrobial resistance (AMR) has been estimated to cause great current and potential harm to population health

and the global economy (Stewardson *et al.*, 2016). Many programmes designed to tackle AMR aim to decrease selection pressure, based on the premise that the consumption of antimicrobials and levels of resistance are associated (Doron *et al*., 2011); these include antimicrobial stewardship (AMS) programmes.

The concept of stewardship was first introduced in 1970s (Scheckler and Bennett, 1970; McGowan and Finland, 1974); and Antimicrobial Stewardship has been defined as the optimal selection, dosage, and duration of antimicrobial treatment that results in the best clinical outcome for the treatment or prevention of infection, with minimal toxicity to the patient and minimal impact on subsequent resistance. It involves appropriate selection, dosing, route of administration, and duration of antimicrobial therapy [i.e., the prudent use of antibiotics] (Singh and Singh, 2017).

The primary goal of antimicrobial stewardship is to optimize clinical outcomes while minimizing the unintended consequences of antimicrobial use. Reducing health care costs without adversely affecting the quality of care is a secondary goal of antimicrobial stewardship.

Stewardship interventions are typically classified as structural (such as the introduction of new diagnostic tests to guide antibiotic treatment), persuasive (such as expert audit of prescriptions and feedback advice to prescribers), enabling (such as guidelines or education on antibiotic use) or restrictive (such as expert approval for use of certain antibiotics). Often, different interventions are combined in antibiotic stewardship bundles (Davey *et al*., 2017).

Hospitals use antimicrobial stewardship activities to ensure the optimal selection, dose, and duration of an antimicrobial that results in the best clinical out- come for the

treatment or prevention of infection, with minimal toxicity to the patient and minimal impact on subsequent resistance (Gerding, 2001).

Antibiotic stewardship is an interventions designed to optimize use of antibiotics, is therefore one of the key actions of the WHO Global Action Plan to contain antibiotic resistance (WHO, 2015).

Use of antimicrobial stewardship in combination with infection prevention and control efforts limits the emergence and transmission of antimicrobial-resistant pathogens. Evidence on effective and feasible stewardship interventions in low and middle income countries (LMICs) is limited, and challenges for implementation of interventions are numerous. Nevertheless, several initiatives at the international and local levels in Latin America, Africa and Asia have shown that AMS effective interventions are feasible in LMICs, although contextualization is essential (van-Dick *et al*., 2016).

2.14.2 Vaccine developments for the prevention of *S. aureus* infections

Staphylococcus aureus is a highly versatile gram positive bacterium which colonises about 30% of individuals (Giersing *et al*., 2016). The organism is impressively fast in acquiring antibiotic resistance, and multidrug-resistant strains are a serious threat to human health (Pozzi *et al*., 2017). The global burden and spread of *S. aureus* infection is currently unknown and more data from low- and middle-income countries are needed, but in the US alone, *S. aureus* infection is reported as a discharge diagnosis for around 300,000 hospital stays per year. *S. aureus* infection is also associated with a five-fold increased risk of in-hospital death and three-fold higher cost of hospital stay compared to inpatients without infection (Noskin *et al*., 2005). A USA study estimated that *S. aureus* infection accounted for $14.5 billion in all inpatient hospital stays and $12.3 billion for surgical stays (Noskin *et al*., 2007). Prior *S. aureus* infection does not provide protection against subsequent infection, but infections among carriers are less severe, suggesting that

some form of immunity does develop during prolonged colonization. Although all adults have pre-existing binding antibodies to *S. aureus* antigens, including capsule and clumping factor A (ClfA), these do not typically include functional antibodies that have opsonophagocytic or neutralizing properties, and therefore do not provide protection against infection.

Due to resistance or insufficient effectiveness, antibiotics and measures leave a tremendous unmet medical need worldwide, and the need for vaccine against *S. aureus*.

To date, only two vaccines have completed human efficacy, and neither has contemplated target populations or indications that are prevalent in low- and middle-income countries (Schaffer and Lee, 2008). StaphVAX is a bivalent polysaccharide- and protein-conjugated vaccine, directed against *S. aureus* capsular polysaccharide types 5 and 8 (CP5 and CP8), which are associated with approximately 80% of *S. aureus* clinical infections. The candidate was evaluated in two phase III studies to prevent bacteremia in end-stage renal dialysis patients in the 3–54 weeks following immunization. In the first 40 weeks, bacteraemia was reduced by 57% but efficacy dropped to 26% by week 54 (Fattom *et al*., 2004).

There are no licensed vaccines on the market despite the significant efforts done by public and private initiatives (Pocci *et al*., 2017).

2.14.3 The Concept of One-Health

The concept of One-Health is an emerging integrated approach to health management; it is not new (AVMA, 2008), and was promoted by William Osler and Rudolf Virchow in the 1800s and revived by Calvin Schwabe in the 1960s. Coming from the "One Medicine" concept (Schwabe, 1984) that advocates a combination of human medicine and veterinary medicine in response to zoonoses (Zinsstag *et al*., 2011), and it was

created in 2004. The One Health approach focuses on emergent infectious diseases, which looks at health in the context of human, animal and environment relationships.

Multiple definitions of One Health have been proposed. Two of the most relevant to this discussion are as follows:

• The collaborative effort of multiple disciplines working locally, nationally, and globally to attain optimal health for people, animals, and our environment, and

• Cooperation between human and veterinary medicine and other scientific professionals to combat diseases that are shared between people and other animals (zoonotic diseases) to promote health of all species and the environment (Maegaret and Higgins, 2009).

One -Health is predicated on the fact that people, domestic animals, and wildlife share the same planet, the same ecosystems, and many of the same health threats. It covers more than infectious diseases, for example, water and air quality, health consequences of toxins, and effects of climate change on nutrition. Now many human and animal medicine organizations, including the American Medical Association and the American Veterinary Medical Association, have endorsed, and are promoting the concept as a strategic change needed in health care transformation (AVMA, 2008).

This approach would encourage the collaborative efforts of multiple disciplines working locally, nationally, and globally, to attain optimal health for people, animals, and our environment (AVMA, 2008).

Over the last three decades, approximately 75% of new emerging human infectious diseases have been zoonotic (Taylor *et al.,* 2001). Our increasing interdependence with animals and their products may well be the single most critical risk factor to our health and well-being with regard to infectious diseases.

The benefits of a One Health approach include:

• Improving animal and human health globally through collaboration among all the health sciences, especially between the veterinary and human medical professions to address critical needs

• Meeting new global challenges head-on through collaboration among multiple professions - veterinary medicine, human medicine, environmental, wildlife and public health

• Developing centers of excellence for education and training in specific areas through enhanced collaboration among colleges and schools of veterinary medicine, human medicine, and public health

• Adding to our scientific knowledge to create innovative programs to improve health

A study by researchers entitled “Microbial Threats to Health, Emergence, Detection, and Response” identified group of factors that simultaneously converged to create a “perfect microbial storm.” The most important of these factors include: adaptation of microbes, global travel and transportation, host susceptibility, intent to do harm, climate change, economic development and land use, human demographics and behavior, a breakdown of both public and animal health infrastructures, poverty and social inequality (Smolinski *et al*., 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

Weighing balance, centrifuge, incubator, microscope, autoclave, deep freezer, hot air oven, refrigerator, thermocycler (PCR) machine, water bath, heating block, electrophoresis machine, computer, photographic machine, eppendorf centrifuge, vortex machine; others were Bijou bottles, test tubes, measuring cylinders, conical flasks, glass slides, Petri dishes, Bunsen burner and pipettes.

3.2 Methods

3.2.1 Study Area and the selected healthcare facilities

Zaria is situated in the centre of Northern Nigeria, located on a plateau at a height of 2,200 feet (652.5 metres) above sea level (Mortimore, 1970). It is positioned between Latitude 110^0 3' N and 704^0 2' E. The climate is tropical continental characterized by cool humid wet seasons and cold or hot dry seasons. Zaria metropolis comprises two Local Government Areas; each with six districts. Zaria Local Government Area (LGA) consists of Zaria city, Tudun Wada, Gyellesu, Tukur Tukur, Wuciciri and Dutsen Abba. Sabon Gari LGA consists of Sabon Gari, Hanwa, Muciya, Samaru, Basawa and Bomo. Giwa LGA is also included.

Zaria is a very large heterogeneous city with a population of about 1,490,000 (NPC 2006). It is second in size to Kaduna (the State capital). Zaria's tropical continental climate has a pronounced dry season lasting from October to May. The dry season is cold between November and February (Mortimore, 1970). There are a number of Primary Health Care (PHC) Centres, General Hospitals and one tertiary healthcare facility in Zaria.

The five healthcare facilities selected for the study were Ahmadu University Teaching Hospital (ABUTH), Zaria; Ahmadu Bello University (ABU) Medical Centre, Samaru, Saint Luke's Hospital (SLH); Wusasa, Major Ibrahim Bello Abubakar Hospital (MIBA), Sabon Gari and Gambo Sawaba Hospital (GSH), Kofar Gayan, Zaria City. All the hospitals provide secondary health services, except ABUTH, Zaria which provides tertiary health services.

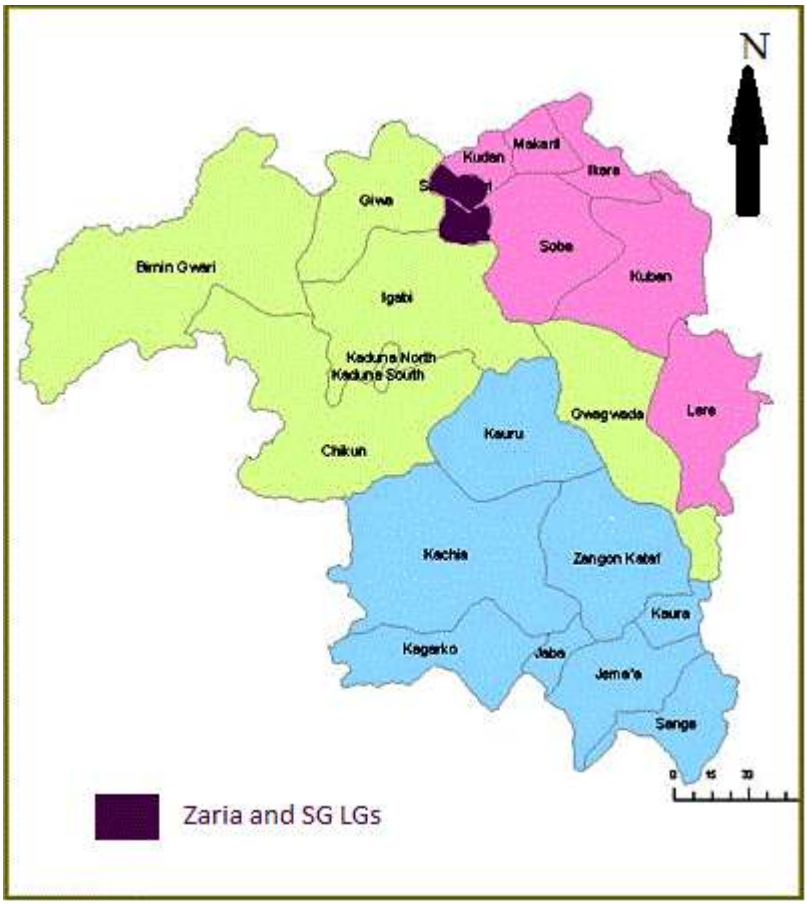


Figure 3.1: Map of Kaduna State showing the 23 LGAs

Source: Googlemap

3.2.2 Study Design

The study was cross-sectional descriptive in design.

3.2.3 Study Population

The study population comprised in-patients and out-patients for investigations at the Microbiology laboratories of the 5 selected healthcare facilities (ABUTH, Shika- Zaria, Major Ibrahim Bello Abubakar (MIBA) Memorial Hospital, Sabon Gari, ABU Medical Centre, Samaru, ST Luke's Hospital, Wusasa and General Hospital, Kofar Gayan, Zaria City.

3.2.4 Sample Size Estimation

The sample size was calculated using Fisher's formula $N= z^2pq/l^2$ and MRSA prevalence (p) of 69% was used as reported in a study in Zaria by Onanuga *et al.* (2005).

- Where N= minimum sample size
- z = 1.96 confidence interval
- p= 69% (Onanuga *et al*., 2005)
- l= 5 (attributable error), Q= 1-P

The calculated sample size was approximately 380; this was rounded up to 420 samples to make room for 10% non-response. The 420 was proportionately divided among the five selected hospitals according to their patient population and status of the hospitals (secondary or tertiary) in the ratio of 2:1 in favour of ABUTH, Zaria (Table 3.1).

3.2.5 Sampling Technique

The patients for the study were selected by purposive sampling technique in each of the five selected hospitals.

3.2.6 Data and clinical sample collection

A structured interviewer administered questionnaire to identify risk factors for *S. aureus* infection and MRSA was administered to the 420 patients that gave consent to participate in the study after explaining to them the aim and benefit of the study. The administered questionnaire had 4 sections- section A: hospital identification information; section B: patient's socio-demographic characteristics; section C: risk factors for *S. aureus* infection and MRSA and section D: sample information (Appendix I).

The 420 respondents were both in-patients and out-patients, whose samples were sent to the Medical Microbiology laboratories of the 5 hospitals for culture during the period of the study.

For the out-patients, the patients that came to the laboratory with their microbiology request forms to collect their sample bottles and sterile swab sticks were recruited for the study. Those that gave consent to participate had their samples collected by the trained laboratory scientist and questionnaire filled by the researcher and laboratory scientist in the respective healthcare facilities. The collected sample and the corresponding filled questionnaires were labelled and matched together. This was continuously done until the required sample size was obtained in each hospital.

For the in-patients in the wards, the microbiology request form was used to select the patients. The in-patients whose samples were submitted at the microbiology laboratories of the 5 selected hospitals were traced to the wards and consent obtained. Those that consented were interviewed to fill the questionnaire and samples collected. The filled questionnaires and the collected samples were then matched.

For both the in-patients and out-patients, only those ≥18 years were asked for consent, for those <18 years, assent was obtained from the parent or guardian. Only one sample was

collected from each patient. Where 2 or more samples were to be collected, balloting was used to select one sample from the many.

