BIOFILM FORMATION AND ANTIBIOTICS RESISTANCE BY*STAPHYLOCOCCUS AUREUS*ISOLATED FROM PATIENTS IN NATIONAL ORTHOPAEDIC HOSPITAL, DALA, KANO, NIGERIA

BY

Dominic Agbo OCHE

DEPARTMENT OF PHARMACEUTICAL MICROBIOLOGY,

FACULTY OF PHARMACEUTICAL SCIENCES,

AHMADU BELLO UNIVERSITY,

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JANUARY, 2020

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Dominic Agbo OCHE,
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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES,
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DEPARTMENT OF PHARMACEUTICAL MICROBIOLOGY,
FACULTY OF PHARMACEUTICAL SCIENCES,
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

JANUARY, 2020

DECLARATION

I declare that the work presented in this dissertation entitled “Biofilm Formation and Antibiotics Resistance by*Staphylococcus aureus*Isolated from Patients in National Orthopaedic Hospital, Dala, Kano, Nigeria” has been performed by me in the Department of Pharmaceutical Microbiology under the supervision of Professor B.O. Olayinka and Professor J.A. Onaolapo. 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 Institution.

Dominic Agbo OCHE		
Name of Student	Signature	Date

DEDICATION

I humbly dedicate this research work to God Almighty for His benevolence and to family, especially my parents.

CERTIFICATION

This dissertation entitled "Biofilm Formation and Antibiotics Resistance by*Staphylococcus aureus*Isolated from Patients in National Orthopaedic Hospital, Dala, Kano, Nigeria" by Dominic Agbo OCHE meets the regulations governing the award of the degree of Master of Science in Pharmaceutical Microbiology of Ahmadu Bello University, Zaria and is approved for its' scientific contribution to knowledge and literary presentation.

Prof. B.O. Olayinka Chairman, Supervisory Committee	Signature	Date
Prof. J.A. Onaolapo Member, Supervisory Committee	Signature	Date
Prof. B.O. Olayinka Head of Department Pharmaceutics and Pharmaceutics Microbiology	Signature	Date
Prof. S. Abdullahi Dean, School of Post Graduate Studies	Signature	Date

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ABSTRACT

Staphylococcus aureus is a Gram-positive pathogens causing a wide range of health-care and community acquired infections. Its ability to form small-colony variants (SCV), biofilm and acquire resistant genes have resulted in its persistence in patients and also putting health-care workers at risk. This study evaluated the antibiotic resistance profile and biofilm forming capacity of *S. aureus* isolated from clinical samples of hospitalized patients in National Orthopaedic Hospital Dala (NOHD), Kano, Nigeria. A total of 189 samples consisting of 49 wound swabs, 49 nasal swab, 49 bed swab and 42 urine samples were collected over a period of 3 months. These samples were cultured on mannitol salt agar (MSA) and the isolates were subjected to Gram staining, catalase test, and coagulase test. And the coagulase positive isolates were further screened using Staphylococcal Microgen identification kit to identify *S. aureus* isolates. A total of 28 *S. aureus* were identified from the189 clinical samples collected. The *S. aureus* isolates were screened for small-colony variants (SCVs) by culturing the isolates on Columbia blood agar (CBA) to detect pin-point or tiny colonies that are non-haemolytic and non-pigmented and they were evaluated for biofilm-formation using the microtiter plate (MTP) method. Also, antibiotics susceptibility pattern and inducible clindamycin (D-test) resistance of the *S. aureus* isolates were determined using the agar disc diffusion method. Molecular analysis of the isolates was done to determine the presence of biofilm associated genes (*icaA* and *bbp*) and antibiotics resistance genes (*mecA* and *vanA*) in the *S. aureus* isolates using polymerase chain reaction (PCR) to amplify the genes if present. Of the 189 samples collected, 28 (14.3%) were confirmed to be *S. aureus*. Biofilm production was observed in 27 (96.4%) of the *S. aureus* isolates with 1 (3.6%) *S aureus* isolate as non-biofilm producer. From the biofilm producers, 3 (10.7%) of the biofilm-producing isolates were strong biofilm-formers, 6

(21.4%) were moderate biofilm-formers and 18 (64.3%) were weak biofilm-formers. Antibiotics susceptibility showed that the *S. aureus* isolates were generally resistant to Amoxicillin-Clavulanic (67.9%), Tetracycline (67.9%), Ciprofloxacin (67.9%), Norfloxacin (64.3%), Cefoxitin (67.9%), Clindamycin (57.1%) and Gentamicin (53.6%). But they were significantly susceptibility to Linezolid (85.7%), Mupirocin (64.3%), Erythromycin (53.6%), Trimethoprim-Sulfamethoxazole (57.1%) and Quinipristin-Dalfopristin (60.7%). High percentage (85.7%) of the *S. aureus* isolates had MAR index> 0.2, and 85.7% of the isolates were multi-drug resistant (MDR) also. A total of 2 (7.1%) *S. aureus* isolates tested positive for inducible clindamycin resistance and 57.1% showed constitutive clindamycin resistance. The high-level mupirocin resistance was found in 21.4% of the isolates while the low-level mupirocin resistance was found in 25% of the isolates. Molecular analysis of these isolates showed that 30% harboured *icaA* gene that was amplified at 188bp, 37.5% harboured *bbp* gene that was amplified at 500bp, 33.3% harboured *mecA* gene that was amplified at 533bp and 20% harboured *vanA* gene that was amplified at 1030bp.

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LIST OF ABBREVIATION, GLOSSARY AND SYMBOLS

S. aureus	*Staphylococcus aureus*
MRSA	Methicillin-resistant *Staphylococcus aureus*
VRSA	Vancomycin resistant *Staphylococcus aureus*
CoNS	Coagulase Positive Staphylococci
CoPS	Coagulase Negative Staphylococci
MSSA	Methicillin Sensitive *Staphylococcus aureus*
MSCRAMMs	Microbial Surface Components Recognizing Adhesive Molecules
CF	Cystic fibrosis
MTP	Microtitre Plate
HA-MRSA	Hospital Acquired Methicillin Resistance *Staphylococcus aureus*
CA-MRSA	Community Acquired-Methicillin Resistance *Staphylococcus aureus*
MDR	Multi-drug Resistance
XDR	Extensively Drug Resistance
PDR	Pan-drug Resistance
MAR index	Multiple Antibiotic Resistance Index
SXT	Trimethoprim-Sulfamethoxazole
SCVs	Small-colony Variants
Thy	Thymidine
Hem	Hemin
Men	Menadione
MGE	Mobile Genetic Elements
WHO	World Health Organization
PIA	Polysaccharide Intercellular Adhesion
ECM	Extracellular Matrix
DNA	Deoxyribonucleic Acid
CRS	Chronic Rhinosinusitis
PCR	Polymerase Chain Reaction
MSA	Manitol Salt Agar
M.I.C.E	Minimum Inhibitory Concentration Evaluator
EUCAST	European Committee on Antimicrobial Susceptibility Testing
NOHD	National Orthopaedic Hospital Dala
IVCs	Intravascular Catheters
ODc	Cut-off Optical Density
Spp	Species
G+C	Guanine-Cytosine
bp	Base Pairs
$\geq$	Equal to or Greater Than
$\leq$	Equal to or Less Than
β	Beta
γ	Gamma
α	Alpha

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Staphylococcus aureus is a facultative anaerobic, non-motile and non-spore forming Gram positive cocci (round) bacterium and is frequently found in the respiratory tract, and on the skin mostly as a commensal organism (Asadi and Jamali, 2017).

Staphylococcus aureus causes a wide variety of infections ranging from mild skin infections, to life-threatening diseases such as necrotizing pneumonia and bacteraemia. *Staphylococcus aureus* is the most common cause of skin and soft tissue (Tong *et al.,* 2015), septic arthritis (Nevius *et al.,* 2016), osteomyelitis (Paulo *et al.,* 2014), bacteremia and lower respiratory tract infections (Tong *et al.,* 2015),toxin-mediated diseases such asgastroenteritis and food poisoning (CDC, 2016).

Its ability to develop drug resistance and the emergence of community-circulating, highly virulent strains has made it a major threat to human health in both the hospital and the community. Studies in the past have shown that Staphylococci have mechanisms for resisting antibiotic therapy that extend beyond the classic forms of resistance (Yagci *et al.,* 2013; Ode *et al.,* 2015; Precit *et al.,* 2016).

Some cases of persistent, difficult-to-treat prosthetic joint infections (PJIs) are associated with a naturally occurring subpopulation of *S. aureus* which exhibits physiologic, biochemical, and colonial morphologies different from usual isolates (Precit *et al.,* 2016). The formation of slow-growing subpopulations of cells that manifest behaviour atypical for *S. aureus*, such as reduced hemolysin production and increased intracellular survival, represent one of such mechanism (Proctor *et al.,* 2014).

This subpopulation designated as “small-colony variants” (SCVs), is characterised with fastidious growth, mainly recovered from clinical specimens, particularly from patients with chronic, persisting, and/or relapsing infections (Maduka-Ezeh *et al.,* 2012; Precit *et al.,* 2016). Staphylococcal SCVs described to date have been known to be primarily auxotrophic typically for hemin, menadione, or thymidine, compounds involved in the synthesis of the electron transport chain components cytochrome and menaquinone respectively (Al-Laham, 2013; Proctor *et al.,* 2014).

Infections caused by SCVs pose a multilevel challenge in terms of identification and management. Difficulties are encountered in identifying SCVs in the clinical microbiology laboratory. On culture media, SCVs grow slowly and form pinpoint colonies such that they may be overlooked or overgrown by wild-type colonies in cases of dual infection (Maduka-Ezeh *et al.,* 2012). Small colony variants demonstrate reduced haemolysis on blood agar. *Staphylococcus aureus* SCVs demonstrate variable results on coagulase testing as well as diminished pigment production (Precit *et al.* 2016), which may result in misidentification. Even when correctly identified, there may be difficulty in antimicrobial susceptibility testing due to a slow growth rate.

The biofilm hypothesis was first promulgated in 1978 by Costerton *et al.*and it states that “bacteria in all nutrient sufficient ecosystems grow predominantly in matrix-enclosed surface-associated communities, within which they are protected from a wide variety of antibiotics”, and extension of this hypothesis into medicine occurred shortly thereafter (Chino *et al.,* 2017), with the description of a biofilm formed by cells of *Staphylococcus aureus* on a pacemaker in a patient with a bacteraemia secondary to an orthopaedic injury (Stoodley *et al.,* 2011). The sessile cells within this biofilm survived 6 weeks of very intensive antibiotic therapy, and the protected microbial community

served as a nidus for recurrent bacteraemia each time that antibiotic therapy was discontinued (Stoodley *et al.,* 2011).

Biofilm infections can cause severe problems in prosthesis, ranging from disfunctioning of the implanted device to lethal sepsis (Jamal *et al.,* 2015). Treatment of biofilm infections is complicated, as biofilms protects the infecting organisms against the natural host defences and antibiotic therapy (Chino *et al.,* 2017). Biofilm forming bacteria are usually 10 - 1,000 times more resistant to antibiotics than their planktonic counterparts (Sharma *et al.,* 2019).

The pathogenesis of many orthopaedic infections is related to the presence of microorganisms in biofilms. The protective mechanisms at work in biofilms appear to be distinct from those that are responsible for conventional antibiotic resistance. The biofilm can act as a shield for the bacteria, making it difficult for them to be reached and destroyed by antibiotics (Chino *et al.,* 2017). Mechanisms for resistance in bacteria biofilms includes inability of antibiotics to reach bacteria present in the deeper layers of biofilms because of the difficulty in penetrating the exopolysaccharide gel (slime), and the fact that bacteria in deeper layers may be in a metabolic state that makes them less susceptible to antibiotics (Chino*et al.,* 2017).The clinical impact of an infection depends on the capacity of the infecting organisms to form a biofilm and the ability of bacterial subpopulations to switch to the dormant metabolic state known as small-colony variants (SCVs)(Percival*et al.,* 2015). Insufficient nutrient and oxygen cause some bacteria in the lower layers of the biofilm to enter a non-growing or "dormant" state: the SCVs, in which they are less susceptible to growth-dependent antibiotic killing(Tong *et al.,* 2015). Many infections are caused by biofilm associated microorganisms, and this has sparked renewed interest in SCVs (Tong *et al.,* 2015). Small colony variants are largely

responsible for the recalcitrance of infections caused by biofilms (Pana, 2012). The significance of SCVs of *Staphylococcus aureus* as causative organisms in chronic and recurrent infections has been demonstrated in patients with chronic osteomyelitis (Tong *et al.,* 2015).

1.2Statement of Research Problem

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged, disseminated globally and has become a leading cause of bacterial infections in both health-care and community settings since the 1960s (Lee *et al.,* 2018).In the United States, MRSA strains are the leading cause of death by an infectious agent with a mortality rate of approximately 20% (Dean *et al.,* 2014).

Difficulty in treating *S. aureus* bone site infections (osteomyelitis and other orthopaedic wounds) are partly due to a subpopulation of *S. aureus* known as small-colony variants (SCVs) that can hide within host cells and are more resistant to antibiotic, even in cases where antibiotic treatment is maintained for long period (Cervantes-García*et al.*, 2015).

The frequency of SCV recovery from clinical specimen ranges from 1 to 30% (Dean*et al.,* 2014). Yagci *et al.* (2013) reported in Ankara, Turkey, prevalence of 16.2% SCVs in 123 *S. aureus* isolated from 248 patients and Ode *et al.* (2015) reported 21.9% prevalence of SCV in 258 Staphylococcal isolates from Ahmadu Bello University Teaching Hospital, Zaria

These SCVs have altered drug resistance profiles, such as an increased resistance to aminoglycosides, and are particularly difficult to detect and treat (Maduka-Ezeh *etal.*,2012). These SCV strains have significant clinical importance and, in some cases, have been shown to persist for a long period of time (Bhattacharyya *et al.,* 2014). These

naturally occurring variants have a survival advantage in their ability to persist within eukaryotic cells (non-phagocytic cells), thereby protecting them from host defences and antibiotics (Gunaratnam*et al.,* 2019).

Staphylococcus aureus strains isolated from different clinical settings display significant genetic variations and it is this difference that is responsible for the strain variation in physiology and stress response (Sabirova *et al.,* 2014). This includes the emergence of multiple drug-resistant *S. aureus* in hospitals and communities that form the basis for MRSA or more specifically Hospital Acquired Methicillin Resistant *S. aureus* (HA-MRSA) and Community Acquired Methicillin Resistant *S. aureus* (CA-MRSA) infections.

Many MRSA isolates have the capacity to form biofilms through an *icaADBG*-independent mechanism, such as fibronectin-binding proteins A and B (FnbpA and FnbpB) as well as major autolysin (Tong *et al.,* 2015).

Tashiro *et al.* reported emergence of SCV under biofilm conditions,which suggests that biofilms induced genetic diversity. Especially, that no SCV was observed in the planktonic culture (Tashiro *et al.,* 2017).

Some organisms in biofilms have been shown to express biofilm-specific antimicrobial resistance genes (Al-Shuneigat *et al.,* 2014). Methicillin-resistant *S. aureus* (MRSA) has been increasingly identified as a causative organism in health care associated infections, including orthopaedic infections (Hideki *et al.,* 2016).

1.3 Justification of the Research

Thousands of bacteria live permanently on the skin (including *S. aureus*). These bacterial population, referred to as "resident" or "normal" flora could be a major source of *S. aureus* contamination in orthopaedic wounds (Webster and Osborne, 2015).

A 2010 study of orthopaedic procedures in general demonstrated that nasal carriage of *S. aureus* increases the risk of *S. aureus* wound infection following orthopaedic surgery (Crowe *et al.,* 2015) and a long term admission to health-care facility increases risk of orthopaedic surgical site infections (SSIs) (Korol *et al.,* 2013).

The clinical relevance of SCVs has been unclear for a long time. However, the high rate of SCV emergence under biofilm condition suggests biofilms induced genetic diversity (Tashiro *et al.,* 2017) and this was consistent with previous findings of Wei *et al.* (2011) obtained from *P. aeruginosa*. Studies have confirmed using scanning electron microscopy and other molecular techniques that wounds are colonized by biofilms (Neopane *et al.,* 2018). This biofilm protects the microorganism from host defences and impedes antibiotics delivery, which may cause impairment in wound healing (Neopane *et al.,* 2018).

The significance of SCVs as causative organisms in chronic and recurrent infections has been demonstrated in patients with chronic osteomyelitis (Tong *et al.,* 2015).

There is limited data on the multi-drug resistance pattern of *S. aureus,* their SCVs and their biofilm forming ability in NOHD. Therefore, evaluation of multi-drug resistance pattern and biofilm formation in *S. aureus* isolates and their SCVs from orthopaedic patients may help with a better understanding of these phenotypes to prevent or

eliminate SCV and biofilm forming ability of *S. aureus*, improve health condition and reduce economic loss.

1.4 Aim of the Study

To determine biofilm forming capacity and antibiotics resistance pattern of *Staphylococcus aureus* isolated from orthopaedic patients on admission in National Orthopaedic Hospital Dala (NOHD), Kano.

1.5 Objectives

Objectives of this study are to

i. Isolate and identify *S. aureus* and their SCVs in samples collected from patients in different wards of National Orthopaedic Hospital Dala, Kano.
ii. Evaluate the biofilm forming capability of *S. aureus* isolates using micro-titre plate method.
iii. Determine the antimicrobial resistance pattern of the *S. aureus* isolates to commonly prescribe antimicrobial agents.
iv. Determine methicillin-resistance among the *S. aureus* isolated using cefoxitin disc diffusion assay.
v. Identify some of the biofilm associated genes, MRSA gene and VRSA gene in the *S. aureus* isolates by the use of polymerase chain reaction (PCR).

1.6Hypothesis

1.6.1Null hypothesis (H_o)

There is no biofilm production and antibiotics resistance in *S. aureus* and their small-colony variants isolated from patients on admission in National Orthopaedic Hospital Dala, Kano.

1.6.2Alternate Hypothesis (H_a):

There is biofilm production and antibiotics resistance in *S. aureus* and their small-colony variants isolated from patients on admission in National Orthopaedic Hospital Dala, Kano.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram positive round-shaped bacterium that is a member of the Firmicutes, and is frequently found in the respiratory tract, and on the skin. It is positive for catalase and nitrate reduction and is a facultative anaerobe that can grow in the need for oxygen (Asadi and Jamali, 2017). Although *S. aureus* is not always pathogenic, it is a common cause of skin infections (e.g abscesses), respiratory infections (e.g sinusitis), osteomyelitis, toxic shock syndrome and infective endocarditis and food poisoning (Bhattacharyya *et al.,* 2012).

2.2 Characteristics of *Staphylococcus aureus*

Staphylococcus aureus is a facultative anaerobic, gram-positive coccal (round) bacterium also known as "golden staph" and "oro staphira". *Staphylococcus aureus* is non-motile and does not form spores (Rekha *et al.,* 2018). *Staphylococcus aureus* appears as Staphylococci (grape-like clusters) when viewed through a microscope, and has large, round, golden-yellow colonies, often with haemolysis, when grown on blood agar plates (Mustafi, 2014).*Staphylococcus aureus* reproduces asexually by binary fission. Complete separation of the daughter cells is mediated by *S. aureus* autolysin, and in its absence or targeted inhibition, the daughter cells remain attached to one another and appear as clusters (Varrone *et al.,* 2014).

Staphylococcus aureus is catalase-positive (meaning it can produce the enzyme catalase). *Staphylococcus aureus* uses catalase enzymeas defence mechanism among others, for cellular detoxification by neutralizing the bactericidal effects of

H_2O_2(Mustafi, 2014). Catalase-activity tests are sometimes used to distinguish Staphylococci from Enterococci and Streptococci.*S. aureus*is differentiated from other Staphylococci by coagulase test.

2.3 Pathogenesis of *Staphylococcus aureus*

Pathogenic strains*Staphylococcus aureus* often promote infections by producing virulence factors such as potent protein toxins, and the expression of cell-surface proteins that bind and inactivate antibodies (Asadi and Jamali, 2017). Among these factors, alpha toxin, also known as alpha-hemolysin (Hla), that have hemolytic, cytotoxic and dermo-necrotic properties and can also provoke cardiovascular collapse and pulmonary edema (François *et al.,* 2018). This toxin allows it to survive within the human host and thereby contribute to its pathogenicity.

Another essential factor in *S. aureus* pathogenesis is its ability to adhere to host cells and/or components present in the host's extracellular matrix (Johannessen*et al.,* 2012; Ajayi*et al.,* 2018). *Staphylococcus aureus* expresses an array of virulence factors such as the Microbial Surface Component Recognizing Adhesive Matrix Molecules (MSCRAMMs), which facilitate its successful adherence and they include surface proteins such as clumping factor (Clf) A, ClfB, serine-aspartate repeat containing protein C (SdrC), SdrD and SdrE (Foster*et al.,* 2014). They share similar structural organization consisting of an N-terminal secretory signal peptide, followed by an A domain, B repeat, and R region containing serine-aspartate repeats. The C- terminal consists of an LPXTG cell wall-anchoring motif, hydrophobic membrane-spanning region and a charged cytoplasmic tail (Foster*et al.,* 2014). These proteins interact with host molecules such as fibrinogen, desmoglein 1 (Dsg 1) (Askarian*et al.,* 2016a) and β-

neurexin (Barbu*et al.,* 2010), resulting in the*S. aureus* virulence, immune evasion and survival within the host (Foster*et al.,* 2014; Ajayi*et al.,* 2018).

Several studies have delineated the function and expression profile of the sdrD protein, further emphasizing its importance in *S. aureus* virulence. SdrD promotes the adhesion of *S. aureus* to keratinocytes via its interaction with Dsg1 (Askarian*et al.,* 2016a). The*sdrD* expression is increased in the presence of blood (Sitkiewicz*et al.,* 2011) and it was recently shown that the protein also promotes bacterial survival and virulence during systemic infection (Askarian*et al.,* 2016b). Moreover, a correlation between the presence of the *sdrD* gene and bone infections has been observed (Ajayi *et al.,* 2018).

Bacterial pathogenesis may be influenced by genetic variation within genes encoding virulence. Polymorphisms in the A domain of FnBP A and B in *S. aureus* have been reported (Murai*et al.,* 2016) and specific single amino acid polymorphisms in FnBP A is associated with infection of cardiovascular devices (Ajayi *et al.,* 2018).Xue *et al.* (2011) observed polymorphisms in the *sdrD* gene from clinical or sub-clinical bovine mastitis associated *S. aureus* isolates. Furthermore, genetic variations within *S. aureus sdrD* sequences obtained from GenBank have also been reported (McCarthy and Lindsay, 2010). Sources of the *S. aureus* isolates used in the aforementioned studies were either from animal or diseased humans. However, genetic variability in the *sdrD* gene in *S. aureus* isolates from a healthy human population has not been reported.

