PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF VANCOMYCIN RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATED FROM IN-PATIENTS IN SELECTED HOSPITALS IN KADUNA METROPOLIS, KADUNA STATE NIGERIA

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY,
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DECLARATION

I declare that the work in this dissertation entitled "PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF VANCOMYCIN RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATED FROM IN-PATIENTS IN SELECTED HOSPITALS IN KADUNA METROPOLIS, KADUNA STATE NIGERIA" has been carried out by me in the Department of Microbiology. 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 at this or any other Institution.

_____ _____

Aisha USMAN Date

CERTIFICATION

This dissertation entitled "PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF VANCOMYCIN RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATED FROM IN-PATIENTS IN SELECTED HOSPITALS IN KADUNA METROPOLIS, KADUNA STATE NIGERIA" by Aisha USMAN meets the regulations governing the award of Master of science degree of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Staphylococcus aureus is an important pathogen associated with various hospital-and community-acquired infections as such it is regarded as a public health problem. Vancomycin was an approved and highly recommended antibiotic worldwide for the treatment of infections caused by *S. aureus* especially methicillin resistant *S. aureus*. This study was carried out in order to phenotypically and genotypically characterize vancomycin resistant *S. aureus* isolated from patients on admission in some selected hospitals in Kaduna metropolis, Kaduna state Nigeria. A total of 300 skin swab samples were collected from patients on admission. The swab sticks were inoculated into brain-heart infusion broth, transported to the laboratory and then inoculated on to mannitol salt agar and blood agar. Isolates with the characteristic colonial morphology of the *S. aureus* were then characterized microscopically and identified biochemically. The susceptibility of *S. aureus* isolates to seven antibiotics was determined using Kirby-Bauer method. Vancomycin minimum inhibitory concentration for the isolates was determined by broth dilution method. The VRSA were confirmed by the detection of *vanA* gene in the isolates by polymerase chain reaction. A total of 52 (17.33%) *S. aureus* isolates were obtained from skin swabs of in-patients in selected hospitals in Kaduna metropolis based on microscopic and biochemical identification. Multiple antibiotic resistance index analysis showed that the MAR indices of the isolates were between 0.14 and 0.71. Most of the isolates had MARI values greater than 0.2 suggesting that the isolates were from high risk environment where antibiotics are frequently used. Among the 52 isolates, 2 were vancomycin intermediate *S. aureus* (MIC value 4-8µg/ml) another 2 were vancomycin resistant *S. aureus* (MIC values ≥ 16µg/ml) while the remaining 48 isolates were vancomycin susceptible *S. aureus* (MIC value ≤2µg/ml). PCR result for the detection of *vanA* gene showed that all the 2 VRSA had the *vanA* gene. It then means the resistance of the VRSA isolates was mediated by *vanA* gene. VRSA was characterized both phenotypically (by determination of vancomycin susceptibility) and genotypically (by detection of *Van A* gene) in this study. The occurrence of *S. aureus* and VRSA in this study was found to be 17.33% and 3.85% respectively.

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ABBREVIATIONS AND SYMBOLS

C	Cytosine.
CDC	Centers for Disease Control and Prevention.
CLSI	Clinical and Laboratory Standards Institute.
df	degree of freedom.
DNA	Deoxyribonucleic Acid.
G	Guanine.
Glc	Glucose.
KDa	KiloDalton.
MAR I	Multiple Antibiotic Resistance Index.
MDR	Multi Drug Resistant.
MIC	Minimum Inhibitory Concentration.
MRSA	Methicillin Resistant *S. aureus*
NAc	N-acetylglucosamine.
PBP	Penicillin Binding Protein.
PCR	Polymerase Chain Reaction.
RNA	Ribonucleic Acid
UDP	Uridine Diphosphate
VISA	Vancomycin Intermediate *S. aureus*
VRE	Vancomycin Resistant *Enterococcus*
VRSA	Vancomycin Resistant *S. aureus*
VSSA	Vancomycin Susceptible *S. aureus*
WHO	World Health Organisation.
β	Beta
χ^2	Chi square
σ	Sigma.

CHAPTER ONE

1.0 INTRODUCTION

1.1 **Background of the Study**

Staphylococcus aureus is one of the most common causes of serious infections in community and hospital settings (Lowry *et al*., 1998). *Staphylococcus aureus*, is a major human pathogen causing large variety of infections worldwide and predominates in surgical wound infections with prevalence rate ranging from 4.6% - 54.4% (Bannerman, 2003).

It is one of the species of the genus *Staphylococcus.* It is a Gram positive, non-motile, catalase positive, mostly coagulase positive, facultative anaerobe, involved in causing a number of diseases including boils, pustules, impetigo, osteomyelitis, mastitis, septicemia, meningitis, pneumonia and toxic shock syndrome,post operation wound infection, food borne intoxication and nosocomial bacteremia (Talaro and Talaro, 2002;Jun *et al*., 2004;Cheesbrough, 2006).

It is considered the most resistant of all non-spore forming pathogens, with well-developed capacities to withstand high salt concentrations (7.5 – 10%), extremes in pH and high temperatures up to 60^{o}C for 60 minutes(Jun *et al*., 2004).

Staphylococcus aureus colonizes the skin and nasal carvities, it occurs in about 25-30% of healthy people. It is a versatile human pathogen responsible for nosocomial (HA) and community acquired (CA) infection, with clinical manifestation of superficial and systemic diseases, associated with high morbidity and mortality rates (Jun *et al*., 2004).

The unique characteristic of *S. aureus* is the production of virulence factors responsible for the establishment of staphylococcal diseases and propensity to develop resistance to multiple antibiotics (Jun *et al*., 2004).

Patients subjected to broad-spectrum antibiotics and immunosuppressive therapies have higher risk of infection by this microorganism (Prasad, 2014).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is now endemic in health care facilities, with rates up to 15.0% in some health care settings (Fridkin *et al*., 1999). Also, recent reports describe MRSA carriage in persons in the community who do not have health care–associated risks. The increased incidence of MRSA has led to more frequent use of vancomycin, the drug commonly relied on for treating MRSA infections (Salgado *et al*., 2003).

Vancomycin is a glycopeptide antibiotic effective against majority of Gram positive bacteria, particularly against multiple drug resistant enterococci and staphylococci which are resistant to β-lactum antibiotics (Reynolds, 1998). These pathogens have acquired resistance to this compound by virtue of their intrinsic property especially in clinical isolates. This leads to severe complications in immunocompromised as well as surgical patients. Vancomycin resistant enterococci (VRE) were first reported in 1988 in Europe and USA (Prasad, 2014).

Vancomycin acts by binding with the C-terminal D-alanyl-D-alanine (D-Ala-D-Ala) residue of pentapeptide, thereby blocking the addition of precursors by transglycosylation to peptidoglycan chain and hence inhibits cross linking of cell wall by transpeptidation (Courvalin, 2006). Resistance to vancomycin is caused by synthesis of precursors with low affinity for these antibiotics conferred by operons present on *Van* gene clusters that encode enzymes which synthesize low affinity precursors wherein C-terminal D-Ala residue is replaced by D-lactate (D-Lac) or D-serine (D-Ser) that modify vancomycin binding site. These genes confer high or low level resistance to vancomycin and teicoplanin which may be inducible or constitutive (Prasad, 2014).

In vitro conjugative transfer of the *vanA* gene from enterococci to *Staphylococcus aureus* was demonstrated in 1992 (Noble *et al*., 1992). However, it was not until 1996, when the first case of vancomycin- intermediate *Staphylococcus aureus* (VISA; MIC, 8–16 mg/mL) was detected, and since then decreased susceptibility to vancomycin became a clinical reality (CDC,1997). None of the VISA strains identified contained the *vanA* gene or any of the other vancomycin-resistant genes found in VRE (Oliveira *et al*., 2001). *In vitro* studies suggest that, with prolonged vancomycin exposure, VISA organisms produce a thickened cell wall matrix, limiting drug penetration (Hanaki *et al*., 1998).

For many years, vancomycin has been considered the drug of choice for the treatment of *Staphylococcus aureus* infections due to strains that had become resistant to methicillin (Oliveira *et al*., 2001). However, in July 2002, things changed when the Centers for Disease Control (CDC) in the USA published the first documented report of *Staphylococcus aureus* that was resistant to vancomycin as well as being resistant to methicillin. The infection occurred in a diabetic patient with chronic kidney (renal) failure who was undergoing peritoneal dialysis in a hospital in Michigan. Approximately 5 years earlier, the Japanese had reported the first strain of *Staphylococcus aureus* with reduced (or intermediate) susceptibility to vancomycin followed by two additional cases from the USA. These earlier isolates were termed Vancomycin Intermediate *Staphylococcus aureus* (VISA) (CDC, 2003).

The definitions of VRSA and VISA are based on the results of laboratory testing which determine the minimum concentration of vancomycin that is required to inhibit the growth of *Staphylococcus aureus* in an *in vitro* study. It should be noted that these definitions are not universal and some countries lump all strains of *Staphylococcus aureus* that require increased concentrations of vancomycin to inhibit their growth into a single VRSA category. Regardless

of the specific categorization of these isolates, the potential clinical impact of these strains on the management of patients is enormous (Cosgrove *et al*., 2004).

The mechanisms of resistance that have been identified for VISA and VRSA strains are quite different and are not fully understood. For VISA strains, the proposed mechanism resulting in reduced susceptibility to vancomycin is believed to be a thickening of the bacterial cell wall such that vancomycin is trapped within the bacterial cell wall and is thus unable to reach its target on the surface of the bacterial cytoplasmic membrane (Cosgrove *et al*., 2004). The VRSA strain that was isolated from the patient in Michigan as well as a second isolate from a patient in Pennsylvania contained the *vanA* gene which codes for an altered target such that the binding of vancomycin to the target is significantly reduced and thus, it cannot carry out its normal function of inhibiting bacterial cell wall synthesis. The source of the *van A* gene isolated in VRSA appears to have come from co-infection with vancomycin resistant *Enterococcus* (VRE) (Applebaum, 2006).

The future of VISA and VRSA strains is not clear and much research is needed to help further understand all aspects of these organisms including their epidemiology, Microbiology, clinical and infection control implications and optimal treatment. As well, in addition to VISA and VRSA strains, there appear to be strains of *Staphylococcus aureus* that are referred to as "heteroresistant." These strains appear to be susceptible to vancomycin based on standard laboratory testing, but contain subpopulations of *Staphylococcus aureus* that have intermediate susceptibility to vancomycin. The clinical significance of these strains requires further investigation (Chang *et al*., 2003).

1.2 Statement of Problem

Vancomycin is commonly used for patients with Methicillin-resistant*Staphylococcus aureus* infections, and reduced susceptibility to vancomycin is becoming increasingly common. Therefore, the treatment of *Staphylococcus aureus* infections is becoming increasingly more complicated due to the emergence of various types of antibiotic resistance (Prasad, 2014).

The high prevalence rate of Vancomycin resistant *Staphylococcus aureus* can be attributed to the high rate of indiscriminate abuse of antibiotics. This lead to antibiotic resistant strains such as MRSA, VISA and VRSA

Vancomycin was considered to be the best alternative for the treatment of multi-drug resistant MRSA. However, there are increasing numbers of reports indicating the emergence of VRSA strains exhibiting two different resistant mechanisms namely modification of Vancomycin-binding target and removal of Vancomycin-binding target. Multi-drug resistant VRSA is a major cause of nosocomial and community acquired infections and is on the rise in humans (Jun *et al*., 2004).

The number of cases of VISA and VRSA reported so far has remained relatively small and thus the epidemiology and risk factors associated with infection with these organisms is not completely known. However, there is evidence that prior exposure to vancomycin, particularly repeated or prolonged courses. For patients infected with VISA strains, mortality appears to increase significantly compared to patients who are infected with vancomycin susceptible *Staphylococcus aureus* (Chang *et al*., 2003).

A prevalence of 5.3% of VRSA was observed in a study carried out at Abakaliki. This high prevalence rate was attributed to the high rate of indiscriminate abuse of antibiotics (Moses *et*

al., 2013). So also a significant rise in the number of strains of *Staphylococcus aureus* with reduced susceptibility to vancomycin, teicoplanin and oxacillin was found by Tiwari and Sen (2006).

Presence of vancomycin resistant *S. aureus* among in-patients and hospital environment posses serious danger, because in such environment there is easy of transmission of the VRSA among patients and health care personnel through contact.

1.3 Justification

S. aureus causes a wide range of infections, from a variety of skin, wound and deep tissue infections to more life-threatening conditions such as pneumonia, endocarditis, septic arthritis and septicemia. This bacterium is also one of the most common species in nosocomial infections. In addition, *S. aureus* may also cause food poisoning, scalded-skin syndrome and toxic shock syndrome, through production of different toxins (Winn Washington 2006).

Staphylococcus aureus is an important pathogen that causes health care and community acquired infection in every region of the world. Although *S. aureus* is a common commensal of the skin, there is need to investigate the prevalence of the organism on the skin of patients on admission especially MRSA and VRSA because most infections, both community and nosocomial, occur as a result of auto-inoculation even though nosocomial infections may also result from person-person transmission. Cross-contamination by *Staphylococcus aureus* among patients, professionals and medical supplies in health facilities is a constant concern (Breves *et al.,* 2015).

Staphylococcus aureus, especially methicillin-resistant *S. aureus* is a major cause of nosocomial infections worldwide. Patients subjected to broad-spectrum antibiotics and immunosuppressive

therapies have higher risk of infection by this microorganism. *S. aureus* infections are often extremely difficult to treat due to the large population heterogeneity, phenotypic switching, intra-strain diversity, hypermutability and most importantly the small colony variants. It is very important to emphasise that host immune responses against persistent infections by *S. aureus* is insufficient resulting normally into chronic infections, which in turn can lead to life threatening situations (Prasad, 2014).

It has also been reported that *S. aureus* strains have a wide variety of multi-drug resistance genes on plasmids, which can be exchanged and spread among different species of *Staphylococcus* and can be transferred to new bacterial hosts by transduction, conjugation or transformation method (Lujan *et al*., 2007). *S. aureus* is known to be notorious in its acquisition of resistance to new drugs and continues to defy control measures (Talaro and Talaro, 2002). Consequential effects of *S. aureus* multidrug resistance include prolonged hospitalization of patients, difficulty in patient management and treatment, and problem in infection control (Kleven 2007).

The treatment of infection caused by *Staphylococcus aureus* is a problem due to the emergence and spread of methicillin resistance gene. Vancomycin is commonly used for treatment of methicillin resistant *Staphylococcus aureus* (MRSA) (Prakash *et al.,* 2006). But the incidence of vancomycin intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) has been increasing in various parts of the world (Tiwari *et al*., 2008).

There is dearth of information on the prevalence of VRSA in Kaduna metropolis. It is therefore important to determine the susceptibility pattern of *Staphylococcus aureus* to vancomycin to be investigated.

1.4 Aim and Objectives

1.4.1 Aim

The study was aimed at phenotypic and genotypic characterization of vancomycin resistant *Staphylococcus aureus* (VRSA) isolated from in-patients in some selected hospitals in Kaduna Metropolis, Kaduna State Nigeria.

1.4.2 Objectives

The objectives were to:

1. Isolate and characterize *Staphylococcus aureus* from skin swabs using standard microbiological techniques.

2. Determine the antibiotic susceptibility pattern of the isolates using Kirby-Bauer method and the Minimum Inhibitory Concentration of Vancomycin.

4. Screen the Vancomycin Resistant *Staphylococcus aureus* (VRSA) isolates for *VanA* gene using Polymerase Chain Reaction.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Microbiology of *Staphylococcus aureus*

2.1.1The genus *Staphylococcus*

The genus *Staphylococcus* is made up of Gram-positive cocci with diameters of 0.5-1.5 μm, characterized by individual cocci that divide in more than one plane to form grape-like clusters (Costa *et al*., 2013) hence the name "*Staphylococcus*" meaning grape like cluster of cocci. They are non-motile, non-spore forming facultative anaerobes with a complex nutritional requirement for growth (Wilkinson, 1997; Plata*et al*., 2009; Costa *et al.*, 2013), a low GC content of DNA (in the range of 30-40 mol%) (Foster, 1996). They can tolerate high concentrations of salt (Plata *et al.*, 2009) and can also resist heat (Kloos and Lambe, 1991).