The samples collected using sterile swab sticks were wound swabs, ear swabs, eye swabs, high vaginal swabs, throat swabs, urethral discharges and abscesses using standard protocol as described by Chessbrough (2006). Midstream urine and semen were collected using sterile sample bottles and under aseptic condition. Blood for culture was also collected under aseptic condition after cleaning the skin with iodine. One sample was collected per patient and appropriately labelled. All the collected samples were conveyed within 1 hour to Bacterial zoonoses laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria and the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, where they were processed.

The questionnaires were filled, and the clinical samples were collected by the researcher and the 5 research assistants trained on the research protocol (laboratory scientists in the 5 healthcare facilities).

Table 3.1: Names of healthcare facilities and the number of samples collected

S/No	Name of Hospitals	Number of samples collected
1	ABUTH, Zaria	140
2	ABU Medical Centre, Samaru	70
3	MIBA, Sabon Gari	70
4	ST Luke's Hospital, Wusasa	70
5	Gambo Sawaba Hospital, Kofar Gaya	70
Total		420

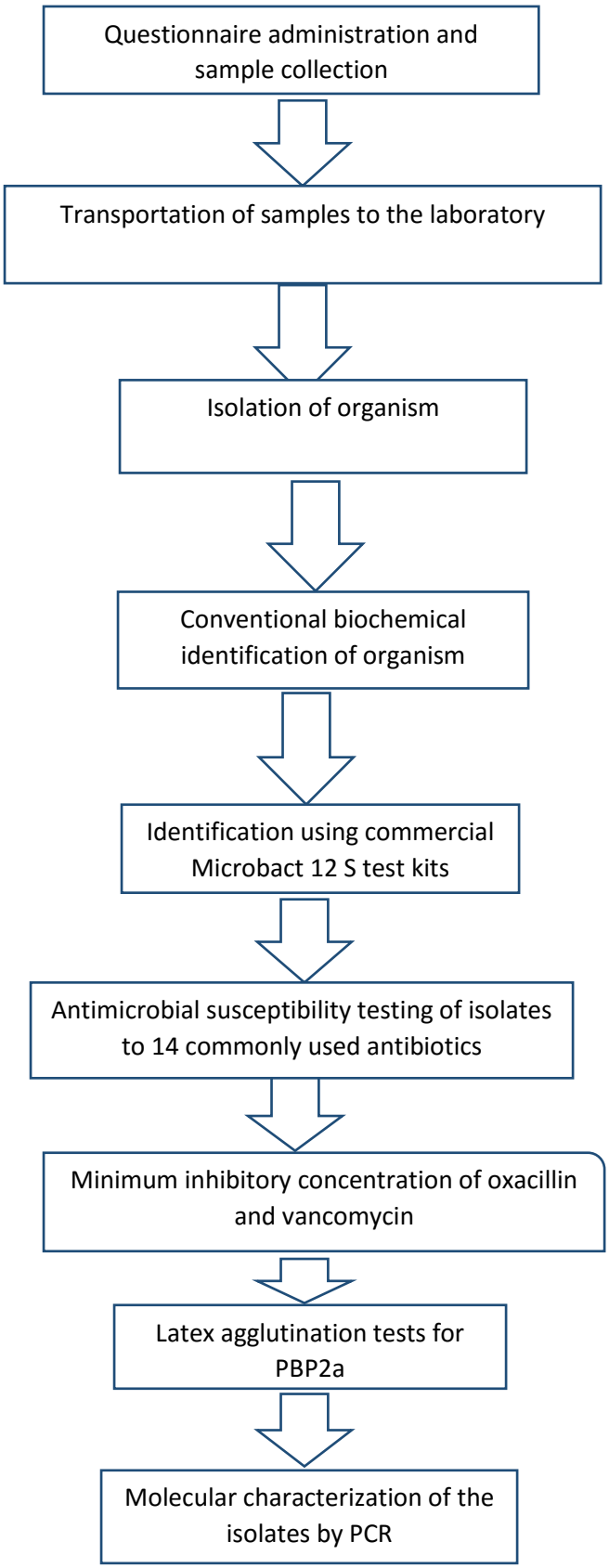


Figure 3.2: A flow chart of the methodology

3.2.7 Ethical Considerations

Ethical clearance was sought and obtained from the Health Research Ethics Committee of Ahmadu Bello University Teaching Hospital, Zaria with reference number- ABUTH/HREC/A22/2012 (Appendix II) and permissions also obtained from the management of the four other Hospitals selected for the study. Informed consent was obtained from the patients after thoroughly explaining the aim of the study. They were made to understand that they have the right to decline from participating in the study without any problem. In the case of children, assent was obtained from the parents or guardians. The questionnaire and the samples were marked and a register kept. All information collected was treated as highly confidential.

3.2.8 Isolation and characterization of Staphylococcal spp using conventional methods

3.2.8.1 Enrichment

The collected swab samples were inoculated into 5ml Brain –heart infusion broth containing 6.5 % sodium chloride for enrichment and incubated for 24 hr at 37^0C.

3.2.8.2 Conventional culture

A loopful of broth was inoculated onto Baird Parker agar and incubated for 48 hr at 37^0C, following which suspected colonies were subcultured onto tryptic soy agar and incubated at 37^0C for 24 hr so as to obtain pure colonies.

3.2.8.3 Morphological identification and Gram staining

Characteristic growths on each medium were subjected to bacteriological identification; the size, shape and colour were noted. On microscopic examination, isolates that appeared as cocci in clusters were selected for further identification procedures.

Gram Staining Reaction

Gram staining reaction of the isolated colonies was carried out. Using sterile wire loop, clean slide and a dropper, a drop of sterile normal saline was placed on a clean grease free slide and thin smear of the isolate made and allowed to air dry. The smear was fixed by heat, flooded with crystal violet (primary stain) for 1min and washed with water, then flooded with iodine (mordant) for 1 min and again washed with water, decolorized with acetone for 30 seconds and then washed immediately with water. It was stained with Safranin (secondary stain) for 30 seconds and washed with water. The preparation was inverted, air dried and observed using oil immersion objective lens (X100) (Cheesbrough, 2006).

3.2.8.4 Haemolysis on blood agar

The isolate was plated onto 5% sheep blood agar and incubated for 24hrs at 37^0C. Complete clearing of the sheep blood was indicative of beta haemolysis, partial clearing was alpha haemolysis while no clearing was indicative of no haemolysis.

3.2.8.5 Biochemical characterization

Suspected staphylococcal isolates from various samples were identified and confirmed using standard biochemical procedures (Cheesbrough, 2006). *S. aureus* strain (ATCC 25923) was used as control.

Each isolate was stained using Gram staining and positive isolates were subjected to sugar fermentation (glucose, xylose, maltose and mannitol), catalase test, the tube coagulase test and Dnase test.

Sugar utilization

The sugars used were glucose, maltose, xylose and mannitol (obtained from Oxoid, UK). One percent of each of the sugars and 1.5% Andrate's peptone water were prepared by taking 1 gram and 1.5 gram of each of the sugars and Andrate's peptone water

respectively put into 100ml and mixed properly. Five (5) ml of each of the sugars was dispensed into bottles and sterilized by autoclaving at 115^0C for 15 min. A single colony from the selective medium (Baird Parker agar) was inoculated into each of the sugars and then incubated at 37C for 24- 48 hr. Pink or red colour indicated positive reaction (fermentation), while yellow colour indicated negative reaction (no fermentation).

Fermentation of Mannitol

Mannitol salt agar (MSA) has a high salt concentration, so only salt tolerant organisms will grow on it. Phenol red is the indicator used in MSA. It is red at a neutral pH but turns yellow if condition in the media becomes acidic.

Interpretation of the result: two different characteristics of the organism are determined with the agar. The first is the organism's ability to tolerate a high salt environment. Evidence of growth on the agar indicates the organism can grow in a high salt environment.

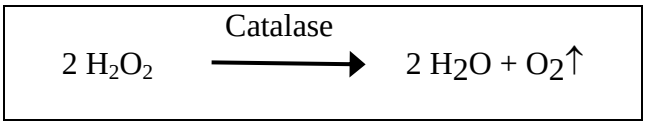
Organisms that ferment the sugar mannitol produce an acid end product that changes the red pH indicator in the media to yellow. Any yellow in the media was considered a positive test for mannitol fermentation. It is possible for organisms to grow on the media and not ferment mannitol.

Coagulase test

This test is used to identify *S. aureus* which produces the enzyme coagulase. The tube method for coagulase test was carried out as follows: 0.5 ml of rabbit serum was diluted at 1:9 ml of normal saline. It was then mixed with the test isolates in a tube and incubated at 37^0C, then examined after 1, 3, 6 and 24 hr respectively for coagulation (clotted plasma) (Cowan, 1995). Clotted plasma indicated positive result. Known positive and negative control strains were tested parallel.

Catalase test

A drop of hydrogen peroxide (3%) was placed on a microscope slide and a small smear of the isolate was placed and then emulsified. Effervescence caused by liberation of oxygen as gas bubbles indicated the presence of catalase in the test organism (Benson, 2002).



3.2.8.6 Deoxyribonuclease (DNase) test

Dnase test was carried out by streaking the isolates on the surface of Dnase agar medium and incubated at 37C for 24hrs. After incubation the plate was flooded with 1N HCl and a zone of clearing around the streaked areas was indicative of Dnase activity.

3.2.8.7 Storage and shipment of *S. aureus* isolates to United Kingdom

All isolates positive for coagulase, catalase, Gram stain, glucose, mannitol, Dnase were stored on nutrient agar after preparation. The nutrient agar medium was prepared according to the Manufacturer's instruction. Five mililitres (5ml) was transferred to Bijou bottles, which were slanted and allowed to solidify after autoclaving. The isolates were then inoculated onto the nutrient agar slants and stored in refrigerator after overnight growth at 37^0C. The *S. aureus* isolates were also stored at – 20^0C in 15 % glycerol in BHI broth, in addition to those kept on nutrient agar slants for further studies.

A duplicate copy of the *S. aureus* isolates were also inoculated onto Mannitol Salt agar and Tryptic Soya agar slants in cryo vials and also spotted on Whatman filter Paper placed in a container with ice and shipped to The Wellcome Trust, Sanger Institute, United Kingdom for the molecular characterization of the isolates. International regulations for shipment of infectious materials were observed.

3.2.9 Confirmation of *Staphylococcus* isolates

3.2.9.1 Microbact 12S identification

The 64 isolates were subjected to further identification using Microbact^{TM} staphylococcal 12S identification system test to confirm staphylococcal species. The test was conducted according to the Manufacturer's instructions (Oxoid Ltd., Basingstoke, United Kingdom [UK]).

The test was carried out and interpreted based on the Manufacturer's instruction. A homogenous suspension was prepared by mixing 2-5 isolated colonies in 3ml staphylococci suspending medium provided in the kit (Microbact 12S Identification system, UK), 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 2 two drops of mineral oil. The inoculated test strips were incubated at $37^0C \pm 2^0C$ for 24 hr. 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 5-10 seconds to purple was observed for positive test. After the incubation, results were read and recorded onto the Microbact^{TM} organism identification report form provided in the test kits. The interpretations of the results were aided by colour charts provided in the test kits. On entering the results on the report form, each block of three reactions was converted into a numeric value. The three numbers were then added together to obtain five digits of the Microbact^{TM} code which was entered into the computer aided software for identification.

3.2.10 Antimicrobial susceptibility testing

The isolates were further tested for their antimicrobial susceptibility patterns. This was conducted on the staphylococcal isolates against 14 antimicrobial agents using the Kirby-Bauer disk diffusion method (Bauer *et al*., 1966).

The antimicrobial agents and concentrations used were: Ampicillin (10µg), Amoxycillin/clavulanate (30µg), Cefoxitin (30µg), Chloramphenicol (30µg), Cotrimoxazole (25µg), Ciprofloxacin (5µg), Gentamicin (10µg), Kanamycin (30µg), Erythromycin (5µg), Oxacillin (5µg), Penicillin G (10iu), Nitrofurantoin (50µg), Tetracycline (30µg) and Vancomycin (30µg).

Procedure**:** One or two colonies of the isolates were 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, 8 antibiotics each were dispensed onto the Mueller-Hinton agar plate. The plates were incubated at 37^0C ± 2^0C for 24 hr. After incubation, zones of inhibition around each antibiotic disc was measured to the nearest millilitres and recorded as susceptible (S), intermediate (I) and resistant (R) based on the Clinical and Laboratory Standards Institute guideline (CLSI, 2011) (Appendix III). *S. aureus* ATCC 25923 was used for quality control.

3.2.11 Minimum Inhibitory Concentration (MIC) of vancomycin and oxacillin

The Minimum Inhibitory Concentration was determined using MIC evaluation strips (E strip) and interpreted as recommended by the Manufacturer (Oxoid, UK). Minimum Inhibitory Concentration is defined as the lowest concentration of an antimicrobial agent that produces complete inhibition of visible growth of an organism. *Staphylococcus aureus* ATCC 25923 was run in parallel with the test as the positive control while sterile BHI served as negative control.

The M.I.C was determined as follows: A 0.5 McFarland standard bacterial suspension was made from an overnight culture and used to uniformly inoculate the surface of a Mueller Hinton agar plate to generate a uniform lawn of growth. Subsequently, E-test strips were placed one on each plate using a forcep. The plates were incubated at 37^0C for

24 hr and after the period, an eclipse corresponding to the antimicrobial concentration no longer inhibiting the growth of the organism. The corresponding concentrations of antibiotic at the point of intersection on the strip were read as MIC in μg /ml.

If the growth intersects on the line below 2 sections, the MIC was read as the value in the lower section. If the growth was along the entire length of the strip, the MIC was read as greater than the highest value on the MIC E-scale. The read MIC values were interpreted as susceptible (S), intermediate (I) and resistant (R) using CLSI, 2011 guidelines.

3.2.12 Screening for Methicillin-Resistant *Staphylococcus aureus*

3.2.12.1 Minimum Inhibitory Concentration (MIC) of Oxacillin and Vancomycin resistant strains of S. aureus

Minimum Inhibitory Concentration is defined as the lowest concentration of an antimicrobial agent that produces complete inhibition of visible growth of an organism (*Staphylococcus aureus*).