2.4 Biofilm Formation by *Staphylococcus aureus*

Biofilm is a community of micro-organisms adhering to each other on a living or non-living surface within a self-produced matrix of extracellular polymeric substances (Jamal *et al.,* 2015). Biofilms protect bacteria from stress or stimuli by providing a thick

layer of extracellular proteins (Chino *et al.,* 2017).Biofilm thickness can range from a single cell layer to a substantial community encased by a viscous polymeric milieu (Chino *et al.,* 2017). Structural analyses have shown that in some cases unique pillar or mushroom-shaped structures can be formed by the micro-colony architecture of these dense biofilms; however, other structures do form depending on the environmental conditions (Wei and Ma, 2013). Intricate channel networks flow through these complex structures and provide some accessibility to essential nutrients even in the deepest regions of the biofilm.

Biofilms are of great clinical significance because bacteria in a biofilm have been reported to possess increased resistance to environmental stress, antimicrobial agents and host immunological defences (Chino*et al.,* 2017).Biofilm in bacterial communities is formed through initial and irreversible attachment, micro-colony formation, biofilm maturation, and biofilm dispersion (Wei and Ma, 2013). Biofilm matrix comprises polysaccharides, proteins, and nucleic acids that can be dissolved by enzymatic degradation (Fong and Yildiz, 2015). Proteinaceous components, such as the non-ribosomally generated peptide aureusimine (phevalin) in *S. aureus* (Paharik and Horswill, 2016) and three exopolysaccharides (Psl, Pel, and alginate) in *Pseudomonas aeruginosa* (Wei and Ma, 2013; Fong and Yildiz, 2015), play important roles in biofilm structural maintenance and are highly resistant to antibiotics and disinfectants.

Staphylococcus aureus biofilms have been widely implicated in many implant-based and chronic infections including those associated with prosthetic heart valves, central venous catheters, urinary catheters, orthopaedic prostheses, penile prostheses and contact lenses, endocarditis, otitis media, osteomyelitis and sinusitis (Precit *et al.,* 2016). These biofilms contributes to the persistence of bacterial infections,

therebyposing serious threats to global public health, mainly due to their resistance to antibiotics penetration and escaping innateimmune attacks by phagocytes (Hu *et al.,* 2017).The enhanced antibiotic resistance of biofilms has been attributed to the presence of exopolysaccharide matrix, a slow growth rate, spatial heterogeneity and biofilm specific drug resistant or drug tolerant physiologies including the presence of persister cells and small-colony variants (Gilbert *et al.,* 2002).

Two major methods for phenotypic identification of biofilm-producing strains involve the use of Congo red agar (CRA) and microtiter plate (MTP) (de Castro Melo *et al.,* 2013). The MTP method was developed to replace the test tube method which was first used for macroscopic estimation of bacterial biofilm on the surface of plastic tube. The CRA plate test uses a solid medium, called Congo red agar for direct analysis of the colonies and identification of slime-forming strains that appear as black colony on the red agar and non-slime-forming strains that appear red-coloured colonies (de Castro Melo *et al.,* 2013). Studies have reported quantification of biofilm using polystyrene microtiter plate with crystal violet staining in a method known as Mircotiter Plate (MTP) technique (Stepanovic *et al.,* 2007; Marinho *et al.,* 2013).

Biofilm formation is considered to be a two-step process in which the bacteria first adhere to the surface by adhesin factors, followed by growth, multiplication and cell aggregation to form multi-layered cell clusters encased within a slimy matrix (Abraham and Jefferson, 2010).

The adhesion stage of *S. aureus* is mediated by a protein family of staphylococcal microbial surface components recognizing adhesive matrix molecules (MSCRAMMs),including extracellular matrix protein known as fibronectin binding proteins (*Fnb*A and *Fnb*B), collagen binding protein (*Cna*), laminin binding protein

(*Eno*), elastin binding protein (*Ebp*S), fibrinogen binding protein (*Fib*) and biofilm associated protein (*Bap*) (Atshan *et al.,* 2012), while the, aggregation stage is conducted under certain conditions, by the synthesis of polysaccharide intercellular adhesin (PIA) molecule (Xue *et al.,* 2014). It has been found that the intracellular adhesion (*ica*) operon is essential for the control of biofilm production (Grinholc *et al.,* 2000). The *ica*locus, consisting of the gene *ica*ADBC, encodes the proteins mediating the synthesis of polysaccharide intercellular adhesion (PIA) molecule (Arciola *et al.,* 2012).

2.5 Carriage of *Staphylococcus aureus*

Although *S. aureus*are found present on the skin of the host as normal flora, they arealso found as normal flora of moist squamous epithelium of the anterior nares, is intermittently carried by majority of human populations (60 %) while 20 % of the population is always colonized with *S. aureus* and the remaining 20 % of populations never carry this organism (Ansari *et al.,* 2016). The evidence suggests that the populations harbouring *S. aureus* and its methicillin resistant (MRSA) strains are at higher risk for developing invasive infection (von Eiff *et al.,* 2001). The ability of the nasal passages to harbour *S. aureus* results from a combination of a weakened or defective host immunity and the bacterium's ability to evade host innate immunity (Quinn and Cole, 2007). Nasal carriage of MRSA contributes as a major risk factor for subsequent infection and transmission of this pathogen (Wertheim *et al.,* 2005). In recent otologic cultures from 173 patients with acute otitis externa (AOE), *S. aureus* was the second most commonly isolated pathogen 53 (30.6%)(Daurte *et al.,* 2017).

2.6 InfectionsCaused by *Staphylococcus aureus*

Staphylococcus aureus usually acts as a commensal bacterium, asymptomatically colonizing about 30% of the human population, and can sometimes cause disease (Tong *et al.,* 2015). *Staphylococcus aureus* cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteraemia and sepsis (Clinical Leadership and Infection,2014).

2.6.1Skin infections

Skin infection is the most common form of *S. aureus* infection, and can manifest in various ways, including small benign boils, folliculitis, impetigo, cellulitis, and more severe, invasive soft-tissue infections (Tong *et al.,* 2015).

Staphylococcus aureus is prevalent in persons with atopic dermatitis that is found in fertile, active places, including the armpits, hair, and scalp. Large pimples that appear in those areas may exacerbate the infection if lacerated can lead to staphylococcal scalded skin syndrome (SSSS), a rare disorder with clinical features varying from superficial localized blisters to generalized exfoliation that predominantly affects children, causing a spectrum of skin lesions, a severe form of which can be seen in new born (Meshram *et al.*, 2018).

The presence of *S. aureus* in persons with atopic dermatitis is not indicative that it be treated with oral antibiotics, because evidence has shown that oral antibiotics are not of help to the patient (American Academy of Dermatology, 2013), because the relationship

between *S. aureus* and atopic dermatitis has not been established (American Academy of Dermatology, 2013).

2.6.2Bone and joint infections

Bacteria of the genus *Staphylococcus* are the principal causative agents of two major types of infection affecting bone (septic arthritis and osteomyelitis), which involve the inflammatory destruction of joint and bone (Wright and Nair, 2010). *Staphylococcus aureus* is commonly responsible for all major bone and joint infections. These infections cause serious morbidity and are often difficult to manage (Claro *et al.,* 2013).The principal routes of infection for both osteomyelitis and septic arthritis are either haematogenous, resulting from bacteraemia; contiguous, when the infection is transmitted from local tissue; or direct, resulting from infiltration of bone, often following injury, surgery or implantation of a foreign body, such as joint replacement (Claro *et al.,* 2013). Infections may be acute or chronic and affect native joints, especially the hip and knee, or prosthetic joints, long bones, vertebrae and almost any other bone (Wright and Nair, 2010).

Septic arthritis is a joint disease typified by bacterial colonization and rapid articular destruction. Infiltration and growth of bacteria within the synovium results in inflammation with infiltration of leukocytes into the joint fluid (Nevius *et al.*, 2016).

Osteomyelitis describes a range of infections in which bone is colonized with microorganisms, with associated inflammation and bone destruction (Wright and Nair, 2010). Acute osteomyelitic foci are characterized by pus-forming inflammation at the site of microbial colonization. Damage to bone matrix and compression and destruction

of vasculature is also observed as the infection spreads to surrounding soft tissues, which can further exacerbate bone necrosis (Paulo *et al.,* 2014).

2.6.3Bacteraemia

Staphylococcus aureus is a leading cause of bloodstream infections (Rasmussen *et al.,* 2011). Infection is generally associated with breakages in the skin or mucosal membranes due to surgery, injury, or use of intravascular devices such as catheters, haemodialysis machines, or injected drugs (Tong *et al.,* 2015). Once the bacteria enter the bloodstream, they can infect various organs, causing infective endocarditis, septic arthritis and osteomyelitis (Rasmussen *et al.,* 2011). These diseasesare particularly prevalent and severe in the very young and very old (Tong *et al.,* 2015).

Without antibiotic treatment, *S. aureus* bacteraemia has a case fatality rate around 80% (Tong *et al.,* 2015). With antibiotic treatment, case fatality rates range from 15% to 50% depending on the age and health status of the patient, as well as the antibiotic resistance of the *S. aureus* strain (Tong *et al.,* 2015).

2.6.4Food poisoning

Staphylococcal food poisoning (SFP) is one of the most common food-borne diseases in the world following the ingestion of staphylococcal enterotoxins (SEs) that are produced by enterotoxigenic strains of coagulase-positive Staphylococci (CPS), mainly *S. aureus* and very occasionally by other Staphylococci species such as *S. intermedius* (Hennekinne *et al*., 2012). *S. aureus* is capable of causing food poisoning in humans by producing toxins that are deleterious to humans (CDC, 2016). Incubation periods ranged from 3 to 4.5 hours (mean 3.5 hours) with the illness itself lasting anywhere from thirty minutes to three days (Fletcher *et al.,* 2015).

2.6.5 *Staphylococcus aureus*Infections in Animal

*Staphylococcus aureus*has been reported to cause diseases such as osteomyelitis, bumble foot, and arthritis in poultry. The pathogen can be isolated from the joints, tendon sheaths and bone of affected animals(Argudín*et al.,* 2013).

Staphylococcus aureus is one of the causal agents of mastitis in dairy cows. Its large polysaccharide capsule protects the organism from recognition by the cow's immune defences (Cenci-Goga, 2003).

2.7 *Staphylococcus aureus*Virulence Factors

The ability of *S. aureus* to cause infections is attributed to the virulence factors they possess. These virulence factors include, enzymes, toxins, adhesion proteins, cell-surface proteins, factors that help the bacteria to evade the innate immune defence, and antibiotic resistance mediate survival of the bacteria and tissue invasion at the site of infection (Zecconi and Scali, 2013). It is important to note that certain toxins cause specific disease entities.

2.7.1Enzymes

Staphylococcus aureus produces various enzymes such as coagulase (bound and free coagulases) which clots plasma and coats the bacterial cell, probably to prevent phagocytosis (Hynes and Walton, 2006). For many years, the term hyaluronidase has been synonymous with spreading factors. *Staphylococcus aureus* produces the enzyme hyaluronidase that are able to breakdown the substrate hyaluronate (hyaluronic acid, hyaluronan), hence, facilitating the spread of bacteria or toxins through tissues (Hynes and Walton, 2006). *S. aureus* also produces deoxyribonuclease, which breaks down the

DNA, lipase to digest lipids, staphylokinase to dissolve fibrin and aid in spread, and beta-lactamase for drug resistance (Kira, 2015).

2.7.2Toxins

Depending on the strain, *S. aureus* is capable of secreting several exotoxins, including:

Super-antigens

Antigens known as super-antigens can induce toxic shock syndrome (TSS). This group includes the toxin TSST-1, enterotoxin type B, which causes TSS associated with tampon use (van Hal *et al.,* 2012). This is characterized by fever, erythematous rash, hypotension, shock, multiple organ failure, and skin desquamation. Lack of antibody to TSST-1 plays a part in the pathogenesis of TSS. Other strains of *S. aureus* can produce an enterotoxin that is the causative agent of *S. aureus* gastroenteritis. Onset of this gastroenteritis takes few minutes to hours from the time of ingesting the enterotoxin. Symptoms include nausea, vomiting, and major abdominal pain, cramp anddiarrhoea (Pinchuk*et al.*, 2010).

Exfoliative toxins

These are exotoxins implicated in the disease staphylococcal scalded skin syndrome (SSSS), which occurs most commonly in infants and young children (Dayan*et al.,* 2016). It may also occur as epidemics in hospital nurseries. The protease activity of the exfoliative toxins causes peeling of the skin observed with SSSS (Liang *et al.*, 2016).

Othertoxins

Staphylococcal toxins that act on cell membranes include alpha toxin, beta toxin, delta toxin, and several bi-component toxins. Strains of *S. aureus* can host phages, such as the

prophage Φ-PVL that produces Panton-Valentine leukocidin (PVL), to increase virulence. The bi-component toxin PVL is associated with severe necrotizing pneumonia in children (Kitur *et al.,* 2015). The genes encoding the components of PVL are encoded on a bacteriophage found in community-associated MRSA strains.

Small RNA

There is a growing list of small RNAs involved in the control of bacterial virulence in *S. aureus.*For example, RNAIII (Chevalier *et al.,* 2010), SprD (Chabelskaya *et al.,* 2010), RsaE, SprA1 (Sayed *et al.,* 2012), SSR42 (Morrison *et al.,* 2012), ArtR, SprX and Teg49 (Kim *et al.,* 2014).

2.7.3Other Immuno-evasive Strategies

Protein-A

Protein-A, an IgG-binding protein, binds to the Fc region of an antibody. Studies involving mutation of genes coding for protein-A resulted in a lowered virulence of *S. aureus* as measured by survival in blood, which has led to speculation that protein-A contributed virulence requires binding of antibody Fc regions (Zainab*et al.,* 2013).

2.8 Drug resistance in *Staphylococcus aureus*

The rapid acquisition of antibiotic resistance by *S. aureus* is a significant problem for treatment of human infections caused by this organism (McGuiness *et al.,* 2017). A timeline illustrating emergence of antibiotic-resistant *S. aureus* following the introduction of key antibiotics is provided in Fig. 2.1.

Mobile genetic elements (MGEs) play an integral part in the ability of *S. aureus* to adapt to environmental stresses, which include exposure to antibiotics. MGEs are a

primary means by which genetic information is exchanged between bacteria via horizontal gene transfer(McGuiness *et al.*, 2017). *Staphylococcus aureus* strains in general contain a relatively large variety of MGEs, including plasmids, transposons, bacteriophages, pathogenicity islands, and staphylococcal cassette chromosomes. Plasmids and staphylococcal cassette chromosomes in particular have played a central role in conferring resistance to β-lactam antibiotics and vancomycin (Holden *et al.*, 2014).

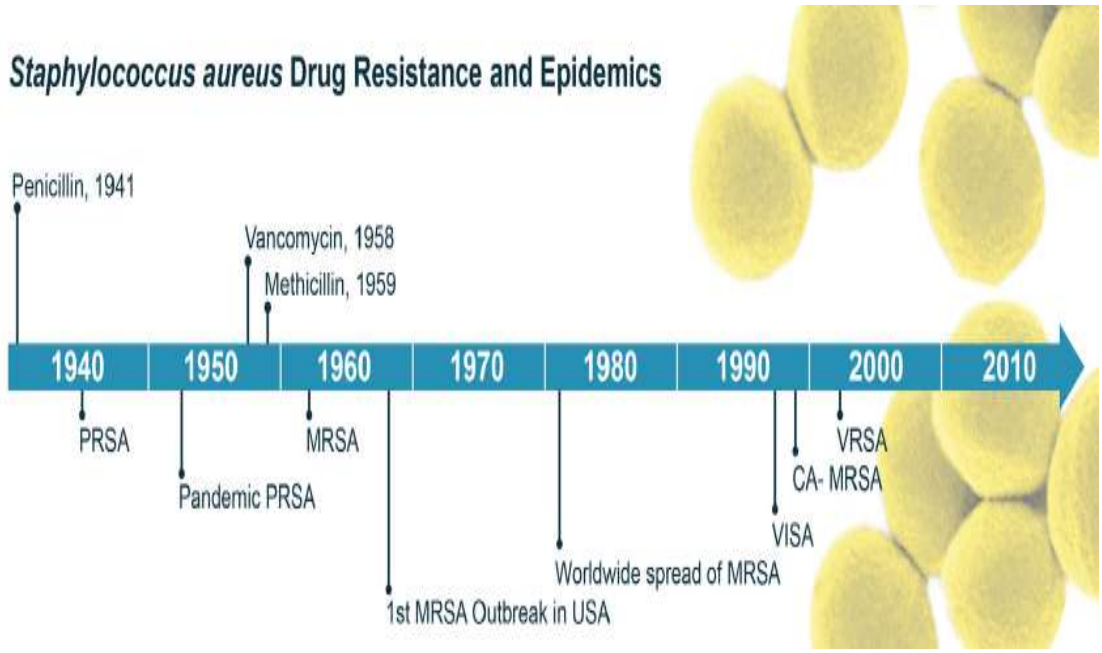

Figure 2.1:Timeline delineating the advent of antibiotic therapies and subsequent emergence of antibiotic-resistant *S. aureus* (McGuiness *et al.*, 2017).

2.9 Multi-Drug Resistance in *Staphylococcus aureus*

Multi-drug resistance (MDR) literally means 'resistance to more than one antimicrobial agent', but no standardized definitions for MDR have been agreed upon yet by the medical community (Magiorakos *et al.,* 2012).The absence of specific definitions for MDR in clinical study protocols gives rise to data that are difficult to compare.

One of the methods used by various authors and authorities to characterize organisms as MDR is based on *in vitro* antimicrobial susceptibility test results, when they test 'resistant to multiple antimicrobial agents, classes or subclasses of antimicrobial agents' (Kallen *et al.,* 2010). The definition most frequently used for Gram-positive and Gram-negative bacteria is 'resistance to three or more antimicrobial classes'(Kallen *et al.,* 2010).

According to Magiorakos *et al.* (2012) MDR is determined by one or more of the following criteria:

(i) an MRSA is always considered MDR by virtue of being an MRSA,

(ii) non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories.

(iii) The isolate is non-susceptible to at least 1 agent in ≥ 3 antimicrobial categories.

Multi-drug resistance is defined as non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos *et al.,* 2012).

2.10 Methicillin Resistant *Staphylococcus aureus*(MRSA)

Methicillin resistance in *S. aureus* is defined as the strains of *S. aureus* that are resistant to the isoxazoyl penicillins such as methicillin, oxacillin and flucloxacillin. Methicillin

Resistance *Staphylococcus aureus* (MRSA) are cross-resistant to all currently licensed β-lactam antibiotics (Taria *et al.,* 2013).

The expression of methicillin resistance in *S. aureus* strains is by virtue of acquired penicillin binding protein PBP2a, encoded by *mecA* gene (Loomba *et al.,* 2010). Structurally, PBP2a possesses both transglycosylase and transpeptidase, and confer resistance to all β-lactam antibiotics. However, the origin of *mecA* gene is unknown. Expression of methicillin resistance in *S. aureus* is commonly under regulatory control by *mec* I or by *Bla* I gene. The *mec* I and *bla* I repressors are controlled by the *mec* RI and *bla* RI transducers. Methicillin resistance expression in *S. aureus* is also influenced by the expression of other genetic loci called *fem* ("factors essential for methicillin resistance") or *aux* ("auxiliary") genes (Loomba *et al.,* 2010). So far, many *fem* and *aux* factors have now been identified, which are involved in formation of the staphylococcal cell wall. The *mecA* gene is located within a larger region of chromosome known as the staphylococcal cassette chromosome *mec* (SCC*mec*) region (21-67 kb) (Loomba *et al.,* 2010). The SCC*mec* is a mobile element, with mobility conferred by the presence of the *ccr*A and *ccr*B genes. The basic elements of SCC*mec* are the *mec*RI-*mec*I-*pbp*2a region and *ccr*A. Healthcare-associated isolates have larger SCC*mec,* owing to the accumulation over time of integrated plasmids or transposons that contribute to the multi-drug resistance (Loomba *et al.,* 2010). There are five currently described SCCmec types (types I, II, III, IVa, IVb, V). Types I, II and III are found predominantly in healthcare-associated MRSA, whereas type IV is commonly found in the more susceptible community-associated MRSA (Loomba *et al.,* 2010). Type IV SCC*mec* element is small and transferable by transduction. Types I to III SCC*mec* elements are large and hence do not transfer by bacteriophage (Loomba *et al.,* 2010).

2.10.1Methods of Detecting Methicillin Resistant *Staphylococcus aureus*

Clinical and Laboratory Standards Institute (CLSI) recommends the cefoxitin-disk (30 µg) screen test, the latex agglutination test for PBP2a, or a plate containing 6 µg/mL of oxacillin in Mueller-Hinton agar supplemented with NaCl (4% w/v; 0.68 mol/L) as alternative methods of testing for MRSA and *mecA* detection based on PCR or hybridization. For *S. aureus*, the cefoxitin-disk (30 µg) test is comparable to the oxacillin-disk (1 µg) test for prediction of *mecA* mediated resistance to oxacillin. This is because, cefoxitin is a better inducer of the *mecA* gene, and disk-diffusion test using cefoxitin gives clearer endpoints and is easier to read and thus is the preferred method than oxacillin (CLSI, 2018).

2.10.2Clinical Importance of Methicillin Resistant *Staphylococcus aureus*

Higher case fatality rates have been observed for certain MRSA infections, including bacteraemia, post-sternotomy mediastinitis and surgical-site infections (Loomba *et al.*, 2010). Researches have reported an association between MRSA infections and increased length of stay, as well as healthcare costs (De Angelis *et al.*, 2010).

Once MRSA is introduced into a healthcare setting, transmission and persistence of the resistant strain is determined by the availability of vulnerable patients, selective pressure exerted by antimicrobial use, increased potential for transmission from larger numbers of colonized or infected patients, and the impact of implementation and adherence to prevention efforts (Odom-Forren, 2017). Patients vulnerable to colonization and infection include those with severe disease, especially those with compromised host defences from underlying medical conditions, recent surgery, or indwelling medical devices (e.g., urinary catheters or endotracheal tubes) (Odom-

Forren, 2017). Hospitalized patients, especially ICU patients, tend to have more risk factors than non-hospitalized patients and have the highest infection rates.

Drugs approved for the treatment of MRSA infections are vancomycin, linezolid, daptomycin, teicoplanin, quinupristine-dalfopristine and tigecycline. The glycopeptide vancomycin has been regarded as the drug of choice for the treatment of infections due to methicillin-resistant strains (McGuiness *et al.*, 2017).