Based on their ability to produce coagulase (an enzyme that causes clots blood), the genus *Staphylococcus* is divided in two groups: the coagulase-positive staphylococci, which includes the most known species *Staphylococcus aureus*; and the coagulase-negative Staphylococci (CoNS), which are common commensals of the skin. Presently there are 44 species of *Staphylococcus* (Foster, 1996; Ray and Ryan, 2003).

2.1.2 *Staphylococcus aureus*

Staphylococcus aureus is the most pathogenic species of the genus *Staphylococcus*, it is implicated in both community-acquired and nosocomial infections. It often asymptomatically colonizes the skin and mucous membranes of healthy individuals, in particular the anterior nares (Harris *et al.*, 2002; Wertheim *et al*., 2005). In effect, it has been estimated that about 20-30 % of the population are permanently colonized by this bacterium, while other 30 % are transient carriers (Peacock *et al.*, 2001). This colonization represents an increased risk of infection by

providing a reservoir from which bacteria are introduced when the host defense is compromised. Due to the importance of *S. aureus* infections and the increasing prevalence of antibiotic-resistant strains, this bacterium has become the most studied Staphylococcal species (Kluytmans *et al.,* 1997).

On a rich medium, *S. aureus* forms medium size "golden" colonies. On sheep blood agar plates, colonies of *S. aureus* often cause β-hemolysis. The golden pigmentation of *S. aureus* colonies is caused by the presence of carotenoids as oppose the pale, translucent, white colonies formed by Coagulase Negative *Staphylococcus*(CoNS) (Ryan and Ray, 2004).

2.1.3 Biochemistry and molecular genetics of *Staphylococcus aureus*

Staphyloccoci are facultative anaerobes capable of generating energy by aerobic respiration and by fermentation which yields mainly lactic acid. Species of *Staphylococcus* are catalase-positive, a feature differentiating them from species of *Streptococcus,* and they are oxidase-negative and require complex nutrients, e.g. many amino acids and vitamins B, for growth. *S. aureus* is very tolerant of high concentrations of (up to 1.7M) sodium chloride. Another feature of the *Staphylococcus* genus is the cell wall peptidoglycan structure that contains multiple glycine residues in the crossbridge, which causes susceptibility to lysostaphin (Crossley and Archer, 1997).

S. aureus produces coagulase which interacts with prothrombin in the blood causing plasma to coagulate by converting fibrinogen into fibrin. Blood coagulation is used to distinguish *S. aureus* from most members of the genus, which are collectively designated as coagulase-negative staphylococci (Ryan and Ray, 2004). Since it is a frequent etiological agent of human diseases and exhibits resistance to a growing number of therapeutic agents, *S. aureus* is also one of the most intensively studied bacterial species (Wertheim *et al.,* 2005).

There are six types of vancomycin resistance based phenotypic and a genotypic characterization viz Van A, B, C, D, E and G. Out of which Van A, B, D, E, and G are acquired resistance while Van *C* is an intrinsic resistance. Strains with the *Van A*- type of resistance display high levels of inducible resistance to both vancomycin and teicoplanin, however Van B-type strains have variablelevels of inducible resistance to vancomycin only (Arthur, 1996).

2.1.4 Virulence factors of *Staphylococcus aureus*

S. aureus is equipped with a great variety of virulence factors, which include both structural and secreted products participating in pathogenesis of infection. It is known to cause a broad range of important infections in humans, this is related to the expression of an array of factors that participate in pathogenesis of infection, allowing it adhere to surfaces/tissues, avoid or evade the immune system, and cause harmful toxic effects to the host (Lowy, 1998; Foster and Höök, 1998; Dinges*et al.*, 2000). These factors are known as virulence determinants and are either cell-surface-associated (adherence) or secreted (exotoxins) factors.

2.1.4.1 Cell surface factors (Attachment-improving agents)

S. aureus expresses several cell surface factors that play a role in its virulence. These include numerous surface protein called “microbial surface components recognizing adhesive matrix molecules” (MSCRAMMs), capsular polysaccharides, and staphyloxanthin (carotenoid pigment) (Lin and Peterson, 2010).

Others are:

i. Fibronectin binding proteins A and B (FnbpA and FnbpB) which participate in attachment of bacterial cells to an extra-cellular matrix component, fibronectin, and to plasma clot (Patti *et al.*, 1994).

ii. Collagen binding protein (Cna) which is necessary for adherence of *S. aureus* to collagenous tissues and cartilage (Switalski *et al.*, 1993) and it has been shown that antibodies against Cna block the bacterial attachment to those tissues (Patti *et al.*, 1994).

iii. Clumping factors A and B (ClfA and ClfB) that mediate clumping and adherence of bacterial cells to fibrinogen in the presence of fibronectin. Clumping factors are thought to play a significant role in wound and foreign body infections and it has been shown that *clfA* mutant is less virulent than the wild type isogenic strain (Foster and Hook, 1998).

iv. Plasma-sensitive surface protein (Pls), once processed by plasmin, participates in binding to both fibrinogen and fibronectin (Hauck and Ohlsen, 2006).

v. Protein A is a hallmark of *S. aureus* which is encoded by the *spa* gene and is a cell wall-associated protein that binds to the Fc domain of immunoglobin G (IgG). Protein A binds IgG in "wrong orientation" on the surface of *S. aureus* cells which is thought to disrupt opsonization and phagocytosis (Switalski *et al.*, 1993). Protein A also exhibits an ability to bind to von Willebrand factor, a protein present at sites of damage of endothelium, and as a result, it can play a role in adherence and induction of endovascular diseases by *S. aureus* (Hartleib *et al.*, 2000).

vi. Biofilms are complex bacterial populations which are surface-attached and enclosed in a polysaccharide matrix, composed of poly-*N*-acetylglucosamine (PNAG). PNAG production depends on proteins encoded by the *ica* (intracellular adhesion) operon (Fitzpatrick *et al.*, 2005).Implanted biomedical device-related *S. aureus* infections depend on the pathogen's ability to attach to the surface of the biomaterial and consequently to form a mucoid biofilm.

2.1.4.2 Secreted factors (exotoxins)

One of the important characteristics of *S. aureus* is its capability to secrete toxins that disrupt membranes of host cells. Cytolytic toxins form β-barrel pores in the cytoplasmic membranes and cause leakage of the cell's content and lysis (Foster, 2005).

S. aureus secrets several cytolytic toxins, among them alpha-hemolysin, beta-hemolysin, gamma-hemolysin, leukocidin, and Panton-Valentine leukocidin (PVL) (Kaneko and Kamio, 2004). Alpha-hemolysin, encoded by the *hla* gene, inserts into eukaryotic membranes and oligomerizes into a β-barrel that forms a pore which causes osmotic cytolysis. Alpha-hemolysin is particularly cytolytic toward human platelets and monocytes (Menestrina *et al.*, 2001).

PVL is classified as a bicomponent cytolysin because it is dependent on two secreted proteins (LukF-PV and LukS-PV) that insert into the host's cytoplasmic membrane and hetero-oligomerize to form a pore (Kaneko and Kamio, 2004). PVL exhibits a high affinity toward leukocytes and is mostly associated with community-acquired methicillin resistant

S. aureus (CA-MRSA) which causes severe necrotizing pneumonia and contagious skin infections (Foster, 2005). Other bicomponent toxins, gammahemolysin (Hlg) and leukocidin (Luk), are cytotoxic toward erythrocytes and leukocytes, respectively (Kaneko and Kamio, 2004).

These secreted factors can be divided into four categories: superantigens, cytolytic (poreforming) toxins, various exoenzymes and miscellaneous proteins (Lin and Peterso, 2010).

a. Superantigens

Superantigens are a group of powerful secreted immune-stimulatory proteins capable of inducing a variety of human diseases, including toxic shock syndrome (TSS).*S. aureus* generates a group of powerful immuno-stimulatory proteins implicated in gastroenteritis and

toxic shock syndrome (Novick, 2003). They are resistant to heat denaturation and proteases. These toxins have the ability to cross-link MHC class II molecules located on antigen-presenting cells with T-cell receptors, forming a trimolecular complex. Formation of the complex induces intense T-cell proliferation in an antigen-independent manner resulting in massive cytokine production and release which causes capillary leak, epithelial damage and hypotension (Baker and Acharya, 2004). The primary function of superantigens is thought to weaken the host's immune system sufficiently to allow the pathogen to propagate and the disease to progress (Kotzin *et al.*, 1993). The staphylococcal enterotoxins A, B, C, D, E, G, Q are responsible for staphylococcal food borne diseases and toxic shock syndrome, while TSST-1 is the cause of toxic shock syndrome (Baker and Acharya, 2004).The superantigen toxins are typically encoded by mobile genetic elements (Novick, 2003).

b. Cytolytic (pore-forming) toxins

S. aureus secretes a large number of cytolytic toxins that, although structurally diverse and with different target specificity, share a similar function on host cells. These toxins form β-barrel pores in the cytoplasmic membranes of target cells and cause leakage of the cell's content (when at low doses) and cell lysis (at high doses) (Foster, 2005; Lin and Peterso, 2010).

c. Various exoenzymes

Nearly all strains of *S. aureus* secrete several extracellular enzymes whose function is thought to be the disruption of host tissues and/or inactivation of host antimicrobial mechanisms (e.g. lipids, defensins, antibodies and complement mediators) to acquire nutrients for bacterial growth and facilitate bacterial dissemination (Dinges, 2000; Lin and Peterson, 2010). These exoenzymes include lipases, nucleases, proteases [serine, cysteine (e.g. staphopain), aureolysin],

hyaluronidase, and staphylokinase (SAK) (Dinges, 2000; Bokarewa*et al.,* 2006; Lin and Peterson, 2010).

d. Miscellaneous proteins

S. aureus also has other specific proteins that can have a profound impact on the innate and adaptative immune system. These proteins include staphylococcal complement inhibitor (SCIN) (Rooijakkers*et al.,* 2006), extracellular fibrinogen binding protein (Efb) (Lee 2004a; 2004b), chemotaxis inhibitory protein of *S. aureus* (CHIPS) (Foster, 2005), formyl peptide receptor-like-1 inhibitory protein (FLIPr) (de Haas *et al.*, 2004; Prat *et al.,* 2006), and extracellular adherence protein (Eap) (Harraghy *et al.,* 2003).

2.1.5. Regulatory mechanisms of virulence determinants in *S. aureus*

The diverse array of cell wall and extracellular components involved in *S. aureus* virulence implies that the pathogenicity of this bacterium is a complex process requiring the tightly coordinated expression of these factors during different stages of infection (i.e. colonization, avoidance of host defense, growth and cell division, and bacterial spread) (Novick and Jiang, 2003; Torres *et al.,* 2010).

Indeed, the regulation of the virulence genes in *S. aureus* appears to follow a strategy that begins with the establishment of the bacterium in the host, followed by the attack of its defenses. For this, *S. aureus* begins by up regulating the expression of genes coding for surface proteins involved in adhesion and defense against the host immune system; and only late in infection it starts to up-regulate the production of toxins that facilitate tissue spread (Cheung *et al.,* 2004; Novick and Geisinger, 2008; Bien *et al.,* 2011).

To control the production of the virulence determinants during infection, *S. aureus* has several regulatory systems that respond to bacterial cell density (quorum-sensing) and environmental

cues (e.g. nutrient availability, temperature, pH, osmolarity, and oxygen tension) (Cheung *et al.*, 1992; Novick *et al.*, 1993; Wu *et al.*, 1996; Torres *et al*., 2010). These systems can be divided into two broad categories: two component signal transduction systems and global transcriptional regulators (Cheung *et al.*, 2002, 2004).

2.1.5.1 Two-component regulatory systems

The two-component regulatory systems in *S. aureus* include the accessory gene regulator (*agr*) and the staphylococcal accessory element (*sae*) (Costa *et al.*, 2013). The *agr* locus regulates more than 70 genes, of which 23 are related to virulence. It is responsible for up regulating the expression of many exoproteins (e.g. α-hemolysin, serine proteinase, TSST-1, enterotoxins, and proteases), and down-regulating the synthesis of cell wall-associated proteins (e.g. FnbpA, FnbpB, and SpA).The *sae* locus codes for another two-component system that regulates the expression of many virulence factors involved in bacterial adhesion, toxicity and immune evasion(Costa *et al.*, 2013). This includes the up-regulation of α-, β- and γ- hemolysins and the down-regulation of SpA (Costa *et al.*, 2013).

2.1.5.2 Global regulatory systems

Several global regulatory systems have been identified in *S. aureus*, including the staphylococcal accessory regulator A (*sarA*) and its several homologues. *sarA* up-regulates the expression of some virulence factors (e.g. Fnbps, α- and β-hemolysins) and down-regulates others (e.g. SpA and proteases). Additional genes with homology to *sarA* have been described, including *sarS* and *sarT* (Costa *et al*., 2013).

The regulation of virulence determinants may also involve sigma factors (σ), which are proteins that bind to the core RNA polymerase to form the holoenzyme that binds to specific promoters. *S. aureus* have two sigma factors: the primary sigma factor, σA, which is responsible for the

expression of housekeeping genes essential for growth; and the alternative sigma factor σB, which regulates the expression of different genes involved in cellular functions (e.g. stress response) and at least 30 virulence genes. It up-regulates capsule, FnbpA and coagulase, and down regulates hemolysins and serine protease A (Costa *et al.*, 2013).

2.1.6 Infections caused by *S. aureus*

S. aureus is a commensal and a pathogen. The anterior nares are the major site of colonization in humans. About 20–30% of individuals are persistent carriers of *S. aureus*, which means they are always colonized by this bacterium, and 30% are intermittent carriers (colonized transiently) (Wertheim *et al.*, 2005). Colonization significantly increases the risk of infections since it provides a reservoir of the pathogen from which bacteria are introduced when host defense is compromised (Kluytmans *et al.*, 1997). Patients with *S. aureus* infections are usually infected with the same strain that they carry as a commensal (Plata *et al.*, 2009).

S. aureus is one of the main causes of hospital- and community-acquired infections which can result in serious consequences (Diekema *et al.*, 2001). Nosocomial *S. aureus* infections affect the bloodstream, skin, soft tissues and lower respiratory tracts. *S. aureus* can be a cause of central venous catheter-associated bacteremia and ventilator- assisted pneumonia (Costa *et al.*, 2013). It also causes serious deep-seated infections, such as endocarditis and osteomyelitis (Schito, 2006). In addition to the infections listed above, *S. aureus* is often responsible for toxin-mediated diseases, such as toxic shock syndrome, scalded skin syndrome and staphylococcal food borne diseases (SFD) (Costa *et al.*, 2013). Hospitalized patients are particularly exposed to *S. aureus* infections due to their compromised immune system and frequent catheter insertions and injections. Aside the ability of *S. aureus* to cause life-

threatening infections, it is also important because of it remarkable potential to develop antimicrobial resistance (Lindsay and Holden, 2004).

2.1.7 Small-colony variants of *S. aureus*

Small-colony variants (SCVs) represent a subpopulation of naturally occurring, slow growing *S. aureus* with distinct phenotype and pathogenetic features. SCVs have been reported to cause recurrent, persistent infections many years after the initial infection had been cured (Proctor *et al.*, 1995).

Very often they reside inside human cells avoiding host defenses and antimicrobial chemotherapeutics. SCVs are defective in their electron transport pathways and usually form non-pigmented, non-hemolytic tiny colonies on agar (Kaneko and Kamio, 2004).

The small-colony variants display marked auxotrophisms for thymidine, menadione and/or hemin (Clements *et al.*, 1999; Bates *et al.*, 2003; Lannergard *et al.*, 2008; von Eiff, 2008). 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.*, 2006).

2.1.8 Organization of the *S. aureus* genome

The first genome sequences of *S. aureus* strains Mu50 and N315 were published in 2001 (Kuroda *et al.*, 2001). At present, complete genomic sequences of*S. aureus* strains are available, and the genomes of several others have been partially determined (Kuroda *et al.*, 2001; Baba *et al.*, 2002; Gill *et al.*, 2005; Diep *et al.*, 2006). The genome of *S. aureus* is a circular chromosome that is 2.8–2.9 Mbp in size, with a G+C content of about 33% (Crossley and Archer, 1997).

The chromosome encodes approximately 2700 coding sequences as well as structural and regulatory RNAs. It has been proposed that the *S. aureus* genome is composed of the core genome, accessory component and foreign genes. The core genes are present in more than 95% of isolates, represent 75% of any *S. aureus* genome and determine the backbone of the genome. The organization of the core component is highly conserved and the identity of individual genes between isolates is 98–100%. The majority of core genes are associated with fundamental functional categories of housekeeping functions and central metabolism (Plata*et al.*, 2009).