Minimum Inhibitory Concentrations of oxacillin and vancomycin were determined using the Epsilometer strip (E strip) (Oxoid). This was carried out as described by the Manufacturer and interpreted using the CLSI guidelines (CLSI, 2011) as follows: A 0.5 McFarland standard bacterial suspension was made from an overnight culture spread on Mueller-Hinton agar using sterile swabs on which the E-test strips were placed with a forcep one on each plate. The plates were incubated for 24 hr at 37^0C. After the period of incubation, an eclipse corresponds to the antimicrobial concentration no longer inhibitory to the growth of the organism. The corresponding concentrations 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).

Guidelines of the Clinical and Laboratory standards Institute (CLSI, 2011) was used to interpret the growth inhibition zones and classification of isolates as susceptible, intermediate and resistant strains. *S. aureus* ATCC 25923 and 43300 were used for quality control for *S. aureus* and MRSA strains respectively.

3.2.12.2 Oxacillin Disk Diffusion

Oxacillin disc (1µg) susceptibility testing was performed according to CLSI (2011). A bacterial suspension adjusted to 0.5 MacFarland Standard was inoculated onto Mueller-Hinton agar (Lib. Biotech). A filter paper disk containing 1µg Oxacillin (Oxoid, UK) was placed on the inoculated Mueller-Hinton agar. All the plates were incubated at 35^0C overnight, after which the plates were held a few inches above a black, non-reflective surface illuminated with reflected light. A ruler was used to measure each zone with the unaided eye while viewing the back of the petri dish. The result was recorded and compared with the zone diameter standard of CLSI 2011.

3.2.12.3 Oxacillin Resistance Screening Agar Base (ORSAB)

All confirmed coagulase-positive *S. aureus* were screened for methicillin/oxacillin resistance by growing on Oxacillin Resistance Screening Agar Base (ORSAB) (Oxoid, Basingstoke, United Kingdom).

The isolates were grown in broth enrichment comprising of Mueller-Hinton broth supplemented with 6.5% NaCl, prepared according to the Manufacturer's instructions. All inoculated plates were then incubated at 37^0C for 24 hr. Then the medium was prepared according to the Manufacturer's instructions, and all plates were inoculated with the samples. The plates were then incubated at 37^0C for 24 hr. Growth of colonies that

showed blue coloration was considered MRSA, while those with no growth or colonies with colour other than blue were considered as negative.

3.2.12.4 Penicillin Binding Protein (PBP2a) Latex Agglutination Test

This is a rapid test latex agglutination assay detecting PBP2a in isolate of staphylococci as an aid in identifying MRSA and methicillin resistant coagulase negative *staphylococci*. The test was conducted according to the Manufacturer's instructions (Oxoid Ltd., Basingstoke, UK).

PBP2a extraction procedure

Four drops of the extraction reagent 1 was added to the micro centrifuge tube. Five micro litre (5ul) of the suspension was picked using 5ul loop and suspended in the micro centrifuge tube, vortexed and observed for clumps. The tube was placed into boiling water and heated for 3 minutes. The micro centrifuge was removed and allowed to cool at room temperature. One drop of the extraction reagent 2 was added to the tube and mixed thoroughly. The tube was then centrifuged at 3000 rpm at 15cm rotation radius and the supernatant separated.

Latex agglutination test procedure

One circle of the test card was labeled for each suspension for testing with Test Latex and another for testing with Control latex. The latex reagent was mixed thoroughly by inversion several times, and one drop of Latex Test and Control Test was added to each of the labeled circles. Five (5) micro litre of the supernatant was placed on the Test circle and Control circle, and then mixed thoroughly with a mixing stick. The card was then rocked for 3 minutes and agglutination was observed under lighting conditions. The results for the tests and control reactions were then recorded.

Interpretation of results**:**

No agglutination in either Latex reagent within 3 minutes- PBP2a negative (MSSA)

Agglutination seen with the Control/Latex within 3 minutes- Positive

Strength of agglutination:

Negative (-) = a homogenous suspension of particle with no visible clumping.

Weak positive (+) = small but definite clumps against a clouded background.

Strong positive (+) = large and small clumps against a slight clouded background or large clump against a much clouded background.

3.2.13 Molecular Characterisation of *Staphylococcus aureus* isolates

Detection of *mec* A, *pvl* and *spa* genes

3.2.13.1 Staphylococcal DNA extraction

This was carried out as described by Stegger *et al*. (2012). Two or three colonies of a fresh overnight culture of *S. aureus* cells was suspended in 100µl lysis buffer (InstaGene Matrix, Biorad) in 1.5ml eppendorf tube, vortexed for 15 min and incubated for 56^0C for 1 hr. Then mixed well by vortexing and incubated at 95^0C for 1 hr. mixed well by vortexing and centrifuged at 13200 rpm for 5 min. The DNA suspension was vortexed and centrifuged (13200 rpm for 5 min) before use. The eluted DNA was stored at -20^0C.

3.2.13.2 PCR Amplification of Staphylococcal Genes

Multiplex Polymerase Chain Reaction to amplify *mec* A, *pvl* and *spa* genes was carried out using DNA template as described by Stegger *et al*., 2012, with the following amplification mixture- dNTPs, magnesium chloride, forward and reverse primers of each gene, and 5µl of template in a total volume of 25µl. The sequence of the primers, the thermocycler program and their corresponding references are summarized in Table 3.2.

Table 3.2: Primers used in the study to detect and characterize *mec*A, *spa* and *pvl* genes

Primer name	Gene	Sequence (5'-3')	Amplicon size (bp)	References
MecA P4	*mec* A	TCCAGATTACAACTTCACCAGG	162	Oliveira and de Lencastre, 2002
MecA P7	*mec* A	CCACTTCATATCTTGTAACG		
Pvl-FP	*luk*F-PV	GCTGGACAAAACTTCTTGGAATAT	83	Deurenberg *et al*., 2004
Pvl-RP		GATAGGACACCAATAAATTCTGGATTG		
Spa-113f	*spa*-250bp repeat, 350b, 400b, 450bp	TAAAGACGATCCTTCGGTGAGC	Variable (180-600bp)	Harmsen *et al*., 2003
Spa-1514r		CAGCAGTAGTGCCGTTTGCTT		

3.2.13.3 Thermal Cycling Procedure for various programme

DNA amplification was carried out in a Thermocycler (Techneprogene Thermodux, Wertheim, Germany): An initial denaturing step of 15 min at 94^0C, followed by 30 cycles of 94^0C for 30 seconds, 59^0C for 1 min and 72^0C for 1 min and then a final extension at 72^0C for10 min.

PCR Controls: spa- *S. aureus* ATCC 29213; *mec* A- mec A positive *S. aureus* 50A247; *pvl*- PVL positive *S. aureus*.

PCR Master Mix: This included distilled water (6.5µl), reaction buffer, deoxynucleoside triphosphate (dNTPs), $MgCl_2$, forward and reverse primers, template DNA and Taq DNA polymerase to add up to 25 µl. Each PCR reaction mixture contained 6.5µl PCR water, 12.5µl 2xGreen PCR Master Mix, 0.5µl of each primer 1, 0.5µl of each primer 2 and 2µl of the DNA template preparation.

3.2.13.4 Agarose Gel Electrophoresis of PCR products

Polymerase chain reaction products were visualized on electrophoresis gels, parallel with a 100bp ladder molecular weight marker on a 2% agarose gel in TAE (40Mm Tris, 20mM Acetate and 2mm EDTA pH8.1) 1x ran for 1hr at about 130V. The gel was stained in Ethidium bromide (10mg/ml) for 30 min and de-stained briefly. Pictures were taken in the transilluminator under UV light, bands observed and results interpreted accordingly.

Detection of *mec* A, *mec* C and *fem* B genes by multiplex polymerase chain reaction carried out at Wellcome Trust Sanger Institute, United Kingdom

3.2.13.5 DNA extraction

Two pure colonies of bacterial cultures were picked from overnight growth cultures on blood agar and suspended in 40µl water (DNase free water). This was then heated for 5 minutes at 95º C in a thermocycler (McLauchlin *et al.*, 2000; Ali, 2016). Cured lysate mixture (2.5 µl) was used as a DNA template for Multiplex PCR procedures.

PCR Procedures

Reaction Mixture:

	x 1 reaction	
MyTaq Red Reaction Buffer 5U/μL(Bioline, UK)*	5μL	
2W2X [Primer *mecA*-F + *mecA*-R (10μM)]	1μL	3μL**
1A1B [Primer *mecC*-F + *mecC*-R (10μM)]	1μL	
1I1J [Primer *femB*-F + *femB*-R (10μM)]	1μL	
MyTaq DNA polymerase5U/μL (Bioline, UK)	0.5μL	
Dist. MilliQ H_2O	14.5μL	
DNA template (~25-50ng/μl)	2μL	
Total volume	**25μL**	

* The MyTaq Red Polymerase Buffer included in the PCR reaction mixture contains an inert red dye that enables users to load samples directly onto a gel after the PCR without the need to add loading buffer.

** Primers were synthesized at Sigma-Aldrich and stocks were stored at -20°C at a concentration of 100μM/μL. Solutions of 10μM/μL were prepared from the stocks with distilled water as diluents. For multiplex reactions, a mixture containing equal volumes of primers was used so that a 1x reaction contained 0.5μL of each Primer. This mixture of primers was stored at -20°C.

3.2.13.6 Polymerase Chain Reaction carried out at Sanger Institute, United Kingdom

Multiplex Polymerase Chain Reaction to amplify *mec* A, *mec* C and *fem* B genes were performed using DNA template, with the following amplification mixture: 200 μM of dNTPs, 1.5 mM magnesium chloride (Promega Corporations, USA), 0.25 μM of both forward and reverse primers of each gene, and 5 μL of template in a total volume of 50 μL. The amplification was carried out in MyCycler Thermal Cycler (Bio-Rad) with an initial denaturation at 94 °C for 30 s, followed by 30 cycles of 94 °C for 45 s, annealing at

55 °C for 1 min, and extension at 72 °C for 2 min, and final extension at 72 °C – 5 min. *S. aureus* ATCC 43300 and ATCC 25932 were used as positive and negative controls respectively.

3.2.14.7 Agarose Gel Electrophoresis

Molecular biology grade Agarose (2% w/v) was dissolved by heating in a microwave in 1×TAE buffer (40mM Tris, 20mM Acetate and 2mM EDTA pH 8.1). Ethidium bromide was not used to stain the gel; instead, Sybr® Safe DNA gel stain (Thermo Fisher, UK) was added to the melted and cooled agarose solution. Each 100ml of 2% agarose contained 2.5µL of Sybr® Safe which was poured into the gel tray (with 1mm 25 well comb from PeqLab) to set. A 10µL of PCR reaction mixture was loaded on to each well. The molecular marker (5µL) used was HyperLadder™ 100bp, consisting of 100-1000bp ladder from Bioline, United Kingdom. Electrophoresis was performed in a Sigma-Aldrich electrophoresis tank with 1 ×TAE at 80V for 75 minutes. Electrophoresed gels were visualised under blue-light and their images captured using the GelDoc™ XR System Imager (BioRad).

Table 3.3: Oligonucleotide primers used for the detection of *mec* A, *mec* C and *fem B* genes

Name	Sequence (5'-3')	Size (bp)	gene	Reference
mecCA-F	CAT TAA AAT CAG AGC GAG GC	188	*mec*C	(Paterson *et al.,* 2014)
mecCA-R	TGG CTG AAC CCA TTT TTG AT			
mecA-F	TGG TAT GTG GAA GTT AGA TTG GGA T	155	*mecA*	(Nakagawa *et al.*, 2005)
mecA-R	CTA ATC TCA TAT GTG TTC CTG TAT TGG C			
femB-F	CAT GGT TAC GAG CAT CAT GG	531	*femB*	(Paterson *et al.*, 2014)
femB-R	AAC GCC AGA AGC AAG GTT TA			

3.2.14 Multilocus Sequence Typing (MLST)

Primers were purchased commercially and the DNA sequences of 7 housekeeping genes supplied by Sanger Institute, United Kingdom. Each primer pair amplified an internal fragment of the housekeeping gene (about 500 bp) and was allowed accurate sequencing of ~450-bp fragments of each gene on both strands (Enright *et al.,* 2000)**.**

Table 3.4: Sequences of primers used in the PCR for the house keeping genes

Gene	Primer	Sequence (5′-3′)
Carbamate kinase (*arcC*)	*arcC*-Up	TTGATTCACCAGCGCGTATTGTC
	arcC-Dn	AGGTATCTGCTTCAATCAGCG
Shikimate dehydrogenase (*aroE*)	*aroE*-Up	ATCGGAAATCCTATTTCACATTC
	aroE-Dn	GGTGTTGTATTAATAACGATATC
Glycerol kinase (*glpF*)	*glpF*-Up	CTAGGAACTGCAATCTTAATCC
	glpF-Dn	TGGTAAAATCGCATGTCCAATTC
Guanylate kinase (*gmk*)	*gmk*-Up	ATCGTTTTATCGGGACCATC
	gmk-Dn	TCATTAACTACAACGTAATCGTA
Phosphate acetyl transferase (*pta*)	*pta*-Up	GTTAAAATCGTATTACCTGAAGG
	pta-Dn	GACCCTTTTGTTGAAAAGCTTAA
Triosephosphateisomerase (*tpi*)	*tpi*-Up	TCGTTCATTCTGAACGTCGTGAA
	tpi-Dn	TTTGCACCTTCTAACAATTGTAC
Acetyl coenzyme A acetyl transferase (*yqiL*)	*yqiL*-Up	CAGCATACAGGACACCTATTGGC
	yqiL-Dn	CGTTGAGGAATCGATACTGGAAC

Polymerase Chain Reaction was carried out with 50-µl reaction volume containing 0.5 µl of chromosomal DNA (approximately 0.5 µg), 0.5 µM of each primer, 1 U of *Taq* DNA polymerase (Qiagen, Crawley, United Kingdom), 5 µl of 10× buffer (supplied with the *Taq* polymerase), and 0.2 Mm deoxynucleoside triphosphates (Perkin-Elmer Applied Biosystems; Foster City, Calif.). The PCR was performed in a PTC-200 DNA engine (MJ Research, Boston, Mass.) with an initial 5-min denaturation at 95°C, followed by 30 cycles of annealing at 55°C for 1 min, extension at 72°C for 1 min, followed by a final extension step of 72°C for 5 min. The amplified products was precipitated with 20% polyethylene glycol–2.5 M NaCl, re-suspended in cold 70% ethanol, and re-precipitated;

and the sequences of both strands were determined with an ABI Prism 377 DNA sequencer with Big Dye fluorescent terminators and the primers used in the initial PCR amplification (Enright *et al.*, 2000).