2.11 Vancomycin Resistant *Staphylococcus aureus* (VRSA)

After the approval of vancomycin for human use in 1958, it became an antibiotic of choice for treatment of MRSA infections in hospital settings in the late 1980s (Walters *et al.*, 2015). Resistance to vancomycin was discovered in enterococci in the 1980s, and this finding elicited significant concern with regard to the future use of vancomycin as an effective treatment for MRSA (Walter *et al.*, 2015). Vancomycin resistant *S. aureus* (VRSA) was reported for the first time in the United States in 2002 (CDC, 2002; Chang *et al.*, 2003), and since that time, there have been a total of 14 isolates reported in the United States (Walters *et al.*, 2015).

2.11.1Mode of Vancomycin Action

The synthesis of peptidoglycan in the production of bacterial cell walls requires several steps. In the cytoplasm, a racemase converts L-alanine to D-alanine (D-Ala), and then 2 molecules of D-Ala are joined by a ligase, creating the dipeptide D-Ala-D-Ala, which is then added to uracil diphosphate-*N*-acetylmuramyl-tripeptide to form uracil diphosphate-*N*-acetylmuramyl-pentapeptide. Uracil diphosphate-*N*-acetylmuramyl-pentapeptide is bound to the undecaprenol lipid carrier, which, after the addition of GlcNAc from uracil diphosphate – GlcNAc, allows translocation of the precursors to

the outer surface of the cytoplasmic membrane. *N*-acetylmuramyl-pentapeptide is then incorporated into nascent peptidoglycan by transglycosylation and allows the formation of cross-bridges by transpeptidation (McGuiness *et al.,* 2017).

Vancomycin binds with high affinity to the D-Ala-D-Ala C-terminus of the pentapeptide, thus blocking the addition of late precursors by transglycosylation to the nascent peptidoglycan chain and preventing subsequent cross-linking by transpeptidation (Courvalin*et al.,* 2006). Vancomycin does not penetrate into the cytoplasm; therefore, interaction with its target can take place only after translocation of the precursors to the outer surface of the membrane(McGuiness *et al.,* 2017).

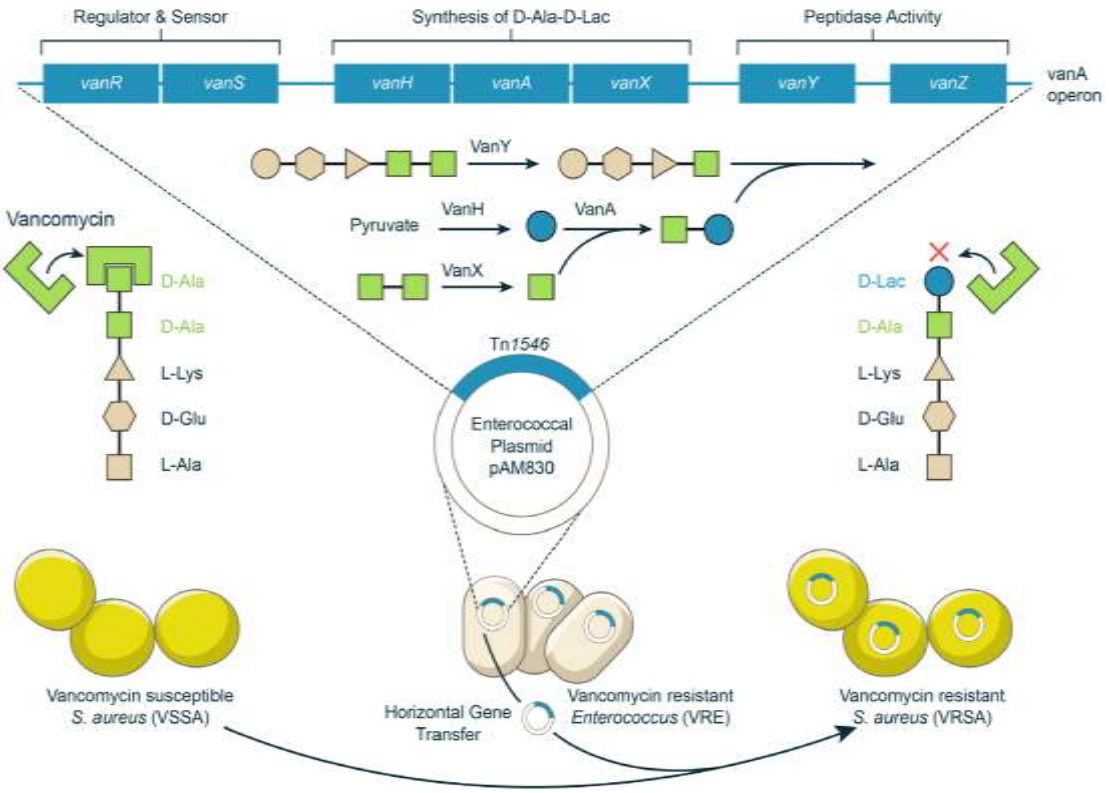

Figure 2.2:Schematic model illustrating the acquisition and molecular mechanism of *vanA*-type vancomycin resistance in *Staphylococcus aureus* (McGuinness *et al.*, 2017).

2.11.2Mechanism of *Staphylococcus aureus*Resistance to Vancomycin

Vancomycin, rather than interact with cell wall biosynthetic enzymes, forms complexes with peptidoglycan precursors, its activity is determined by the substrate specificity of the enzymes that determine the structure of peptidoglycan precursors and the affinity for a target enzyme. Vancomycin owes its resistance to the presence of operons that encode enzymes:

a. for synthesis of low-affinity precursors, in which the C-terminal D-Ala residue is replaced by D-lactate (D-Lac) or D-serine (D-Ser), thus modifying the vancomyin-binding target, and
b. for elimination of the high-affinity precursors that are normally produced by the host, thus removing the vancomycin-binding target (McGuiness *et al.,* 2017).

Complete vancomycin resistance in *S. aureus* (MIC ≥ 16 μg/ml) is conferred by the *vanA* operon encoded on transposon Tn*1546*, originally a part of vancomycin resistant enterococci (VRE) conjugative plasmid. *S. aureus* can acquire these enterococcal plasmids during discrete conjugation events. Vancomycin resistance in *S. aureus* is then maintained by retaining an original enterococcal plasmid or by a transposition of Tn*1546* from the VRE plasmid into a staphylococcal resident plasmid (McGuiness *et al.,* 2017). This is demonstrated in Fig. 2.2.

In Gram-positive bacteria, vancomycin interferes with late-stage peptidoglycan synthesis by forming non-covalent hydrogen bonds with the penultimate D-Ala-D-Ala residues of newly synthesized UDP-MurNAc-pentapeptides, thereby disrupting downstream peptidoglycan assembly. Ultimately, cell wall synthesis is inhibited and bound vancomycin-pentapeptide complexes accumulate within the cell (McGuiness *et*

al., 2017). Two key events are necessary for *vanA* operon-mediated vancomycin resistance:

a. hydrolysis of dipeptide D-Ala-D-Ala peptidoglycan precursors, which bind vancomycin,
b. synthesis of D-Ala-Dlactate peptidoglycan precursors, which cannot bind vancomycin (McGuiness *et al.,* 2017).

A schematic diagram that depicts acquisition and molecular mechanism of *vanA*-type vancomycin resistance is provided by Fig. 2.2.

The *vanA* operon comprise of *vanA, vanH, vanX, vanS, vanR, vanY,* and *vanZ* genes. The *vanA* operon is controlled via a two-component sensor- regulator system encoded by *vanS* and *vanR* that sense vancomycin and activate transcription of the operon respectively (McGuiness *et al.,* 2017). VanA, VanH, and VanX together are essential for the vancomycin resistance phenotype. VanA and VanH are responsible for synthesizing the depsipeptide D-Ala-D-Lac. VanA is a ligase that catalyzes the ester-bond formation of the D-Ala-D-Lac depsipeptide and VanH is a dehydrogenase that forms D-Lac by reducing pyruvate (McGuiness *et al.,* 2017). VanX is a D,D dipeptidase that hydrolyzes the D-Ala-D-Ala ester bond, ensuring the newly formed D-Ala-D-Lac depsipeptide has little competition to bind the UDP-linked tripeptide peptidoglycan precursor (Reynolds *et al.,* 1994). VanY is a D,D-carboxylpeptidase that performs a similar, but not essential function by facilitating the cleavage of D-Ala-D-Ala dipeptides already attached to the C-terminal end of stem entapeptide structures (McGuiness *et al.,* 2017). The role of VanZ is not well understood, but it may confer *S. aureus* resistance to teicoplanin. Incorporation of altered D-Ala-D-Lac into peptidoglycan yields a cell wall that is no longer susceptible to vancomycin.

2.12 Vancomycin Intermediate *Staphylococcus aureus* (VISA)

In 1997, Hiramatsu *et al.* reported the first *S. aureus* with intermediate-level resistance to vancomycin (VISA), which raised the threat of incurable staphylococcal infections (Hiramatsu *et al.,* 1997). Since then, a number of cases have been reported worldwide, with eight confirmed cases in the United States as of June 2002 (Chang *et al.,* 2003; Fridkin *et al.,* 2003). The majority of these cases have occurred in patients who have had prolonged exposure to vancomycin (Fridkin *et al.,* 2003).

Vancomycin resistance is defined by an MIC of ≥ 32 μg/ml. Three criteria for identifying VISA strains according to the CDC (2002) include:

(i) broth microdilution vancomycin MICs of 4 to 8 μg/ml,

(ii) E-test vancomycin MICs of ≥6 μg/ml, and

(iii) growth of > 1 colony within 24 hours on commercial brain heart infusion agar (BHIA) screen plates containing 6 μg of vancomycin per ml (CDC, 2002; Walters *et al.*,2015).

2.12.1 Resistance Mechanisms of Vancomycin Intermediate *Staphylococcus aureus*

The mechanism of intermediate resistance in *S. aureus* is unknown. However, conjugative transfer appears to be the mechanism of resistance in the two VRSA strains isolated (CDC, 2002) but none of the VISA strains have been shown to have any of the *van* determinants (*vanA*, *vanB*, *vanC1*, *vanC2*, or *vanC3*) that are present in VRE. Thus, interspecies transfer of resistant genes is not responsible for intermediate resistance to vancomycin in *S. aureus*. The VISA strains have been observed to have lower growth rates and thicker cell walls than fully susceptible strains (Smith *et al.,* 1999).Cui *et al.* (2003) noted that cell wall thickening correlated with increased vancomycin MICs and

was a common phenotype observed in VISA strains. Increased cell wall thickness appears to play a role in resistance by sequestering vancomycin molecules in the cell wall peptidoglycan, thus reducing the susceptibility of *S. aureus* to vancomycin.

2.12.2Clinical Significance of Vancomycin Intermediate *Staphylococcus aureus*

The clinical significance of Vancomycin Intermediate *Staphylococcus aureus*(VISA) has been difficult to assess. It is unknown whether these strains are fully virulent or perhaps even more virulent than vancomycin-susceptible strains of *S. aureus* and whether levels of resistance are responsible for treatment failures.

2.13 Inducible Clindamycin Resistance

Clindamycin is an excellent pharmacokinetics agentand useful as alternative treatment option for patients who are allergic to Penicillin for treatment oflocalized as well as systemic infections caused by drug resistant *Staphylococcus aureus* (Paul *et al.,* 2019). One macrolide resistance mechanism, modification of a drug binding site on the ribosome, results in resistance to macrolides, azalides, lincosamides, and group (B) streptogramins (MLSB). MLSB phenotypes can be either constitutive ($MLSB_c$) or inducible ($MLSB_i$) resistant.Inducible resistance phenotypes ($MLSB_i$) are those resistant to erythromycin and having a clindamycin zone of inhibition ≥ 21 mm, and are characterized by a D-shape zone of inhibition around clindamycin disc when placed at a distance of 12 – 20 mm away from an erythromycin disc on Mueller-Hinton agar plate. While the constitutive resistance phenotype ($MLSB_c$) are those resistant to both erythromycin and clindamycin (CLSI, 2016; Adhikari *et al.,* 2017(a)).

The inducible resistance to clindamycin in MRSA can severely compromise therapy and can result in failure of clindamycin treatment of MRSA infections when non-

suitable therapy (e.g. erythromycin) is given (Ahmed *et al.,* 2010). $MLSB_i$ strains can be successfully treated with clindamycin; however $MLSB_i$ can complicate therapy when $MLSB_i$ phenotype-switching into $MLSB_c$ occurs possibly due to mutation, in the absence of macrolide inducers (Ahmed *et al.,* 2010). Clindamycin can still be used for MRSA infections in hospitals (Ahmed *et al.,* 2010). However, susceptibility testing for the detection of inducible resistance to clindamycin should be routinely performed (Ahmed *et al.,* 2010).

2.14 High-Level and Low-Level Mupirocin Resistance

Mupirocin is an effective antibiotic for the elimination of MRSA that colonize the nasopharynx (Anderson*et al.,* 2014). It is a topical antibacterial agent made up of pseudomonic acid that is produced by *Pseudomonas fluorescens,* and its ability to inhibit bacterial synthesis of protein by reversibly binding to isoleucyl-tRNA has made an excellent active agent in eradication of *S. aureus* colonization, resulting in decreased number of infections among patients in high-risk settings such as intensive-care units (ICUs), haemodialysis, surgical theaters and long term-care centers (Septimus and Schweizer,2016). Mupirocin resistant strains are grouped into two distinct categories: low level (MupRL), with MICs of 8 – 256 μg/ml, and high level (MupRH), with MICs ≥ 512 μg/ml (de Oliveira*et al.,* 2007). Susceptible strains are defined as those with a MIC ≤ 4 μg, showing zone diameters of ≥ 14 mm around 5 μg mupirocin discs (EUCAST, 2016). High-level mupirocin resistance has been associated with failure to clear the organism from patients. However, it has been suggested that MupRL nasal isolates can still be controlled with mupirocin therapy, as the ointment used contains a much higher mupirocin concentration (20,000 μg/ml) than the MupRL MICs (Anderson*et al.,* 2014).

2.15 *Staphylococcus aureus*Small-colony Variants

A new era of small colony variants (SCV) started since 1994 when Proctor and his colleagues characterized biological and pathogenic traits of *S. aureus* SCV strains on molecular level. So far, SCV recoveryfrom *S. aureus* isolates have reported in various studies (Yagci *et al.,* 2013; Kahl, 2014, Ode *et al.,* 2015; Precit *et al.,* 2016).

Staphylococcus aureus Small-colony variants (SCVs) are a slow growing sub-population of*S. aureus* with distinct phenotypic and pathogenic features atypical to their parent strains, including small-colony size, slow growth, and down-regulated virulence genes (Kahl *et al.,* 2016). These SCVs have been reported to cause re-current infections that can cause persistent infections due to their ability to persist intracellularly (Bhattacharyya *et al.,* 2014). Very often, they reside inside human cells avoiding host defences and antimicrobial chemotherapeutics.

The small-colony variants are defective in their electron transport pathways, hence, are auxotrophicto thymidine, menadione and/or hemin. And they are non-pigmented and non-haemolytic tiny colonies on blood agar (Proctor *et al.,* 2014). They exhibit reduced rate of metabolism and are less virulent, but due to their slow growth and reduced cell wall synthesis, they are more tolerant to β-lactam antibiotics than their wild-type parents. Their low membrane potential makes them also resistant to aminoglycoside antibiotics (Proctor *et al.,* 2014).Figure 2.3 shows a plate from the work of Precit *et al.,* 2016 showing in comparison, the normal colony of *S. aureus* and its SCV.

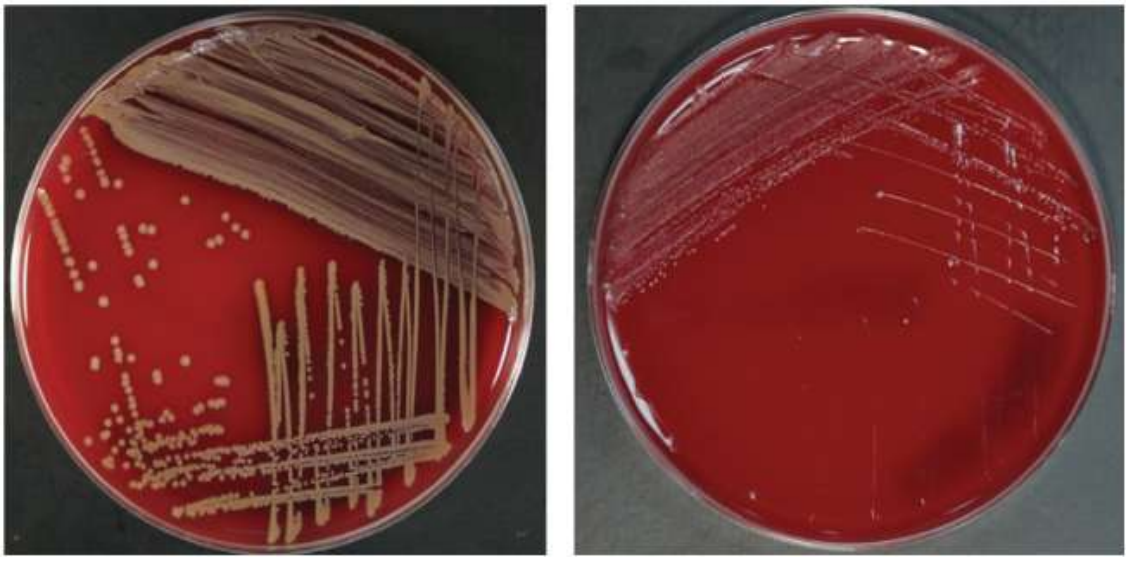

Figure 2.3: Culture of *Staphylococcus aureus* (left) and *Staphylococcus aureus* SCV (right) on Columbia blood agar (Precit *et al.*, 2016).

2.16 Occurrence of *Staphylococcus aureus* Small-Colony Variants

Staphylococcus aureus SCVs are found to occur commonly in chronic diseases like cystic fibrosis (Morelli *et al.,* 2015) and osteomyelitis(Tong *et al.,* 2015). *Staphylococcus aureus* SCVs have been known to occur during the normal growth cycle in *in vitro* studies (Edwards, 2012), but their occurrence during acute *S. aureus* infections has not been described. However, SCVs have been reported to be cultured from patients with chronic re-current infections, indicating their selection and optimized fitness compared to the normal *S. aureus* phenotype during persistent infections and antibiotic therapy.

Many SCVs have been characterized in terms of their underlying auxotrophism, meaning that specific substrates, such as hemin, menadione, or thymidine, support the growth of these SCVs or give enhanced growth (Proctor *et al.,* 2014). Based on the antibiotics used to treat the patients, SCVs with particular genetic mutations can be expected. While aminoglycoside therapy is associated with the emergence of menadione or hemin dependent SCVs, trimethoprim-sulfamethoxazole (SXT) therapy is strongly associated with the emergence of thymidine-dependent (TD) SCVs (Wolter *et al.,* 2013). However, for many SCVs, such an association of treatment and auxotrophism is still not clear. For example, it is not known if a special antibiotic regimen induces and selects for CO_2-dependent SCVs. Recent researches suggest that additional antimicrobial compounds, such as moxifloxacin and clindamycin, promote the formation of SCVs (Tuchscherr *et al.,* 2016).

2.17 Pathogenesis of *Staphylococcus aureus* Small-Colony Variants

An intracellular location provides a survival niche for bacteria, because the microorganisms are protected against antibiotic therapy and host defences. Several studies have shown in the past that *S. aureus* is not only an extracellular pathogen but also an intracellular pathogen, owing to effective uptake of these bacteria by non-professional phagocytes, such as endothelial and epithelial cells, fibroblasts, osteoblasts and keratinocytes(Gunaratnam*et al.*, 2019). This intracellular location itself can trigger the emergence of SCVs (Cano *et al.*, 2003), and this might be a mechanism for persistence even when there is no exposure to antibiotics.

Internalized normal *S. aureus* strains readily lyse endothelial cells, owing to expression of α-toxin and a pore-forming toxin. Researches have shown that α-toxin is necessary and sufficient for the induction of apoptosis of Jurkat T cells and primary mononuclear cells (Bantel *et al.*, 2001; Haslinger *et al.*, 2003). Clinical hemin-auxotrophic, menadione-auxotrophic and thymidine-dependent SCVs persist longer in eukaryotic cells than in the corresponding wild-type strains (von Eiff *et al.*, 2001). This could be explained by the expression of less α-toxin by SCVs than by the normal phenotype, as determined by transcriptional analysis (kahl *et al.*, 2005). Interestingly, keratinocytes with internalized SCVs had a healthy appearance 48 hours after infection, whereas cells infected by the corresponding normal *S. aureus* underwent apoptotic or necrotic cell death (von Eiff *et al.*, 1999). Taken together, these data indicate that the SCV phenotype might be one of the survival strategies that *S. aureus* uses for optimal internalization and survival in the host.

2.18 *Staphylococcus aureus* Small-Colony Variant as Intracellular Pathogen

S. aureus has various strategies for resisting therapy that extend beyond classic mechanisms. Such strategies include the potential for evading the effect of a given antibiotic even though it tested susceptible by production of diffusion barriers, e.g. biofilm production, or by withdrawal into the intracellular milieu. The latter mechanism has been documented for SCVs. Indeed, in several assays using various non-professional phagocytes such as endothelial or epithelial cells, these variants were able to persist intracellularly(Gunaratnam*et al.,* 2019).

Becker *et al.* carried out a research to identify the intracellular location of SCVs by infecting primary human endothelial cells with various strain pairs displaying either the normal or the SCV phenotype (Becker *et al.,* 2004). Subsequently, maturation of phagosomes using live cell imaging was visualized. Within one hour, all internalized Staphylococci accumulated in lysosomal organelles and remained there for up to 5 days. Whilst an effective bactericidal activity of human endothelial cell lysosomes towards Staphylococci was observed, these studies provided evidence that SCVs of selected strains are able to withstand this activity.

2.19 Clinical Infections Caused by *Staphylococcus aureus* Small-Colony Variants

Infections caused by *Staphylococcus aureus* SCVs includes

2.19.1Foreign Body-Related Infections:

These are infections that occur in individuals who received foreign or artificial biological body part such as pacemakers and heart assist devices, bones and joints replacement, indwelling catheter, etc. These devices provide surface for adherence by

the infecting bacteria. Examples are Pacemaker-related infections, Heart-assist device infection (Maduka-Ezeh *et al.*, 2012), Prosthetic Joint Infections (Tuchscherr *et al.*, 2010) and Subacute/Chronic Bone Infections (Tong *et al.*, 2015).