The accessory component includes genetic regions present in 1–95% of isolates and accounts for about 25% of any *S. aureus* genome. It typically consists of mobile genetic elements that have or previously had the ability of horizontal transfer between strains. These genetic elements include pathogenicity islands, genomic islands, prophages, chromosomal cassettes and transposons (Lindsay and Holden, 2004).

2.1.9 Pathogenicity islands of *S. aureus*

The family of staphylococcal pathogenicity islands that carry genes for superantigen toxins (SaPIs) are 15–20 kb elements located at constant positions in the chromosome. SaPIs possess certain bacteriophage- related attributes: genes coding for integrases, helicases and terminases, and flanking direct repeats (Novick, 2003b). The archetype of this family, SaPI1, codes for toxic shock syndrome toxin TSST (*tst*) and is excised and induced to replicate as well as transduced at high frequency by phage 80α. DNA of SaPI1 is encapsulated into 80α phage-like particles for transfer (Lindsay *et al.*, 1998; Ruzin *et al.*, 2001).

Another member of SapI family, SapI3, encodes enterotoxin B and is thought to be mobilized and encapsulated by phage 29 (Novick, 2003b). Certain bovine isolates of *S. aureus* carry SaPIbov1 which encodes toxic shock syndrome toxin (*tst*) and can be induced by three phages:

80α, ϕ11 and ϕ147 (Ubeda *et al.*, 2005). Members of the SaPI family have been found in almost all strains of *S. aureus* sequenced so far (Kuroda *et al.*, 2001; Baba *et al.*, 2002; Diep *et al.*, 2006).

In addition to SaPIs, *S. aureus* strains contain genomic islands from the *v*Sa family. These islands carry genes coding for about half of the *S. aureus* toxins and virulence factors, and greatly contribute to the pathogenicity of this species (Gill *et al.*, 2005).

They are found in all sequenced strains in the same locations and some of the genes carried by them are highly conserved (Lindsay and Holden, 2004). They encode their own integrase and usually are spontaneously excised from the host chromosome (Baba *et al.*, 2002). Members of this family of genomic islands include, but are not limited to, *v*Sa1 (carrying enterotoxin genes *seb*, *tsst*, *ear*), *v*Sa2 (containing genes encoding enterotoxine (*sec*) and toxic shock syndrome toxin — (*tsst*)) (Gill *et al.*, 2005). Other examples include *v*Saα and *v*Saβ, which are found in all sequenced isolates and have a defective transposase gene, and therefore are not excised from the chromosome. Additionally, they carry a cassette encoding a restriction-modification system and genes encoding leukocidin (*lukDE*) (Baba *et al.*, 2002).

2.1.10 Cell wall structure and biosynthesis

The staphylococcal cell wall is a dynamic, semi-rigid structure. It is composed of three components: peptidoglycan, teichoic acids and surface proteins. Of these three constituents, peptidoglycan is the major component which builds the murein sacculus. Structurally, peptidoglycan forms a macromolecular net in which glycan strands are cross-linked by short peptides. The glycan strands are composed of repeating disaccharide units of β-1-4 linked *N*acetylglucosamine and *N*-acetylmuramic acid (Labischinski, 1992). The majority of glycan chains have a length of 3–10 disaccharide units. Extending from the carboxyl residue of the β-1-

4 linked acetylglucosamine moiety is the stem peptide with the sequence: l-alanyl-d-isoglutaminyl-l-lysyl-D-Alanyl-D-Alanine (Scheffers and Pinho, 2005). A series of five l-glycine residues are attached to the l-lysine of the stem peptide which is a characteristic feature of the *S. aureus* cell wall (Rohrer *et al.*, 2003). The pentaglycine cross-bridge is synthesized in a sequential manner by a family of FemABX non-ribosomal peptide transferases (Berger-Bachi and Tschierske, 1998; Rohrer *et al.*, 2003).

The first step of peptidoglycan synthesis takes place in the cytoplasm and leads to the synthesis of nucleotide sugar-linked precursors: UDP-*N*-acetylmuramyl- pentapetide (UDP-murNAc-pentapeptide) and UDP-*N*-acetylglucosamine (UDP-GlcNAc). In the second stage, which occurs at the cytoplasmic membrane, UDP-murNAc-pentapeptide is transferred to the membrane-bound acceptor (bactoprenol) yielding lipid I and is followed by addition of UDP-GlcNAc resulting in lipid II formation. Bactoprenol (undecaprenol phosphate) is a lipophilic molecule that enables the cell to translocate hydrophilic precursors from the aqueous cytoplasm across the hydrophobic membrane to the externally localized sites of polymerization of peptidoglycan (Scheffers and Pinho, 2005; Bouhss *et al.*, 2008).

The final step of biosynthesis takes place outside the cytoplasmic membrane and involves incorporation of the recently synthesized disaccharide-peptide units into the peptidoglycan. The last step of peptidoglycan synthesis is carried out by penicillin-binding proteins (PBPs) which catalyze the transglycosylation and transpeptidation reactions, i.e., formation of the glycosidic and peptide bonds, respectively (Plata *et al.*, 2009).

The transglycosylation is catalyzed by multimodular penicillin-binding proteins, particularly PBP2 and by the monofunctional glycosyltransferase, Mtg, and results in incorporation of the lipidlinked precursors into the glycan polymer (Wang *et al.*, 2001; Barrett *et al.*, 2005). In the

transpeptidation (cross-linking) reaction, which is also catalysed by PBPs, the terminal l-glycine of the pentaglycine interpeptide bound to l-lysine of one stem peptide is attached to the D-Alanine of another stem peptide. The terminal D-Alanine of this stem peptide is cleaved off during this reaction. Thus, a flexible pentaglycine cross-bridge is formed between two peptidoglycan moieties, resulting in a strong and flexible cell wall structure (Gally and Archibald, 1993). *S. aureus* typically possesses four PBPs which are able to catalyze the transpeptidation reaction. PBPs 1, 2 and 3 are high molecular mass proteins (87, 80 and 75 kDa, respectively) and PBP4 is a low molecular mass protein (41 kDa)(Scheffers and Pinho, 2005).

Another component of the *S. aureus* cell wall are teichoic acids, which are polymers of ribitol residues or polymers of glycerol phosphate. Teichoic acids contribute to the negative charge present on the cell surface that plays a role in acquisition of ions, also and have been reported to be a component of *S. aureus* phage receptor (Plata*et al.*, 2009).

The last component of the *S. aureus* cell wall are surface proteins, including microbial surface components recognizing adhesive molecules (MSCRAMMs). These proteins contain a signal sequence directing their secretion and the LPXTG motif.The LPXTG motif is cleaved by sortase and then the proteins are covalently attached to the peptidoglycan. Protein A, fibronectin binding protein, collagen binding protein and clumping factor A, among many others, are components of the cell wall and are attached in this manner (Foster and Hook, 1998; Plata *et al.*, 2009).

2.1.11 Epidemiology of *Staphylococcus aureus*

In healthy individuals, the carrier rate of *S. aureus* range between 15% to 35% with a risk of 38% of individuals developing infection followed by a further 3% risk of infection when colonized with methicillin susceptible *Staphylococcus aureus* (MSSA) (File, 2008). Certain

groups of individuals are more susceptible to *S. aureus* colonization (because of their exposure to the bacterium) than others including health-care workers, nursing home inhabitants, prison inmates, military recruits and children (Ben-David *et al*., 2008; Ho *et al*., 2008).

In a study carried out in 2013 to determine the prevalence of vancomycin resistant *S. aureus* among clinical isolates in a tertiary health care facility in Abakaliki metropolis, the capital of Ebonyi State, South Eastern Nigeria, 5.3% prevalence rate of VRSA was observed. A higher prevalence of VRSA (46.2%) was reported in Zaria, Kaduna State, Nigeria by Otalu *et al* . in 2011

In a study, conducted in 2007 by the University of the Witwatersrand and the University Hospital of Geneva, health-care workers accounted for 93% of personnel to patient transmission of methicillin resistant *S. aureus* (MRSA) (Albrich and Harbath, 2008). Previously several outbreaks have been reported in Northern-Taiwan in 1997 that suggested MRSA transmission associated with health-care workers, including surgeons (Wang *et al*., 2001). Grundmann and colleagues (2006) reported a prevalence of > 50% in countries such as Singapore (1993-1997), Japan (1999-2000) and Colombia (2001-2002) while countries with a prevalence of 25% to 50% included South Africa (1993-1997), Brazil (2001), Australia (2003), Mexico and United States. The lowest prevalence of less than 1% were found in Norway, Sweden and Iceland (1993-1997) (Grundmann *et al*., 2006). In 2007, a prevalence of more than 50% of MRSA strains isolated from Cyprus, Egypt, Jordan and Malta was reported by Borg and colleagues (2007). This high prevalence was attributed to overcrowding and poor handhygiene facilities in the hospitals (Borg *et al*., 2007).

2.1.12 *Staphylococcus aureus* Carriage and Disease

Staphylococcus aureus is found as a commensal organism on the squamous epithelium of the anterior nares up to 20% of the population at any one time, however, it has been estimated that *S. aureus* can transiently colonize up to 60% of the human population (Foster, 2005). *S. aureus* can cause a wide range of infections ranging from minor skin abscesses to more serious invasive diseases. *S. aureus* commonly causes boils, carbuncles, furuncles and impetigo, but after gaining access to the blood, may also be a major cause of endocarditis, osteomyelitis, pneumonia, toxic shock syndrome and septicaemia (Lowy, 1998). Many invasive staphylococcal infections are correlated with nasal carriage of infecting strains. Although immunocompromised patients may be at greater risk for developing an invasive staphylococcal infection, healthy individuals may be also susceptible, especially if they are carriers (Peacock *et al*., 2001).

2.1.13 Emergence of Antibiotic Resistance in *Staphylococcus aureus*

Staphylococcus aureus is a major cause of life-threatening infections acquired in healthcare and community settings. It has developed resistance to most classes of antimicrobial agents. Penicillin was the first choice of antibiotics to treat staphylococcal infection. In 1944, by destroying the penicillin by penicillinase (beta-lactamase), *S.aureus* become resistant to penicillin. Most *S. aureus* strains (≥90%) are resistant to penicillin (Neu, 1994). Methicillin, a semisynthetic penicillins was used to treat Penicillin Resistant *Staphylococcus aureus* but resistance finally emerge in 1962 (Livermore, 2001; Lowy, 1998). Methicillin-resistance in *S. aureus* is mediated by the presence of penicillin-binding protein 2a (PBP-2a) which is expressed by an exogenous gene, *mecA*. This gene is carried by a genetic element, designated as staphylococcal cassette chromosome *mec (SCCmec),* which is inserted near the chromosomal

origin of replication (Hiramatsu, 2001). High prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) in hospitals has been reported from many states of India (Rajaduraipandi *et al*., 2006). Methicillin resistance among *S. aureus* isolates has reached phenomenal proportions in Indian hospitals, with some cities reporting 70% of the strains to be resistant to methicillin (Anupurba *et al*., 2003). High prevalence of vancomycin resistant *S. aureus* (46.2%) was reported in Zaria (Otalu *et al.,* 2011)

Vancomycin continues to be an important antimicrobial agent for treatment of Methicillin Resistant *Staphylococcus aureus* (MRSA) infections but resistance finally emerges. In 1996, a *S. aureus* strain with intermediate resistance to vancomycin (VISA) (vancomycin MIC= 8 Sg/ml) was first isolated from a patient in Japan (Hiramatsu *et al*., 1997). Shortly afterward, VISA strains were isolated in USA, Europe and other Asian countries (Hamilton-Miller, 2002), arousing considerable concern regarding the emergence of *S. aureus* strains for which there will be no effective therapy. Characterization of these VISA strains indicates that the mechanisms of resistance are complex and involve changes in cell wall content and composition (Avison *et al*., 2002).

In June 2002, the world's first reported clinical infection due to *S. aureus* with high resistance to vancomycin (VRSA) (vancomycin MIC>128 μg/ml) was diagnosed in a patient in the USA (Sievert *et al*., 2002). This isolate contain the *vanA* genes from enterococci and the methicillin-resistance gene *mecA*. The possible emergence and dissemination of VRSA strains is a serious health threat and makes it absolutely necessary to optimize prevention strategies and fast detection methods. Till today only five VRSA have been found all over the world, first in USA in 2002 (Sievert *et al*., 2002), second in Michigun in 2002 (Chang *et al*., 2003), third in Pennsylvania in 2002 (Tenover *et al*., 2004), fourth in New York in 2004 (Kacica, 2004), fifth

in New York in 2005 (Perichon and Courvalin, 2006), and the sixth in Kolkata (India) in 2005 (Saha *et al*., 2008).

2.1.14 Drug of last resort

Due to the nature of MRSA, when patients are infected with it, the resulting treatment inevitably involves the use of vancomycin. As such, vancomycin is thought of as a drug of last resort. While vancomycin is currently an effective treatment in the battle against MRSA, past experience has shown us that this should not lead to complacency and with the increasing use of vancomycin in the treatment of such super-bugs the appearance of vancomycin resistance is only to be accelerated. To compound these fears, vancomycin resistance is already well documented in vancomycin-resistant enteriococci (VRE), and has been know about for many years. This, and the emergence of MRSA with resistance to vancomycin (VRSA) in a small number of isolated cases, is definitely caused for alarm. If new antibiotics cannot be found then such infections will once again be without cure and the consequences deadly. As such, the current situation of looking for new antibiotics or ways to circumvent the current methods of resistance employed by bacteria has led to the comparison to an arms race, with the hope that we can develop the cures faster than the bacteria evolve (Chakraborty *et al.*, 2012).

2.1.15 Vancomycin Intermediate *Stapylococcus aureus* and Vancomycin Resistant *Staphylococcus aureus*.

The glycopeptide vancomycin exert it antimicrobial effects by binding irreversibly to the terminal D-Alanyl-D-Alanine (D-Ala-D-Ala) of bacterial cell wall precursors, inhibiting the synthesis of the *S. aureus* cell wall. The reduced susceptibility to vancomycin in VISA strains is due to the synthesis of an unusually thickened cell wall containing dipeptides (D-Ala-D-Ala) capable of binding vancomycin, sequestering them effectively, thereby reducing availability of

the drug for intracellular target molecules. The genetic basis for these cell wall alterations has not yet been determined. On the other hand, the vancomycin resistance in VRSA is due to the plasmid-mediated transfer of the *van A* gene cluster (*van*R, *van*S, *van*H, *vanA* and *van*X) carried by the mobile genetic element Tn1546 from vancomycin-resistant enterococci (VRE). The *vanA* cluster confers vancomycin resistance due to the synthesis of an alternative cell wall terminal peptide (D-Ala-D-Lac) with a reduced affinity for vancomycin that replaces the normaldipeptide D-Ala-D-Ala in peptidoglycan synthesis. Fortunately, these strains did not spread substantially, possibly due to increased fitness cost associated with high-level resistance to vancomycin (Costa *et al.,* 2013).

2.2 Vancomycin

Vancomycin was isolated in 1953 by Edmund Kornfeld from a soil sample collected from the interior jungles of Borneo by a missionary. It is a glycopeptide antibiotic used in the prophylaxis and treatment of infections caused by Gram-positive bacteria (Chakraborty *et al.,* 2012). It has traditionally been reserved as a drug of "last resort", used only after treatment with other antibiotics had failed, although the emergence of vancomycin-resistant organisms are increasingly being displaced from this role by linezolid and daptomycin. The organism that produced it was eventually named *Amycolatopsis orientalis*. The original indication for vancomycin was for the treatment of penicillin-resistant *Staphylococcus aureus*. The compound was initially labelled compound 05865, but was eventually given the generic name, vancomycin (Levine, 2006). Eli Lilly first marketed vancomycin hydrochloride under the trade name Vancocin (Moellering, 2006) and as COVANC (India, Marketed by Nucleus) (Chakraborty *et al.*, 2012).

Vancomycin has been in clinical use for nearly 50 years as a penicillin alternative to treat penicillinase-producing strains of *Staphylococcus aureus*. It is one of the most widely used antibiotics in the United States for the treatment of serious gram-positive infections involving methicillin-resistant *S. aureus* (MRSA) (Moellering, 2006).