For each locus, the sequences obtained from all the isolates were compared and the different sequences were assigned allele numbers. For each isolate, the alleles at each of the seven loci define the allelic profile which corresponded to its ST. The clustering of isolates were achieved by the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) from the matrix of the percentage of pair wise differences between the allelic profiles of the isolates by using Statistica (StatSoft, Tulsa, Okla.). The non-randomness in the distribution of variable sites along the sequence of each gene fragment was examined by the method of Sawyer (Sawyer, 1989). Polymorphic sites were displayed by using Sequence Output, a Macintosh program available from the MLST website (*http://mlst.zoo.ox.ac.uk)*.

The evolutionary origins were examined by the algorithm eBURST, in order to group the received allelic profiles into clonal complexes. Grouping was based on the similarity between sequence types in six of seven loci and singleton STs defined as not sharing six out of seven loci with any other STs in the data set.

3.2.15 Phylogenetic Studies and Analysis of *S. aureus*

The phylogenetic relationship was established using the 16 rRNA sequences of the type strains defining the genus *Staphylococcus* (BX571857, BA000033, AP015012, CP017115 and CP01780). The BLAST search for previously reported sequences that are identical to the three (3) local isolates was done using NCBI GenBank *(http://www.ncbi.nlm.hlh.gov/).* Multiple alignments were carried out using cluster algorithm. Neighbor-joining trees (Saitou and Nei, 1987) were constructed on the basis of

genetic distances, estimated by Kimura's (1980) two-parameter method, using MEGA 5 (Kumar *et al*., 2001; *http://www.megasoftware.net*). The reliability of the trees was estimated by bootstrap confidence values (Felsenstein, 1985) and 1000 bootstrap replications were used. A bootstrap value of 70% was considered significant evidence for phylogenetic grouping.

The nucleotides of the *Staphylococcus aureus* 23448_1#126, 23448_1#127 and 23448_1#130 genes were used for the final construction of both Neighbor-joining (NJ) phylogenetic trees. *Escherichia coli* was used as outgroup in the tree.

3.2.16 Data analyses

The collected data was manually cleaned, then keyed into Microsoft excel and SPSS version 21 (Chicargo, USA). Descriptive statistics were generated from the quantitative data and Chi-square used to test for association. A p value of less or equal to 0.05 ($p < 0.05$) was considered statistically significant.

Associations between *S. aureus*, MRSA, antimicrobial susceptibility, MDR, *mec* A, *pvl*, *spa* and MLST were assessed. Sequence alignments, translations and comparisons were carried out using BIOEDIT (version 7.0.9.0, Ali, 2016). The BLAST algorithm was used to search the NCBI GenBank *(http;//www.ncbi.nlm.hth.gov/)* database for homologous sequences.

Neighbor-joining trees (Saitou and Nei, 1987) were constructed on the basis of genetic distances, estimated by Kimura's (1980) two-parameter method, using MEGA 5 (Kumar *et al*., 2001; *http://www.megasoftware.net).* The reliability of the trees was estimated by bootstrap confidence values and 500 bootstrap replications were used.

The MLST genomic gene sequences used to construct the neighbor-joining tree were by NCBI GenBank accession numbers, the *S. aureus* MLST genomic genes sequences are *S. aureus* (23448_1#126, 23448_1#127 and 23448_1#130).

CHAPTER FOUR

4.0 RESULTS

A total of 420 samples collected from patients in the 5 selected healthcare facilities in Zaria metropolis were studied.

4.1 Socio-demographic characteristics of the patients

Majority of the respondents (54.3%) were within the age range of 21-40 years. The mean age was 26.0 ± 12.0 years and age range of 1-60 years. More than half (58.6%) were females, 57.6% Muslims, and 58.3% married. About 1/3 (36.0%) had secondary education and 32% were housewives. Slightly more than 2/3 (69.8%) gave history of use of antimicrobials before seeking medical care at the hospitals, 8.8% were in-patient and 6% had history of surgery (Table 4.1). The mean admission duration for the in-patient was 4.16 ± 2.4 days, with range 2-14 days.

The most common surgery the patients had was appendicectomy (28%) followed by caesarian section (24%), while incision and drainage constituted 24% each (Figure 4.1).

Table 4.1: Socio-demographic characteristics of the patients (N=420)

Variable	Frequency	Percent
Age (in years)		
1-10	56	13.3
11-20	85	20.2
21-30	175	41.7
31-40	53	12.6
41-50	28	6.7
51-60	23	5.5
Sex		
Male	174	41.4
Female	246	58.6
Religion		
Christianity	178	42.4
Islam	242	57.6
Marital Status		
Child	74	17.6
Divorced	1	0.2
Single	86	20.5
Married	245	58.3
Widow	6	1.4
Widower	8	1.9
Educational status		
Child	69	16.4
No formal education	78	18.6
Primary	83	19.8
Secondary	151	36.0
Tertiary	39	9.3
Occupation		
Applicant	10	2.4
Civil servant	57	13.6
Student	68	16.2
Child	72	17.1
Business man	12	2.9
Business woman	1	0.2
Trader	32	7.6
Farmer	30	7.1
Artisan	3	0.7
Housewife	135	32.1
History of use of Antibiotics		
Yes	293	69.8
No	127	30.2
Type of patient		
Out-patient	383	91.2
In-patient	37	8.8
History of surgery		
Yes	25	6.0
No	395	94.0

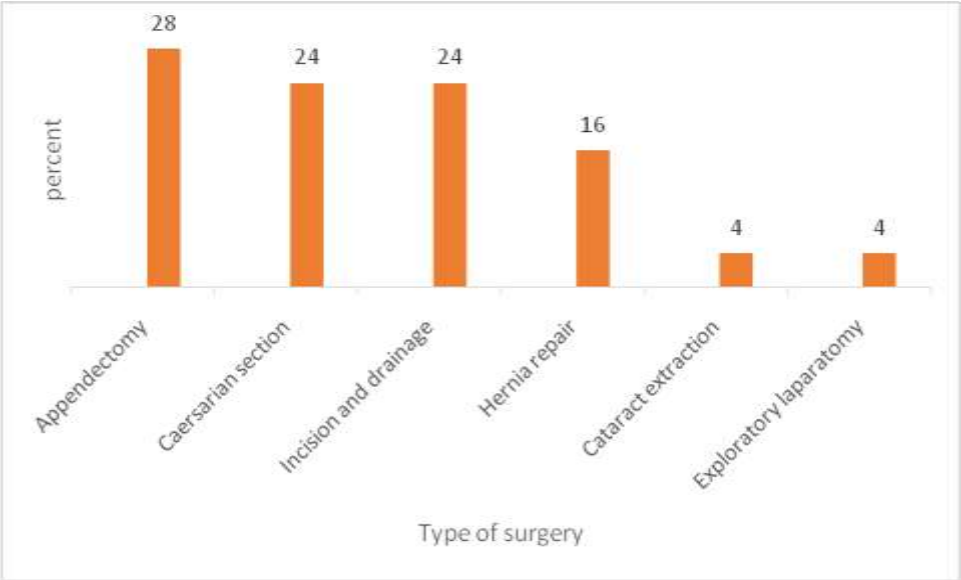


Figure 4.1: Types of surgery had by the patients (N=25)

4.2 Antibiotic use

The most common antimicrobial used by the patients was ampicillin-cloxacillin (19.5%), followed by sulphamethoxazole-trimethoprim (10.0%). About 10% of the respondents did not know the names of the antimicrobials they took. Tetracycline (7.6%) and Amoxycillin (3.8%) were also commonly used by the patients (Table 4.2). Of the 293 (69.8%) who used antimicrobials, the mean duration of use was 3.5 ± 1.2 days, with antimicrobial use range of 1-7 days and a mode of 3 days. The distribution of the clinical samples collected were urine 29.3%, abscesses 16.0%, High Vaginal Swabs (HVS) 15.0% and ear swabs 13.1%, among others (Table 4.3).

Table 4.2: Distribution of antimicrobials used by the patients before consultations (N=293)

Antimicrobial	Frequency	Percent
Name unknown	40	9.5
Amoxicillin	16	3.8
Ampicillin	27	6.2
Ampicillin/cloxacillin	82	19.5
Amoxicillin/clavulanate	7	1.7
Ciprofloxacin	12	2.9
Chloramphenicol	4	1.0
Cefuroxime	2	0.5
Erythromycin	5	1.2
Ceftriaxone	3	0.7
Tetracycline	32	7.6
Gentamicin	1	0.2
Sulphamethoxazole -Trimethoprim	42	10.0
Spectinomycin	1	0.2
Metronidazole and ampicillin-cloxacillin	8	1.9
Gentamicin and ampicillin-cloxacillin	1	0.2
Amoxicillin/clavulanate and gentamicin	1	0.2
Penicillin injection	6	1.4
Doxycycline	5	1.2

Table 4.3: Distribution of the clinical samples collected from the 5 hospitals (N=420)

Sample collected	Frequency	Percent
Blood	4	1.0
Ear swab	59	13.1
High Vaginal Swab (HVS)	63	15.0
Sputum	33	7.9
Seminal fluid	1	0.2
Throat swab	11	2.6
Eye swab	3	0.7
Wound swab	59	14.0
Abscess	67	16.0
Urine	123	29.3
Urethral swab	1	0.2
Total	420	100.0

4.2.1 Detection rate of *S. aureus* and MRSA

The detection rate of the *S. aureus* from ABUTH was 20 (14.3%), 7 (10.0%) each for ABU Medical centre and MIBA, 5 (7.1%) and 3 (4.3%) for Saint Luke's hospital and Gambo Sawaba hospital respectively. The overall detection rate for *S. aureus* was 10% (42/420).

The corresponding detection rates for MRSA were 9 (2.1%), 4 (0.9%), 5 (1.2%), 2 (0.5%), and 2 (0.5%) for ABUTH, ABU Medical centre, MIBA, Saint Luke's and Gambo Sawaba hospitals respectively; with the overall detection rate of 5.2% (22/420) (Table 4.4). The detection rates showed no statistically significant differences as shown in Table 4.4.

Table 4.4: Detection rate of *S. aureus* and MRSA from clinical isolates

Hospital	No of samples examined	No (%) positive for *S. aureus*	No (%) positive for MRSA
ABUTH	140	20 (14.3)	9 (2.1)
ABU Medical centre	70	7 (10.0)	4 (0.9)
MIBA	70	7 (10.0)	5 (1.2)
Saint Luke's Hospital	70	5 (7.1)	2 (0.5)
Gambo Sawaba Hospital	70	3 (4.3)	2 (0.5)
Total	420	42 (10.0)	22 (5.2)
Test statistics			
χ^2 =		17.668	8.066
df=		4	4
p value		0.819	0.427

4.2.2 Antimicrobial susceptibility and multiple antimicrobial indices of *S. aureus*

The antimicrobial resistance of the clinical isolates of *S. aureus* were ampicillin 42 (100%), cefoxitin 15 (35.7%), penicillin G 39 (92.9%), oxacillin 19 (45.2%) and 13 (30.9%) for vancomycin and chloramphenicol. The lowest rate of resistance was to gentamicin (11.9%), followed by ciprofloxacin (21.4%) and sulphamethoxazole-trimethprim (26.2%) (Table 4.5).

The multiple antibiotic resistance indices ranged from 0.2 to 0.9 for the *S. aureus*. MAR index of 0.2 had % isolate of 9.5% and the remaining 0.3 – 0.9 was 90.5%.; 0.6 MAR index had the highest percentage of 28.5%, followed by 0.7 with 19.5% and 0.4 with 16.6% (Table 4.6**).**

4.2.3 Antimicrobial Susceptibility of MRSA

All MRSA (100%) showed resistance to ampicillin, 95.5% to penicillin G, 86.4% to oxacillin, 77.3% and 72.7% to cefoxitin and erythromycin respectively. Gentamicin had the lowest resistance (9.1%), followed by ciprofloxacin (22.7%) and sulphamethoxazole-trimethoprim (31.8%) (Table 4.7).

Table 4.5: Antimicrobial susceptibility testing of clinical isolates of *S. aureus* from clinical samples (N=42)

Antimicrobial	No (%) Susceptible	No (%) Intermediate Resistant	No (%) Resistant
Ampicillin (AMP 10)	0 (0)	0 (0)	42 (100)
Augmentin (AMC 30)	27 (64.3)	0 (0)	15 (35.7)
Cefoxitin (FOX 30)	5 (11.9)	5 (11.9)	32 (76.2)
Chloramphenicol (C 30)	25 (59.5)	4 (9.5)	13 (30.9)
Ciprofloxacin (CIP 5)	20 (47.6)	13 (59.0)	9 (40.9)
Erythromycin (E 5)	13 (30.9)	4 (9.5)	25 (59.5)
Gentamicin (CN 10)	29 (69.0)	8 (36.4)	5 (11.9)
Kanamycin (K 30)	19 (45.2)	7 (16.7)	16 (38.1)
Nitrofurantoin (F 50)	22 (52.4)	8 (19.0)	12 (28.6)
Penicillin G (P 10)	3 (7.1)	0 (0)	39 (92.9)
Septrin (SXT 25)	25 (59.5)	6 (14.3)	11 (26. 2)
Tetracycline (TE 30)	17 (40.5)	1 (2.4)	24 (57.1)
Oxacillin (OX 5)	19 (45.2)	4 (9.5)	19 (45.2)
Vancomycin (VA 30)	28 (66.7)	1 (2.4)	13 (30.9)

Table 4.6: Multiple antibiotic resistance (MAR) index of *S. aureus* from clinical samples (N=42)

MAR Index	No of isolates	% isolate
0.2	4	9.5
0.3	1	2.4
0.4	7	16.6
0.5	4	9.5
0.6	12	28.5
0.7	8	19.0
0.8	4	9.5
0.9	2	4.7
Total	42	100.0

Table 4.7: Antimicrobial Susceptibility testing of MRSA from clinical samples (N=22)

Antimicrobial	No (%) Susceptible	No (%) Intermediate	No (%) Resistant
Ampicillin (AMP 10)	0 (0)	0 (0)	22 (100)
Augmentin (AMC 30)	11 (50.0)	0 (0)	11 (50.0)
Cefoxitin (FOX 30)	2 (9.1)	3 (13.6)	17 (77.3)
Chloramphenicol (C 30)	14 (63.6)	0 (0)	8 (36.4)
Ciprofloxacin (CIP 5)	10 (45.5)	7 (31.8)	5 (22.7)
Erythromycin (E 5)	3 (13.6)	3 (13.6)	16 (72.7)
Gentamicin (CN 10)	15 (68.2)	5 (22.7)	2 (9.1)
Kanamycin (K 30)	8 (36.4)	4 (18.2)	10 (45.5)
Nitrofurantoin (F 50)	9 (40.9)	3 (13.6)	10 (45.5)
Penicillin G (P 10)	1 (4.5)	0 (0)	21 (95.5)
Septrin (SXT 25)	11 (50.0)	4 (18.2)	7 (31.8)
Tetracycline (TE 30)	7 (31.8)	0 (0)	15 (68.2)
Oxacillin (OX 5)	1 (4.5)	2 (9.1)	19 (86.4)
Vancomycin (VA 30)	11 (50.0)	1 (4.5)	10 (45.5)

4.2.4 Multiple antimicrobial resistance profile and antimicrobial classification of *S. aureus* from clinical isolates

Forty one (41) different antimicrobial profiles were displayed by the isolates, with only one profile (ampicillin, cefoxitin, penicillin) displayed by 2 isolates and the rest were shown by one isolate each (Table 4.8).