2.19.2Osteomyelitis

Proctor *et al.* demonstrated in 1995 the impact of SCV in chronic bone and joint infection by analyzing five patients with chronic bone and joint infection. Since then, there has been worldwide reports showing the development of SCVs in different staphylococcal species, and reduced susceptibility to aminoglycosides by this strain was mentioned earlier and later confirmed in other studies (Maduka-Ezeh *et al.*, 2012).

2.19.3Cystic Fibrosis Airway Infection

In the last 40 years, various prospective studies has been carried out, dealing with the culture of *S. aureus* SCVs from the airways of almost 2,000 cystic fibrosis (CF) patients (the numbers of patients in the different studies ranged from 14 to 594) during a period of 3 to 34 months (mean, 13 months). These reports are summarized in Table 2.1, showing their various auxotrophies.

Table 2.1: Reports of *Staphylococcus aureus* Small-Colony Variants recovered from airway specimens from Cystic Fibrosis patients

References	No. of investigated isolates or patients	No. (%) of patients with SCVs	Study period (month)
Sparham *et al.*(1978)	14	7(50)	7
Gilligan *et al.*(1987)	200	20(10)	12
Kahl *et al.*(1998)	78	26(33)	34
Vergison *et al.*(2007)	627	25(4)	7
Besier *et al.*(2007)	252	20(8)	12
Schneider *et al.*(2008)	98	8(8)	3
Green *et al.*(2011)	260	17(6)	6
Yagci *et al.* (2013)	248	20(16.2)	11
Wolter *et al.*(2013)	100	24(24)	24
Morelli *et al.*(2015)	222	28(13)	Not known
Ode *et al.* (2015)	258	48(21.9)	6

2.19.4Infections Involving Skin, Mucous Membranes, Soft Tissues and Wounds

Staphylococcus aureus has been linked to the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP), and the pathogen has beendemonstrated in the mucosa of CRSwNP patients by peptide nucleicacid-fluorescence *in situ* hybridization (PNA-FISH) (Corriveau *et al.,* 2009). However, the role of *S. aureus* SCVs in CRS is unclear. While somestudies failed to detect SCVs in nasal lavage fluid and mucosalbiopsy specimens from CRS patients (Gitomer *et al.,* 2015), other groups wereable to cultivate intra mucosal SCVs (*S. aureus* and other species)from respective sinonasal tissues of CRS patients (Tan *et al.,* 2014).

Chronic wound infection caused by *S. aureus* SCV has beenreported for a patient subsequent to herniotomy (Abele-Horn *et al.,* 2000).

2.20 Clinical Importance of *Staphylococcus aureus* Small-Colony Variants

The incidence of SCVs in clinical specimens has been found to range from 1% to more than 30% in different studies(Proctor *et al.,* 2006; Yagci *et al.,* 2013; Ode *et al.,* 2015). Analysis of sputa from 72 patients with CF has shown that more than 70% (52 of 72) are chronically colonized with *S. aureus,* and of these samples, 46% (24 of 52) contained SCVs (Kahl *et al.,* 2003). In a similar manner, *S. aureus* SCV have also been reported in researches to be responsible for Cystic Fibrosis (Yagci *et al.,* 2012; Wolter *et al.,* 2013; Morelli *et al.,* 2015).

A study in 1997 by von Eiff *et al.,* showed *S. aureus* recovered from bone specimens or deep-tissue aspirates of patients with osteomyelitis, *S. aureus* SCVs were found in 29% of patients (4 of 14). Chronic wound infection caused by *S. aureus* SCV has

beenreported for a patient subsequent to herniotomy (Abele-Horn *et al.,* 2000). Baltch *et al.* also have in 2008, recovered *S. aureus* SCV from a case of chronic osteomyelitis.

Staphylococcus aureus SCV co-occurring with normal phenotype isolate from the skin specimen of Darier's disease patient has been reported (von Eiff *et al.,* 2001), and also have been implicated in the pathogenesis of Chronic Rhinosinusitis with Nasal Polyps (Corriveau *et al.,* 2009).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1Materials

3.1.1Equipment

The equipment used in study includes an incubator (Natural appliance: Aheinicke Company Portland, Oregon, U.S.A. Model-630 Serial No., 1-81-1550-1), Electronic weighing balance (Top balance digital, U.S.A. Ohaus, PA313-model), Hot-Air-Oven (Baird and Tatlock London limited), Wire loop (or inoculating loop), Micro-titre plate reader, Autoclave (Adelphi MFG Co Ltd, Portland autoclave), Microscope (Wild M11, Switzerland), Micropipette and Pasteur Pipette, Refrigerator (NAPCO Model 630 Portland, Oregon, U.S.A.), Antibiotic Test Strips, Staphytech Plus (Oxoid Ltd., Basingstoke, Hampshire, England), Micro-titre plate and Microgen™ Staph-ID System.

3.1.2Culture Media

Agars used in the course of this study include the following: Columbia Blood Agar, Mueller Hinton Agar, Mannitol Salt Agar, Brain Heart Infusion Agar, Nutrient agar and Broth, Luria and Bertani (LB) broth, and Agar Powder as Agar purified (all from Oxoid Ltd., Basingstoke, Hampshire, England).

3.1.3Chemicals and Reagents

The following chemicals and reagents were used in this study: Crystal violet (May and Baker Ltd. Dagenham England), Lugol's iodine (May and Baker Ltd. Dagenham England), Acetone, Methyl-red, Hydrogen peroxide (SKG Pharma Ltd. Ikeja Lagos,

Nigeria), Sterile deionized water, Ethanol-acetone, oil immersion (BDH), Staphytech Plus Test Kit (Oxoid Ltd., Basingstoke, Hampshire, England), Sucrose, Normal Saline.

3.1.4Glass wares

The following glass wares were used in this study: Universal bottles, Microscope glass slides and Petri dishes (Pyrex, England), Test tubes (Pyrex, England), Measuring Cylinder (Pyrex, England), Beakers (Pyrex, England), and conical flask(Pyrex, England).

3.1.5Software

Software used to analyse data in this study include:

i. Microgen Identification System Software
ii. Statistical Package for Social Sciences (SPSS) Version 21

3.2 Methods

3.2.1Ethical Consideration

Ethical clearance was obtained from the ethical committee of National Orthopaedic Hospital Dala (NOHD) to enable collection of demographic data and clinical specimen from hospitalized patients by hospital medical personnel.

Consent form was designed and translated to the local language (Hausa; for those that don't understand English) by Dr. Shu'aibu Hassan from the Department of African Languages and Cultures, Faculty of Arts, Ahmadu Bello University, Zaria. Only patients who gave their consent by personally filling or guided to fill the consent form were included in this research. Each patient was expected to give 4 samples when possible, and these samples were coded with the serial number on the patients consent form,

followed by the first letter of the sample source. For example, patient with serial number 01 would have his/her samples labelled as 01w for wound sample, 01N for nasal sample, 01U for urine sample and 01B for bed sample.

3.2.2Collection of Demographic Data

Patient's data such as age, gender, antibiotics used and length of stay in the hospital were collected.

Inclusion Criteria: Patients on admission in the different wards of the hospital within a period of 10 weeks (September, 2017 and December, 2017), and gave consent for their nasal swab, wound swab, swab from patient's bed and urine samples to be collected were included in this study.

Exclusion Criteria: Patients that did not give consent, and out patients (i.e. patients that were not on admission) were excluded from this study.

3.2.3Research Limitation

This study was limited to orthopaedic patients on admission in all the hospital wards and available within a period of 10 weeks that spanned between September, 2017 and December, 2017.

3.2.4Determination of Sample size

In this study, samples were collected using the convenience sampling method. Wound sab, nasal swab, bed swab and urine samples were collected from the patients in the various wards.

3.2.5Collection of Samples

A total of 189 clinical specimens comprising 42 urine samples, 49 wound swabs, 49 nasal swabs and 49 swabs from patient's bed were collected from patients on admission in the National Orthopaedic Hospital, Dala – Kano. The swab sticks were moistened with physiological saline. A moistened swab stick each was used to gently swab the patient's wound, nostril and bed respectively, and then returned into its case to avoid contamination. Patients were instructed on how to collect mid-stream urine into sterile bottles and closed tightly.

The specimens collected were transported to the Microbiology Laboratory, Department of Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria in a sterile Ziploc plastic bag for microbiological evaluation.

Collection of samples was done with the assistance of the hospital nurses, after they agreed to assist and were taught how to collect these set of samples as described above.

3.2.6Preparation of Culture Media

The media were prepared according to the manufacturer's specification.

3.2.7Isolation of Test Organism

The clinical samples were inoculated into freshly prepared nutrient broth and incubated at 37^{o}C for 24 hours. A loop-full of growth from the nutrient broth medium was streaked onto Mannitol Salt Agar plate and incubated for 24 hours at 37^{o}C as described by UK Standards (2017).

Discrete, single, golden-yellow colonies were presumptively identified as Staphylococci, and they were inoculated onto nutrient agar in a slant bottle, incubated at 37^{o}C24 hours and then preserved in refrigerator for further analysis.

3.2.8Identification of the Isolates

Gram Staining

This was carried out to determine the morphology of the organisms and differentiate cocci isolates in cluster from streptococci and bacilli in accordance to Acharya(2015).

Smear was prepared from the growth of the isolates by placing a drop of distilled water at the centre of a clean glass slide, and a sterile wire-loop was used to pick discrete colony of the bacteria culture of not more than 24 hours and mixed. The smear was fixed on the slide by passing it over a flame and allowed to air-dry. The smear was flooded with crystal violet (primary stain) for 30 seconds and washed off with running tap water, then Lugol's iodine was added for 1 minute (to act as mordant) and was washed off with running tap water. Again, the smear was flooded with 70% alcohol for 30 seconds for decolourization and then rinsed off with tap water. Smear was counter stained with methyl red (secondary stain) for 1 minute and rinsed under running tap water. The smear was then allowed to air-dry and then observed under the microscope using × 100 oil immersion.

All cocci isolates that retained the primary stain (blue) were identified as Gram positive isolates. They were selected and subjected to the next set of tests.

Catalase Test

Catalase test was carried out using method described by Aditi(2017)(to detect presence of catalase enzyme in the isolate). A drop of 3% hydrogen peroxide (H_2O_2) was placed

on a clean glass slide; using a sterile applicator, a loopful of a 24 hours culture of gram positive cocci was picked and mixed with the drop of hydrogen peroxide on the slide. A positive test is indicated by bubbling and frothing.

Coagulase Test

Coagulase test was done to differentiate *S. aureus* (coagulase positive) from coagulase negative *Staphylococcus* (CONS) using DrySpot Staphytech Plus Test Kit to detect bound coagulase(clumping factor).

As described by DrySpot Staphytech Plus Test Kit manufacturer, a drop (50 µl) of Staphytech Plus Test Kit was dropped within one of the circles on the reaction card provided, and a drop of control reagent was dropped on the opposite circle. With a sterile wire-loop, a 24 hours culture of catalase positive isolate was picked and mixed with the Staphytech Plus Test Kit on the reaction card respectively, and the reaction card was picked up and rocked. Clumping within 5 - 10 seconds in the test circle indicated positive agglutinationwhile no clumping indicated negative result.

3.2.9Identification of Isolates using Microgen™ Staph-ID System

Gram positive cocci, catalase positive and coagulase positive isolates were subjected to the Microgen™ Staph-ID System test. This test is to confirm that the isolates belong to the genus of *Staphylococcus,* and also to determine the species of *Staphylococcus* that the isolatesare.

Colony pigment production by each isolate on Mannitol Salt agar (MSA) was observed and recorded. A golden-yellow single colony from a 24 hours culture on MSA was emulsified in the suspending medium supplied in the kit and mixed thoroughly. The adhesive tape sealing the microwell test strip was removed, and using a sterile Pasteur

pipette, 3 drops (100µl) of the bacterial suspension was added to each well of the strips. Well 10 and 11 were overlaid with 3 drops of mineral oil which are highlighted with black circle. The top of the microwell was sealed with the adhesive tape and incubated at 37^{o}C for 24 hours.

After incubation, the adhesive tape was removed and the microwells were examined for colour change. Positive reactions were recorded for microwells 1 to 11 with the aid of the colour chart and substrate reference table supplied.

Exactly 1 drop of PYR reagent was added to well 12 and it was read after 10 minutes. Formation of a very deep pink/red colour indicated positive result, while nitrate reduction test on well 9 was performed after reading and recording β-glucuronidase reaction by adding 1 drop each of Nitrate A and B reagents to the well and read after 60 seconds. Red colour indicated that nitrate has been reduced to nitrite.

The results were read using colour chart provided by the manufacturer, and further interpreted using Microgen identification system software. And the identified *S. aureus* were then stored in the refrigerator for further analysis.

3.2.10Isolation and Identification of *Staphylococcus aureus* Small-colony Variants

Columbia blood agar supplemented with 5% sheep blood was used to identify and isolate *S. aureus* small-colony variants (SCVs).

The *S. aureus* isolates identified using the Microgen™ Staph-ID System were cultured on Columbia blood agar (Oxoid Ltd., Basingstoke, Hampshire, England) at 35^{o}C for 24 – 48 hours. Non-haemolytic, non-pigmented, pinpoint or fried-egg colonies on Culombia blood agar were considered as *S. aureus* small-colony variants (Precit*et al.*, 2016).

3.2.11Biofilm Assay of *Staphylococcus aureus* Isolates

In order to test for biofilm production by the *S. aureus* isolated from orthopaedic patients, the micro-titre plate method as described by Christensen *et al.,* (1985) and as reported by Neopane*et al.,* (2018) was used.

A colony of *S. aureus* was isolated from a fresh agar plate and inoculated in 2 mL of trypticase soy broth. The broth was incubated overnight at 37 °C. The culture was then diluted to 1:100 with fresh medium. A sterile individual plate with 96 flat-bottom polystyrene wells was filled with 200 µL of the diluted culture. The plate was incubated at 37 °C for 24 hours. After incubation, the contents of each well were removed by gentle tapping. The wells were washed with 200 µL of phosphate buffer saline (pH 7.3) to remove free-floating bacteria. Biofilms formed by bacteria adherent to the wells were stained with 0.1% crystal violet (CV). Excess stain was washed gently, and the plate was kept for drying.A positive result was seen as the presence of a layer of stained materials adhered to the inner wall of the wells.

The quantitative assay of the biofilm production was performed by adding 250µl of ethanol-acetic acid (95:5 vol/vol) to dissolve the stained substance on the wall of the wells obtained from the preceding test, then 100µl from each well was transferred to a new micro-titre plate and the optical density (OD) of the solutions was measured at a wavelength of 630 nm using micro-ELISA auto-reader (HUMAN). The experiment was performed in triplicate. The un-inoculated medium was used as control. The cut-off OD (ODc) was defined as the average OD value of negative control + (3 x standard deviations of negative control). The biofilm forming ability of the tested strains were classified into four categories based on their OD: non-adherent (OD<ODc), weakly

adherent (OD >ODc, but OD< 2 x ODc), moderately adherent (OD > 2 x ODc < 4 x ODc), and strongly adherent (OD > 4 x ODc) (Stepanovic *et al.*, 2007).

3.2.12Determination of Antibiotic Susceptibility of *Staphylococcus aureus* Isolates

The disc diffusion method was used to determine the antibiotic susceptibility of *S. aureus* strains, using the method described in EUCAST (2018).

Discrete colonies of *S. aureus* isolates on Mannitol salt agar plates was emulsified in 5ml of sterile physiological saline and the turbidity adjusted to 0.5 McFarland standard (approximately a cell density of 1.5×10^8 cfu/ml). The standardized suspension was inoculated on Mueller Hinton agarand spread using a sterile swab stick to ensure even distribution and confluent growth. Using a sterile antibiotics disc dispenser (Oxoid Ltd., Basingstoke, Hampshire, England) the sensitivity discs of the selected antibiotics were aseptically placed on the dried inoculated agar surface using a sterile disc dispenser. Then, the plates were incubated at 37^oC for 18 hours at an inverted position. After incubation, the plates were examined for the zones of inhibition. Then the diameter of the zones of inhibition around the antibiotics were measured in millimetre (mm), recorded and result interpretation according to EUCAST (2018).

Selected antibiotics were in accordance to EUCAST (2018). They include: Cefoxitin (30µg), Gentamicin (10µg), Erythromycin (15µg), Clindamycin (2µg), Norfloxacin (10µg), Ciprofloxacin (10µg),Vancomycin (30µg), Linezolid (30µg), Quinupristin-dalfopristin (15µg), Mupirocin (5µg and 200µg), Tetracyclinee (30µg), Amoxycilin/clavulanic acid (30µg) and Trimethropin-sulfamethoxazole (1.25µg+23.75µg).

Determination of Inducible Clindamycin Resistance in Staphylococcus aureus Isolates

Isolates that showed resistance to erythromycin (no zone of inhibition), but susceptible (showed zone of inhibition) to clindamycin were subjected to inducible clindamycin resistance test.

Inducible clindamycin resistance was carried out using the D-zone test method according to EUCAST (2018) guidelines. Erythromycin (15µg) disc was placed at a distance of 12 – 20mm edge to edge from clindamycin (2µg) disc on a Mueller Hinton agar plate inoculated with 0.5 McFarland standard equivalent bacterial suspensions. This was incubated at 37^{o}C for 18 hours at an inverted position.

After culturing at 37°C for 18 hours, flattening of the zone of inhibition adjacent to the erythromycin disc (referred to as a D-zone) indicated *erm*-mediated inducible clindamycin resistance (positive D-test). While the absence of D-shaped and clindamycin growth inhibition zone diameter ≤14 was indicative of constitutive clindamycin resistance (Deresinski, 2005; Yilmaz *et al.*, 2007).

Determination of High- Level and Low-level Mupirocin Resistance in Staphylococcus aureus Isolates

Test for High- and low-level mupirocin resistance were carried out according to EUCAST (2016) guidelines. Mupirocin (200µg) and Mupirocin (5µg) discs were placed on a Mueller Hinton agar plate that was previously inoculated with 0.5 McFarland standard bacterial suspension. After culturing at 37°C for 24 hours, light growth within the zone of inhibition are carefully examined with transmitted light. No zone of inhibition around the 200 µg discs indicates high-level Mupirocin resistance, while any zone observed indicated absence of high-level of resistance.

Absence of a zone for the 5 μg disc indicates low-level Mupirocin resistance, while if any zone is observed, it indicates absence of low-level Mupirocin resistance (EUCAST, 2016).

3.2.13Molecular Characterization of Isolates of *Staphylococcus aureus* Isolates.

Bacterial Cell Preparation

Preparation of the bacterial cells that were found to be biofilm former and resistant isolates of *S. aureus* were carried out by the method described by Lephoto and Gray, (2013). Pure colonies from the overnight culture on Mannitol Salt Agar were inoculated into 5 ml Luria and Bertani (LB) broth and incubated at 37^{o}C for 24 hours. Bacterial cells were harvested by centrifugation at 4^{o}C, 8000 rpm (6800 ×g) in a micro-centrifuge for 2 minutes at room temperature in an Eppendorff's tube, the supernatant was discarded and cells harvested. The step was repeated for higher yield of cells (Lephoto and Gray, 2013).

Genomic DNA Extraction

QIAGEN Genomic DNA extraction kit was used for the extraction of the bacteria DNA from the step of bacteria cell preparation above.A QIAGEN Genomic-tip 20/G was equilibrated with 4 ml of Buffer QBT, and the QIAGEN Genomic-tip was allowed to empty by gravity flow. Then the sample (the bacteria cell preparation) was vortexed for 10 sec. at maximum speed and applied to the equilibrated QIAGEN Genomic-tip, allowing it to enter the resin by gravity flow. The QIAGEN Genomic-tip was then washed with 2 x 7.5 ml of Buffer QC, and the genomic DNA was eluted with 1 x 5 ml of Buffer QF.

The DNA was precipitated by adding 3.5 ml of room temperature isopropanol to the eluted DNA. And the precipitated DNA was mixed and recovered by centrifuging immediately at >5000 x *g* for at least 15 min at 4°C, then the supernatant was carefully removed. The centrifuged DNA pellet was washed with 4 ml of cold 70% ethanol, vortexed briefly and centrifuged at >5000 x *g* for 10 min at 4°C. The supernatant was carefully removed without disturbing the pellet. The DNA pellet was air-dried for 5 - 10 min, and re-suspended in 0.1 - 2 ml of a suitable buffer (10 mM Tris·Cl, pH 8.5). The DNA was dissolved overnight on a shaker, and was ready to be used for amplification.

Polymerase Chain Reaction (PCR) Amplification of Genomic DNA

Amplification of biofilm associated genes (*icaA* and *bbp*), MRSA gene (*mecA*) and VRSA gene (*vanA*) were done using PCR after an external optimization of the reaction to ensure a better amplification. The following process was carried out; The thin walled PCR tubes were marked and the following components was added for each isolate for single reaction of 50µl Viz: 25µl of Dream Taq™ PCR master mix was added in the PCR tube, 1.0µl of forward primer, 1.0 of reverse primer, 7.0µl of temple DNA (genomic DNA), nuclease-free water (16µl) was added in the PCR tube to make up a total volume of 50µl. The samples was spun down as PCR is performed using the thermal cycling conditions as stated by Zymo Research UK (Lephoto and Gray, 2013).

Agarose Gel Electrophoresis of PCR Products

At completion of the amplification of each gene, the PCR amplicons were resolved on agarose gel electrophoresis. Agarose gel (1%), into which 0.5µL ethidium bromide had

been added was prepared. The gel plates were then placed inside an electrophoresis tank (Weal Tek Corp., Taiwan) which contained 1 x TBE solution. A 5µL of amplicon was mixed with 5µLOrange G (loading buffer) and loaded to the well of the agarose gel. The power was adjusted to 100 volts for 25 minutes. For each run, a hundred base-pair molecular weight DNA standard (New England Biolabs "NED") was used to determine the size of each PCR amplicon. The DNA bands were then visualized with a short wave ultraviolet trans-illuminator and photographed using gel bio-imaging system (UVP Imaging System, Upland, CA, USA) (Lephoto and Gray, 2013).