Early use of vancomycin was associated with a number ofadverse effects, including infusion-related toxicities, nephrotoxicity and possible ototoxicity. Upon further investigation,it appears that the impurities in early formulations of vancomycin caused many of these adverse events. Its over all use was curtailed significantly with the development of semisynthetic penicillins (e.g., methicillin, oxacillin, nafcillin) that were considered less toxic. However, the steady rise in the number of MRSA infections since the early 1980shas once again brought vancomycin into the forefront as the primary treatment for infections caused by this organism (Virgincar and MacGowan, 2004; Murray and Nannini, 2005; Levine, 2006; Moellering, 2006).

Over the years, vancomycin has been one of the moststudied antibiotics. Extensive pharmacokinetic studies in a variety of patient populations and the availability of commercial drug assays have allowed clinicians to target serum vancomycin concentrations precisely in a relatively narrow range. This approach has been advocated to lessen the potential for nephrotoxicity and ototoxicity and to achieve therapeutic concentrations. However, it should be noted that the practice of routine monitoring and adjusting of serum vancomycin drug concentrations has been the subject of intense debate for many years. The controversy has resulted from conflicting evidence regarding the use of serum vancomycin concentrations to predict and prevent drug-induced toxicity and as a measure of effectiveness in treating infections (Lodise *et al*., 2008).

Further, data derived from more recent studies appear to suggest that vancomycin has little potential for nephrotoxicity or ototoxicity when used at conventional dosages (e.g., 1 g every 12 hours [15 mg/kg every 12 hours]), unless it is used concomitantly with known nephrotoxic drugs or at very high dosages (Lodise *et al*., 2008).

2.2.1 Mode of Action of Vancomycin

Peptidoglycan synthesis during bacterial cell walls formation requires several steps. In the cytoplasm, the enzyme racemase converts L-alanine to D-Alanine (D-Ala), and then 2 molecules of D-Ala are joined by the enzymeligase, creating the dipeptide D-Ala-D-Ala, which is then added to uracil diphosphate–*N*-acetylmuramyl-tripeptideto form uracil diphosphate–*N*-acetylmuramyl-pentapeptide. Uracil diphosphate–*N*-acetylmuramyl-pentapeptideis 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 cytoplasmicmembrane. *N*-acetylmuramyl-pentapeptide is then incorporated into nascent peptidoglycan by transglycosylation and allows the formation of cross-bridges by transpeptidation. 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.

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 (Couvalin, 2006).

2.2.2 Pharmacology and Chemistry

Vancomycin is a branched tricyclic glycosylated non-ribosomal peptide. It has multiple chemically distinct rotamers owing to the rotational restriction of some of the bonds. The form

present in the drug is the thermodynamically more stable conformer and, therefore has more potent activity.

2.2.3 Mechanism of Resistance to Vancomycin

Since vancomycin does not interact with cell wall biosynthetic enzymes but rather forms complexes with peptidoglycan precursors, its activity is not determined by the affinity for a target enzyme but by the substrate specificity of the enzymes that determine the structure of peptidoglycan precursors. Resistance to vancomycin is due to the presence of operons that encode enzymes (Arthur *et al*., 1996):

(1) for synthesis of low-affinity precursors, in which the C-terminalD-Ala residue is replaced by D-lactate (D-Lac) or D-Serine (D-Ser), thus modifying the vancomyin-binding target; and

(2) for elimination of the high-affinity precursors that are normally produced by the host, thus removing the vancomycin-binding target (Arthur *et al*., 1996).

Vancomycin resistant strains have been isolated in Nigeria, other African countries, Japan, The USA, France, Korea, South Africa, Brazil, Scotland and other parts of the world (Tenover *et al.*, 1998; Moses *et al.*, 2013). Vancomycin resistant *S. aureus* strains have increased the yearning of the world at large for the development of new drugs that will be effective in the treatment of multidrug resistant bacteria, *S. aureus* being one of them. Especially considering the widespread infection caused by *S. aureus* in developing countries (Moses *et al.*, 2013).

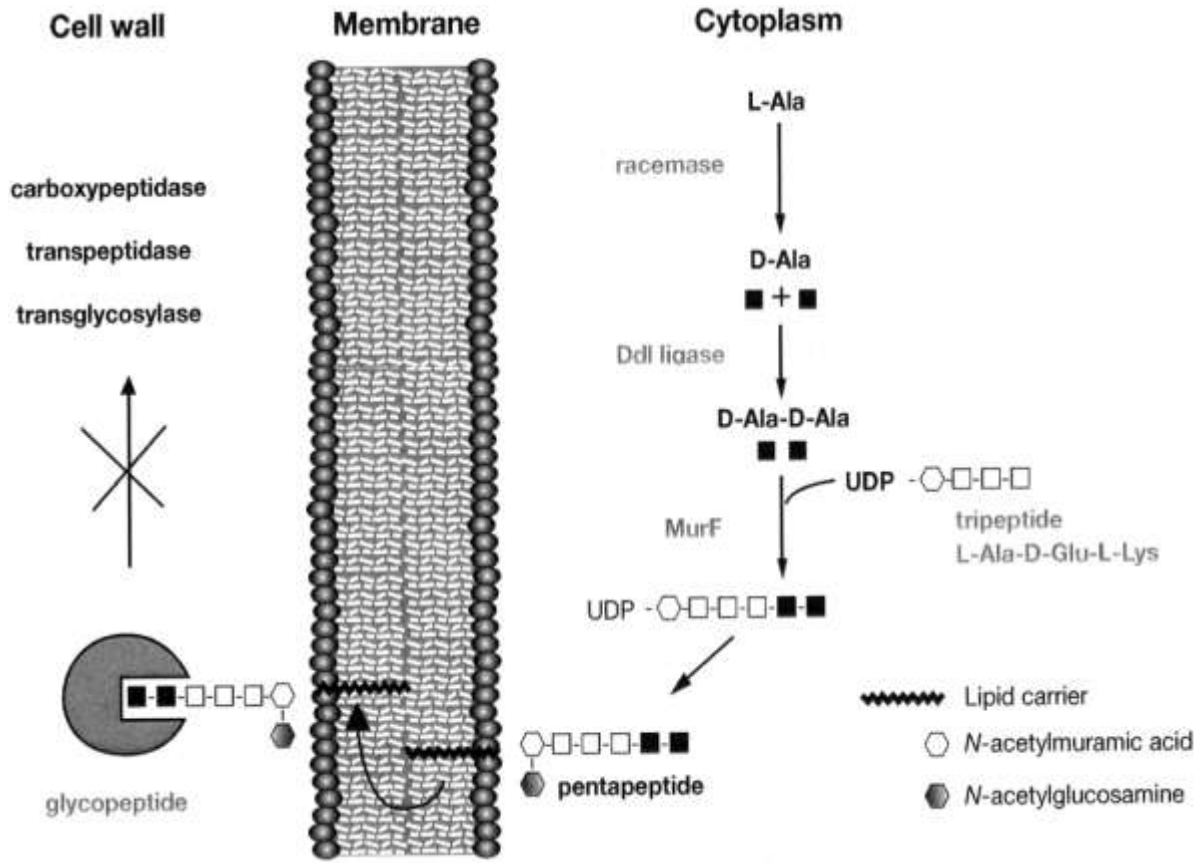


Figure 2.1: Peptidoglycan biosynthesis and mechanism of action of vancomycin.Binding of the antibiotic to the C-terminal D-Ala–D-Ala of late peptidoglycan precursors prevents reactions catalyzed by transglycosylases, transpeptidases, and the D,D-carboxypeptidases. Ddl, D-Ala:D-Ala ligase; MurF, a synthetase protein; UDP, uracil diphosphate (Couvalin, 2006)

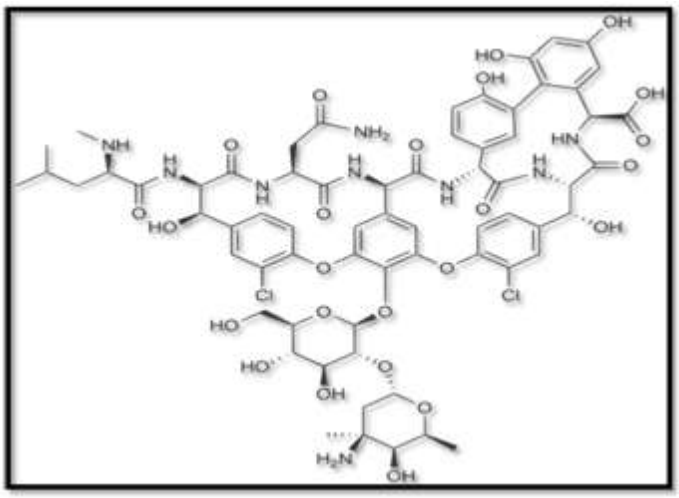


Figure 2.2: Chemical structure of vancomycin.

Since it emergence in 1997, the threat of vancomycin resistance in *S. aureus* has been the topic of intensive research and discussion. Although vancomycin resistance in *S. aureus* is said to be extremely rare, there is widespread concern that vancomycin-resistant *S. aureus* poses, by far, the greatest risk to patients, given the virulence of the organism (Srinivasan *et al.,* 2002). The presence of *van-A* genes in VRSA suggests that the resistance determinate was acquired from a vancomycin resistant *Enterococcus* (Noble *et al.,* 1992).

The resistance mechanisms used by *S. aureus* to resist to antibiotics include (i) inactivation of the antibiotic due to structural modification by enzymatic action, (ii) prevention of access to target by altering the outer membrane permeability, (iii) alteration of the antibiotic target site, (iv) efflux pump which pumps out the antibiotic, (v) target enzyme bypass or over production.

2.2.3.1 Target modification.

VanA-type resistance, which is characterized by inducible high levels of resistanceto vancomycin and teicoplanin, was the first type of resistance described and is mediated by transposonTn*1546* and elements closely related to it. The transposon encodes a dehydrogenase (VanH), which reduces pyruvate to D-Lac, and the *VanA* ligase, which catalyzesthe formation of an ester bond between D-Ala and D-Lac (Arthur*et al*., 1996; Couvalin, 2006). The resulting D-Ala-D-Lac depsipeptide replaces the D-Ala-D-Ala dipeptide in peptidoglycan synthesis, a substitution that decreases the affinity of the molecule for glycopeptides considerably (Bugg*et al.,* 1991; Couvalin, 2006).

The VanC resistance phenotype was described first in *Enterococcusgallinarum* (Leclercq*et al.,* 1992) and then in the *Enterococcus casseliflavus–Enterococcus flavescens* (Navarro and Courvalin, 1994) species, which possess intrinsic low levels of resistance to vancomycin and are susceptible to teicoplanin. Three genes are required for VanC type resistance. *vanT* encodes

the VanT membraneboundserine racemase, which produces D-Ser ; the *vanC* gene product VanC synthesizes D-Ala-D-Ser , which replaces D-Ala-D-Ala in late peptidoglycan precursors; and *vanXYc* encodes the VanXYC protein, which possesses both D,D-dipeptidase andD,D-carboxypeptidase activities and allows hydrolysis of precursor sending in D-Ala (Reynolds and Courvalin, 2005). Substitution of the ultimate D-Ala by a D-Ser results in steric hindrance that reduces its affinity for vancomycin (Billot-Klein *et al.,* 1994).

2.2.3.2 Removal of the susceptible target

The simultaneous production of precursors ending in D-Ala or D-Lac does not lead to resistance (Arthur *et al.,* 1996). Under these conditions, binding of glycopeptides to precursors that contain D-Ala-D-Ala inhibits peptidoglycan synthesis. The interaction of vancomycin with its target is prevented by the removal of the susceptible precursors that terminate in D-Ala (Reynolds, 1998). Two enzymes are involved in thisprocess: the VanX D,D-dipeptidase, which hydrolyzes the D-Ala-D-Ala dipeptide synthesized by the host D-Ala:D-Alaligase (Ddl) (Reynolds *et al.,* 1994), and the VanY D,D-carboxypeptidase, whichremoves the C-terminal D-Ala residue of late peptidoglycan precursors when elimination of D-Ala-D-Ala by VanX is incomplete. As opposed to *VanA*-type resistance, in whichthe VanX and VanY activities are catalyzed by 2 enzyme (Arthur *et al.,* 1998), VanXYC has both D,D-dipeptidase and D,D-carboxypeptidase activity (Reynolds *et al.,* 1999).

2.2.4 Types of resistance

Six types of vancomycin resistance have been characterized on both a phenotypic and a genotypic basis in enterococci viz *VanA*, B, C, D, E and G. Five of these types (*VanA*, B, D, E and G) correspond to acquired resistance; one type (VanC) is an intrinsic resistance. Classification of glycopeptide resistance is currently based on the primary sequence of the

structural genes for the resistance ligases rather than on the levels of resistance to glycopeptides, because the MIC ranges of vancomycin and teicoplanin against the various types overlap. *VanA*-type strains display high levels of inducible resistance to both vancomycin and teicoplanin, whereas VanB-type strains have variable levels of inducible resistance to vancomycin only (Arthur, 1996).

VanD-type strains are characterized by constitutive resistance to moderate levels of the 2 glycopeptides. VanC-, VanE- and VanG-type strains are resistant to low levels of vancomycin but remain susceptible to teicoplanin (Reynolds and Courvalin, 2005).

Although the 6 types of resistance involve related enzymatic functions, they can be distinguished by the location of the corresponding genes and by the mode of regulation of gene expression. The *vanA* and *vanB* operons are located on plasmids or in the chromosome, whereas the *vanD*, *vanC* ,*vanE* , and *vanG* operons have, thus far, been found only in the chromosome (Couvalin, 2006).

2.2.4.1 Van A

VanA is the most frequently encountered type of glycopeptides resistance in enterococci and, to date, is the only one detected in *Staphylococcus aureus*. The prototypeTn*1546 VanA*-type resistance element, which was originally detected on a plasmid in an *Enterococcus faecium* clinical isolate, is an 11-kb transposon. It encodes 9 polypeptides thatcan be assigned to various functional groups: transposition (ORF1 and ORF2), regulation of resistance gene expression (VanR and VanS), synthesis of the D-Ala-D-Lac depsipeptide (VanH and *VanA*), and hydrolysis of peptidoglycan precursors (VanX and VanY); the function of VanZ remains unknown. The VanR and VanS proteins are part of a 2-componentregulatory system that modulates transcription of the resistance gene cluster. This system is composed of a cytoplasmic

VanR response regulator, which acts as a transcriptional activator and a membrane-bound VanS histidine kinase.The *vanA*gene cluster has been found mainly in *E. faecium* and *Enterococcus faecalis* but also in *Enterococcus avium, Enterococcus durans, Enterococcus raffinosus,* and atypical isolates of *E. gallinarum* and *E. casseliflavus,* which are highly resistant to both vancomycin and teicoplanin (Couvalin, 2006).

2.2.4.2 Van B

As in *Van A*-type strains, acquired VanB-type resistance is due to synthesis of peptidoglycan precursors endingin the depsipeptide D-Ala-D-Lac instead of the dipeptide D-Ala-D-Ala. The organization and functionality of the *vanB* cluster is similar to that of *vanA* but differs in its regulation, because vancomycin, but not teicoplanin, is an inducer of the*vanB* cluster. The *vanB* operon contains genes encoding a dehydrogenase, a ligase, and a dipeptidase, all of which have a high level of sequence identity (67%–76% identity) with the corresponding deduced proteins of the *vanA* operon and the *vanRBSB* regulatory genes that encode a 2-component system only distantly related to VanRS (34% and 24% identity). The function of the additional VanW protein found only in the *vanB* cluster is unknown, and there is no gene related to *vanZ*. On the basis of sequence differences, the *vanB* gene cluster can be divided into 3 subtypes: *vanB1, vanB2,* and *vanB3*. There is no correlation between the *vanB* subtype and the level of resistance to vancomycin (Couvalin, 2006).