Thiry six of the *S. aureus* isolates (36/42=85.7%) exhibited multidrug resistance, while 6 (2/42=14.3%) showed extensive drug resistance (Table 4.9).

Table 4.8: Multiple antimicrobial resistance profile of *S. aureus* from clinical isolates (N=42)

Antibiotics	No. of isolates resistant	% of isolates resistance
AMC, AMP, CIP, CN, FOX, K, OX, P, SXT	1	2.3
AMC, AMP, C,CIP, E, F, FOX, K, OX, P, TE	1	2.3
AMC, AMP,CIP, E, F, FOX, K, OX, P, SXT, VA	1	2.3
AMC, AMP, E, FOX, K, OX, P, SXT	1	2.3
AMC, AMP, C, CN, E, FOX, OX, P, TE, VA	1	2.3
AMP, C,CIP, E , F, FOX, K, OX, P, TE	1	2.3
AMP, E, FOX, K, OX, P	1	2.3
AMC, AMP, C,CIP, E, F, FOX, K,OX, P, SXT,TE	1	2.3
AMP, CN, FOX, K, P, SXT, TE	1	2.3
AMC, AMP, C, E, FOX, OX, P, TE, VA	1	2.3
AMC, AMP, C,CIP, E, FOX, OX, P, TE	1	2.3
AMP, CN, E, F, OX, TE, VA	1	2.3
AMC, AMP, C, E, FOX, K, OX, P, SXT, TE, VA	1	2.3
AMP, CN, E,F,FOX, K, OX, P,TE, VA	1	2.3
AMC,AMP,C,CIP,CN,E,F,FOX, K,OX, P,SXT, TE	1	2.3
AMP, CIP, CN, E,F, FOX, OX, P, SXT, TE	1	2.3
AMP, CIP, E, F, FOX, K, OX,P,TE,VA	1	2.3
AMC, AMP, E, F, K, OX, P , SXT, TE, VA	1	2.3
AMP, F, FOX, OX, P, VA	1	2.3
AMP, CIP, E, F, FOX, OX, P, SXT	1	2.3
AMP, CIP, E, F, FOX, OX, P, TE, VA	1	2.3
AMP, CIP, E , FOX, K, OX, P, SXT, VA	1	2.3
AMP, FOX, P, VA	1	2.3
AMP, C,CIP, F, K, P, SXT, TE	1	2.3
AMP, E , FOX	1	2.3
AMP, E, F, FOX, P, TE	1	2.3
AMP, C, CN	1	2.3
AMC, AMP,CIP, CN, E, F, FOX, K, P, SXT	1	2.3
AMP, E, FOX, P, TE	1	2.3
AMP, CIP, E , FOX, K, OX, P, SXT	1	2.3
AMP, C,CIP, E, F, FOX, K, P, SXT	1	2.3
AMP, C,CIP, E, FOX, K, P, SXT	1	2.3
AMP, C,CIP, FOX, P, TE	1	2.3
AMC, AMP, C,CIP, FOX, K, P,TE	1	2.3
AMP, CIP, CN , F, FOX, K, OX, P, SXT, TE	1	2.3
AMP, E,F, FOX, P, TE, VA	1	2.3
AMP, C, CN, FOX, K,P,TE	1	2.3
AMC, AMP, C,CIP, CN, E, F, FOX, K,P, TE	1	2.3
AMP, FOX, P	2	4.7
AMP, C, CN, E, P, TE	1	2.3
AMC, AMP, CIP, FOX, P, VA	1	2.3
Total	42	100.0

Table 4.9: Multiple antimicrobial resistance profile, antibiotic classification and multiple antibiotic resistance index of MRSA from clinical samples (N=42)

MRSA isolate ID	Resistance Pattern	NARI	ARC	MAR Index
9 blood	AMC, AMP,CIP, CN,FOX, K, OX,P, SXT	9	MDR	0.64
2417	AMC, AMP, C,CIP, E, F, FOX, K, OX,P, TE	11	XDR	0.79
4 semen	AMC, AMP, CIP, E, F, FOX, K, OX, P, SXT, VA	11	XDR	0.79
1B	AMC, AMP, E, FOX, K, OX, P, SXT	8	MDR	0.57
1237	AMC, AMP, C, CN, E, FOX, OX, P, TE, VA	10	MDR	0.71
1212B	AMP, C,CIP, E, F, FOX, K, OX, P , TE	10	MDR	0.71
7191	AMP, E , FOX, K, OX, P	6	MDR	0.43
5061	AMC, AMP, C,CIP, E, F, FOX, K, OX, P, SXT, TE	12	XDR	0.86
5060	AMP, CN, FOX, K, P, SXT, TE	7	MDR	0.50
3284	AMC, AMP, C, E, FOX, OX, P, TE, VA	9	MDR	0.64
3279	AMC, AMP, C,CIP, E , FOX, OX, P, TE	9	MDR	0.64
2316	AMP, CN, E, F, OX, TE, VA	7	MDR	0.50
2137	AMC, AMP, C, E, FOX, K, OX, P, SXT, TE, VA	11	XDR	0.79
1665	AMP, CN, E, F, FOX, K, OX, P, TE, VA	10	MDR	0.71
1212	AMC,AMP, C,CIP,CN, E,F,FOX, K,OX, P,SXT,TE	13	XDR	0.93
1188	AMP,CIP,CN, E,F,FOX,OX,P, SXT,TE	10	MDR	0.71
1180	AMP, CIP, E,F, FOX, K, OX, P, TE,VA	10	MDR	0.71
903	AMC,AMP, E,F, K,OX,P,SXT,TE,VA	10	MDR	0.71
440	AMP, F, FOX, OX, P, VA	6	MDR	0.43
400	AMP, CIP, E, F, FOX, OX, P, SXT	8	MDR	0.57
234	AMP, CIP, E, F, FOX, OX, P, VA, TE	9	MDR	0.64
142	AMP, CIP, E, FOX, K, OX, P, SXT, VA	9	MDR	0.64
U9	AMP, FOX, P, VA	4	MDR	0.29
007	AMP, C, CIP, F, K, P, SXT, TE	8	MDR	0.57
3	AMP,FOX, P	3	MDR	0.21
Nasal 6	AMP, E , FOX	3	MDR	0.21
K03	AMP, E, F, FOX, P, TE	6	MDR	0.43
8 urine	AMP, C, CN	3	MDR	0.21
10A	AMC, AMP, CIP, CN, E, F, FOX, K, P, SXT	10	MDR	0.71
SB34	AMP, E, FOX, P, TE	5	MDR	0.36
4003	AMP, CIP, E , FOX, K, OX, P, SXT	8	MDR	0.57
2339	AMP, C,CIP, E, F, FOX, K, P, SXT	9	MDR	0.64
2008	AMP, C,CIP, E ,FOX, K, P, SXT	8	MDR	0.57
1565	AMP, C,CIP, FOX, P, TE	6	MDR	0.42
1504	AMC, AMP, C,CIP, FOX, K, P, TE	8	MDR	0.57
1213	AMP,CIP, CN, F, FOX, K, OX, P, SXT, TE	10	MDR	0.71
1190	AMP, E,F, FOX, P , TE, VA	7	MDR	0.50
395	AMP, C, CN , FOX, K,P, TE	7	MDR	0.50
370	AMC, AMP, C,CIP, CN , E, F, FOX, K,P, TE	11	XDR	0.79
297	AMP,FOX, P	3	MDR	0.21
233	AMP, C, CN, E, P, TE	6	MDR	0.43
200	AMC, AMP, CIP, FOX, P, VA	6	MDR	0.43

Key:

NARI= No of antibiotic resistant to,

ARC- Antibiotic resistance classification

MAR Index= Multiple antibiotic resistance index

MDR= Multiple drug resistance

XDR= Extensive drug resistance

4.2.5 MIC for oxacillin and vancomycin

All the 22 (100%) MRSA strains had MIC of >256 μg/ml for vancomycin, while for oxacillin 18 (81.8%) had values of >256 μg/ml and 4 (18.2%) of >128 μg/ml.

4.2.6 Penicillin binding protein 2a detection

Penicillin binding protein 2a was detected in 15 out of the 22 MRSA isolates (15/22= 68.2%) (Table 4.10).

Table 4.10: Detection rate of PBP2a among the MRSA isolates in the 5 selected hospitals

Hospital	No. PBP2a positive (%)	No. PBP2a negative(%)	Total
ABUTH	7 (77.8)	2 (22.2)	9
Gambo Sawaba Hospital	2 (100)	0 (0)	2
Major Ibrahim Bello Abubakar Hospital	4 (80)	1(20)	5
ABU Medical centre	1(25)	3(75)	4
ST Luke's Hospital	1(50)	1(50)	2
Total	15 (68.2)	7(31.8)	22(100)

χ^2= 7.876, df=4, p=0.446

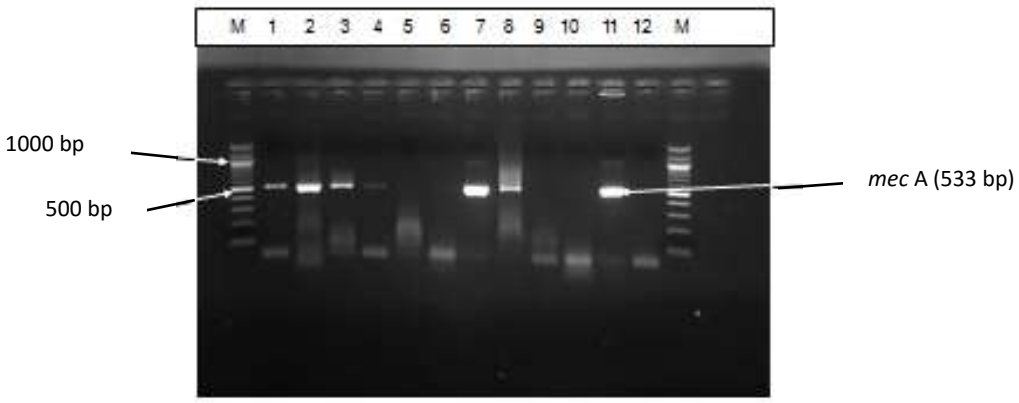


Plate 1: Monoplex PCR detection of *mec* A gene (533bp) from *S. aureus.* Lane 1-10: Clinical isolates: lane 1 (1180), 2(1180), 3(070), 4(1190), 5(078), 6(440), 7(400), 8(142), 9(200), 10(243), lane 11: Positive control; Lane 12: negative control; Lane 1,2,3,4,6,7,8, positive isolates; Lane 9 & 10: negative; Lane M: 100bp DNA Ladder (New England Biolab Inc).

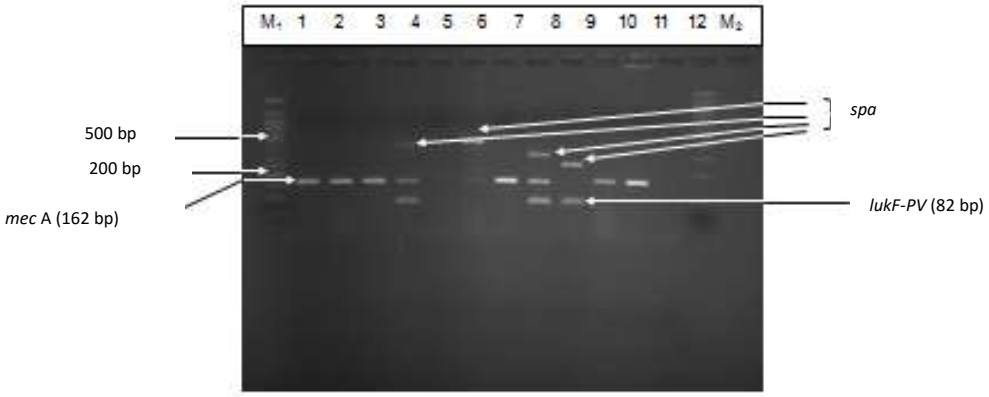


Plate 2: Triplex PCR detection of *mec* A, *spa, lukf-pv* genes from *S. aureus;* Lane M1:50bp ladder; Lane 1-10; Clinical isolates: Lane 11: *mec* A positive control: Lane 12; negative control; M2; 100bp ladder Lane 1: *mec* A positive (162bp); Lane 2; *mec* A positive (162bp): Lane 3; *mec* A positive (162bp): Lane: *pvl* (83bp), *mec* A (162bp); *spa* (400bp) positive; Lane 6; *mec* A (162bp), *spa* (450p) positive; Lane 7; *mec* A (162bp); Lane 8; *pvl* (83bp), *mec* A (162bp), *spa* (350bp) positive; Lane 9; *pvl* (83bp), *spa* (250bp) positive; Lane 10; *mec* A (162bp) positive.