Table 3.1: Primers Used in the Study

Gene	Primers and Probes Description	Sequence	Amplicon Size (bp)	PCR and Real Time PCR Conditions of Cycling	References
bbp	Forward	5ˈ- CGGGATCCGTCTAGGGAACCAAGAAGCT-3′	500	94°C 5 min; 35 × (94°C 60 s, 57°C60 s, 72°C 60 s); 72°C 7 min.	(Tung *et al.,* 2000)
	Reverse	5ˈ- ATAGTTTAGCGGCCGCAACTTCGCCACCATCTGCATC-3′			
icaA	Forward	5ˈ- ACACTTGCTGGCGCAGTCAA -3′	188	95°C 5 min (Hot Start activation); 40 × (95°C 5 s, annealing/extension 60°C 10 s).	(Mirzaee *et al.,* 2014)
	Reverse	5ˈ -TCTGGAACCAACATCCAACA -3′			
mecA	Forward	5′–AAA ATC GAT GGT AAA GGT TGG C -3′	533	94^{o}C 10 min; 40 × (95oC 15 s, 52.9^{o}C 60 s, 72^{o}C 60 s); 72^{o}C 5 min.	(Olowe, *et al.,* 2013)
	Reverse	5′- AGT TCT GCA GTA CCG GAT TTG C -3′			
vanA	Forward	5′- ATGAATAGAATAAAAGTTGC-3′	1030	94^{o}C 5 min; 40 × (94^{o}C 30 s, 72^{o}C 60 s); 72^{o}C 7 min.	(Budati *et al.,* 2016)
	Reverse	5′- TCACCCCTTTAACGCTAATA -3′			

Key: *bbp* (Bone sialoprotein-binding protein)

icaA (Intercellular adhesion gene)

mecA (Methicillin resistance factor gene)

vanA (Vancomycin resistance factor gene)

3.2.14Statistical Analysis

All data analysis was conducted using the statistical package for social sciences (SPSS) program, version 21.

And the statistical analysis was presented as mean ± standard deviation (SD) where it was necessary.

CHAPTER FOUR

4.0 RESULTS

4.1 Demography of Samples Collected

A total of 189 samples were collected from 49 patients on admission within the study period, among which 42 (22.2%) were from urine, 49 (25.9%) were from wound, 49 (25.9%) were from nasal swabs and 49 (25.9%) were collected from patients' bed by swabbing. All these were from 6 different wards in National Orthopaedic Hospital, Dala, Kano over the period of 10 weeks (September, 2017 – December, 2017). Most of the participants were male 42 (85.7%), while 7 (14.3%) were female. Among these participants, 3 (6.1%) were within 1 – 17 years, 38 (77.6%) were within 18 – 40 years and 8 (16.3%) were above 40 years. Table 4.1 shows the age distribution of participants in this study.A total of 23 (46.9%) of the patients were within their first month of admission, 17 (34.7%) within their second month of admission, while 9 (18.4%) were in their third month and above of admission when samples were collected.

4.2Isolation and Identification of *Staphylococcus aureus* Isolates

Preliminary identification by growth on mannitol salt agar and subsequently, Gram reaction showed that only 47 (24.9%) isolates were suspected to be *Staphylococcus aureus* based on the formation of yellow colonies that indicates fermentation of phenol red in MSA and cooci isolates retaining the primary stain when viewed under microscope. Further identification of the 47 cocci positive isolates showed that 28 (59.6%) were coagulase positive while 19 (40.4%) were coagulase negative. Further identification using the

Microgen Staph-ID Kit confirmed all the coagulase positive isolates to be *S. aureus*. The identified *S. aureus* isolates were screened on Columbia blood agar for production of Small-Colony Variants (SCVs), and none of 28 *S. aureus* isolates were SCV former. This is shown in Figure 4.1.

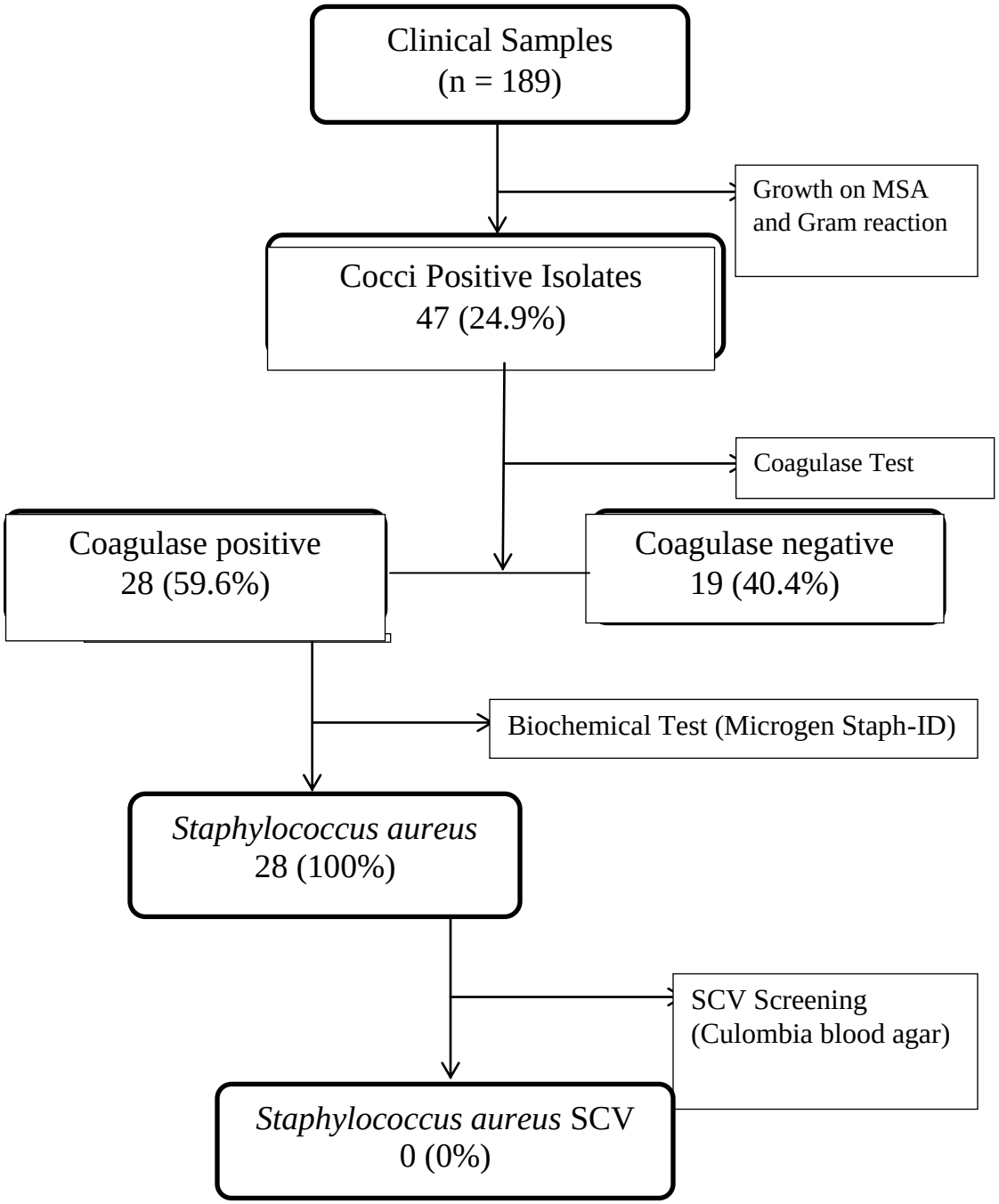

Figure 4.1: Isolation and Identification of *Staphylococcus aureus* from Clinical Samples.

Table 4.1: PercentageDistribution of*Staphylococcus aureus* Isolates by Age-Group and Gender of Patients

Age Range in Years	**Percentage of Patients Recruited**		**All Patients**
	Male	**Female**	
1 – 17	2 (7.1%)	1 (3.6%)	3 (10.7%)
18 – 40	14(50%)	5 (17.9%)	19 (67.9%)
41 – above	6 (21.4%)	0 (0)	6 (21.4%)
Total	22 (78.6%)	6 (21.4%)	**28 (100%)**

4.3Distribution of *Staphylococcus aureus* Isolates by Specimen

The majority of *S. aureus* isolates were from wound 12 isolates (42.9%), while nasal swabs were 8 isolates (28.6%), urine samples have 6 isolates (21.4%) and swabs from patient's bed had 2 isolates (7.1%). A breakdown of the prevalence of (9.7%) in wound samples was recorded as shown in Table 4.2.

Table 4.2: Distribution of the *Staphylococcus aureus* Isolates by Specimen

Isolate Source	***Staphylococcus aureus*** **n(%)**
Bed Swab	2 (7.1%)
Wound Swab	12 (42.9%)
Nasal Swab	8 (28.6%)
Urine Sample	6 (21.4%)

4.4Antibiotic Resistance Profile of the*Staphylococcus aureus* Isolates

The *S. aureus* isolates were generally resistant to Amoxicillin-Clavulanic (67.9%), Tetracycline (67.9%), Ciprofloxacin and Cefoxitin (67.9%), Norfloxacin (64.3%), Clindamycin (57.1%), Gentamicin (53.6%), Erythromycin (46.4%), Trimethoprim-Sulfamethoxazole (42.9%), Quinipristin-Dalfopristin (39.3%), Mupirocin (35.7%) and Linezolid (14.3%) as shown in Figure 4.2. Resistance to Cefoxitin serves as a marker for MRSA.

4.5Antibiotic Resistance Pattern and Classes of Resistance in the*Staphylococcus aureus* Isolates

The *S. aureus* isolates have different pattern of antibiotics resistance as shown in Table 4.3. and are classified based on the pattern of their antibiotics resistance. However, most of the isolates have different antibiotic pattern as shown in Table 4.3.

As for the classes of resistance, a total of 24 (85.7%) were multi-drug resistance (MDR), 4 (14.3%) of them were extensively drug resistance (XDR) and 1 (3.6%) *S. aureus* isolate was pan drug resistance (PDR). The classifications are shown in Figure 4.3.

4.6Multiple Antibiotic Resistance Index of the *Staphylococcus aureus* Isolates

The multiple antibiotic resistance (MAR) index was determined as the ratio of the number of antibiotics to which the *S. aureus* isolates were resistant, to the total number of antibiotics to which the organisms were exposed. A total of 85.7% of the isolates have an MAR index > 0.2 as shown in Table 4.5.

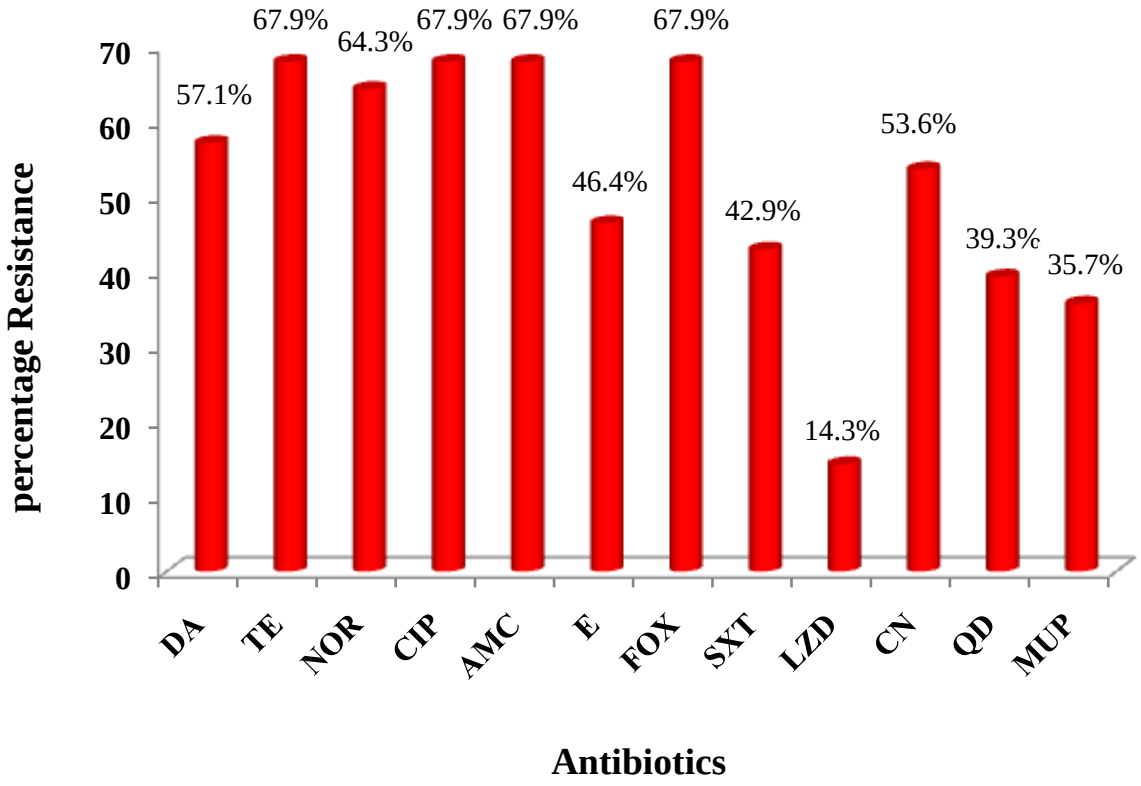

Figure 4.2: Antibiotics Resistance of *Staphylococcus aureus* Isolates to Tested Antibiotics

Key: DA = Clindamycin, TE = Tetracycline, NOR = Norfloxacin, CIP = Ciprofloxacin, AMC = Amoxycilin-clavulanic acid, E = Erythromycin, FOX = Cefoxitin, SXT = Trimethropin-sulfamethoxazole, LZD = Linezolid, CN = Gentamicin, QD = Quinupristin-dalfopristin and MUP = Mupirocin.

Table 4.3: Antibiotics Resistance Phenotype in *Staphylococcus aureus* Isolates

Resistance Phenotype	No of Isolates (n=28)	Percentage (%)
TE	1	3.6
DA, TE	1	3.6
DA, MUP	1	3.6
DA, TE, AMC, E, QD	1	3.6
CIP, AMC, FOX, MUP	2	7.1
DA, TE, NOR, CN, QD	2	7.1
TE, AMC, FOX, CN,QD	1	3.6
TE, NOR, CIP, FOX, SXT	1	3.6
NOR, CIP, FOX, SXT, CN, QD	1	3.6
DA, TE, CIP, AMC, FOX, MUP	1	3.6
DA, AMC, FOX, SXT, LZD, MUP	1	3.6
DA, TE, NOR, CIP, AMC, E, FOX	1	3.6
TE, NOR, CIP, AMC, E, FOX, SXT	1	3.6

NOR, CIP, AMC, E, FOX, SXT, CN	1	3.6
TE, NOR, CIP, AMC, FOX, SXT, CN	1	3.6
TE, NOR, CIP, AMC, E, FOX, CN, QD	1	3.6
TE, NOR, CIP, AMC, E, FOX, SXT, CN	1	3.6
DA, NOR, CIP, AMC, E, FOX, SXT, CN	1	3.6
DA, TE, NOR, CIP, AMC, FOX, SXT, CN	1	3.6
DA, TE, NOR, CIP, AMC, E, FOX, QD, MUP	1	3.6
DA, NOR, CIP, AMC, E, FOX, CN, QD, MUP	1	3.6
DA, TE, NOR, CIP, E, SXT, LZD, CN, QD, MUP	1	3.6
DA, TE, NOR, CIP, AMC, E, FOX, SXT, LZD, CN	1	3.6
DA, TE, NOR, CIP, AMC, E, SXT, LZD, CN, QD, MUP	1	3.6
DA, TE, NOR, CIP, AMC, E, FOX, SXT, LZD, CN, QD, MUP	1	3.6

Key: DA = Clindamycin, TE = Tetracycline, NOR = Norfloxacin, CIP = Ciprofloxacin, AMC = Amoxycilin-clavulanic acid, E = Erythromycin, FOX = Cefoxitin, SXT = Trimethropin-sulfamethoxazole, LZD = Linezolid, CN = Gentamicin, QD = Quinupristin-dalfopristin and MUP = Mupirocin.

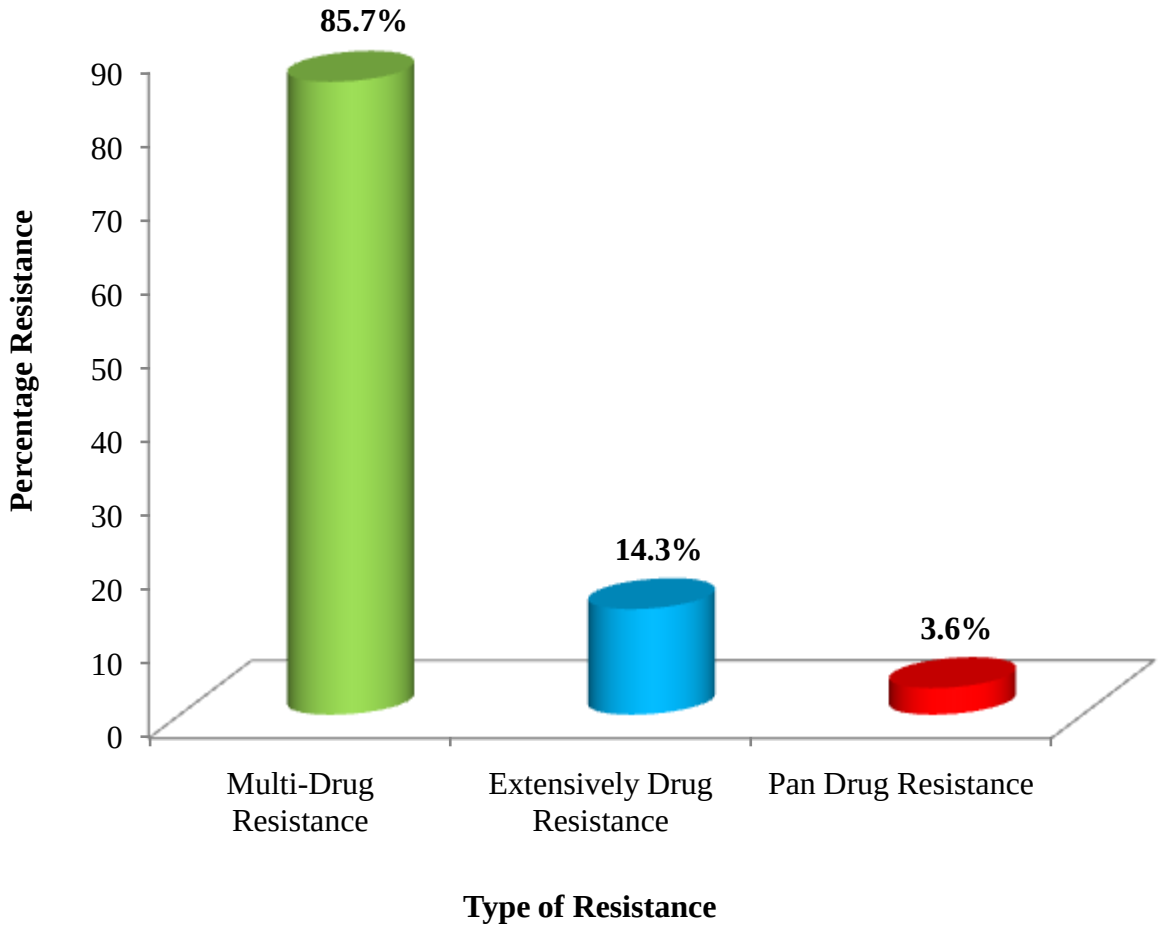

Figure 4.3: Percentage Distribution of Resistance Types in the *Staphylococcus aureus* Isolates

Table 4.4: Distribution of Methicillin-Resistant *Staphylococcus aureus* Isolates

Sample Type	MRSA (%)	MSSA (%)	TOTAL (%)
Bed	2 (7.1)	0 (0)	2 (7.1)
Wound	9 (32.1)	3 (10.7)	12 (42.8)
Nasal	3 (10.7)	5 (17.9)	8 (28.6)
Urine	5 (17.9)	1 (3.6)	6 (21.5)
TOTAL	**19 (67.8)**	**9 (32.2)**	**28 (100)**

Key: MRSA = Methicillin resistant *Staphylococcus aureus*

MSSA = Methicillin susceptibility *Staphylococcus aureus*

Table 4.5: Multiple Antibiotic Resistance (MAR) index of *Staphylococcus aureus* Isolates.

MAR index	**No of Isolates**	**Percentage (%)**
0.17	3	10.7
0.25	1	3.6
0.33	5	17.9
0.42	8	28.6
0.50	4	14.3
0.58	4	14.3
0.66	1	3.6
0.75	1	3.6
0.83	0	0
0.92	0	0

4.7Inducible Clindamycin Resistance in *Staphylococcus aureus* Isolates

From the results of antibiotic susceptibility testing (Fig. 4.2), inducible clindamycin test (D-Test) was carried out on five (5) *S. aureus* isolates that showed resistance to erythromycin (i.e. no zone of inhibition), but susceptible (showed zone of inhibition) to clindamycin.It showed that 2 (7.1%) of the total *S. aureus* isolates were inducible clindamycin resistant isolates. However, 16 (57.1%) of the isolates were constitutive clindamycin resistant*S. aureus* while 7 (25%) showed true sensitivity to clindamycin. The percentage resistance is shown in Figure 4.4.

4.8Distribution of Methicillin Resistant*Staphylococcus aureus* in the Patients

This study used resistance to cefoxitin to classify the isolates as MRSA and methicillin sensitive *Staphylococcus aureus* (MSSA). A total of 19 (67.9%) of *S. aureus* isolates tested were MRSA while 9 (32.1%) were MSSA as shown in Table 4.4. The highest percentage distribution was 9 (32.1%) from wound swabs isolates and 1 (3.6%) from urine sample as the lowest percentage distribution.

4.9Mupirocin Resistance in *Staphylococcus aureus* Isolates

Mupirocin antibiotics disc susceptibility testing was categorized into three (3); mupirocin-susceptible *S. aureus*, *S. aureus* with low-level mupirocin resistance and *S. aureus* with high- level mupirocin resistance. According to CLSI (2016), high-level mupirocin resistance is determined by presence of growth around mupirocin (200µg), while absence of growth signifies susceptible test. And this is the same for mupirocin (5µg). Out of 28

isolates tested, 6 (21.4%) showed high-level resistance, while 7 (25%) showed low-level mupirocin resistance. As shown in Figure 4.5.