2.2.4.3 Van D

Acquired VanD-type resistance is due to constitutive production of peptidoglycan precursors ending in D-Ala-D-Lac. The organization of the *vanD* operon, which is located exclusively in the chromosome in strains that have been studied, is similar to that of *vanA* and *vanB*. However, no genes homologous to *vanZ* or *vanW* from the *vanA* and *vanB* operons, respectively, are

present. VanD-type strains share other characteristics that distinguish them from *VanA*- and VanB type enterococci. Resistance is constitutive and is not transferable by conjugation to other enterococci. VanD-type strains have negligible D, D-dipeptidase activity, which shouldresult in a susceptible phenotype, because these bacteria are unable to eliminate peptidoglycan precursors ending in D-AlaD-Ala, which is the target for glycopeptides. However, in VanD type strains, the susceptible pathway does not function, because the Ddl is inactive as the result of various mutations in the chromosomal *ddl* gene (Couvalin, 2006). The gene can be disrupted by a 5-bp insertion, insertion of the IS*19* element or a point mutation. Consequently, the strains should growonly in the presence of vancomycin, because they rely on the inducible resistance pathway for peptidoglycan synthesis. However, this is not the case because the *vanD* clusters are expressed constitutively as a result of mutations (frame shift or point mutation or insertion-inactivation) in the VanSD sensor or a point mutation in the VanRD regulator (Couvalin, 2006). Another unusual feature of VanD-type strains is their only slightly diminished susceptibility to teicoplanin (MIC, 4 mg/mL) despite their constitutive production of peptidoglycan precursors that terminate mainly in D-Ala-D-Lac. VanD-type strains that constantly activate the *vanD* operon, by mutation in the 2-component regulatory system, and that have eliminatedthe susceptible pathway, by inactivation of the Ddl, provide are markable example of "tinkering" in both intrinsic and acquired genes to achieve higher levels of antibiotic resistance (Couvalin, 2006).

2.2.4.4 Van C

E. gallinarum and *E. casseliflavus–E. flavescens* are intrinsically resistant to low levels of vancomycin but remain susceptible to teicoplanin. The VanC phenotype isexpressed constitutively or inducibly as a result of the productionof peptidoglycan precursors ending in D-

Ser. Three *vanC* genes encoding D-Ala:D-Ser ligases have been described:*vanC-1* in *E. gallinarum, vanC-2* in *E. casseliflavus,* and *vanC-3* in *E. flavescens.* The organization of the *vanC* operon, which is chromosomally located and is not transferable, is distinct from those of *vanA, vanB,* and *vanD.* Three proteins are required for VanC-type resistance: VanT, a membrane bound serine racemase, which produces D-Ser; VanC, a ligase that catalyzes synthesis of D-Ala-D-Ser ; and VanXYC, which possesses both D,D-dipeptidase and D,D-carboxypeptidase activities and allows hydrolysis of precursors ending in D-Ala. In the *vanA, vanB,* and *vanD* clusters, the genes encodingthe 2-component regulatory systems (i.e., VanRS, VanRBSB, orVanRDSD) are located upstream from the resistance genes, whereas, in the *vanC* cluster, these genes are downstreamfrom *vanT.* The deduced proteins of the *vanC-2*operon from *E. casseliflavus* display high degrees of identity(71%–91% identity) with those encoded by the *vanC* operon and those of the *vanC-3* gene cluster from *E. flavescens* display extensive identity with *vanC-2* (97%–100% identity), including the intergenic regions. It is, therefore, difficult to distinguish between *E. casseliflavus* and *E. flavescens* as 2 different species (Couvalin, 2006).

2.2.4.5 Van E

The VanE phenotype corresponds to low-level resistance to vancomycin and susceptibility to teicoplanin due to synthesis of peptidoglycan precursors terminating in D-Ala-D-Ser as in intrinsically resistant *Enterococcus* species. The *vanE* cluster has an organization identical to that of the *vanC* operon (Couvalin, 2006).

2.2.4.6 Van G

Acquired VanG type is characterized by resistance to low levels of vancomycin (MIC, 16 mg/mL) but susceptibility to teicoplanin (MIC, 0.5 mg/mL) and by inducible synthesis of peptidoglycan precursors ending in D-Ala-D-Ser. The chromosomal *vanG* cluster is composed

of 7 genes recruited from various *van* operons. In contrast to the other *van* operons, the cluster contains 3 genes *(vanUG, vanRG,* and*vanSG)* encoding a putative regulatory system. *vanRG* and *vanSG* have the highest similarity to *vanRD* and *vanSD,* and the additional *vanUG* gene encodes a predicted transcriptional activator. A protein of this type has not previously been associated with glycopeptide resistance (Couvalin, 2006).

2.2.5 Glycopeptide-dependent strains

An intriguing and clinically important phenomenon that has developed in some *VanA* and VanB-type enterococci is vancomycin dependence. These strains are not only resistant to vancomycin or to both vancomycin and teicoplanin, but also require their presence for growth. Variants of glycopeptide-resistant *E. faecalis* and *E. faecium* that grow only in the presence of glycopeptides have been isolated *in vitro,* in animal models, and from patientstreated with vancomycin for long periods. In the presence of vancomycin, the *vanA*- or *vanB*-encoded D-Ala:D-Lac ligase is induced, which overcomes the defect in synthesis ofpeptidoglycan precursors ending in D-Ala-D-Ala because of thelack of a functional Ddl following various mutations in the *ddl* gene and, thus, permits growth of the bacteria. Becausethese strains require particular growth conditions, their prevalence is probably underestimated and not easily detected by routine laboratory testing. Reversion to vancomycin independence has been observed and occurs as a result of a mutation in either the VanS or VanSB sensor, which leads to constitutive production of D-Ala-D-Lac and, thus, to teicoplanin resistance, or in the *ddl* gene, which restores synthesis of D-Ala-D-Ala and leads to a VanB phenotype inducible by vancomycin (Couvalin, 2006).

2.2.6 Regulation of Vancomycin Resistance

Expression of glycopeptide resistance is regulated by a VanS/VanR-type 2-component signal transduction system composed of a membrane-bound histidine kinase and a cytoplasmic response regulator that acts as a transcriptional activator. Van S-type sensors comprise an N-terminal glycopeptide sensor domain with 2 membrane spanning segments and a C-terminal cytoplasmic kinase domain. After a signal associated with the presence of a glycopeptide in the culture medium, the cytoplasmic domain of VanS catalyzes ATP-dependent auto phosphorylation on a specific histidine residue and transfers the phosphate group toan aspartate residue of VanR present in the effector domain. VanS also stimulates dephosphorylation of VanR in the absence of glycopeptide. The VanS sensor therefore modulates the phosphorylation level of the VanR regulator: it acts as a phosphatase under non inducing conditions and as akinase in the presence of glycopeptides, leading to phosphorylation of the response regulator and activation of the resistance genes (Couvalin, 2006).

2.2.7 Genetics of the *Van* Operons

2.2.7.1 van A Operon.

The *vanA* gene cluster was detected originallyon the nonconjugative Tn*1546* transposon (Arthur *et al.,* 1993). *VanA*-type resistance in clinical isolates of enterococci is mediated by genetic elements, identical or closely related to Tn*1546,* that are generally carried by self-transferable plasmids and, occasionally, by the host chromosome as part of larger conjugative elements (Handwerger and Skoble, 1995; Couvalin, 2006).

Tn*1546*-like elements are highly conserved, except for the presence of insertion sequences that have transposed into intergenicregions that are not essential for expression of glycopeptide resistance. Conjugal transfer of plasmids that have acquiredTn*1546*-like elements by

transposition appears to be responsible for the spread of glycopeptide resistance in enterococci (Couvalin, 2006).

2.2.7.2 vanB Operon.

Gene clusters related to *vanB* are generally carried by large (90–250-kb) elements that are transferable by conjugation from chromosome to chromosome. Plasmid borne *vanB* clusters have also been detected in clinical isolates of enterococci (Couvalin, 2006). Much of the dissemination of VanB-type resistance appears to have resulted from the spread of *vanB2*clusters carried on Tn*916*-like conjugative transposons. Two related elements, Tn*5382* (27 kb in size) and Tn*1549* (34kb in size), have been identified widely in the United States and in Europe (Couvalin, 2006).

2.2.7.3 vanG Operon.

Transfer of VanG-type resistance is associated with the movement, from chromosome to chromosome, of genetic elements of ~240 kb that also carry *ermB*-encoded erythromycin resistance (Couvalin, 2006).

2.2.8 Overview of Vancomycin Pharmacokinetic and Pharmacodynamic Properties

Sophisticated pharmacokinetic techniques such as Bayesian and non compartmental modeling have been used to derive pharmacokinetic parameters for vancomycin. The serum vancomycin concentration–time profile is complex and has been characterized as one-, two-, and three-compartment pharmacokinetic models (Skhirtladze *et al.*, 2006). In patients with normal renal function, the a-distribution phase ranges from 30 minutes to 1 hour, and the elimination half-life ranges from 6 to 12 hours. The volume of distribution is 0.4–1 L/kg. While reports of the degree of vancomycin protein binding have varied, a level of 50–55% is most often stated (Albrecht *et al.*, 1991). Penetration of vancomycin into tissues is variable and can be affected by

inflammation and disease state. For example, with uninflamed meninges, cerebral spinal fluid vancomycin concentrations ranging from 0 to approximately 4 mg/L have been reported, whereas concentrations of 6.4–11.1 mg/L have been reported in the presence of inflammation. Penetration into skin tissue is significantly lower for patients with diabetes (median, 0.1 mg/L; range, 0.01–0.45mg/L) compared with non diabetic patients based on the median ratio of tissue vancomycin to plasma vancomycin concentrations (median, 0.3 mg/L; range, 0.46–0.94 mg/L) (Skhirtladze *et al.,* 2006).

Vancomycin concentrations in lung tissue ranging from 5% to 41% of serum vancomycin concentrations have been reported in studies of healthy volunteers and patients (Stevens, 2006; Rybak, 2006).Epithelial lining fluid (ELF) penetration in critically injured patients is highly variable, with an overall blood: ELF penetration ratio of 6:1 (Georges *et al.,* 1997; Lamer *et al.,* 1993).

2.2.9 Vancomycin Toxicity

Vancomycin was initially dubbed "Mississippi mud" because of the brown colour of early formulations, which were about 70% pure. The impurities are thought to have contributed to the incidence of adverse reactions (Elting *et al.,* 1998; Darko *et al.,* 2003). In the1960s, purity increased to 75% and in 1985 to 92–95% for Eli Lilly's vancomycin product (Mohammedi *et al.,* 2006). Concurrently, a decrease in the reporting of serious adverse events occurred.

The most common vancomycin adverse effects are unrelated to serum drug concentration and include fever, chills and phlebitis (Darko *et al.,* 2003). Red man syndrome may be associated with histamine release and manifests as tingling and flushing of the face, neck, and upper torso. It is most likely to occur when larger dosages are infused too rapidly (>500 mg over≤30 minutes) (Wilhelm *et al.,* 1999; Darko *et al.,* 2003). Vancomycin should be administered

intravenously over an infusion period of at least 1 hour to minimize infusion-related adverse effects. For higher dosages (e.g., 2 g), the infusion time should be extended to 1.5-2 hours. Less frequent adverse events, such as neutropenia, also appear unrelated to serum drug concentrations. Vancomycin has long been considered a nephrotoxic and ototoxic agent. Excessive serum drug concentrations have been implicated, and it was assumed that monitoring of serum concentrations would allow interventions that decrease toxicity (Darko *et al.*, 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was carried out in Kaduna metropolis, Kaduna, located in the Northwestern part of Nigeria's high land. According to the 2006 census, Kaduna State has a land mass of 46,053 km^2 (17,781sq mi), population of 6,066,562 and population density 130/km^2 (340/sq mi). It consists of 23 local Government Areas and populated by about 59 to 63 different ethnic group (httpa://en.m.wikipedia.org/wiki/kadunastate).

The presence of many institutions both educational and research, favorable weather condition, good agricultural soil and hospitality of its indigenes are among the socioeconomic factors that have attracted people to Kaduna state.

Three (3) hospitals were selected based on convenience. The hospitals include Barau Dikko Teaching Hospital, Yusuf Dantsoho Hospital and Gwamna Awan Specialist Hospital. The hospitals were selected based on convenience because the hospital management gave us approval to carry out the study, their locations were accessible easily and easy of sample collection within the shortest possible time.

3.2 Sample Size

The sample size was determined using the equation described by Naing *et al.* (2006):

$$N=Z^2 P (1-P)/d^2.$$

Where N = sample size

Z = statistics for a level of 95% confidence interval =1.96

p= prevalence of 5.3% in Abakaliki (Moses *et al*., 2013).

d= precision (allowable error) = 5%= 0.05

Therefore:

$N = (1.96)^2 \times 0.053(1-0.053) / (0.05)^2$

= 77.13.

A total of 300 skin swabs (100 from each hospital) were collected for this study to increase statistical precision and reduce error.

3.3 Study Population

The study population comprised of in-patients both male and female of all age groups in the selected hospitals.

3.4 Inclusion Criteria and Exclusion Criteria

3.4.1 Inclusion criteria:

Male and female in-patients of all age groups in the selected hospital who consented.

3.4.2 Exclusion Criteria:

Out-patients and those in-patients who did not consent.

3.5 Ethical Approval

Ethical approval was obtained from the Ethical Committee of The Ministry of Health, Kaduna state. Consent forms were issued out to seek patient consent (Appendices I and II).

3.6 Sample Collection

A total of 300 skin swabs were collected from patients on admission in the children ward (51 skin swabs i.e. 17 from each hospital), female medical ward (51 skin swabs i.e. 17 from each

hospital), female surgical ward (51 skin swabs i.e. 17 from each hospital), male medical ward (51 skin swab i.e. 17 from each hospital), male surgical ward (51 skin swab i.e. 17 from each hospital)and emergency ward of the selected hospitals (45 skin swab i.e. 15 from each hospital). A total of 100 samples were collected from each of the three selected hospitals. The samples were collected as described by Breves *et al.* (2015). Skin swabs of in-patients were taken using sterile swab sticks, which were then inoculated in screwed capped bijou bottle containing brain-heart infusion (BHI) broth and transported immediately after collection in an insulated box with icepack container to Bacteriology Laboratory, Departmentof Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Kaduna state.

3.7 Isolation and Characterization of *Staphylococcus aureus*

3.7.1 Isolation of *Stapylococcus aureus*

A loopful of the inoculated brain-heart infusion broth was inoculated on Mannitol Salt Agar (MSA) and 5% blood agar plates. The inoculated plates were then incubated at 37ºC for 48 hours and 24 hours respectively as previously described by Baba *et al.* (2002). Suspected *S. aureus* colonies which were Mannitol fermenters appeared as yellow on MSA while on blood agar they appeared as creamy white. The suspected colonies from MSA and BA were subcultured on another MSA and BA respectively in order to obtain pure cultures. Thereafter, distinct colonies with characteristic colonial morphology (yellow colonies and creamy white colonies on MSA and Blood agar respectively) were picked aseptically and stored on nutrient agar slants for further analysis (Baba *et al.*, 2002).

3.7.2 Gram Staining

A smear was prepared by picking discrete colony of the suspected *S. aureus* and emulsifyied in a drop of distilled water on a glass slide, and was allowed to dry. The dried smear was then heat-fixed by passing the slide over a Bunsen burner flame. The heat-fixed smear was then covered with crystal violet for 30-60 seconds. It was then rapidly washed off with clean water from running tap. Next the smear was covered with Lugol's iodine for another 30-60 seconds and again washed off with clean water. The smear was then decolorized with acetone for 2 seconds and then washed off immediately, before covering with Safranin to counter stain for 60 seconds. Finally, the slide was washed and air-dried before viewing with the oil immersion objective. *S. aureus* appeared as Gram positive cocci in clusters (Winn *et al*, 2006).

3.7.3 Biochemical characterization

Suspected colonies of *S. aureus* were subjected to the following biochemical tests as described by Cowan and Steel (2003): Catalase, Coagulase, DNAse and Sugar fermentation tests.

3.7.3.1 Catalase test

Catalase test was carried out by using a wooden applicator stick to transfer growth from the centre of a colony to the surface of a glass slide. Then a drop of 3% H_2O_2 was added and observed for bubble formation. A rapid and sustained bubble formation was indicative of a positive test for *S. aureus*

3.7.3.2 Coagulase test

Tube coagulase test

Two (2) well isolated colonies of the test organism were emulsified in 0.5ml of physiological saline in a clean serological tube. Approximately, 1ml of citrated human plasma was then added and incubated at 35^{o}C, and it was observed after 1hr, 2hrs, 3hrs and 4hrs incubation for proper

coagulation. Those that were negative at 4 hours were incubated at room temperature overnight and checked again for clot formation. Formation of clumps (coagulation) after incubation was indicative of a positive test for *S. aureus*.