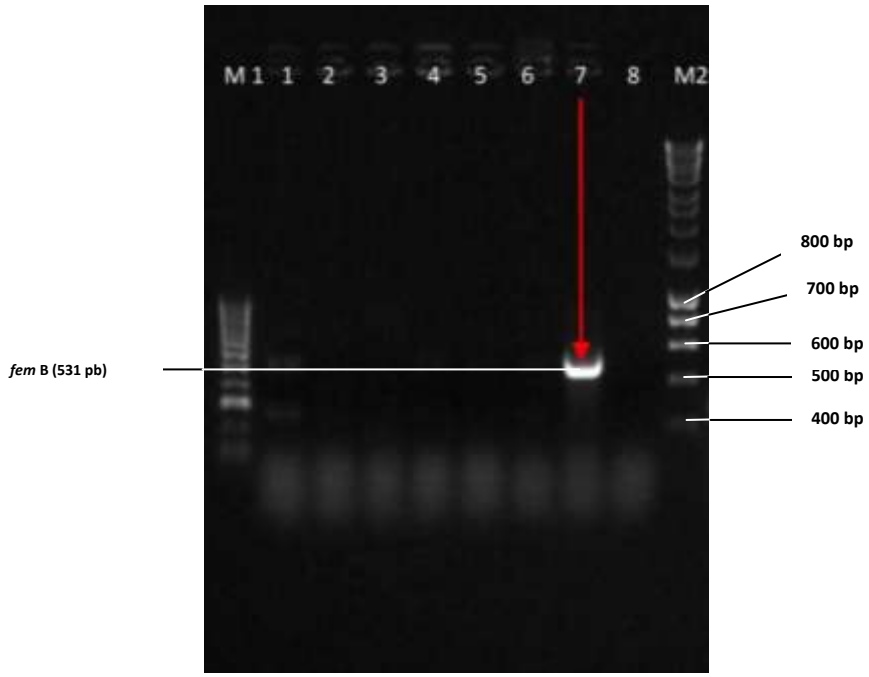


Plate 3: PCR detection of *fem* B gene (531bp). 1= molecular ladder, Lane 7 = *fem* B positive isolate (1B) M2= molecular ladder, Lane 1-8= clinical isolates, M3= Molecular ladder, positive control N1, N2and 3 = *fem* B negative controls.

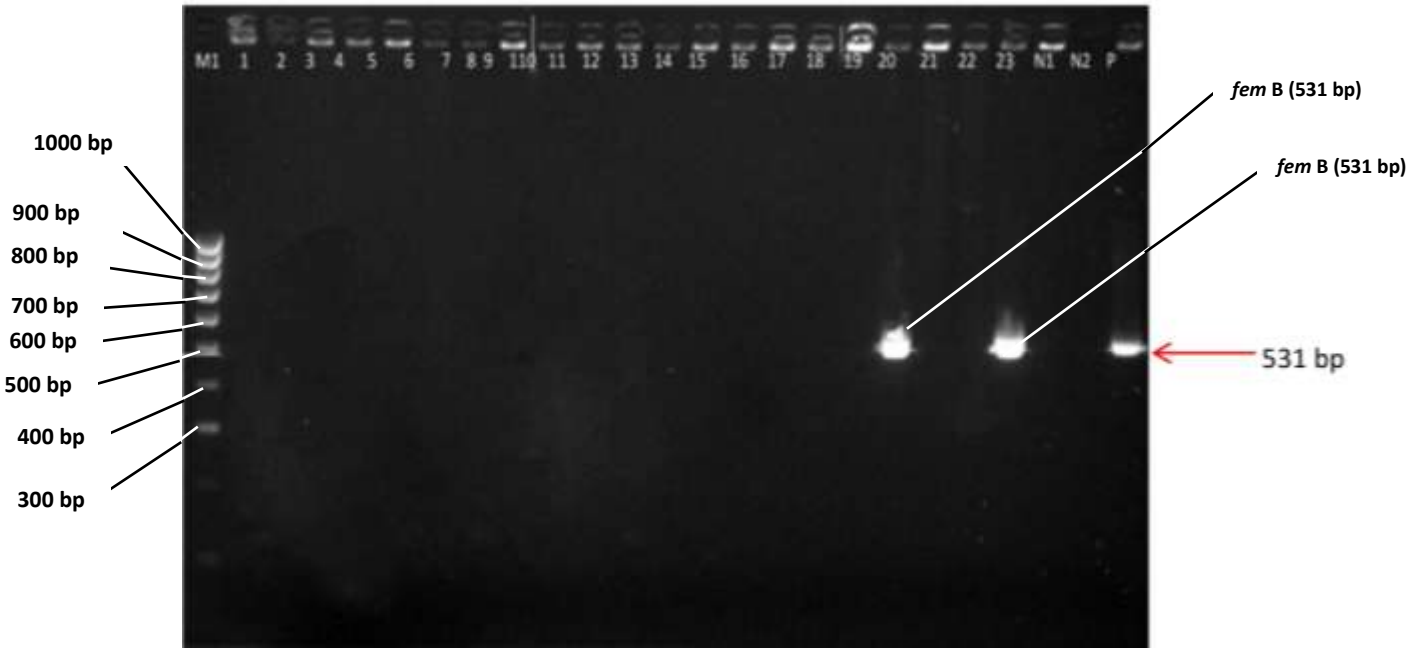


Plate 4: PCR detection of *fem* B gene (531bp). M1= molecular ladder, Lane 1-23= samples, Lane 20 and 23 = poaitive for *fem* (325 and 313) B M2= molecular ladder, N1 and N2= *fem* B negative controls, P= *fem* B positive control.

The detection rates of the targeted genes were *mec* A -15%, *pvl*- 10%, *spa* -10.3% *and* *fem* B- 10%.

4.2.7 MLST results

All the 3 isolates were MLST types 1 (ST 1) with similar allelic profiles at all seven loci and comparison with all *S. aureus* isolates in the database at mlst.net revealed the presence of only one clonal complex CC1.

The three (3) ST1 23448_1#126, 23448_1#127 and 23448_1#130 (Figure 4.6) were of clonal complex (CC) 1 and the isolates (23448_1#126, 23448_1#127 and 23448_1#130) were isolated from cases of urinary tract infection in pregnancy (MIBA), otitis media (ABU Medical Centre) and post-surgical wound infection (ABUTH).

4.2.8 Phylogenetic study

The tree illustrated the close relationship of *S. aureus* 23448_1#126, 23448_1#127 and 23448_1#130 with *S. aureus* isolates from BX571857, BA000033, AP015012, CP017115 ad CP01780. A blastn search of all five copies of 16S rRNA sequence revealed 99 % identity with (BX571857, BA000033, AP015012, CP017115 and CP01780) genomes in the database and they clustered together more tightly as compared to those obtained from Genbank.

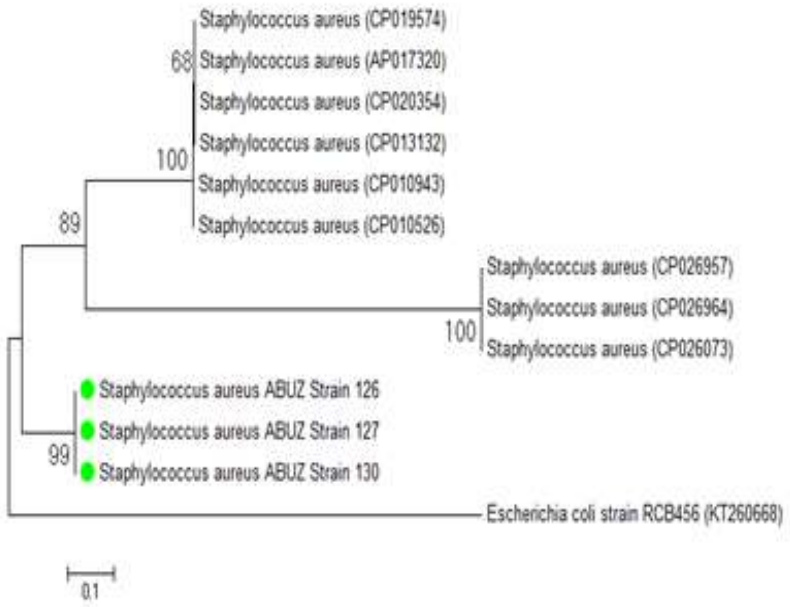


Fig. 4.6: Phylogenetic tree of *S. aureus* based on nucleotide residues of the ABU 126, ABU 127 and ABU 130 genes

CHAPTER FIVE

5.0 DISCUSSION

This study presents phenotypic and molecular characterisation of *S. aureus* isolated from hospital patients in Zaria metropolis of Kaduna State.

Majority of the patients were within the age range of 21-40 years, were females, Muslims, married and most had secondary education. A high proportion of the respondents had history of use of antimicrobials prior to seeking medical attention in the hospitals and a small proportion had surgery. These two variables are known risk factors for *S. aureus* and MRSA infections. Studies by Odugbemi (1980) and Shittu *et al*. (2011) reported use of self-prescribed antimicrobials in Nigeria. This implied that widespread use of unprescribed antibiotics has been a public health problem in Nigeria for very long time, and it is still a challenge today. Inexpensive antibiotics are widely available in developing countries without prescription (Sherma and Rai, 2012). The public health consequence of this is the creation of massive selection pressure environment and development of drug resistance by microbes to the misused/ abused antimicrobials. In Nigeria, 53% of respondents in a survey took incomplete regimen of antimicrobials, a significant proportion of which were self-prescribed for unspecified ailments (Yah *et al*., 2008).

Staphylococcus aureus is the main causative agent of bacterial diseases in the world (dos Santos *et al*., 2018), and it is a member of the ESKAPE pathogens. The ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter* species) are the leading cause of nosocomial infections around the world (Santagit and Indrawattana, 2016). The majority of these pathogens are MDR isolates, which is one of the biggest challenges in clinical practice (Santjit and Indrwanna, 2016).

In this study, ampicillin-cloxacillin and sulphamthoxazole-trimethoprim were the commonly used antibiotics by the patients. This could be as a result of the availability and low cost of the drugs, which are similar to the finding by Risk *et al*. (2013) that reported cotrimoxazole (40%) and penicillin (11.8%) to be commonly abused in Gambia and Gabon (Alabi *et al*., 2013). Nigeria shares similar characteristics with these 2 countries which could be responsible for the similar finding. Nigeria, Gambia and Gabon are developing countries with high burden of infectious diseases and history of antibiotic misuse and poor antibiotic stewardship.

Appendicectomy being the most common surgery reported by the patients could be related to their ages, whereby majority was within the range of 21-40 years. However, appendicectomy if not carried out under aseptic condition could result in wound infection and likely contamination by MRSA.

The majority of the samples collected were urine 123 (29.3%), followed by high vaginal swabs 63 (15%). This could be related to the number of females sampled because urinary tract iinfection is a common infection in this group of persons (Onanuga *et al*., 2005). This is similar to the findings of Onanuga *et al*. (2005) in Zaria that reported a high prevalence of urinary tract infection among women with no symptoms. This is related to the anatomy of the female genital tract. A significant proportion of wound swabs also showed the need for conducting surgeries under aseptic conditions and proper wound management to reduce the chances of developing infections.

The mean duration of antibiotic use of 3.5 ±1.2 days means that majority of the respondents took the drugs for very short time which is a risk factor for the development of drug resistance as against taking antibiotics for the recommended 7- 14 days depending on the infection being treated (Odugbemi, 1980; Shittu *et al*., 2011).

In this study, the highest detection rate of *S. aureus* and MRSA was from ABUTH, Zaria, probably because it is a tertiary referral centre receiving patients from primary and secondary hospitals and also because of the large number of samples tested. A study in Kano by Nwankwo and Nasiru (2011) showed a *S. aureus* detection rate of 28.5% which is different from the result of this study (10%), probably because 38 (62.0%) of the isolates in Kano study were in-patients and 15 (38%) out-patients, whereas in this study only 8.8% of the respondents were in-patients. Similar studies in Jos and Ilorin reported higher rates of 43% and 34.7% respectively (Ikeh, 2003; Taiwo *et al*., 2004).

The global problem of antimicrobial resistance is particularly high in developing countries; where infectious disease burden is high and cost constraints, prevent the wide application of newer, more expensive and effective agents (Osundiya *et al*., 2013). The isolated *S. aureus* showed different degrees of susceptibility to the 14 commonly used antibiotics resistant to penicillin ranging between 92.9% and 100%. Resistance to gentamicin was the least frequent by the isolates (Table 4.5). Penicillins are among the most commonly abused antibiotics in Nigeria (O'Malley *et al*., 2009; Shittu *et al*., 2011). This agrees with findings by Shittu *et al*. (2011), Karmakar *et al*. (2010) and Momoh (2015), and could be as a result of easy access to the drug, affordable cost and because most are taken orally.

The relationship between antimicrobial use and microbial resistance is complex, and an example is the mis-use and abuse of antibiotics in both human and veterinary medicine. Over-use of antimicrobials in humans and animals for treatment leads to the development of antimicrobial resistant strains in the environment (Ghafar *et al.,* 2010). A study showed an annual antimicrobials production of 210 million kilograms in China and 46.1% were used in livestock industries (Yong-Guan *et al*., 2013) which is of great public health significance nationally and internationally.

Multiple drug resistance is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories; extensive drug resistance defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) and pandrug resistance was defined as non-susceptibility to all agents in all antimicrobial categories (Magiorakos *et al*., 2012).

The main causes of MDR bacteria are the over-use and mis-use of antimicrobials in human medicine, veterinary medicine, agriculture and aquaculture (McMamm and Stockwill, 2001). The multiple antimicrobial resistance (MAR) indices of the *S. aureus* ranged between 0.2 and 0.9; with majority (28.5%) being 0.6. This agrees with the report of a study by Adenaike *et al*. (2016). Thermazhi *et al*. (2014) stated that MAR index value of greater than 0.2 indicates existence of isolates from high risk contaminated sources with frequent use of several antibiotics, while values ≤0.2 shows bacteria from sources with less antimicrobial usage. This will lead to the release of antimicrobials and antimicrobial resistant bacterial strains into the environment (Ghafar *et al*. 2010), which will increase morbidity, mortality and cost of treatment of infections caused by the isolates (Kleven *et al*., 2007). The findings here implies that the burden of antimicrobial resistance in the study population is high and this may directly or indirectly increase resistance of *S. aureus* and other bacteria in the study area and population. Nasal cavity is the natural ecological niche of staphylococci, and the nasal carriage of multidrug-resistant *S. aureus* is thought to be a major risk factor of staphylococcal transmission among humans (Abulreesh and Organji, 2011; Bettin *et al*., 2012).