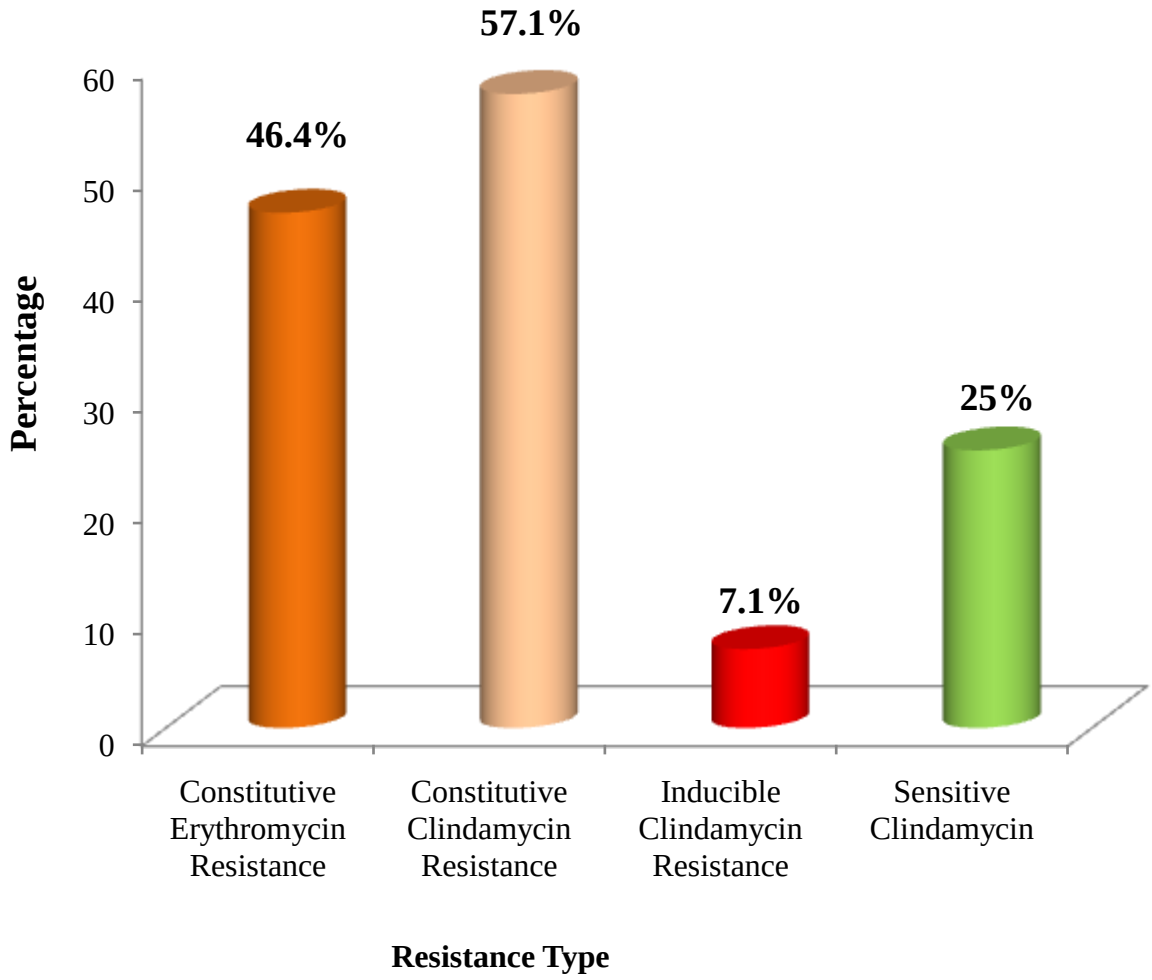

Figure 4.4: Percentage Inducible Clindamycin Resistance in the*Staphylococcus aureus*Isolates.

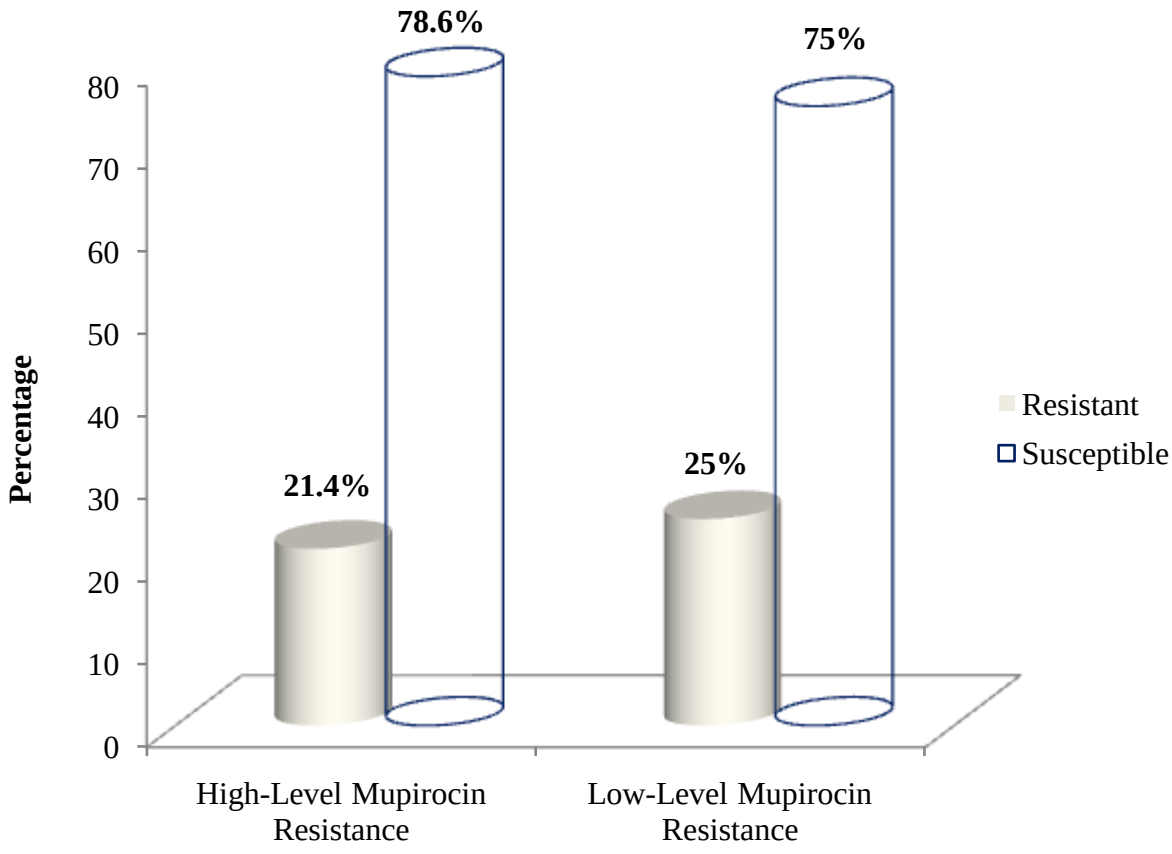

Figure 4.5: Mupirocin Susceptibility Testing of the*Staphylococcus aureus* Isolates.

4.10 Qualitative Analysis of Biofilm Formation in *Staphylococcus aureus*.

Out of 28 *S. aureus* isolates tested for biofilm production, 27 (96.4%) were biofilm formers. The distribution of the biofilm forming*S. aureus* isolates showed that they were from wound swab 12 (42.9%), nasal swab 8 (28.6%). Details of the distribution are seen in Table 4.6.

4.11Quantification of Biofilm Producedby*Staphylococcus aureus* Isolates

Majority of the *S. aureus* isolates whose biofilm forming capacity were quantified (27) were weak formers 18 (64.3%) while only 3 (10.7%) were strong biofilm formers. The classification and distribution of quantity of biofilm produced by the *S. aureus* isolates is shown in Table 4.6.

Table 4.6: Distribution of the Qualitative and Quantity of Biofilm Produced by*Staphylococcus aureus*with Respect to Sample Type

Biofilm Production	**Bed**	**Wound**	**Nasal**	**Urine**	**All Samples**
None Biofilm Former	0 (0%)	0 (0%)	1 (12.5%)	0 (0%)	1 (3.6%)
Weak Biofilm Former	1 (50%)	8 (66.7%)	6 (75%)	3 (50%)	18 (64.3%)
Moderate Biofilm Former	0 (0%)	3 (25%)	0 (0%)	3 (50%)	6 (21.4%)
Strong Biofilm Former	1 (50%)	1 (8.3%)	1 (12.5%)	0 (0%)	3 (10.7%)
Total	**2 (7.1%)**	**12 (42.8%)**	**8 (28.6%)**	**6 (21.4%)**	**28 (100%)**

4.12 Multi-Drug Resistance Pattern of *S. aureus* Compared to Biofilm Formation

The multi-drug resistance pattern in biofilm forming and non-biofilm producing *S. aureus* isolates when compared is shown in the Table 4.7. Among the 27 biofilm forming *S. aureus* isolates, 85.2% were multi-drug resistant while 14.8% were non-multi-drug resistant. Analysing the MDR and non-MDR *S. aureus* isolates and the biofilm- and non-biofilm-forming *S. aureus* isolates using Chi-square to determine the correlational between MDR and biofilm-formation, it was statistically proven that the multi-drug resistance in the isolates were independent of the biofilm-formation at 5% level of confidence.

Table 4.7: Biofilm Production in Multi-Drug Resistant *Staphylococcus aureus* Isolates

Isolate Code	Multi-Drug Resistance	Biofilm Formation
01N	– VE	+ VE
03W	+ VE	+ VE
03N	+ VE	+ VE
03U	– VE	+ VE
05W	+ VE	+ VE
07N	+ VE	+ VE
11W	– VE	+ VE
13N	+ VE	+ VE
13U	+ VE	+ VE
14N	+ VE	+ VE
15W	+ VE	+ VE
17U	+ VE	+ VE
23W	+ VE	+ VE
24U	+ VE	+ VE

26W	+ VE	+ VE
27B	+ VE	+ VE
29W	+ VE	+ VE
32N	– VE	+ VE
33U	+ VE	+ VE
38W	+ VE	+ VE
40W	+ VE	+ VE
41U	+ VE	+ VE
46B	+ VE	+ VE
46W	+ VE	+ VE
46N	+ VE	– VE
49W	+ VE	+ VE
49N	+ VE	+ VE
50W	+ VE	+ VE

Key: N =Nasal swab, W = Wound swab, B = Bed swab, U = Urine sample

4.13Molecular Characterization of *Staphylococcus aureus* Isolates

Four genes including: *icaA, bbp, mecA, vanA* were detected by polymerase chain reaction. The *icaA* gene(188bp) was amplified in ten (10) *S. aureus* isolates (comprising of the 3 strong biofilm-formers, the 6 moderate biofilm formers, and one out of the weak biofilm-formers) as shown in Plate 4.1. The *bbp* gene (500bp) was amplified in eight (8) *S. aureus* isolates (comprising of the 3 strong biofilm formers, and 5 out of the 6 moderate biofilm formers) as shown in Plate 4.2. The *mecA* gene (533bp) was amplified in 9 methicillin-resistant *S. aureus* (MRSA) isolates as shown in Plate 4.3. Out of the nine (9)*S. aureus* isolates tested for the presence of *mecA*gene, 7 were phenotypically resistant to methicillin, while the remaining 2 were methicillin susceptible *S. aureus* (MSSA), but multi-drug resistant. The 2 MSSA isolates were included to see if by chance, *mecA* gene would be detected in isolates that did not express methicillin resistance phenotypically. The *vanA* gene (1030bp) was amplified in ten (10) MRSA isolates that were also multi-drug resistant as shown in Plate 4.4.

4.14The Percentage of*Staphylococcus aureus* Isolates with Biofilm and Resistance Genes

Biofilm associated genes, *icaA* 3 (30%) and *bbp* 3 (37.5%)were detected in the *S. aureus*. And in the resistant genes, *mecA* 3 (33.3%), and *vanA* 2 (20%) were detected in the *S. aureus* isolates as shown in Table 4.8.

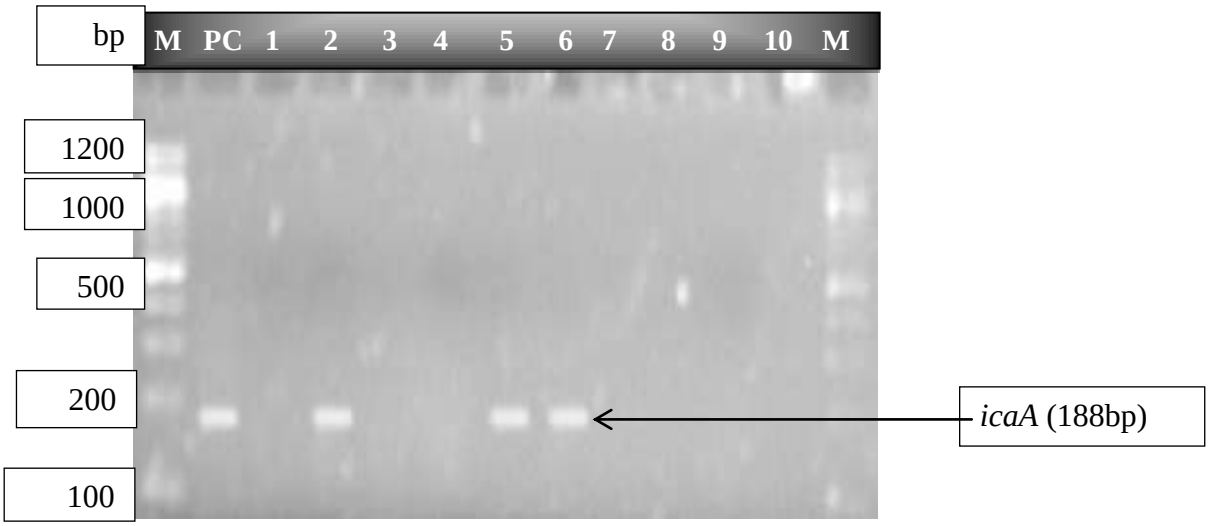

Plate 4.1: Electrophoretograph of Amplified *icaA* gene (188bp) in Biofilm-Producing *Staphylococcus aureus* Isolates.

Lane 2: 03N

Lane 5: 23W

Lane 6: 46B

Lane M: 100bp Molecular DNA ladder

Lane PC: Positive Control

Keys: M = Size marker, bp= Base pair.

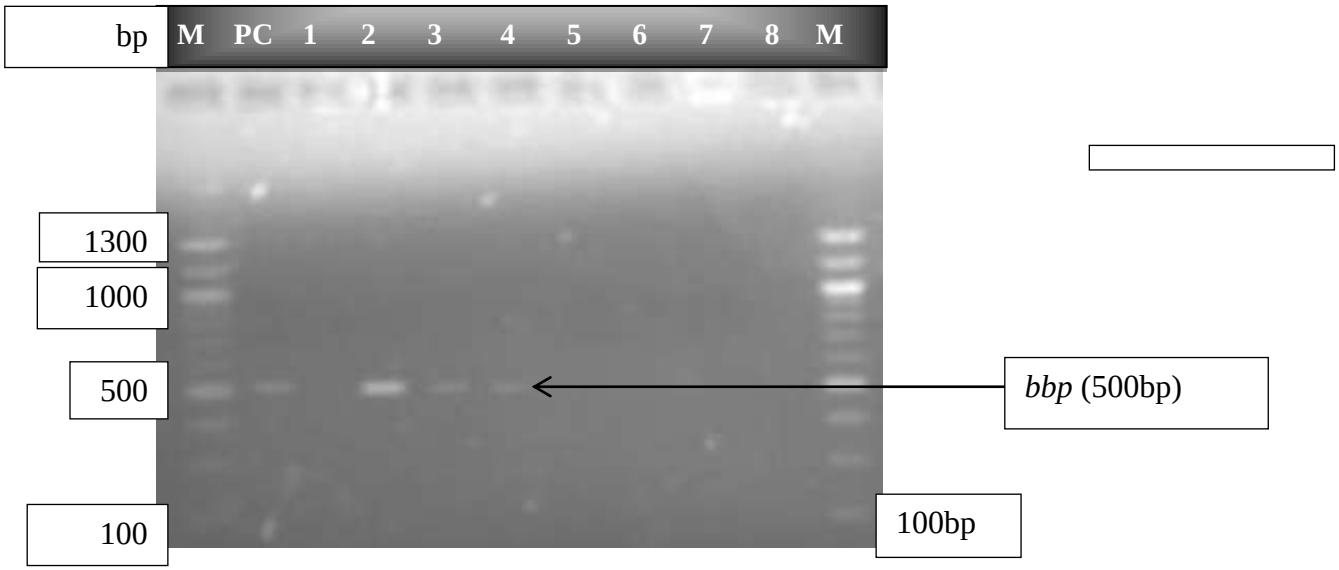

Plate 4.2: Electrophoretograph of Amplified *bbp* gene (500bp) in Biofilm-Producing *Staphylococcus aureus* Isolates.

Lane 2: 03N

Lane 3: 26W

Lane 4: 23W

Lane M: 100bp Molecular DNA ladder

Lane PC: Positive Control

Keys: M = Size marker, bp= Base pair.

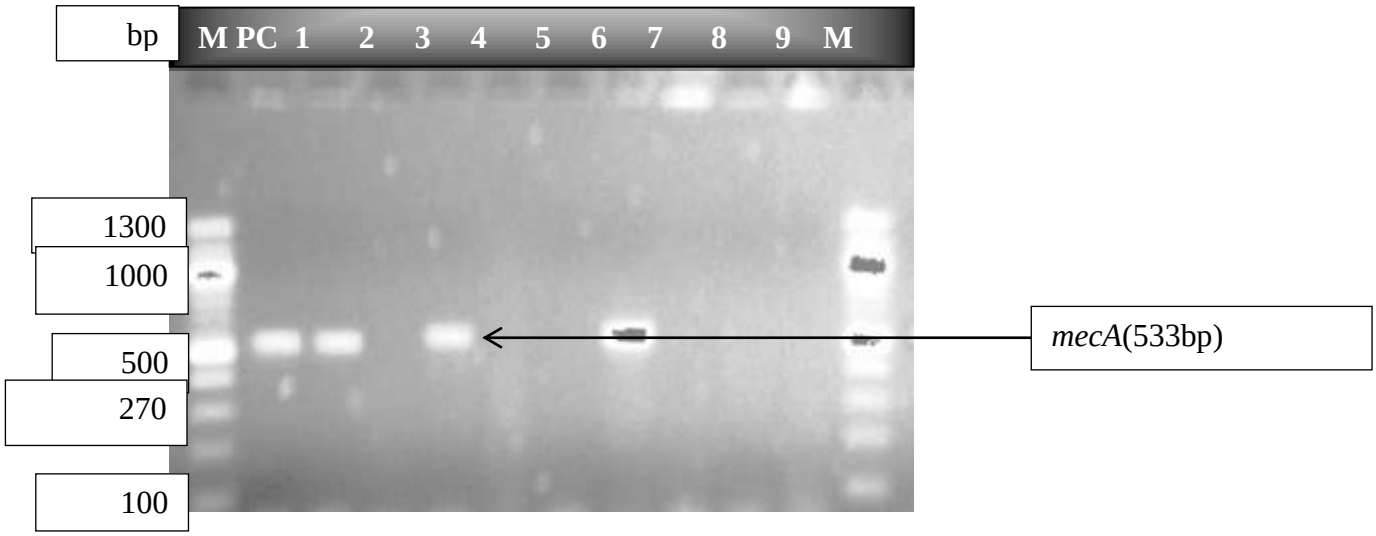

Plate 4.3: Electrophoretograph of Amplified *mecA* gene (533bp) in Methicillin Resistant*Staphylococcus aureus* Isolates.

Lane 2: 49W

Lane 3: 23W

Lane 6: 26W

Lane M: 100bp Molecular DNA ladder

Lane PC: Positive Control.

Keys: M = Size marker, bp= Base pair.

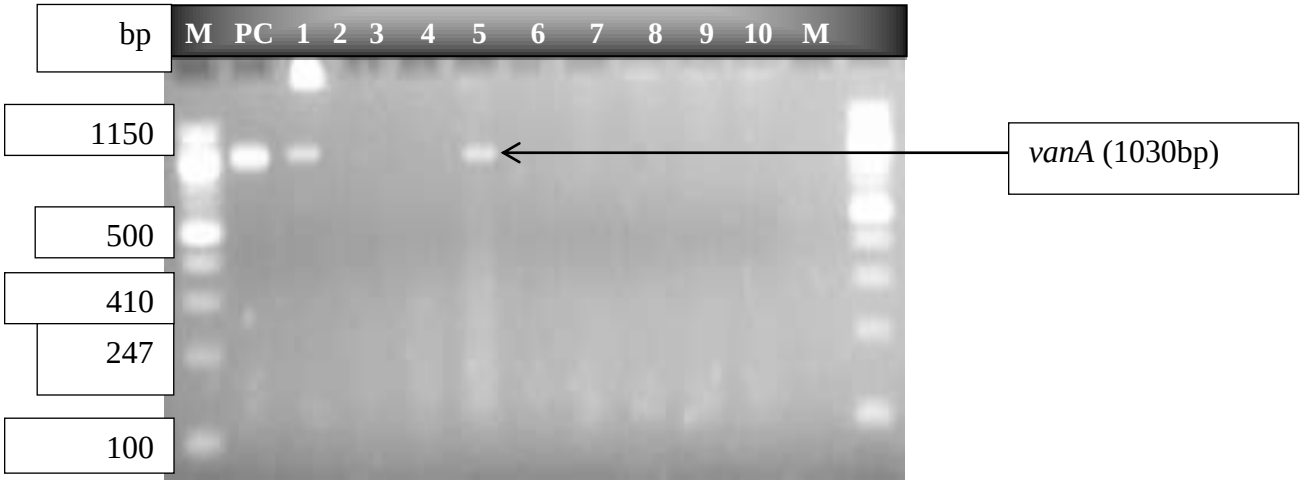

Plate 4.4: Electrophoretograph of Amplified *vanA* gene (1030bp) in Multi-Drug Resistant*Staphylococcus aureus* Isolates.

Lane 1: 23W

Lane 4: 26W

Lane PC: Positive control

Lane M: 100bp Molecular DNA ladder

Lane PC: Positive Control.

Keys: M = Size marker, bp= Base pair.

Table 4.8: Percentage of Adhesin and Resistance genes detected in the *S. aureus* Isolates

Gene	No. of Isolates	Amplification Result
icaA	10	3 (30%)
Bbp	08	3 (37%)
mecA	09	3 (33.3%)
vanA	10	2 (20%)

CHAPTERFIVE

5.0 DISCUSSION

A total of 49 in-patients were recruited to participate in this study carried out in National Orthopaedic Hospital Dala (NOHD). These patients comprise of 42 male and 7 female, giving a ratio of 1:6. The patients recruited in the study were mostly of the age group 18 – 40 years.This age group (18 – 40 years) constitutes the work force population and are easily exposed to environmental and mechanical hazard that can lead to various kind of orthopaedic wound and subsequently, infections.A similar trend was observed by Ribeiro *et al*. (2013) who reported the highest group of participating patients in their study to be within the ages of 18 – 48 years.

The prevalence of *S. aureus* isolated from the clinical samples in this study is similar to the 14.3% reported by Dilnessa and Bitew (2016) in Addis-Ababa, Ethiopia. In this study, most of the *S. aureus* isolates came from wound swab. This is because *S. aureus* being a haemolytic organism, the wound provides more conducive environment for proliferation than the bed, nostril and urine. However, the study of Dilnessa and Bitew (2016) showed different results in terms of their sources; with nasal swab having highest prevalence of 33.3% compared to 28.6% nasal swab reported in this study. The difference between nasal swab results in these two studies could be as a result of geographical area where samples were collected. Similar to this study, Ibrahim *et al.* (2018) reported in the same geographical area (Kano, Nigeria) *S. aureus* isolation rate of 47.3% with the isolates from wound swabs also having the highest prevalence (32.7%).