3.7.3.3 DNAse test

This was carried out by inoculating Deoxyribonuclease agar (Oxoid) with several colonies of the isolates of *Staphylococcus* from the blood agar plate, and then incubated for 24 hours at 35°C. After 24 hours of incubation, the isolates were tested for DNase production by flooding the plate with 1 Normal hydrochloric acid solution. The acid precipitated the unhydrolyzed DNA. Colonies of DNAse-producing isolates were surrounded by clear zone due to DNA hydrolysis and were *S. aureus* isolates.

3.7.3.4 Sugar fermentation tests (Mannitol, Sucrose, Arabinose and Raffinose)

Sugar fermentation test was carried out by preparing series of test tubes containing sterile phenol red, peptone water with 1% each of the fermentable sugar (Mannitol, Sucrose, Arabinose and Raffinose) and inverted Durham's tubes. Each of the test tubes was inoculated with suspected *Staphylococcus aureus* isolate. The inoculated tubes were incubated at 35^{o}C for 24 hours. At the end of the incubation period, all tubes were examined for acid and gas production and then compared with the control for interpretation. Isolates that fermented (produce acid from) Mannitol and Sucrose but not Arabinose and Raffinose were presumptive *S. aureus.*

3.8 Antibiotic Susceptibility Testing

3.8.1 Preparation of McFarlands standard and standardization of inocula

Mc Farlands standard (0.5) was prepared by adding 0.05ml of 1% (volume/volume) solution of Barium chloride ($BaCl_2$) to 9.95ml of 1% (volume/volume) Sulphuric acid (H_2SO_4) (Andrews, 2001).

Inocula of the *Staphylococcus aureus* isolates were standardized by picking discrete colonies and emulsifying in normal saline to a turbidity equivalent to that of 0.5 McFarland's standard (1.5×10^8 cfu/ml) (Andrews, 2001).

3.8.2 Determination of antibiotic susceptibility pattern of the isolates

The antibiotic susceptibility pattern of the isolates was determined by the Kirby Bauer disk diffusion method as described by Acharya (2013). Susceptibility of the isolates to the following antibiotics was determined: oxacillin (1µg), cefoxitin (30µg), chloramphenicol (30µg), gentamicin (30µg), ciprofloxacin (5µg) and trimethoprim/ Sulfamethoxazole (1.25/23.75µg).

About 0.1ml of each of the standardized inocula were inoculated evenly onto Mueller Hinton agar plates with the aid of a sterile swab stick. The antibiotic discs were then placed on the surface of the medium and allowed to diffuse for 10 minutes. The plates were then incubated at 35^{o}C for 18hrs. The diameters of the zones of inhibition were measured, recorded and interpreted as susceptible, intermediate or resistant using the Clinical Laboratory Standards Institute guideline (CLSI, 2015).

3.8.3 Determination of Vancomycin minimum inhibitory concentration.

The MIC of vancomycin was determined by a broth dilution (macrodilution) method using Mueller Hinton broth (MHB) (Andrews, 2001; Chakraborty *et al.,* 2012). About 2ml of the 0.5 McFarland standardized inoculums was transferred into tubes containing2ml of different concentrations (1µg/ml, 2µg/ml, 4µg/ml, 8µg/ml, 16µg/ml, 32µg/ml, 64µg/ml and 128µg/ml)of vancomycin in Mueller Hinton broth to arrive at a final concentrations 0.5µg/ml, 1µg/ml, 2µg/ml, 4µg/ml, 8µg/ml, 16µg/ml, 32µg/ml, 64µg/ml of vancomycin respectively and incubated at 37°C for 18h. The minimum concentration at which there was no visible turbidity was taken as the MIC of vancomycin (Chakraborty *et al.,* 2012). Isolates with MIC $\leq$ 2µg/ml were

considered to be Vancomycin susceptible *S. aureus* (VSSA), those with MIC 4-8µg/ml were considered to be Vancomycin intermediate *S. aureus* (VISA), while those with MIC ≥16µg/ml were considered to be Vancomycin resistant *S. aureus* (VRSA)(CLSI, 2015). Both VISA and VRSA isolates were screened for the presence *vanA* gene.

3.8.4 Determination of the Multiple Antibiotics Resistant (MAR) Index of the Isolates

The Multiple Antibiotics Resistance index of the isolates was calculated using the formula reported by Olonitola *et al*. (2007):

$$\text{MAR Index} = \frac{\text{Number of antibiotics to which the isolate is resistant}}{\text{Total number of antibiotics used}}$$

3.9 Molecular detection of *VanA* gene.

3.9.1 DNA isolation

The *Staphylococcus aureus* isolates were cultured in 5 ml Luria Bertani broth and incubated at 37 °C for 24 hrs. Then 1.5ml of *Staphylococcus aureus* culture was vortexed for 15 seconds and 1 ml was aliquot into a microcentrifuge tube and centrifuged at 14,000 rpm for 15 seconds. The supernatant was poured out and re-suspended in 100 µl of digestion buffer containing lysozyme/lysostaphin mix. The tube was incubated at 37^0C for 45 minutes. After incubation, 300 µl of lysis solution and 10 µl of reconstituted proteinase K was added to the digestion mixture and mixed well by vortexing. The lysate was then incubated at 55^0C for 45 minutes. After incubation, 40µl of binding solution and 180 µl of 96-100% ethanol were added to the lysis mixture, and mixed by vortexing. The sample was spinned for 10 seconds at 14,0000 rpm. Using a pipette, the clear aqueous phase only was carefully transferred to a spin column that has been attached to a collection tube. The column assembly was centrifuged for 3 minutes at 14,000 rpm to bind the bacterial DNA. Five hundred microliter of wash solution I was applied

to the column and centrifuged for 2 minutes at 14,000 rpm. The flow-through was discarded and the column reassembled and the collection tube. Five hundred microliter of wash solution II was added to the column and centrifuged again for 2 minutes at 14,000 rpm. The elution was done by transferring the spin column to the provided 1.7 ml elution tube. Then 75 µl of elution buffer was applied to the column and centrifuge at 6,000 rpm for 2 minutes. This was spinned for an additional 2 minutes at 14,000 rpm to complete the DNA elution (Donabedian *et al.,* 2000).

3.9.2 Detection of *vanA* gene by PCR

The amplification was carried out in a total volume of 50 µl. Amplification was carried out as follows: 20 µl of genomic DNA sample was added to 50 µl of PCR mixture (1.5 mM $MgCl_2$, 200 µM each dNTP, 2 µM each primer, 0.1 µg template DNA, 3% (volume/volume) Dimethylsulphoxide (DMSO) and 1 U *Taq* DNA polymerase). The amplification process was started with an initial denaturation step (98^0C for 2 minutes). Followed by 35 cycles of denaturation at 98^0C for 10 seconds; annealing at 50°C for 1 minute; polymerization at 72^oC for 60 s and final extension at 72^oC for 5 minutes. For the visualization of the product, 10 µl of the each PCR reaction product was mixed with 5 µl of loading dye and loaded on 1.5% agarose gel for electrophoresis and visualization of the amplified PCR products. A 100 bp molecular weight DNA ladder was used to determine the gene size (Donabedian *et al.,* 2000). The primer sequences specific for *van A* used in the study is shown in the table below.

Table 3.1: Nucleotide sequence and amplicon size of the *van A* specific primer.

Gene	Primer sequence (5' – 3')	Amplicon size	Reference
vanA	F- ATGAATAGAATAAAAGTTGC-	1032bp	Donabedian *et al.*(2000)
	R- TCACCCCTTTAACGCTAATA-		

CHAPTER FOUR

4.0 RESULTS

4.1 Occurrence of *Staphylococcus aureus* from skin swabs of in-patients in the selected hospitals

Figure 4.1 shows the occurrence of *S. aureus* isolated from skin swabs of in-patients in selected hospitals in Kaduna metropolis. Out of the 300 samples collected, 52 isolates of *S. aureus* were obtained therefore the rate of isolation of *S. aureus* was found to be 17.33%.

4.2 Occurrence of *Staphylococcus aureus* isolated from skin swab based on gender

Figure 4.2 shows the distribution of *Staphylococcus aureus* isolated from skin swab of in-patients in the selected hospitals by gender. Of the 300 samples collected, 192 were from females out of which 33 were positive while the remaining 108 were from males out of which 19 were positive. The percentage of *S. aureus* was found to be 17.19% and 17.59% for female and male respectively. The difference observed was not statistically significant (p=0.9287).

4.3 Distribution of *Staphylococcus aureus* from skin swab by age group

The distribution of *Staphylococcus aureus* isolated from skin swab based on age group is shown in Figure 4.2. Age group ≤ 10 years had the highest prevalence, followed by 11-20 years. The least was observed among age group 31-40 years. The difference observed within the age groups was statistically significant (p = 0.0001).

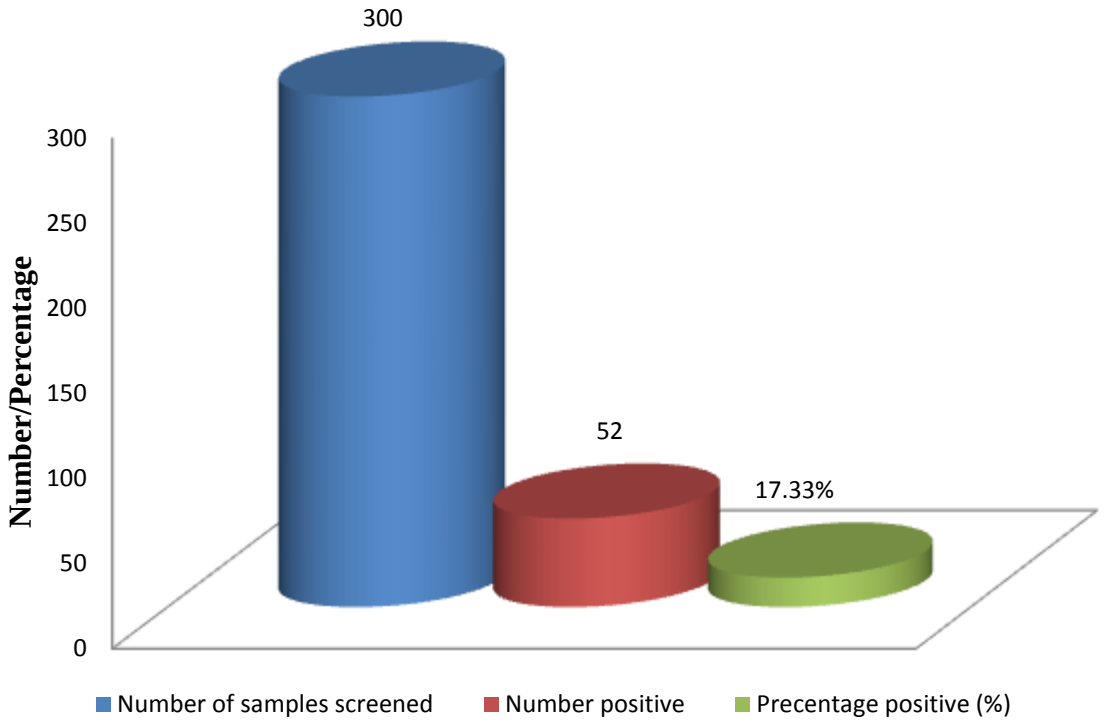
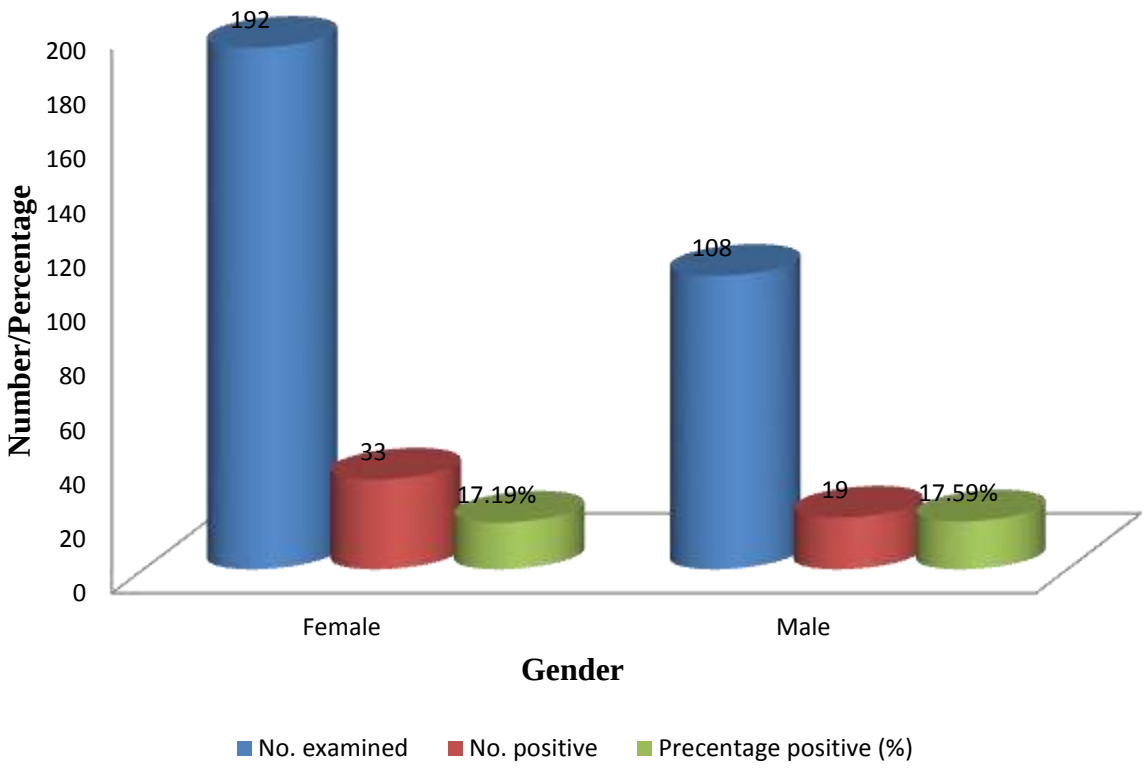
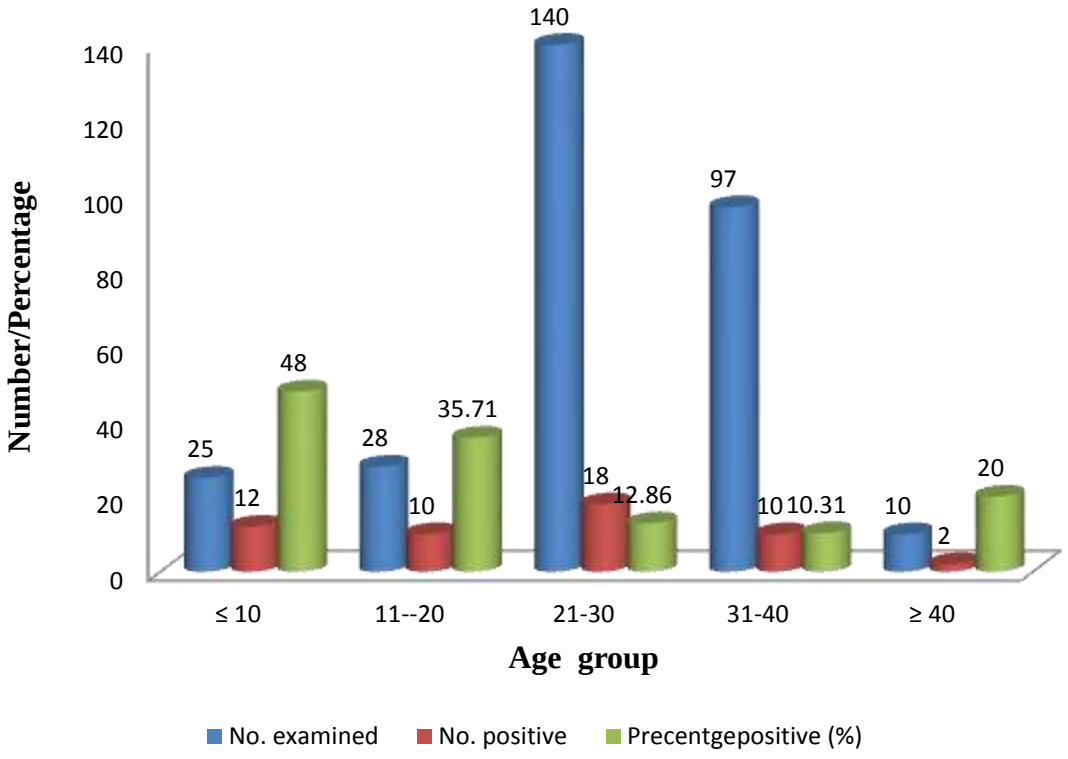


Figure 4.1: Occurrence of *Staphylococcus aureus* isolated from skin swab of in-patients in the selected hospitals



$\chi^2 = 0.008$, df = 1, $p = 0.9287$.