In this study, we observed multiple drug resistance in *S. aureus* isolates from wound swabs, ear swabs, blood cultures, and urine sample among others. Wounds can be the source of staphylococcal infection, where it can be contaminated by nasal *S. aureus* (both

MRSA and non- MRSA) (Almeida *et al*., 2014). The observed multiple-resistance among some of the wound isolates in this study may suggest that these isolates were from hospitalized patients, because multiple-resistant *S. aureus* strains from wounds are more common in the hospital community (Charyulu *et al*., 2012).

The detection rate of 5.2% MRSA in this study is low compared with 12.5% in a study by Abdulazeez and Olayinka (2016) in Ahmad Bello University Teaching Hospital (ABUTH), Zaria. The high prevalence of MRSA in the teaching hospital could be as a result of signifant numbers that were in-patients and the hospital is a major referral centre in the region. Fifty percent of the MRSA isolates were resistant to amoxicillin-clavulanate which agrees with findings of a study in Kano by Nwankwo and Nasiru (2011) but contrary to 96% reported by Karmakar *et al*. (2016). Similarly, the resistance of MRSA isolates to penicillin G and ampicillin (95.5% and 100% respectively) agrees with the findings by Shittu *et al*. (2011) and Falagas *et al*. (2013). About half of the isolates (45.5%) were resistant to vancomycin, which should be worrisome to clinicians. However, gentamicin was the agent to which the least resistance by the organism was detected, probably because the preparation is in injectable form, therefore, not easily abused. Misuse and overuse of drugs, together with lack of control measures in Nigeria, might have been responsible for the high MRSA prevalence (Kesah *et al*., 2003). The high prevalence of medical tourism by Nigerians to countries such as India, South Africa, Egypt, Germany and USA, among others may also be responsible for increasing prevalence of MRSA in Nigeria.

Infections due to MRSA have been associated with a variety of clinical manifestations, including mild skin infection, pneumonia, and sepsis. Furthermore, there have been reports of increasing mortality associated with MRSA bacteremia and ventilator-associated pneumonia associated with MRSA. Generally, the main prognostic factor in

patients with MRSA infections is inadequate antibiotic therapy (Ippolito *et al*., 2010). MRSA is usually transmitted by direct skin to skin contact with colonized or infected individuals and occasionally via fomites. The so-called five Cs have been implicated in MRSA outbreaks namely: contact, lack of **c**leanliness, compromised skin integrity, contaminated object and crowded living conditions (Deleo *et al*., 2011; Goyal *et al*., 2013). Other risk factors for MRSA include HIV infection/AIDS, diabetes mellitus, frequent hospitalization, dialysis and advancing age (Goyal *et al*., 2013).

On the multiple antimicrobial profiles, 2 isolates (No 3and 279) showed resistance to same set of antimicrobial agents- ampicillin, cefoxitin, penicillin; while the remaining 40 isolates showed different resistant profiles. The implication is that many of the 14 selected commonly used antimicrobials will not be effective in the treatment of infections caused by the MRSA isolates, invariably leading to prolonged hospital stay, increased cost of treatment and poor clinical outcomes.

The detection of *S. aureus* classified as multidrug resistant (85.7%) and extensive drug resistant (14.3%), the high MIC values for vancomycin and oxacillin and those bearing PBP2a are of public health importance. The presence of such pathogens in Nigerian health facilities is bound to escalate cost of patient and facility management, eventually elevating overall cost of healthcare. The most important remedial strategy is having a vigorous infection prevention and control (IPC) program. Health system capability in running infection control program plays a role in the prevalence of MRSA isolates (Vaez *et al*., 2011).

The virulence of *S. aureus* depends on a variety of components, such as *mec* A, protein A and Panton Valentine Leukocidin among others. *mec* A has been suggested to be responsible for resistance to methicillin. It encodes the altered PBP2a with a low affinity

for beta lactam antimicrobials (Emran *et al.*, 2012). MRSA strains are the main causes of treatment failure and increase in treatment costs (Kleven, 2007; Emran *et al*, 2012), and also it is associated with a higher mortality rate (Okon *et al.*, 2013).

Studies by Mournir *et al*. (2014), Ibadin *et al*. (2017) and O'Malley *et al*. (2014), reported *mec* A prevalence of 20.7%, 38% and 42% in Serbia, Benin and Lagos respectively which are higher than the 15% in this study. In the Benin study, the MRSA carrying the *mec* A gene were multidrug resistant and the samples collected were both from in-patients and out-patients, though predominantly in-patients. Similar study in Iran showed a much higher prevalence of *mec* A of 53.3% detected by PCR. This could be as a result of the source of the samples, specifically from blood and wounds and also from orthopedic wards of teaching hospital in Iran. Hospital admission and wound infection are risk factors for MRSA, in addition to the fact that all the samples were from teaching hospitals (Referral Centres).

In a study in Zaria by Olayinka *et al.* (2009), no *mec* A gene was detected in all the clinical isolates. The molecular study was carried out in USA, and the isolates had to be shipped and therefore the condition of the shipped isolates may have been responsible for loss of *mec* A gene during the process. Studies have shown *mec* A gene to be lost at storage temperature of -80^0C (van Griethuysen *et al*., 2005).

Olowole *et al.* (2013) and Abdulreesh *et al*. (2017) reported *mec* A gene prevalence of 19.2% in Ekiti State and 14% in Saudi Arabia respectively, which is similar to the finding of this study.

Methicillin resistance in *S. aureus* is conferred by the *mec* A gene, which codes for penicillin-binding protein 2a (PBP2a) (transpeptidase), causing decreased binding affinity for the beta-lactam antibiotics, including the penicillinase-resistant penicillin. The *mec* A

gene resides on a mobile genetic element, the staphylococcal cassette chromosome mec (SCC*mec*). The *mec* A gene complex contains insertion sites for other mobile genetic elements (e.g. plasmids and transposons) that facilitate the acquisition of resistance genes to other antibiotics (Gebremariam and Zelelow, 2014).

The detection rate of *pvl* gene of 10% in this study is not in agreement with the findings of studies by Shittu *et al*. (2012), Shittu *et al*. (2011), Bazzi *et al*. (2015), Arman and Nabi (2016) in which rates of 44.4%, 40%, 30% and 40.6% respectively were reported. The higher prevalences recorded by Shittu *et al*. (2012) could be as a result of the multicentre nature of the study involving 8 tertiary hospitals. The report by Shiitu *et al*. in 2011 could be because most of the samples were from inpatients. A study by Kader *et al*. (2015) in Egypt reported a similar *pvl* prevalence of 10.3% among MRSA.

Africa is now considered to be a pvl-endemic region with high rates of pvl-positive isolates, mainly MSSA, ranging from 17% to 74% (Bruerec *et al*., 2011). This is in stark contrast to Europe, where the prevalence of *pvl*-positive isolates is low (0.9–1.4%) (von Eiff *et al*., 2004). The reasons for the high prevalence of *pvl* are unknown, but might be related to the host (i.e. altered C5a receptors, which have been identified as *pvl* targets), so far unidentified virulence factors of *S. aureus* that facilitate dissemination and the humid environment of tropical Africa (Spaan *et al*., 2013).

The *pvl* gene codes for a cytotoxin, one of the beta pore forming toxins and its presence is associated with the increased virulence of certain strains of *S. aureus.*The Panton-Valentine Leucocidin is a bicomponent leucotoxin composed of S-related and F-related proteins that are secreted separately but act synergistically; the cytotoxin is found to cause leukocyte destruction and tissue necrosis (Lina *et al*., 1999). It is a marker for community associated MRSA, and it causes leukocyte destruction and tissue necrosis, an aggressive

condition that often kills patients within 72 hr (Arman and Nabi, 2016). Contact with colonized and/or infected individuals as well as contaminated fomites with *pvl*-positive *S. aureus* have been described as risk factors for community- associated MRSA (Cataldo *et al*., 2010; Shittu *et al*., 2011). Colonization and transmission of *S. aureus* in hospitals and communities has been attributed to social risk factors such as high population density, urbanization, inadequate infection control policies, antimicrobial use and lack of appropriate healthcare delivery (Nair *et al*., 2013), among others.

The complement system is a part of the immune system that enhance (complements) the ability of antibodies and phagocytic cells to clear microbes and damaged cells from an organism, promotes inflammation and attack the pathogen's cell membrane. It is part of the innate immune system.

The C5a receptor also known as complement component 5a receptor (C5ARI) is a G-protein-coupled receptor for C5a and it modulates inflammatory response. C5a receptor structure and its residues plays role in ligand binding or signaling.

S. aureus pvl is a pore-forming toxin targeting the human C5a receptor (hC5aR), enabling pathogen to battle the immune response by destroying phagocytes through targeted lysis.

Pvl is cytotoxic to neutrophil, monocytes and macrophages, but not to lymphocytes (Witko-Sarsat *et al*., 2000). It is possible that lysis of neutrophil by pvl is responsible for a reduced host defense response allowing the pathogen to spread and cause eventual tissue damage. Neutrophils kill the invading pathogens by phagocytosis (Witko-Sarsat *et al*., 2000).

Being one of the major constituents of the innate immune system, complement plays an important role in protecting the body from pathogens, trauma, or the altered host milieu (Ricklin *et al*., 2016).

The three complement pathways converge on the proteolysis of C3. Complement fragments 3a and 5a (C3a and C5a), also termed anaphylatoxins, are released through the subsequent caspase cascades (Klos *et al.,* 2013). Of which, C5a, the most potent anaphylatoxin, mediates inflammatory immune responses such as chemotaxis, leukocyte degranulation, vascular permeability, cytokine and chemokine production, and functions beyond innate immunity in organ development, tissue regeneration, hematopoiesis, and others (Mastellos *et al*., 2013).

The functions of complement are not restricted to inflammation and immunity. The multifunctional C5a may be involved in tissue repair and regeneration (Zhang *et al*., 2017).

The *fem* B gene was detected in 3 isolates (1B, 1237 and 3279) which were all sequenced and found to belong to clonal complex 1 with multiple drug resistance. This finding of MLST type 1 showed that the isolates were from the community. In a study in Saudi Arabia by Mournier *et al*. (2014), all the MRSA isolates were positive for *fem* A and B genes. Studies have reported the use of *mec* A as marker for MRSA detection and *fem* genes for recognition of *S. aureus* species; and the detection of *fem* B and *mec* A genes may also be helpful in explaining the severity of the infection. *fem* B is involved in pentaglycine side chain formation and interpeptide bridge formation, as well as expression of methicillin resistance (Mournir, 2014). The detection of *fem* A and *fem* B together with *mec* A by PCR was considered to be a more reliable in differentiating MRSA from *mec* A positive coagulase negative *S. aureus* than *mec* A alone.

The multiple drug resistance profile of the isolates has significant public health implications in terms of prolonged hospitalization, increase in medical expenses, and difficulty in patient care (Kleven, 2007; Okon *et al*., 2013).

None of the isolates was found to be carrying the *mec* C gene which is a homologue (70%) of *mec* A gene (Garcia-Alvarez *et al.,* 2011) with similar property of development of methicillin resistance. The absence of this gene and the low detection of the *spa* genes, compared with *spa* prevalence of 88.3% as reported by Haghkhah and Lotfi (2016), could be due to the long storage of the isolates that might have resulted in the loss of the genes. Studies have showed bacterial genes to be lost as result of long or poor storage (van Griethysen *et al*., 2005).

The result of the phylogenetic analyses showed the three isolates whose *fem* B genes were sequenced were the same clade and with 99% similarity with the *S. aureus* strains established in the gene bank. The three isolates- 1B, 1237 and 3279 were obtained from cases of urinary tract infection in pregnancy, otitis media and post-surgical wound infection respectively, which are very common infections in the study area.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study provides information on the phenotypic and molecular characteristics of *S. aureus* isolates from hospital patients in Zaria metropolis, Kaduna State, Nigeria**.** The data generated will contribute to clinical auditing on the antimicrobial resistance patterns and detected virulence genes; of the bacteria in the selected hospitals; and also serve as a baseline for future studies on the epidemiology of *S. aureus* among the study population. Conclusions made from this study are as follows:

Majority of the patients were within the productive age group of 21-40 years (54.8%) with mean age of 26 ± 12years; and risk factors for *S. aureus* and MRSA infections include hospital admission and surgery.

There is high prevalence of use of self-prescibed antibiotics by the patients with mean duration of use of 3.5 ±1.2 days, with commonly abused drugs as ampicllin-cloxacillin (19.5%) and sulfamethoxazole - trimethoprim (10%).

There is low detection rate of *S. aureus* and MRSA with rates of 10.0% and 5.2% respectively and they showed various degrees of resistance to ampicillin, penicillin, cefoxitin, oxacillin and vancomycin among others.

Majority of the isolates were multi-drug resistant and some extensive drug resistant which could lead to increase cost of treatments of the patients and poor clinical outcomes.

All the isolates had MAR index of 0.2 - 0.9, which is indicative of isolates from environment where antibiotics are commonly used.

The confirmation of 3 MRSA by MLST to be ST 1 (CC 1) strains are indicative of community associated MRSA. The isolates are from cases of urinary tract infection in pregnancy (isolate 1B from MIBA), otitis media (isolate 1237 from ABU Medical Centre) and post-surgical wound infection (isolate 3279 from ABUTH).

There was high prevalence of resisitance to oxacillin and vancomycin from the MIC results; and most of the MRSA isolates are carrying PBP2a.

Approximately 95% (40/42) of the isolates exhibited resistance to different drugs giving them different antimicrobial patterns, with the exception of 2 isolates (No3 and 279) that showed same resistance pattern to ampicillin, cefoxitin and penicillin.

The study confirmed the presence of *mec* A (15%), *pvl (10%), spa (10.3%), fem* B (10%) genes in *S. aureus* isolated from the 5 selected hospitals in Zaria metropolis, and some of the isolates carried 2 or 3 of these virulence genes.

The isolates were 99% similar to BX571857, BA000033, AP015012, CP017155 and CP01780 established in the Genbank and the phylogenetic analysis showed the 3 typed isolates 1B, 1237 and 3279 to be from same clade.