Small colony variants (SCVs) are selected *in-vitro*following exposure to certain antibiotics, and antibiotic treatment has been associated with subsequentdetection of SCVs (Kahl, 2014). None of the 28 *S. aureus* isolates screened for the formation of SCVs was positive. This is contrary to studies that reported SCV prevalence of 16.2% (Yagci *et al.,* 2013) and 21.1% (Ode *et al.,* 2015) in *S. aureus* isolates. Production of SCV in *S. aureus* could be in response to environmental stress, such as prolong use of antibiotics such as the aminoglycosides, particularly Gentamicin which affects the electron transport chain of the bacteria, and in turn induces hemin and menadione dependent SCVs and Trimethoprim-Sulfamethoxazole (SXT) that induces thymidine dependent SCVs (Wolter*et al.,* 2013). From the records of NOHD where these samples were collected, patient's infections were not treated with Gentamicin and SXT. Hence, the probable reason for the absence of SCV in the *S. aureus* isolates in this study.

The *S. aureus* isolated in this study showed resistance to antimicrobial agents tested.The resistance rate of *S. aureus* isolates was above 60% for Tetracycline, Norfloxacin, Ciprofloxacin, Amoxicillin-clavulanic acid, and Cefoxitin. The *S. aureus* isolates obtained from the samples that were collected from orthopaedic patients showed the least percentage resistance to linezolid (14.3%). One of the *S. aureus* isolates was completely resistant to all the antibiotics tested. However, one isolate was susceptible to almost all the antibiotics tested, except to two antibiotics that it had intermediate resistance against. Linezolid, which is the least resistant antimicrobial agent tested in this study happens to have been listed among drugs approved for treatment of MRSA infections (McGuiness *et al.,* 2017), as such it can be used in NOHD as drug of last resort in severe cases of MRSA. *Staphylococcus aureus* has been reported resistant in northern Ethiopia to penicillin G (90%), amoxicillin

(82.9%) and vancomycin (0%) (Taddesse, 2014), and this is contrary to the result of this study. In contrast to this study, Udobi *et al*. (2013) reported in Kaduna, Nigeria, *S. aureus* resistance to ampicillin (100%), perfoxacin (90.9%) from an orthopaedic wound.

In order to analyse the health risk, multiple antibiotic resistance (MAR) index is a very helpful tool, and it (MAR index) is also used to check the antibiotic resistance (Thenmozhi*et al*., 2014).

In this study, 85.7% of the isolate had MAR index> 0.2 when tested to 12 classes of antibiotics. Bacteria having MAR index > 0.2 originates from a high risk contaminated source where antibiotics are frequently being used, while values ≤ 0.2 shows that bacteria are from sources with less antibiotic usage(Bauer *et al.,* 1966; Thenmozhi *et al.*, 2014).This is suggesting that the 85.7% of *S. aureus* isolates that had MAR index> 0.2would have spread from a niche of high antibiotic use. Hence, selection of antibiotics is made more difficult. And this is a call for vigilant surveillance and remedial measures.

Classification of resistance profile of the isolates as MDR (85.7%), XDR (14.3%) and PDR (3.6%) in this study shows the risk posed by infection caused by these isolates, which may not be easily treated resulting in longer hospital stay, increased treatment and health care cost. A report in India showed 37.1% MDR which is far less than this study, 13.8% XDR that is almost the same as 14.3% of this study, and no PDR was found among the bacterial strains tested (Basak *et al*., 2016). Even though resistance profiles are expected to vary with different studies form different communities, a study conducted in Limpopo Province, South Africareported high antibiotic resistance among *S.aureus* isolates (Samie and Shivambu, 2011).

Using cefoxitin and its breakpoint as a standard for determining methicillin resistance in *S. aureus* as recommended by EUCAST (2018), this study showed a total of 19 (67.9%) isolates to be MRSA, while 9 (32.1%) were MSSA. The MRSA prevalence of 62% among in-patients in Kano, Nigeria as reported by Nwankwo *et al.,* (2010) is lower than that of this study, while Udobi *et al.*(2013) reported a higher MRSA prevalence of 75% among Staphylococcal isolates from the orthopaedic wards of Ahmadu Bello University Teaching Hospital. The high prevalence of MRSA infection in the hospital studied may not be unconnected with the hospital environment, for example, arrangement of people in the wards which makes transfer of these organisms among in-patients easier. Also, prolonged hospitalization, open wounds, long term indwelling catheter, living in area or staying in hospital with high prevalence of CA-MRSA and HA-MRSA may be possible reasons for this high prevalence (Siddiqui and Koirala,2018).

Inducible clindamycin resistance in this study made up 7.1% of the *S. aureus* isolates, implying that these 2 isolates possess the *erm* gene that encodes for enzymes conferring either inducible or constitutive resistance to clindamycin. Nevertheless, the *S. aureus* isolates also showed constitutive resistance to erythromycin (46.6%) and clindamycin (57.1%). Kumurya (2015) reported inducible clindamycin resistance (ICR) of 18.1% from staphylococcal isolates in Kano and 46.9% resistance to erythromycin and clindamycin(Kumurya, 2015).

In this study, all isolates that showed inducible clindamycin resistance are MRSA and 57.1% of the *S. aureus* isolates have constitutive clindamycin resistance. Clindamycin is kept as a reserve drug, and depending on the antimicrobial susceptibility results, use of clindamycin in severe in-patient MRSA infections is usually advocated. And proper use

ofclindamycin in severe MRSA can reduce the use of vancomycin (Paul*et al*., 2019). The results of this study suggest that clindamycin should be recommended, especially for orthopaedic patients in National Orthopaedic Hospital Dala, Kano, Nigeria. This is because clindamycin possesses exceptionally high bone penetration.

Mupirocin is an effective antibiotic for the elimination of methicillin-resistant *S. aureus* (MRSA) that colonize the nasopharynx and has been used to control spread of MRSA among patients during outbreaks (Anderson*et al.,* 2014).

This study, reported 6 (21.4%) high-level mupirocin resistance and 7 (25%) low-level mupirocin resistance, contrary to report of Jayakumar *et al*. (2013) among *Staphylococcus* spp where 3 (2%) were high-level mupirocin resistant and 2 (1.3%) were low-level mupirocin resistant. All the high-level and low-level mupirocin resistant isolates in this study were MRSA. As such, mupirocin may not be the best choice of pre-operation and nasal decolonization of MRSA among orthopaedic patients in NOHD.

The high- and low- levelmupirocin resistance in *S. aureus* isolates is usually an indication of carriage of *mupA* gene in their genome (Park *et al.,* 2015).

Treatment of orthopaedic infections remains challenging owing to the inability of antibiotics topenetrate biofilms and prevent their regrowth (Marque`s *et al.,* 2015). In this study, the qualitative analysis showed that 27 (96.4%) of the 28 *S. aureus* isolates were biofilm formers, leaving only 3.6% of the isolates as non-biofilm former. Also, quantitative classification of the 27 biofilm-forming *S. aureus* isolates showed that only 3 (10.7%) were strong biofilm formers, while 6 (21.4%) were moderate biofilm formers and 18 (64.3%) were weak biofilm formers. This result is in line with the study of Eyoh *et al*. (2014)

wheremost of the *S. aureus* isolates (35.6%)from medical and non-medical personnel were weak biofilm-formers.

Comparing the biofilm formation by the isolates with their various antibiotics resistance pattern, it was seen that majority of the biofilm-forming*S. aureus* isolates were MDR (85.2%). And from these biofilm forming isolates, all the strong biofilm producers are MDR, and 83.3% of the moderate biofilm producers (i.e. 5 out of 6 isolates) are MDR. Also, out of the 18 weak biofilm producers, 15 (83.3%) are MDR. This result has a high implication that the high rate of multi-drug resistance in the *S. aureus* isolates from patients in NOHD Kano, Nigeria may not be as a result of biofilm formation by these isolates. Statistical analysis using Chi-square to determine the correlation between multi-drug resistance and biofilm formation showed that multi-drug resistance in the *S. aureus* isolates is independent of Biofilm formation at 5% level of significance. The result of this study is similar to the work of Lihua *et al.*(2016) which reported that87.6% of the weak biofilm producers were MDR. Similar to the result of this study where the only non-biofilm former was MDR, almost all the non-biofilm producing *S. aureus* isolates (91.3%) in the study of Lihua *et al.* (2016) were MDR.

From the result of polymerase chain reaction carried out on ten (10) biofilm-producing *S. aureus* isolates, *icaA* gene showed 30% prevalence. This gene (*icaA*) is very important for biofilm formation as it enables the production of polysaccharide intracellular adhesin (PIA) in *S. aureus,* which mediates intercellular adherence and the accumulation of multilayer biofilms (Batistão *et al.*, 2016). Nourbakhsh and Namvar (2016) in comparing biofilm cells with planktonic cells showed that the *icaA*gene is essential for initiation of biofilm development.

According to this study, the prevalence of *bbp* is 37%. However, only 66.7% of the isolates that showed presence of *icaA* gene have the *bbp* gene. The *bbp* gene encodes for microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), particularly, the bone sialoprotein binding protein (Nemati *et al.,* 2009). The *bbp* gene was detected in this study, probably due to the fact that the clinical samples were of orthopaedic source. However, *icaA* and *bbp* are both involved in biofilm formation. Result obtained in this study is much lower than 74% of bone sialoprotein-binding (*bbp*) and collagen-binding (*cna*) genes co-occurrence in orthopaedic implant infections as reported by Montanaro *et al.,* (2016). However, studies have failed to find *bbp*gene in their *S. aureus* isolates, although, not of orthopaedic source (Serray *et al*., 2016).

Among the isolates tested for the presence of *mecA* gene by the use of PCR, only 3 (33.3%) of isolates possessed the *mecA* gene, and they were all among those that expressed phenotypical methicillin resistance. Hyper-production of*β*-lactamase may be responsible for methicillin resistance in organisms that does not possess *mecA* (Adhikari *et al*., 2017(b)). This might be the reason why *mecA* gene was not detected in other isolates that were phenotypically resistant to methicillin. The result from this study (33.3% *mecA*) is in close agreement with the study of Adhikari *et al*. (2017(b)) where they observed *mecA* in 29.1% of *S. aureus* isolated from pus/wound swab samples of the patients attending a tertiary care hospital in Kathmandu, Nepal. Similar to this study where *mecA* gene was not detected in some of the phenotypically expressed MSRA isolates, Elhassan *et al.* (2015) reported in Shendi City, Sudan that out of 200 *S. aureus* isolates, 123 (61.5%) were phenotypically MRSA, but all showed absence of *mecA* gene. Also Olayinka *et al.* (2009) also reported in Zaria, Nigeria, the absence of *mecA* gene in 36 *S. aureus* isolates that were

phenotypically MRSA positive and most of these isolates were *β*-lactamase hyper-producer.

Vancomycin resistant *Staphylococcus aureus* (VRSA) isolates have been reported from countries, including India, Iran, Pakistan (Moravvej *et al.*, 2013), and Portugal (Melo-Cristino *et al.,* 2013). These have raised concern among health care providers and in public health. The emergence of *vanA*-mediated vancomycin resistance among MRSA strains that are well adapted to transmission in community settings could potentially increase the health risks associated with VRSA (Limbago *et al.,* 2014). This study detected *vanA* gene in 2 (20%)out of 10 MDR *S. aureus* isolates tested. This is contrary to study of Farhadian *et al.,* (2014) in Iran where they failed to detect vancomycin resistance isolate and *vanA* gene respectively.Bamigboye *et al.*(2018) phenotypically, reported 11 vancomycin-intermediate *Staphylococcus aureus* (VISA) and 1 VRSA isolates, but didn't detect *vanA* in any of them. In North-Central Nigeria, Okolie *et al.,* (2015) reported that out of 155 *S. aureus* isolates, 22 (14.2%) were identified as VRSA and*vanA* gene was detected in all the VRSA isolates. Also, Olonitola *et al.,* (2006) reported no VRSA in*S. aureus* isolated from patients in Ahmadu Bello University Teaching Hospital Zaria-Nigeria.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1Summary

From the samples collected, 28 isolates were identified as *Staphylococcus aureus* giving an isolation rate of 14.3%, and none of the *S. aureus* isolates were small-colony variants (SCV).

A total of 27 (96.4%) were positive for biofilm formation using the microtiter plate in a qualitative test. Quantitative analysis showed that 3 (10.7%) were strong biofilm formers, 6 (21.4%) of the isolates were moderate biofilm formers and 18 (64.3%) of the isolates were weak biofilm formers.

From the antibiotic susceptibility testing, methicillin-resistant *S. aureus* isolates made a total of 19 (67.9%). Also, 85.7% MDR, 14.3% XDR, 3.6% PDR were reported and a total of 23 (85.7%) of the isolates had MAR index>0.2. The highest percentage of resistance was to cefoxitin, ciprofloxacin and amoxicillin-clavulanic acid (67.9%) while the lowest was to linezolid (14.3%). A total of 2 (7.1%) of the *S. aureus* isolates tested positive to inducible clindamycin resistance, but 16 (57.1%) showed constitutive clindamycin resistance. High-level mupirocin resistance was detected in 21.4% of the *Staphylococcus aureus* isolates.

Molecular characterization of identified *S. aureus* detected adhesion and adhesin genes such as *bbp* (30%) and *icaA* (37%). While *mecA* (33.3%) and *vanA* (20%) that confer resistance to methicillin and vancomycinwere also detected.

6.2 Conclusion

This study showed that majority of the *Staphylococcus aureus*(96.4%) isolated from National Orthopaedic Hospital Dala, Kano were biofilm formers and some harbour genes responsible for biofilm production and genes that confer resistance to antibiotics. However, this research has shown that multi-drug resistance in the *S. aureus* isolates is independent of biofilm formation at 5% level of significance.

6.3 Contribution to Knowledge

This study established that:

i. All the *S. aureus* isolates screened for formation of small-colony variants (SVCs) were negative for SCV formation.

ii. There was a High-level mupirocin resistance in the *Staphylococcus aureus* isolates, whose high percentage was 21.4%.

iii. There was no difference in the multi-drug resistance (MDR) pattern of the biofilm forming and non-biofilm forming isolates.

iv. Isolates from the nasal swabs of orthopaedic patients have been found to be virulent, having 10.7% MRSA and 22.2% biofilm formation. And isolates from the bed swabs have been found to possess 7.1% MRSA and 3.7% biofilm formation.

v. Out of 10 MDR *S. aureus* tested, 2 (20%) possessed *vanA* gene.

6.4 Recommendations

Based on the observations made in this study, the following recommendations are made:

i. It will be important to collect and analyse follow up samples from patients and monitor the trend of biofilm formation in order to make conclusive remarks.

ii. Further research in this field should consider multi-centre study and longer sampling time to increase the number of patients.

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8.0 APPENDICES

Appendix 1: Ethical Approval from National Orthopaedic Hospital Dala, Kanu State

NATIONAL ORTHOPAEDIC HOSPITAL,
DALA - KANO

NOHD/RET/ETHIC/43

29th August, 2017

OCHE DOMINIC AGBO
(Pi5PHPM8019),
Department of Pharmaceutics and Pharmaceutical-Microbiology,
Faculty of Pharmaceutical Sciences,
Ahmadu Bello University - Zaria,
Kaduna.

ETHICAL CLEARANCE

RE: BIOFILM FORMATION AND ANTIBIOTICS RESISTANCE IN STAPHYLOCOCCUS AUREUS SMALL COLONY VARIANTS ISOLATED FROM ORTHOPAEDIC PATIENTS IN NATIONAL ORTHOPAEDIC HOSPITAL, DALA - KANO, NIGERIA

Following receipt and consideration of your Research Proposal by the HOSPITAL RESEARCH ETHICS COMMITTEE, I am directed to inform you that Ethical Clearance is hereby granted to you for the conduct of the above titled study.

I am further directed to advise you to adhere strictly to the approved methodology and to kindly make available a copy of the final approved Research to the Hospital, please.

Best wishes.

MURTALA RASHEED
For: Chairman, Hosp. Research Ethics

Appendix 2: Information Consent Form Template for Research Participants

DEPARTMENT OF PHARMACEUTICAL MICROBIOLOGY, FACULTY OF PHARMACEUTICAL SCIENCES, AHMADU BELLO UNIVERSITY (ABU) ZARIA, NIGERIA.

INFORMED CONSENT FORM (ICF)

Serial No …… Age …… Sex …….. Phone No ……

This informed consent form is for the biofilm formation and antibiotics resistance by*Staphylococcus aureus* in orthopaedic patients of National Orthopaedic Hospital Dala (NOHD), Kano State, Nigeria. I am inviting you to participate in this research work titled "**Biofilm Formation and Antibiotics Resistance by *Staphylococcus aureus*Isolated from Patients in National Orthopaedic Hospital, Dala, Kano, Nigeria**". The research will involve the collection of medical specimens and nasal swab samples.

Supervisors: Prof. B.O Olayinka and Prof. J. A. Onaolapo

Name of Institution: Ahmadu Bello University (ABU) Zaria, Kaduna-Nigeria.

Sponsor: Self

This informed consent form consist of two sections

(a) Information sheet

(b) Certificate of consent

SECTION A: INFORMATION SHEET

Introduction

I am Oche Dominic Agbo, a postgraduate student from the department of Pharmaceutical Microbiology, ABU Zaria, Kaduna State. I am carrying out a prospective study on the

prevalence of *Staphylococcus aureus* Small-colony Variants in orthopaedic patient in NOHD, Kano State.

Aim of Research

This research is aimed at determining the biofilm production and antibiotics resistance, in *Staphylococcus aureus* Small-colony Variants isolated from orthopaedic patients in NOHD, Kano-Nigeria.

Procedure and Protocol

If you agree to participate in this research, samples such as urine, nasal swab and wound swab if you have will be taken by medical personnel for microbiological analysis. Biohazard safety bin will be used to discard the remaining specimens and there will be no follow up for sample collection.

Risk

From the best of my knowledge, within the context of this research there will be no health hazard or whatsoever that would be detrimental to you as a volunteer. In an event of any complain, I will take full responsibility.

Benefits

If you participate in this research you will have the following benefits

i. The microorganism that pass through your nostril and contribute to persistence of your infection will be identified.

ii. The antibiotic which will kill the microorganism better with lowest toxicity will be identified.

iii. It will help reduce the risk of developing antibiotic resistance.

iv. This research will sensitize patient on adherence to aseptic/hygienic practices to reduce distribution of microorganism with particular virulent genes among patients.

v. This will also be beneficial to the doctors, nurses and other health workers that are taking care of you in the hospital.

Confidentiality

Information and results obtained from this research will be used strictly for research purpose and your confidentiality will be respected. Results obtained will be shared only with management of the hospital and perhaps the state ministry of health for the purpose of surveillance.

SECTION B: CERTIFICATE OF CONSENT

Participant

I …… hereby consent to participate in this study. The full procedure of the test/study have been explained to me by the investigator. I therefore give this consent voluntarily without being subjected to any pressure.

Signature of Participant/Thumb Print …… Date ..……

Witness

I …… hereby give consent that the full procedure of the test/study have been explained to the participant and no financial support or subjected pressure is used to influence his/her participation.

Signature /Thumb Print ……Date …….

Researcher

I confirmed that sufficient information about the research, including risk and benefits have been fully explained to the participant. Individual will not be forced into giving consent, and the consent was giving freely and voluntarily.

Name of Researcher ……..