Figure 4.2: Occurrence of *Staphylococcus aureus* isolated base on gender



$\chi^2 = 28.357$, df = 4, $p = 0.0001$.

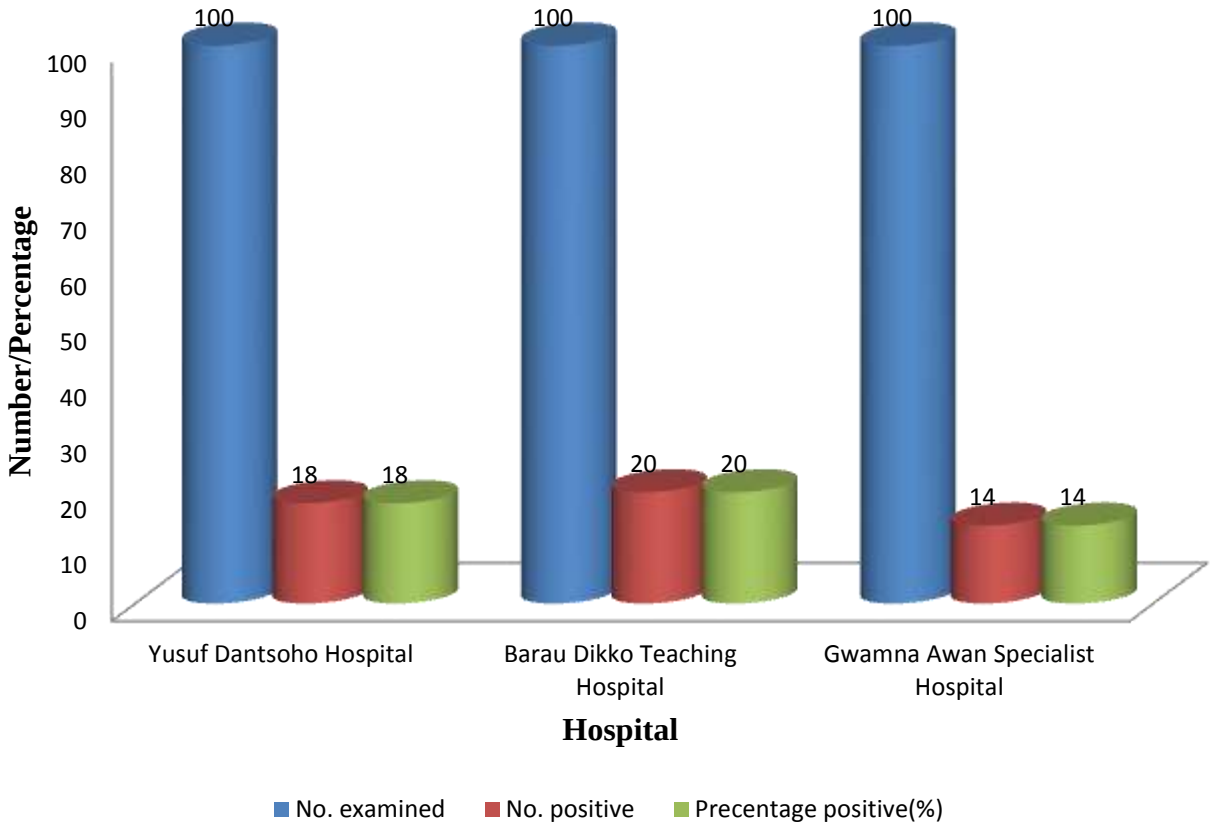
Figure 4.3: Occurrence of *Staphylococcus aureus* isolated from skin swab based on age group

4.4 Occurrence of *Staphylococcus aureus* based on the hospitals selected

Occurrence of *Staphylococcus aureus* isolated from skin swab based on the hospitals selected is presented in Figure 4.4. Gwamna Awan Specialist hospital had a prevalence of 14.0%, Yusuf Dantsoho hospital had a prevalence of 18.0% and Barau Dikko Teaching hospital had a prevalence of 20.0%.

4.5 Antibiotics susceptibility of *S. aureus*

Out of the 52 isolates, high level of resistance was recorded to oxacillin (98.08%) and cefoxitin (53.85%). Moderate level of resistance was observed to chloramphenicol (36.54%), gentamicin (34.62%), ciprofloxacin (46.15%) and trimethoprim/sulfamethoxazole while low level of resistance was observed to vancomycin (3.85%) (Table 4.1, Table 4.2, Table 4.3 and Figure 4.5). Table 4.4 shows the MAR indices of the isolates, number of isolates resistant, Number and antibiotics resisted. Ten isolates were resistant to only 1 antibiotic (MAR index 0.14), so also another 10 were resistant to 2 antibiotics (0.29). While 6, 5, 16 and 5 isolates were resistant to 3(MAR index 0.43), 4 (MAR index 0.57), 5 (MAR index 0.71) and 6 (MAR index 0.86) antibiotics respectively. Nineteen different susceptibility patterns were observed out of which the most common was OX (10 times).



$\chi^2 = 1.303$, df = 2, $p = 0.5213$.

Figure 4.4: Occurrence of *Staphylococcus aureus* isolated from skin swab based on the hospitals

Table 4.1: Antibiotic susceptibility pattern of *Staphylococcus aureus* isolates

Antibiotic disc (concentration)	Number (percentage) of isolates (n = 52)		
	Susceptible	Intermediate	Resistant
Vancomycin	48(92.30)	2(3.85)	2(3.85)
Oxacillin (1µg)	1(1.92)	0(0.00)	51(98.08)
Cefoxitin (30µg)	24(46.15)	0(0.00)	28(53.85)
Chloramphenicol (30µg)	29(55.77)	4(7.69)	19(36.54)
Gentamicin (30µg)	30(57.69)	4(7.69)	18(34.62)
Ciprofloxacin (5µg)	27(51.93)	1(1.92)	24(46.15)
Trimethoprim/ Sulfamethoxazole (1.25/23.75µg).	27(53.85)	3(5.77)	22(38.46)

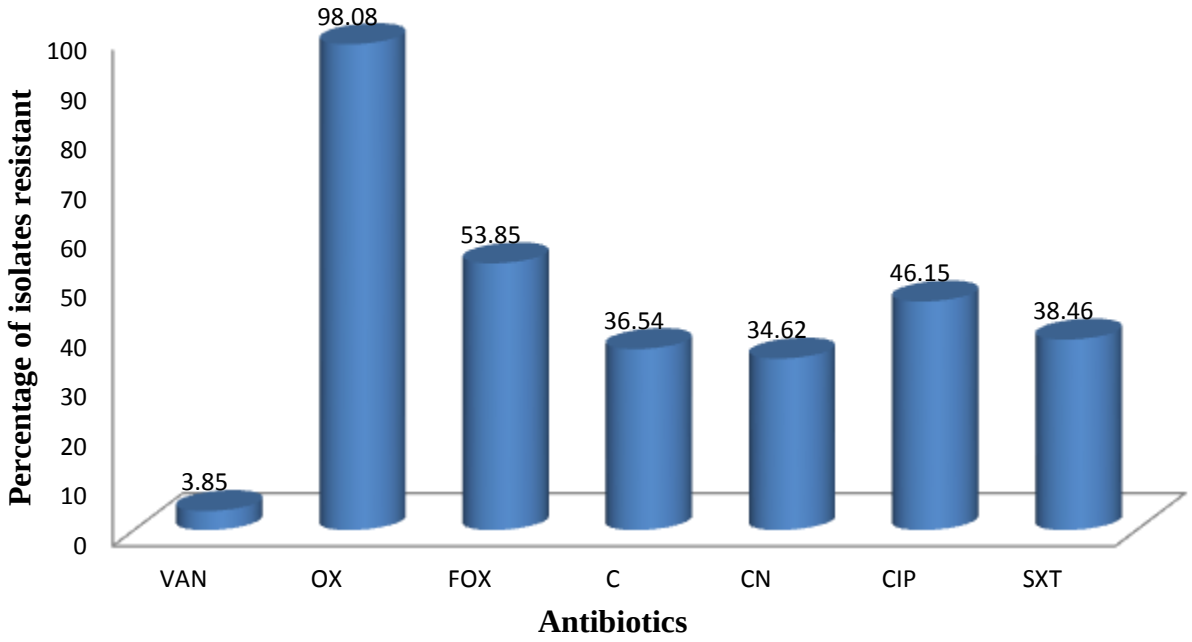


Figure 4.5 : Percentage resistance of *S. aureus* to some selected antibiotics

Key: VAN = vancomycin,OX = oxacillin, FOX = cefoxitin, CIP = ciprofloxacin, CN = Gentamicin, C = chloramphenicol, SXT = Trimethoprim/Sulfamethoxazole

Table 4.2: Minimum Inhibitory Concentrations (MICs) of Vancomycin for the 52 isolates of *S. aureus*

MIC Values	Number of isolates	Inference*
1μg/ml	32	VSSA
2 μg/ml	16	VSSA
4 μg/ml	1	VISA
8 μg/ml	1	VISA
16 μg/ml	2	VRSA

* Isolates with MIC values ≤ 2 μg/ml are Vancomycin Susceptible *S. aureus* (VSSA), those with MIC values between 4-8 μg/ml are Vancomycin Intermediate *S. aureus* (VISA) while those with MIC values ≥ 16 μg/ml are Vancomycin Resistant *S. aureus* (VRSA) (CLSI, 2015).

Table 4.3: *In vitro* susceptibility result of the 52 isolates from the selected hospitals to the antibiotics

Hospital of isolation		Number (%) of isolates susceptible to the antibiotics						
		VAN	OX	FOX	C	CN	CIP	SXT
Yusuf Dantsoho (18)		17(94.44)	0(0.00)	5(27.78)	10(55.56)	8(44.44)	9(50.00)	9(50.00)
Barau Dikko (20)		18(90.00)	1(5.00)	10(50.00)	9(45.00)	12(60.00)	11(55.00)	8(40.00)
Gwamna Awan (14)		13(92.86)	0(0.00)	9(64.29)	10(71.43)	10(71.43)	7(50.00)	10(71.43)
Total	52	48(92.31)	1(1.92)	24(46.15)	29(55.77)	30(57.69)	27(51.92)	27(51.92)

Key: OX = oxacillin, VAN = vancomycin, FOX = cefoxitin, CIP = ciprofloxacin, CN = gentamicin, C = chloramphenicol, SXT = Trimethoprim/Sulfamethoxazole

Table 4.4: Multiple Antibiotic Resistance patterns and indices of 52 isolates of *S. aureus* from skin swab tested against 7 antibiotics

Resistant patterns	**Number of isolates (%)**	**MAR indices**
OX	10(19.23)	0.14
OX,VA	2(3.84)	0.29
OX,C	7(13.46)	0.29
OX,CN	1(1.92)	0.29
OX,CIP,C	1(1.92)	0.43
CIP,C,SXT	1(1.92)	0.43
OX,FOX,SXT	1(1.92)	0.43
OX,FOX,CN	1(1.92)	0.43
OX,C,SXT	1(1.92)	0.43
OX,CN,SXT	1(1.92)	0.43
OX,FOX,CIP,SXT	3(5.77)	0.57
OX,FOX,CN,SXT	2(3.84)	0.57
OX,FOX,CIP,CN,SXT	7(13.46)	0.71
OX,FOX,CIP,CN,C	4(7.69)	0.71
OX,FOX,CIP,C,SXT	2(3.84)	0.71
OX,FOX,CN,C,SXT	2(3.84)	0.71
OX,VA,FOX,CIP,CN	1(1.92)	0.71
OX,FOX,CIP,CN,C,SXT	4(7.69)	0.86
OX,VA,FOX,CIP,CN,C	1(1.92)	0.86

Key: OX = oxacillin, VA = vancomycin, FOX = cefoxitin, CIP = ciprofloxacin, CN = gentamicin, C = chloramphenicol, SXT = trimethoprim/sulfamethoxazole

4.6 Vancomycin Susceptibility Pattern of the isolates

Figure 4.6 shows the Occurrence of the isolates in terms of their susceptibility to vancomycin. Two (3.85%) of the isolates were VRSA (MIC ≥ 16μg/ml), so also 2(3.85%) of the isolates were VISA (MIC values between 4-8μg/ml) and the remaining 48 (92.30%) were VSSA (MIC ≤ 2μg/ml).

4.7 Vancomycin Resistant *S. aureus* Isolated from Skin Swab of in-atients in the Hospitals selected

Of the 52 *Staphylococcus aureus* isolated, 2 were found to be Vancomycin Resistant *Staphylococcus aueus* (VRSA). The percentage of VRSA among in-patients in the selected hospitals in Kaduna metropolis was found to be 3.85% (Figure 4.7).

4.8 Detection of *VanA* gene by PCR

The *van A* gene was detected in all the two isolates that were vancomycin resistant. Plate I shows the agarose gel electrophoresis result of the PCR amplicons for *VanA* gene.

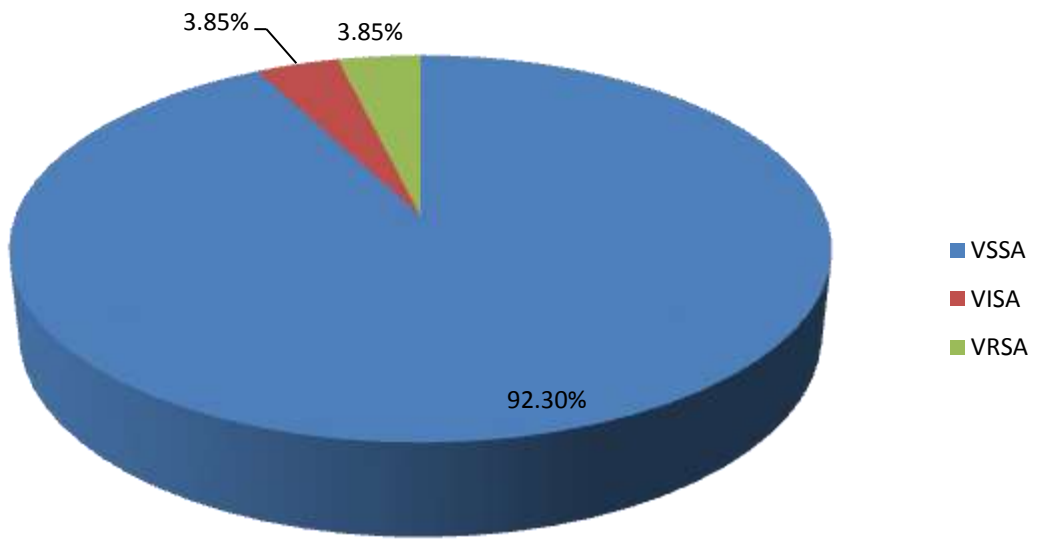


Figure 4.6: Percentage Occurrence of *S. aureus* according to vancomycin susceptibility pattern

VSSA = Vancomycin Susceptible *S. aureus*, VISA = Vancomycin Intermediate *S. aureus* VRSA = Vancomycin Resistant *S. aureus.*