6.2 Recommendations

The following recommendations are made based on the findings of this study-

1. There should be continuous health education of the general population on hand hygiene by health workers and other relevant stakeholders both at hospital and community levels inorder to reduce the spread of infections cause by the organism.

2. There is need for intensification of infection prevention and control in all the hospitals including use of isolation, barrier nursing, hand hygiene, frequent

cleaning and periodic disinfection of environmental surfaces in hospitals by hospital management.

3. There should be health education on rational use of antimicrobials and for the duration recommended by the health worker. The importance of antimicrobial stewardship should be stressed always.

4. The Government should fully implement the National Action Plan for combatting antimicrobial resistance in order to control irrational use of antimicrobials in human, veterinary medicine and aquaculture.

5. There should be periodic clinical and laboratory auditing in hospitals to determine antimicrobial resistance profiles for quality control and rational antimicrobial prescription.

6. There is need for regular molecular epidemiological surveys on clinical isolates in the hospitals to monitor changes in antimicrobial resistance over time and to detect the emergence or re-emergence of resistant clones in the country.

7. The concept of one health should be encouraged by the various professional bodies. This will strengthen the collaboration between the veterinary and human medicine practitioners and other relevant stakeholders for effective control of zoonotic infections.

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APPENDICES

APPENDIX I: QUESTIONNAIRE

Phenotypic and Molecular Characterisation of *S. aureus* isolated from patients in secondary and tertiary healthcare facilities in Zaria Metropolis, Kaduna State, Nigeria

This questionnaire is intended to gather information on patients' socio-demographic characteristics and potential risk factors for infection with methicillin-resistant *S. aureus* (MRSA); a bacterium that can cause a wide spectrum of diseases in humans and animals.

Date…….....

A) Hospital Identification information

1. Name of hospital…….

2. Hospital type: Primary health care [ ] Secondary health care [ ] Tertiary health care [ ]

3. Local Government Area……

4. Patient's Hospital number……

B) Patient's Socio-demographic Characteristics

5. Gender: Male [ ] Female [ ]

6. Age (in years): …….

7. Religion: Christianity [ ] Islam [ ] others (specify)……

8. Marital Status: Single [ ] Married [ ] Widow [ ] Widower [ ] Divorced [ ] separated [ ]

9. Educational Status: No formal education [ ] Primary [ ] Secondary [ ] Tertiary [ ]

10. Occupation: Business [ ] Student [ ] child [ ] Housewife [ ] civil servant [ ] health worker [ ] others (specify) …….

C) Risk factors for *Staphylococcus aureus* infection and MRSA

11. History of antibiotic (s) use prior to present consultation: Yes [ ] No [ ]

11a. If yes to Q11 above, who prescribed the antibiotic (s)? .....

11b.Name (s) of the used antibiotic(s):…..……..

11c. Duration of use of the above named antibiotic (s): ……..

12. Patient type: In-patient [ ] Out-patient [ ]

13. If in-patient:

13a. Provisional diagnosis: ……..

13b. Ward patient was admitted into ……

13c. Duration on admission……

13d. History of surgery: Yes [ ] No [ ]

13e. If yes to Q13d, type of surgery? ……..

13f. If there is history of antibiotic (s) use on admission: antibiotic(s) name (s)……

…….

13g. Duration of the antibiotic use…….

14. If out-patient:

14a. Provisional diagnosis: ……

14b. Clinic patient is attending: …….

14c. History of recent (≤ 6 months) surgery: Yes [ ] No [ ]

14d. If yes to Q14c, type of surgery? ……..

14e. If there is history of presently using antibiotic (s): antibiotic name (s)…….

…….

14f. Duration of the antibiotic use…….

D) Sample Information

15a. Type of sample collected: ……

15b. Site of the body where sample was collected: ……

15c. Sample label …….

APPENDIX II: ETHICAL CLEARANCE

Ahmadu Bello University Teaching Hospital

P.M.B. 06, Shika - Zaria, Kaduna State, Nigeria. 069-876305

website: www.abuth.org abuthshika@yahoo.com Abuthshika@gmail.com

Chairman of Board:

Chief Medical Director: **DR. LAWAL KHALID,** *MBBS, FMCS, FWACS, FRCS(ED) mni*

Chairman, Medical Advisory Committee: **DR. ABDULLAHI MOHAMMED,** *MBBS, FWACP FICS*

Director of Administration: **BARR. ISHAK BELLO,** *LL.B, BL, LL.M, PGDM, AHAN, FCAI*

Our Ref: ABUTH/HREC/TRG /36

30th May, 2013

FULL ETHICAL CLEARANCE AFTER COMMITTEE REVIEW

Re: ***"Phenotypic and molecular characterization of staphylococcus aureus isolated from patients in Zaria"***

ABUTH Ethics Committee assigned number:	-	ABUTH/HREC/A22/2012
Name of the principal Investigator:	-	Dr. Joshua, Istifanus Awekoson
Address of the Principal Investigator:	-	Dept. of Vet. Medicine, A.B.U Zaria
Date of receipt of valid application:	-	21st May, 2013
Date of meeting when final determination on ethical approval was made:	-	30th May, 2013

This is to inform you that the research described in the submitted protocol, the consent forms, and other participant information materials have been reviewed and ***given full approval by the ABUTH Ethics Committee.***

Please note: this approval dates from ***30th May, 2013*** to ***30th May, 2014***.

No participant recruitment into this research may be conducted outside these dates.

All informed consent forms in this study must carry the ABUTH HREC number assigned to this research and the duration of ABUTH HREC approval of the study.

This HREC expects that you submit your application as well as an annual report for ethical clearance renewal 3 months prior to expiration of study dates. This is to enable you obtain renewal of your approval and avoid interruption of your research.

If there is delay in starting the research, please inform the ABUTH HREC so that starting dates can be adjusted accordingly.

No changes are permitted in the research without prior approval by ABUTH HREC, except in circumstances outlined in national code for Health Research Ethics:http://www.nhrec.net.

ABUTH HREC reserves the right to conduct compliance assessment visits to your research site without prior notification.

Prof. J.U. Okpapi
Chairman, ABUTH

APPENDIX III: CLSI GUIDELINES (2011) FOR INTERPRETATION OF ANTIBIOTIC ZONE OF DIAMETER FOR *S. AUREUS* AND CONS

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

Key> S= Susceptible

I= Intermediate

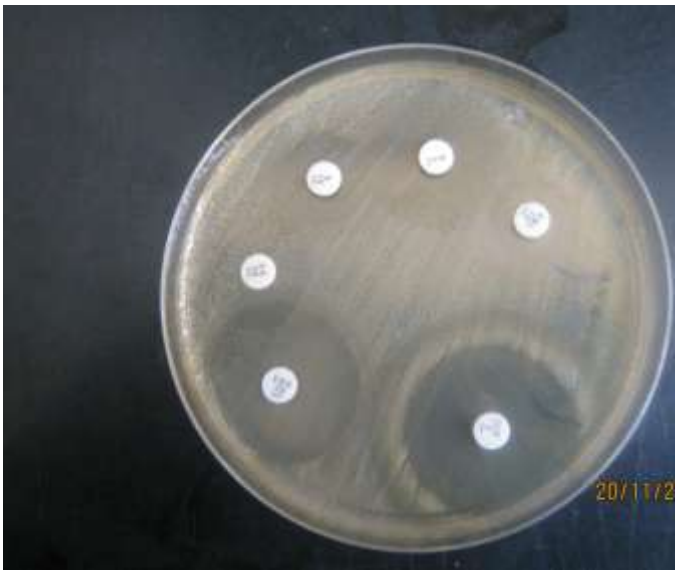
R= Resistance

M.I.C = Minimum Inhibitory Concentration

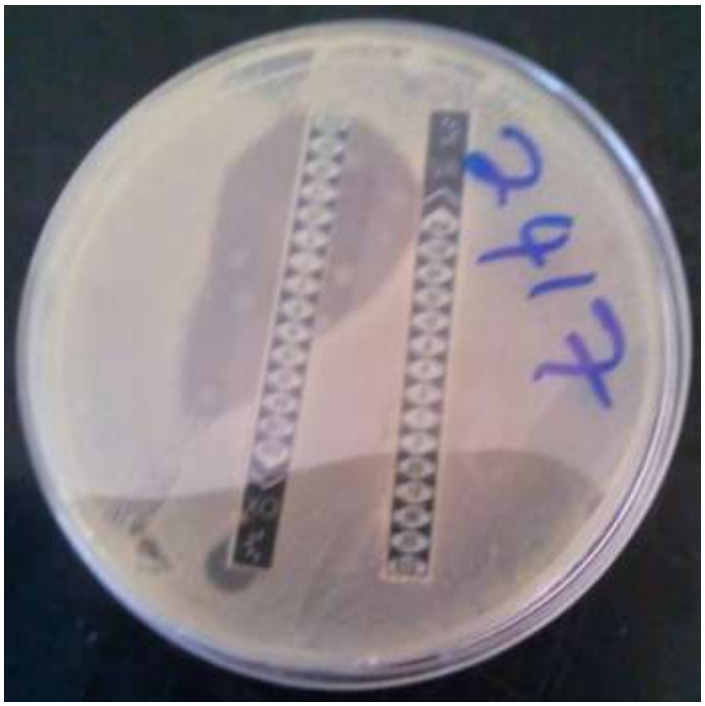
APPENDIX IV

Bacterial isolates on Oxacillin Resistance Screening Agar Base

Prepared 0.5 Mcfarland Standard of Isolate for antimicrobial susceptibility testing

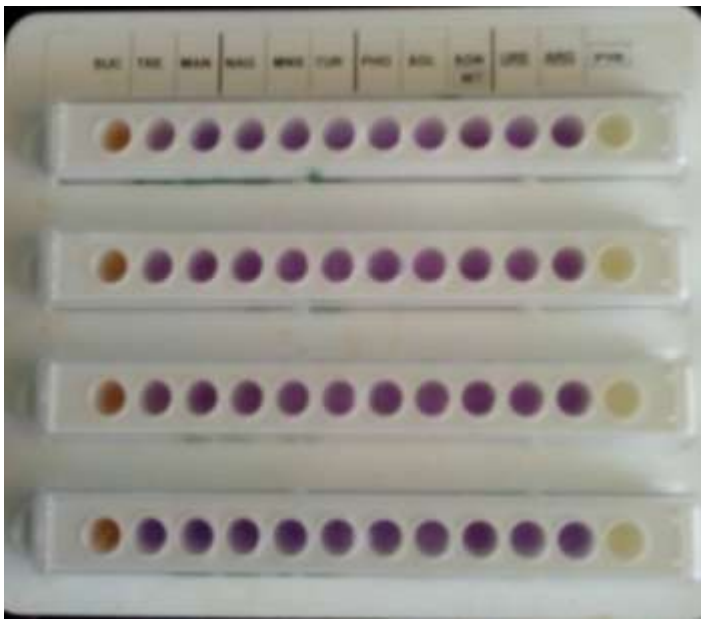


Antimicrobial susceptibility test showing zones of inhibition

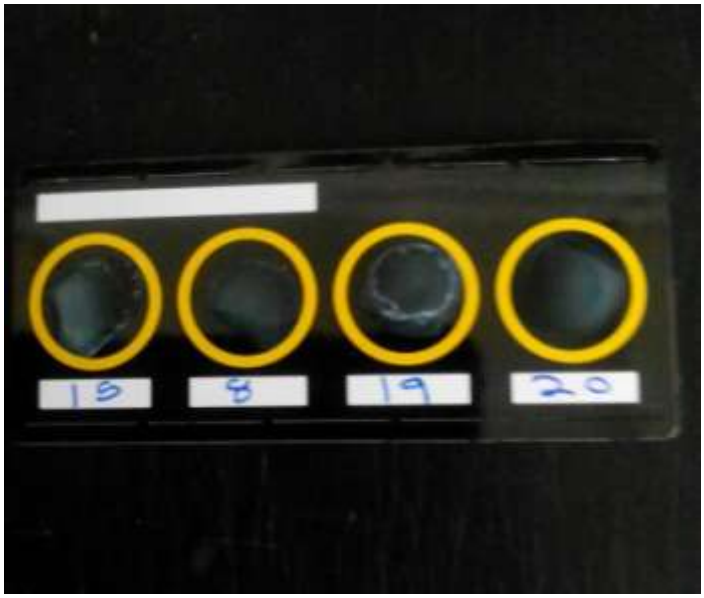


Measurement of Mininum Inhibitory Concentration using E-strip

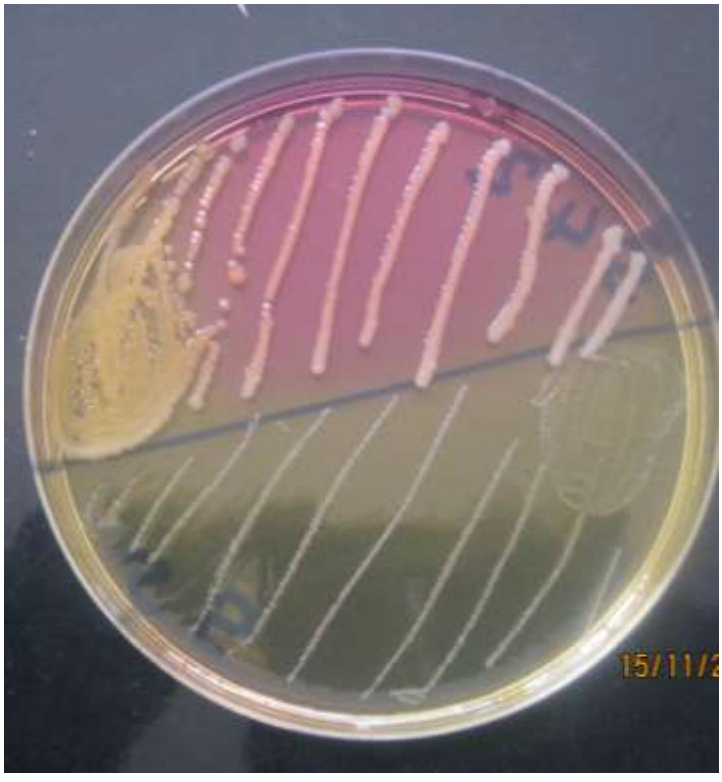
Microbact 12S Test Kit



Microbact 12S Testing of Isolates



Positive Latex Agglutination Test for PBP2a



Isolates on Mannitol Salt Agar