Signature /Thumb Print ……Date ……

Appendix 3: Morphology, Biochemical and Identification of Isolates

KEYS:
G – Y: Golden-yellow
C +ve: Gram positive cocci
C –ve: Gram negative cocci
R +ve: Gram positive rods
R –ve: Gram negative rods
+ve: Positive
-ve: Negative

Isolate Code		Growth on Mannitol Salt Agar	Gram Reaction	Catalase test	Coagulase Test	Microgen test	**SCV Screening** on Columbia Blood Agar
S/No	Source						
1.	**B**	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	U	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
2.	**B**	**P**	**R + VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
3.	**B**	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	N	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	U	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
4.	**B**	**P**	**R + VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	N	**P**	**R – VE**	**-**	**-**	**-**	**-**
	U	**P**	**R – VE**	**-**	**-**	**-**	**-**
5.	**B**	**P**	**R + VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**

	N	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	U	**P**	**R – VE**	**-**	**-**	**-**	**-**
7.	**B**	**P**	**R + VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	U	**P**	**R + VE**	**-**	**-**	**-**	**-**
8.	**B**	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	W	**N.G**	**-**	**-**	**-**	**-**	**-**
	N	**P**	**R + VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**C – VE**	**-**	**-**	**-**	**-**
9.	**B**	**P**	**R – VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	U	**P**	**R – VE**	**-**	**-**	**-**	**-**
10.	**B**	**P**	**R + VE**	**-**	**-**	**-**	**-**
	W	**N.G**	**-**	**-**	**-**	**-**	**-**
	N	**G –Y**	**R – VE**	**-**	**-**	**-**	**-**
	U	**P**	**R – VE**	**-**	**-**	**-**	**-**
11.	**B**	**P**	**R + VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	N	**P**	**R – VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
12.	**B**	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	W	**P**	**R – VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	U	**-**	**-**	**-**	**-**	**-**	**-**
13.	**B**	**N.G**	**-**	**-**	**-**	**-**	**-**
	W	**G –Y**	**R – VE**	**-**	**-**	**-**	**-**

	N	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	U	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
14.	**B**	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	U	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
15.	**B**	**P**	**R + VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	N	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	U	**-**	**-**	**-**	**-**	**-**	**-**
16.	**B**	**P**	**R + VE**	**-**	**-**	**-**	**-**
	W	**P**	**R – VE**	**-**	**-**	**-**	**-**
	N	**G –Y**	**R + VE**	**-**	**-**	**-**	**-**
	U	**P**	**R – VE**	**-**	**-**	**-**	**-**
17.	**B**	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	W	**P**	**R – VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
18.	**B**	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	W	**P**	**R – VE**	**-**	**-**	**-**	**-**
	N	**P**	**R + VE**	**-**	**-**	**-**	**-**
	U	**-**	**-**	**-**	**-**	**-**	**-**
19.	**B**	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
	U	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
20.	**B**	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
	W	**G – Y**	**C + VE**	**+ VE**	**-VE**	**-**	**-**

	N	**G – Y**	**C – VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
22.	**B**	**P**	**R + VE**	**-**	**-**	**-**	**-**
	W	**P**	**R + VE**	**-**	**-**	**-**	**-**
	N	**P**	**R – VE**	**-**	**-**	**-**	**-**
	U	**-**	**-**	**-**	**-**	**-**	**-**
23.	**B**	**P**	**R – VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	N	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	U	**P**	**R + VE**	**-**	**-**	**-**	**-**
24.	**B**	**G – Y**	**C – VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
25.	**B**	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
26.	**B**	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	N	**G – Y**	**C – VE**	**-**	**-**	**-**	**-**
	U	**-**	**-**	**-**	**-**	**-**	**-**
27.	**B**	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	W	**P**	**R + VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	U	**N.G**	**-**	**-**	**-**	**-**	**-**
28.	**B**	**P**	**R + VE**	**-**	**-**	**-**	**-**
	W	**P**	**R + VE**	**-**	**-**	**-**	**-**

	N	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
29.	**B**	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	N	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	U	**-**	**-**	**-**	**-**	**-**	**-**
30.	**B**	**G – Y**	**C – VE**	**-**	**-**	**-**	**-**
	W	**P**	**R – VE**	**-**	**-**	**-**	**-**
	N	**P**	**R + VE**	**-**	**-**	**-**	**-**
	U	**P**	**R – VE**	**-**	**-**	**-**	**-**
31.	**B**	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	N	**P**	**R + VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
32.	**B**	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	W	**N.G**	**-**	**-**	**-**	**-**	**-**
	N	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	U	**-**	**-**	**-**	**-**	**-**	**-**
33.	**B**	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	W	**P**	**R + VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
34.	**B**	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
	W	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
	U	**G – Y**	**C – VE**	**-**	**-**	**-**	**-**
35.	**B**	**P**	**R + VE**	**-**	**-**	**-**	**-**
	W	**P**	**R + VE**	**-**	**-**	**-**	**-**

	N	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
	U	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
36.	**B**	**P**	**R + VE**	**-**	**-**	**-**	**-**
	W	**P**	**R – VE**	**-**	**-**	**-**	**-**
	N	**P**	**R + VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
37.	**B**	**G – Y**	**C – VE**	**-**	**-**	**-**	**-**
	W	**P**	**R – VE**	**-**	**-**	**-**	**-**
	N	**P**	**R + VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
38.	**B**	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	N	**G – Y**	**C – VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**C – VE**	**-**	**-**	**-**	**-**
39.	**B**	**P**	**R + VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	N	**P**	**R – VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
40.	**B**	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	N	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
41.	**B**	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
	W	**P**	**R + VE**	**-**	**-**	**-**	**-**
	N	**P**	**R – VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
42.	**B**	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
	W	**P**	**R – VE**	**-**	**-**	**-**	**-**

	N	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
	U	**N.G**	**-**	**-**	**-**	**-**	**-**
43.	**B**	**N.G**	**-**	**-**	**-**	**-**	**-**
	W	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**C – VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
44.	**B**	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	W	**N.G**	**-**	**-**	**-**	**-**	**-**
	N	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**C – VE**	**-**	**-**	**-**	**-**
45.	**B**	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
	W	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	N	**P**	**R + VE**	**-**	**-**	**-**	**-**
	U	**N.G**	**-**	**-**	**-**	**-**	**-**
46.	**B**	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	W	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	N	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	U	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
47.	**B**	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**C – VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**C – VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
48.	**B**	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	W	**P**	**R + VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**C – VE**	**-**	**-**	**-**	**-**
	U	**G – Y**	**C – VE**	**-**	**-**	**-**	**-**
49.	**B**	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**

	N	**G – Y**	**C + VE**	**+ VE**	**+ VE**	***S. aureus***	**- VE**
	U	**G – Y**	**C + VE**	**+ VE**	**- VE**	**-**	**-**
50.	**B**	**P**	**R + VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**C + VE**	**+VE**	**+ VE**	***S. aureus***	**- VE**
	N	**P**	**R – VE**	**-**	**-**	**-**	**-**
	U	**N.G**	**-**	**-**	**-**	**-**	**-**
51.	**B**	**G – Y**	**R – VE**	**-**	**-**	**-**	**-**
	W	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	N	**G – Y**	**R + VE**	**-**	**-**	**-**	**-**
	U	**P**	**C + VE**	**+ VE**	**- VE**	**-**	**-**

SUMMARY

49 Patient gave consent
07 Patients that didn't give urine sample
189 Samples collected
62 Cocci isolates
47 Cocci positive isolates
15 Cocci negative isolates
47 Catalase positive
00 Catalase negative
28 Coagulase positive isolates
34 Coagulase negative isolates

Appendix 4: Isolation of *Staphylococcus aureus* Small-colony Variants

Isolate Code	Morphology	Pigmentation	Haemolysis	Presumed SCV
01N	Smeared	P	B	–VE
03W	Smeared	N.P	α	–VE
03N	Smeared	P	B	–VE
03U	TC	N.P	α	–VE
05W	Smeared	P	α	–VE
07N	TC	N.P	α	–VE
11W	TC	N.P	α	–VE
13N	Smeared	N.P	β	–VE
13U	Smeared	P	α	–VE
14N	Smeared	N.P	β	–VE
15W	Smeared	N.P	α	–VE
17U	TC	P	γ	–VE
23W	TC	N.P	β	–VE
24U	Smeared	P	β	–VE
26W	Smeared	P	α	–VE
27B	Smeared	P	α	–VE
29W	Smeared	P	α	–VE
32N	Smeared	N.P	β	–VE
33U	Smeared	P	α	–VE
38W	Smeared	N.P	α	–VE
40W	Smeared	P	γ	–VE
41U	TC	N.P	β	–VE
46B	Smeared	P	α	–VE
46W	Smeared	N.P	α	–VE
46N	Smeared	P	β	–VE
49W	Smeared	N.P	α	–VE
49N	Smeared	P	α	–VE
50W	Smeared	P	β	–VE
Control	-	-	-	-

KEYS:

β: Complete haemolysis

α: Partial haemolysis

γ: Non haemolytic

P: Pigmented

N.P: Non pigmented

TC: Tiny colony

Appendix 5: Auxotrophy Test for Supposed *Staphylococcus aureus* Small-colony Variants

Isolate code	Hemin	Menadione	Thymidine
01N	– VE	– VE	– VE
03W	– VE	– VE	– VE
03N	– VE	– VE	– VE
03U	– VE	– VE	– VE
05W	– VE	– VE	– VE
07N	– VE	– VE	– VE
11W	– VE	– VE	– VE
13N	– VE	– VE	– VE
13U	– VE	– VE	– VE
14N	– VE	– VE	– VE
15W	– VE	– VE	– VE
17U	– VE	– VE	– VE
23W	– VE	– VE	– VE
24U	– VE	– VE	– VE
26W	– VE	– VE	– VE
27B	– VE	– VE	– VE
29W	– VE	– VE	– VE
32N	– VE	– VE	– VE
33U	– VE	– VE	– VE
38W	– VE	– VE	– VE
40W	– VE	– VE	– VE
41U	– VE	– VE	– VE
46B	– VE	– VE	– VE
46W	– VE	– VE	– VE
46N	– VE	– VE	– VE
49W	– VE	– VE	– VE
49N	– VE	– VE	– VE
50W	– VE	– VE	– VE
Control	-	-	-

Appendix 6: Antibiotic Susceptibility Interpretative Chart According (EUCAST, 2016; EUCAST, 2018)

Groups	Antibiotics	Disk content	Zone diameter breakpoint (mm)		
			Susceptibility	Intermediate	Resistance
Penicillins	AMC	30(µg)	≥ 18	–	< 18
Lincosamides	Clindamycin	02(µg)	≥ 22	19 – 21	< 19
Tetracyclinees	Tetracyclinee	30(µg)	≥ 22	19 – 21	< 19
Fluoroquinolones	Norfloxacin	10(µg)	≥ 17	–	Test other agents
	Ciprofloxacin	05 (µg)	≥ 20	–	< 20
Macrolides	Erythromycin	15(µg)	≥ 21	18 – 20	<18
Glycopeptides	Vancomycin	256(µg)	< 02	–	≥ 02
	M.I.C.E				
Cephalosporins	Cefoxitin	30(µg)	≥ 22	–	< 22
Folate Pathway	SXT	25(µg)	≥ 17	14 – 16	< 14
Aminoglycosides	Gentamicin	10(µg)	≥ 18	–	< 18
Streptogramins	Q.D	15(µg)	≥ 21	18 – 20	< 18
Oxazolidinones	Linezolid	30(µg)	≥ 21	–	< 21
Bactroban	Mupirocin	200(µg)	≥ 30	18 – 29	< 18

Appendix 7: *S. aureus* Antibiotics Susceptibility Test Result Interpretation According to Eucast (2016 & 2018)

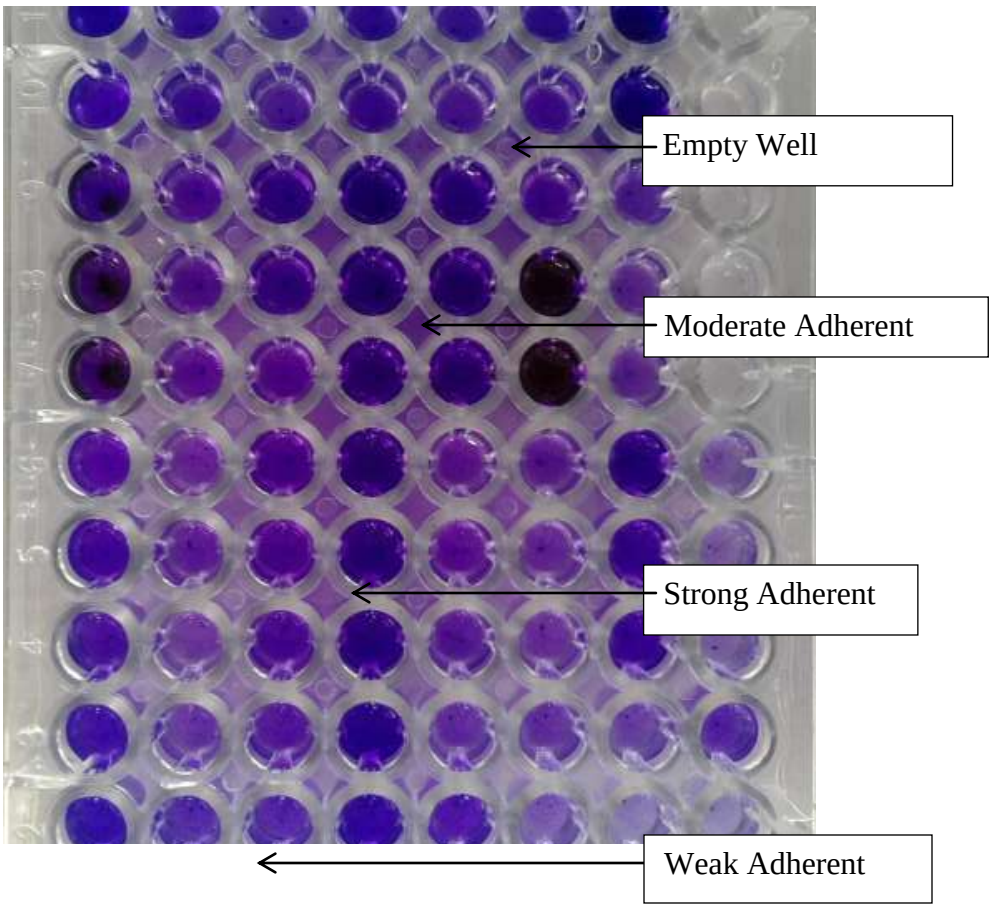
S. aureus Code No.	DA 02	TE 30	NOR 10	CIP 05	AMC 30	E 15	VA 30	FOX 30	SXT 25	LZD 30	CN 10	QD 15	MUP 200	MUP 05	MDR	MAR index
01N	**R**	**I**	**S**	**S**	**S**	**I**	**-**	**S**	**S**	**S**	**S**	**S**	**R**	**10mm**	**– VE**	**0.1**
03W	**R**	**R**	**R**	**R**	**R**	**R**	**-**	**R**	**R**	**S**	**R**	**S**	**S**	**27mm**	**+ VE**	**0.6**
03N	**R**	**R**	**S**	**S**	**R**	**R**	**-**	**S**	**S**	**S**	**S**	**R**	**S**	**26mm**	**+ VE**	**0.4**
03U	**I**	**I**	**S**	**S**	**S**	**S**	**-**	**S**	**S**	**S**	**S**	**S**	**S**	**31mm**	**– VE**	**0**
05W	**S**	**R**	**R**	**R**	**S**	**I**	**-**	**R**	**R**	**S**	**S**	**S**	**I**	**18mm**	**+ VE**	**0.3**
07N	**R**	**R**	**R**	**R**	**R**	**R**	**-**	**S**	**R**	**R**	**R**	**R**	**R**	**07mm**	**+ VE**	**0.7**
11W	**R**	**R**	**S**	**S**	**S**	**S**	**-**	**S**	**S**	**S**	**S**	**S**	**I**	**30mm**	**– VE**	**0.1**
13N	**R**	**R**	**S**	**R**	**R**	**S**	**-**	**R**	**S**	**S**	**S**	**I**	**R**	**0mm**	**+ VE**	**0.4**
13U	**R**	**R**	**R**	**R**	**R**	**R**	**-**	**R**	**I**	**S**	**S**	**R**	**R**	**23mm**	**+ VE**	**0.6**
14N	**S**	**R**	**R**	**R**	**R**	**R**	**-**	**R**	**R**	**S**	**S**	**S**	**S**	**28mm**	**+ VE**	**0.4**
15W	**S**	**R**	**R**	**R**	**R**	**R**	**-**	**R**	**R**	**S**	**R**	**I**	**S**	**17mm**	**+ VE**	**0.5**
17U	**R**	**R**	**R**	**R**	**R**	**R**	**-**	**R**	**R**	**R**	**R**	**R**	**R**	**0mm**	**+ VE**	**0.8**
23W	**R**	**R**	**R**	**R**	**R**	**R**	**-**	**R**	**S**	**S**	**S**	**S**	**I**	**17mm**	**+ VE**	**0.4**
24U	**I**	**I**	**S**	**R**	**R**	**S**	**-**	**R**	**S**	**S**	**S**	**I**	**R**	**0mm**	**+ VE**	**0.3**
26W	**R**	**I**	**R**	**R**	**R**	**R**	**-**	**R**	**S**	**S**	**R**	**R**	**R**	**0mm**	**+ VE**	**0.6**
27B	**S**	**I**	**S**	**R**	**R**	**I**	**-**	**R**	**S**	**S**	**S**	**S**	**R**	**0mm**	**+ VE**	**0.3**
29W	**I**	**R**	**R**	**R**	**R**	**S**	**-**	**R**	**R**	**S**	**R**	**S**	**S**	**16mm**	**+ VE**	**0.4**

32N	**S**	**R**	**S**	**S**	**S**	**S**	**-**	**S**	**S**	**S**	**S**	**S**	**S**	**19mm**	**– VE**	**0.1**
33U	**S**	**S**	**R**	**R**	**R**	**R**	**-**	**R**	**S**	**S**	**R**	**S**	**S**	**24mm**	**+ VE**	**0.4**
38W	**R**	**R**	**R**	**S**	**S**	**I**	**-**	**S**	**S**	**S**	**R**	**R**	**I**	**23mm**	**+ VE**	**0.3**
40W	**R**	**R**	**R**	**S**	**S**	**S**	**-**	**S**	**S**	**S**	**R**	**R**	**I**	**21mm**	**+ VE**	**0.3**
41U	**R**	**S**	**R**	**R**	**R**	**R**	**-**	**R**	**R**	**S**	**R**	**S**	**I**	**26mm**	**+ VE**	**0.5**
46B	**I**	**R**	**S**	**S**	**R**	**S**	**-**	**R**	**S**	**S**	**R**	**R**	**I**	**22mm**	**+ VE**	**0.4**
46W	**S**	**R**	**R**	**R**	**R**	**R**	**-**	**R**	**S**	**S**	**R**	**R**	**S**	**22mm**	**+ VE**	**0.5**
46N	**R**	**R**	**R**	**R**	**S**	**R**	**-**	**S**	**R**	**R**	**R**	**R**	**R**	**09mm**	**+ VE**	**0.6**
49W	**R**	**R**	**R**	**R**	**R**	**S**	**-**	**R**	**R**	**S**	**R**	**S**	**S**	**20mm**	**+ VE**	**0.5**
49N	**I**	**S**	**R**	**R**	**S**	**I**	**-**	**R**	**R**	**S**	**R**	**R**	**S**	**27mm**	**+ VE**	**0.4**
50W	**R**	**S**	**S**	**S**	**R**	**S**	**-**	**R**	**R**	**R**	**S**	**S**	**R**	**29mm**	**+ VE**	**0.2**
R (%)	**57.1**	**67.9**	**64.3**	**67.9**	**67.9**	**46.4**	**-**	**67.9**	**42.9**	**14.3**	**53.6**	**39.3**	**35.7**	**-**	**85.7**	
S (%)	**25.0**	**14.3**	**35.7**	**32.1**	**32.1**	**35.7**	**-**	**32.1**	**53.6**	**85.7**	**46.4**	**50.0**	**39.3**	**-**	**14.3**	
I (%)	**17.9**	**17.9**	**-**	**-**	**-**	**17.9**	**-**	**-**	**03.6**	**-**	**-**	**10.7**	**25.0**	**-**	**-**	

KEY:

S = Susceptible, I = Intermediate, R = Resistant, B = Bed, W = Wound, N = Nasal, U = Urine, DA 02 = Clindamycin (2µg), TE 30 = Tetracyclinee (30µg), NOR 10 = Norfloxacin (10µg), CIP 05 = Ciprofloxacin (05µg), AMC 30 = Amoxicillin-Clavulanic (30µg), E 15 = Erythromycin (15µg), VA 30 = Vancomycin (30µg), FOX 30 = Cefoxitin (30µg), SXT 25 = Trimethoprim-sulfamethoxazole (1.25µg+23.75µg), MUP 200 = Mupirocin (200µg), MUP 05 = Mupirocin (05µg), CN 10 = Gentamicin (10µg), QD 15 = Quinupristin-dalfopristin (15µg), LZD 30 = Linezolid (30µg), MDR = Multi drug resistance and MAR index = Multi Antibiotics Resistance Index.

Appendix 8: *Staphylococcus aureus* Biofilm Quality and Quantity in Microtitre Plate



Appendix 9: Microtitre Plate Reading for the Quantitative of *Staphylococcus aureus* Isolates.

Isolate Code	Replicates (nm)			Mean	Standard Deviation	Category of Biofilm Formed
	Well 1	Well 2	Well 3			
01N	0.405	0.523	0.462	0.463	0.048	weak biofilm former
03W	0.405	0.470	0.395	0.423	0.033	weak biofilm former
03N	2.385	1.969	1.474	1.942	0.372	strong biofilm former
03U	0.385	0.585	0.479	0.483	0.082	moderate biofilm former
05W	0.453	0.385	0.272	0.370	0.075	weak biofilm former

07N	0.231	0.367	0.254	0.284	0.059	weak biofilm former
11W	0.302	0.332	0.299	0.311	0.015	weak biofilm former
13N	0.378	0.361	0.426	0.388	0.028	weak biofilm former
13U	0.439	0.355	0.269	0.354	0.069	weak biofilm former
14N	0.333	0.343	0.383	0.353	0.022	weak biofilm former
15W	0.317	0.516	0.486	0.439	0.088	weak biofilm former
17U	0.261	0.304	0.289	0.284	0.018	weak biofilm former
23W	0.522	0.474	0.675	0.557	0.086	moderate biofilm former
24U	0.709	0.749	0.905	0.787	0.085	moderate biofilm former
26W	0.774	0.857	0.938	0.856	0.067	moderate biofilm former
27B	0.309	0.366	0.377	0.350	0.030	weak biofilm former
29W	0.329	0.330	0.245	0.301	0.040	weak biofilm former
32N	0.298	0.191	0.226	0.238	0.045	weak biofilm former
33U	0.665	0.665	0.489	0.606	0.083	moderate biofilm former
38W	0.220	0.288	0.368	0.292	0.060	weak biofilm former
40W	0.360	0.259	0.334	0.317	0.043	weak biofilm former
41U	0.245	0.250	0.313	0.269	0.031	weak biofilm former
46B	2.581	2.461	2.509	2.517	0.049	strong biofilm former
46W	0.255	0.338	0.481	0.358	0.093	weak biofilm former
46N	0.206	0.205	0.208	0.206	0.001	non biofilm former
49W	0.444	0.440	0.725	0.536	0.133	moderate biofilm former
49N	0.268	0.269	0.301	0.279	0.015	weak biofilm former
50W	1.099	1.160	1.434	1.231	0.146	strong biofilm former
Negative Control	0.184	0.200	0.196	0.193	0.007	–

Cut-off optical density (ODc) = 0.214 nm

Appendix 10: Statistical Analysis Using Chi-Square to Determine the Correlation Between Multi-Drug Resistance and Biofilm.

Set hypothesis:-

H_o : Multi-drug resistance is independent of Biofilm formation

H_i : Multi-drug resistance is dependent of Biofilm formation

α : 0.05 level of significance

Test statistics: $\chi^2 = \Sigma(O - E)^2$

E

Computation:

n_{ij}– Observed value

I x J	Positive	Negative	Total ($n_{j.}$)
Multi-drug resistance	24	4	28
Biofilm formation	27	1	28
Total (n,j)	51	5	56
			n..

m_{ij}– Expected value

I x J	Positive	Negative	Total ($m_{i.}$)
Multi-drug resistance	25.5	2.5	28.0
Biofilm formation	25.5	2.5	28.0
Total (n,j)	51.0	5.0	56.0
			m..

Formula for estimating expected values

$$M_{ij.} = \frac{n_{i.}\, n_{.j}}{n..}$$

$$\chi^2_{\text{calculated}} = \sum_{i}^{2} \sum_{ji=1}^{2} (\text{observed value} - \text{expected value})^2$$

$$= \frac{(24 - 25.5)^2}{25.5} + \frac{(4 - 2.5)^2}{2.5} + \frac{(27 - 25.5)^2}{25.5} + \frac{(1 - 2.5)^2}{2.5}$$

$$= 0.088 + 0.9 + 0.088 + 0.9 = 1.976$$

$\chi^2_{\text{tabulated}} = \chi^2_{(2-1)(2-1)(0.05)} = \chi^2_{1(0.95)} = 3.841$

Decision role: reject H_o if $\chi^2_{\text{calculated}} > \chi^2_{\text{tabulated}}$ otherwise, accept H_o.

Decision: Since $\chi^2_{\text{calculated}} = 1.976 < \chi^2_{\text{tabulated}} = 3.841$, there is no enough evidence at 5% level of significance to reject H_o.

Conclusion: Multi-drug resistance is independent of Biofilm formation at 5% level of significance.