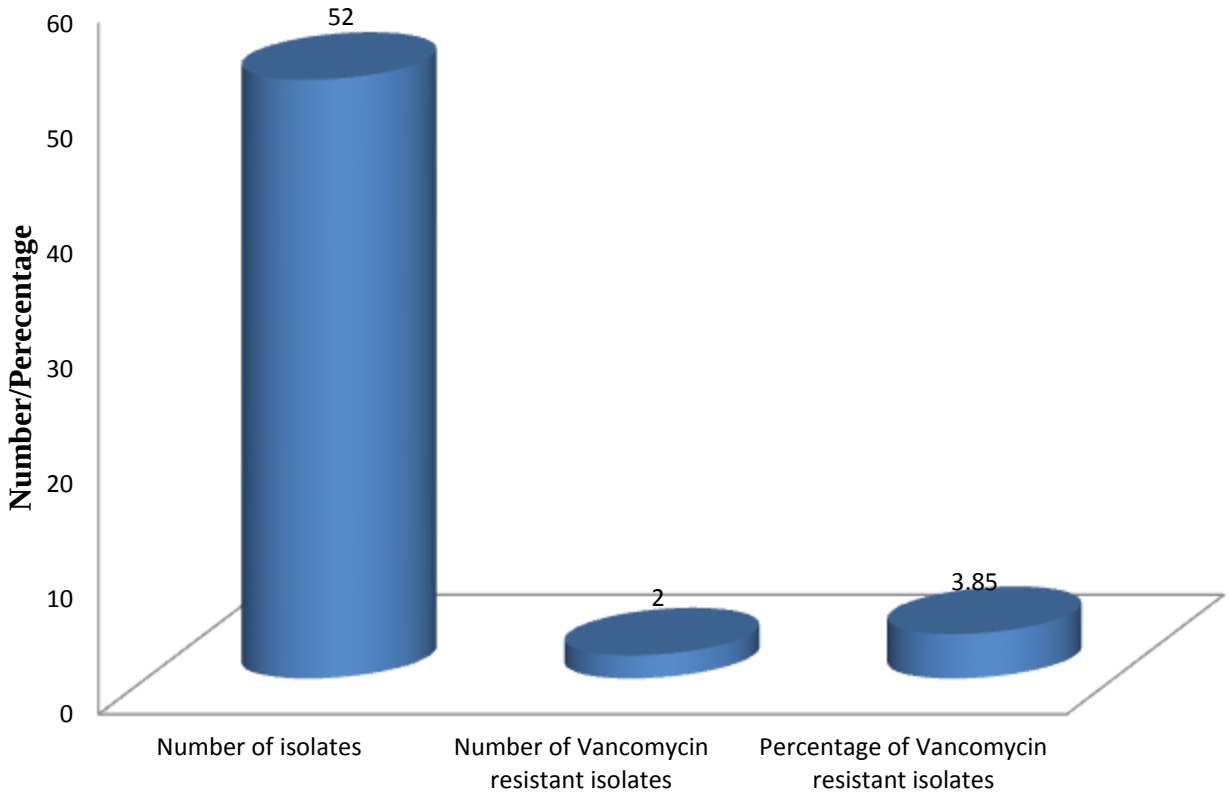


Figure 4.7: Occurrence Of Vancomycin Resistant *S. aureus* Isolated from Skin Swab of in-patients in the Hospital

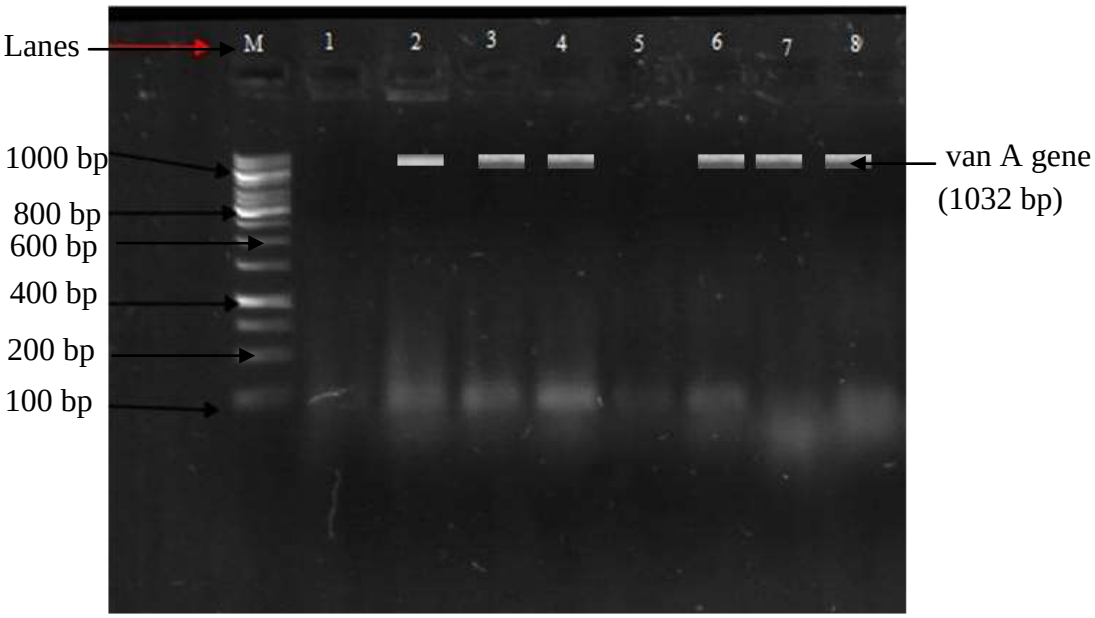


Plate I: Amplicons of *S. aureus* van A gene, size 1032pb.Lane M 100 bp DNA ladder, lanes 1 and 5 are negative controls, lanes 2,3 and 4 are replicate of PCR amplicons of the 1st sample and 6,7 and 8 are replicate of PCR amplicons of the 2nd sample.

CHAPTER FIVE

5.0 DISCUSSION

The prevalence of *S. aureus* from the skin of in-patients in selected hospitals in Kaduna state was found to be 17.33%. This means that a significant percentage of the study population was having *S. aureus* colonization. The incidence of high concentration of *S. aureus* on sin of human may represent potential health hazards, especially if these isolates possess determinants of antibiotic resistance and are able to produce enterotoxins (Percival *et al*., 2004). A higher rate of *S. aureus* (26.12%) was reported by Ghoniem *et al*. (2014). The possible reasons for the higher prevalence in their study may be because different types of samples were used in the studies. Ghoniem *et al*. (2014) isolated *S. aureus* from sputum, nose swab, blood, urine and environmental swabs. So also their study design included patients, medical staff and hospital environments.

The occurrence of *S. aureus* according to gender showed that the percentage among females and males were 17.19% and 17.59% respectively. This suggests that *S. aureus* colonizes both genders at almost the same rate. Ghoniem *et al.* (2014) and Primo (2012) reported a higher prevalence of *S. aureus* in males compared to females probably due to life style (out door activities and contacts with formates) of the male which exposes them more to *S. aureus* colonization. The difference observed between the result of our study and theirs may be due to difference in geographical location, study population and type of sample used.

Distribution based on age group: ≤ 10 years (48%), 11-20 years (35.71%) and ≥40 years (20.0%) showed higher percentage of *S. aureus* compared to age groups 31-40 years (10.31%) and 21-30 years (12.81%). The higher percentage observed in the two extreme age groups may

be due to low hygiene and low immune statues of the patients within these age groups. Higher percentage among the elderly patients (above 55years) was also reported by Ghomiem *et al.* (2014) and Warren (2012). They attributed this to weakened immunity of the older patients.

The differences observed in the occurrence of *S. aureus* according to the hospitals may be attributed to the differences in location and category of patients attending these hospitals since all the hospitals maintain good hygiene practices. Since all the hospitals use disinfectants in cleaning the contact surfaces and the health care workers there practice hand washing hygiene then it is likely that the difference may be attributed to the categories of patients attending those hospitals.

The level of resistance observed to oxacillin (98.08%) and cefoxitin (53.85%) can be attributed to the emergence and spread of β-lactamase producing *S. aureus*. The overall high resistance observed against the antibiotics tested in this study can be attributed to abuse and misuse of antibiotics by the populace and the use of antibiotic in animal feeds. This creates selective pressure for the resistant strain of *S. aureus.*

In this study, ten (19.23%) of the isolates had MAR index ≤ 0.2 whereas the remaining 42 (80.77%) isolates had MAR index ≥ 0.2. This indicates that isolates were recovered from individuals who have had contact with significantly contaminated or high risk site. This result is worrisome as it shows that antibiotic resistance is on the increase and is spreading fast within the study area.

Multiple Antibiotic Resistance (MAR) index is a good tool for risk assessment. Isolates with MAR index value higher than 0.2 are considered to have originated from high risk sources where antibiotics are abused frequently (Hemen *et al.,* 2012). The MAR index gives an insight

on the number of isolates showing antibiotic resistance and the risk zones in routine susceptibility testing.

In many developing countries including Nigeria, virtually all drugs are sold in local drug stores called ‘chemists’ without proper control, hence antibiotics are readily available to the population and abused. So also, the traders in these ‘chemists’ prescribe drugs to their customers with reckless abandon and worst still, there is high rate of empirical therapy prescription, whereby clinicians prescribe antibiotics to patients without obtaining antibiotic sensitivity results from the medical laboratories. These factors increase the rate of drug abuse and consequently increase the rate of development and spread of bacterial resistance to antibiotics in a geometric rate higher than that observed in developed countries (Moses *et al.,* 2013).

CLSI (2015) recommends that MIC tests should be performed to determine the susceptibility of all isolates of staphylococci to vancomycin. This is because the disk test does not differentiate vancomycin susceptible isolates of *S. aureus* from vancomycin intermediate isolates of *S. aureus.*

In this study, a relatively low rate of VRSA (3.85%) occurrence was observed. This may be attributed to the fact that use of vancomycin is still low in the study area because it is expensive and is reversed for the treatment of infections caused by MRSA. So also these will limit the misuse and abuse of vancomycin. Moreover, resistance to vancomycin is due the presence of operon that encodes enzymes for the synthesis of low-affinity precursors in which the C-terminal D-Alanine residue is replaced by D-lactate/D-Serine or enzymes for the elimination of the high affinity precursors, this mechanism is complex when compared to resistance

mechanism for other drugs such as efflux and reduced permeability and as such it will not be easily achieved.

This prevalence however, is lower than that reported by Moses *et al.* (2013) in Abakaliki, who reported a higher prevalence of 5.3% of VRSA. A significant increase in the number of strains of *S. aureus* with reduced susceptibility to vancomycin was found by Tiwari and Sen (2006) in Northern part of India.

Infections caused by vancomycin-resistant *S. aureus* are associated with high morbidity and mortality rates due to limited treatment options. Vancomycin is the main antimicrobial agent available to treat serious infections with MRSA but unfortunately, decrease in vancomycin susceptibility of *S. aureus* and isolation of vancomycin-intermediate and resistant *S. aureus* have recently been reported from many countries (Thati *et al.,* 2011). A prevalence as high as 14.2% was reported by Okolie *et al.* (2015) in North central Nigeria.

The MIC values (≥16μg/ml) from this study indicated that 2(3.85%) of the isolates were resistant to vancomycin. Two other isolates with MIC values between 4μg/ml and 8μg/ml(3.85%) were intermediately resistant. The remaining 48 (92.30%) isolates were vancomycin susceptible with MIC values ≤ 2μg/ml. The low resistance rate of vancomycin resistance observed in this study suggests the relatively low level of vancomycin abuse and misuse among the study population.

Thati *et al.* (2011) reported a lower occurrence rate of VRSA (1.96%) and higher occurrence rates of VISA (4.46%) and VSSA (93.57%) from intensive care units of tertiary care hospitals in Hyderabad, India. The difference observed in the occurrence rates in our study compared to

that of Thati *et al.* (2011) may be due to difference in sample types, regional difference in infection control, antibiotic use policies and vancomycin resistance detection technique use.

The vancomycin resistance of VRSA isolates was confirmed by the presence of *van A* gene (an amplicon of 1000 bp (1kb)) following PCR amplification. This was confirmed by the presence of *vanA* gene in the two VRSA isolates.

The detection of *vanA* gene in the 2 VRSA isolates (100%) from this study varies with the findings of Ghoniem *et al.* (2014), where they detected the *van A* gene in only 51.9% of VRSA isolates. Thati *et al.* (2011) reported PCR amplification for *van* A in six out of seven VRSA isolates. The difference in the protocol used in the determination of vancomycin MIC may account for the differences in the detection of *van A* gene.

Studies have demonstrated variability in vancomycin MICs among available testing methods. One of such studies is that carried out by Kruzel *et al.* in 2011 to determine vancomycin MICs using different methodologies. Ghoniem *et al.* (2014) used E-test to determine the MIC of isolates in their study, whereas in this study macrotube dilution method was used to determine the MIC while Thati *et al.* (2011) used agar dilution method.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The study has established that 52 *Staphylococcus aureus* isolates were obtained from the skin swabs of in-patients in selected hospitals giving a prevalence of 17.33%. Result of the antibiotic susceptibility test shows that the level of resistance was higher to oxacillin (98.08%).

Staphylococcus aureus was isolated from female and male at the rate of 17.19% and 17.59% respectively. So also based on age group, *S. aureus* was isolated more from the extreme age groups: ≤ 10 years (48%), 11-20 years (35.71%) and ≥ 40 years (20.0%).

Results of the antibiotic susceptibility test show that the level of resistance was higher to oxacillin (98.08%). Out of the 52 isolates, 2(3.85%) were vancomycin resistant (VRSA) with MIC value of ≥16μg/ml, 2(3.85%) were vancomycin intermediate (VISA) with MIC value of 4-8μg/ml while the remaining 48(92.31%) were vancomycin susceptible (VSSA) with MIC value of ≤ 2μg/ml . the prevalence of VRSA therefore in the study was found to be 3.85%. The vanA gene detected in this study was 1032bp in size which is the expected amplicon of vanA.

6.2 Recommendations

1. Antibiotic therapy should be based on results of culture and sensitivity testing.
2. Response of patients to treatment should be monitored during therapy for early detection of resistance.
3. Surveillance programme for VRSA should be carried out to enhance the ability of the public health system to rapidly address this resistant pathogen.

4. Using proper infection control practices and good antimicrobial agent management will go a long way in limiting the emergence and spread of antibiotic resistant strains.

5. Controlled use of vancomycin should be encouraged in hospitals and among people to control and prevent the spread of vancomycin resistant *S. aureus.*

REFERENCES

Acharya, T. (2013). Modified Kirby-Bauer disc diffusion method for antimicrobial susceptibility testing, *Microbe online.*

Albirich, W. and Harbath, S. (2008).Health-care workers: source, vector, or victim of MRSA? *The Lancet Infectious Diseases*, **8**:289-301.

Albrecht, L. M., Rybak, M.J. and Warbasse, L.H. (1991).Vancomycin protein binding in patients with infections caused by *Staphylococcus aureus*. *Annals of pharmacotherapy,* **25**:713–715.

Andrews, J.M. (2001). Determination of Minimum Inhibitory Concentrations. *Journal of Antimicrobial Chemotherapy*, **48**(1):5-16

Anupurba, S., Sen, M.R., Nath, G., Sharma, B.M., Gulati, A.K. and Mohapatra, T.M. (2003). Prevalence of methicillin resistant *Staphylococcus aureus* in a Tertiary care Referral Hospital in Eastern Uttar Pradesh. *Indian Journal of Medical Microbiology*, **21**: 49-51

Applebaum, P. C. (2006).The emergence of *Staphylococcus aureus* with reduced vancomycin-intermediate vancomycin-resistant *Staphylococcus aureus. Clinical Microbiology and Infection*, **12**(1):16-23.

Arciola, C.R., Baldassarri, L. and Montanaro, L. (2001).Presence of icaA and icaD genes and slime production in a collection of staphylococcal strains from catheter-associated infections. *Journal of Clinical Microbiology,* **39**: 2151–2156.

Arthur, M., Depardieu, F., Cabanie, L., Reynolds, P. and Courvalin, P. (1998).Requirement of the VanY and VanX D,D-peptidases for glycopeptide resistance in enterococci. *Molecular Microbiology,* **30**:819–30.

Arthur, M., Depardieu, F., Reynolds, P. and Courvalin, P. (1996).Quantitative analysis of the metabolism of soluble cytoplasmic peptidoglycan precursors of glycopeptide-resistant enterococci. *Molecular Microbiology,* **21**:33–44.

Arthur, M., Molinas, C., Depardieu, F. and Courvalin, P. (1993).Characterization of Tn1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *Journal of Bacteriology*,**175**:117–127.

Arthur, M., Reynolds, P. and Courvalin, P. (1996).Glycopeptide resistance in enterococci. *Trends in Microbiology,* **4**:401–407.

Avison, M.B., Bennett, P.M., Howe, R.A. and Walsh, T.R. (2002).Preliminary analysis of the genetic basis for vancomycin resistance in *Staphylococcus aureus* strain Mu50. *Journal Antimicrobial Chemotherapy,* **49**:255-260.

Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K., Oguchi, A. (2002).Genome and virulence determinants of high virulence community-acquired MRSA.*Lancet*,**359**: 1819–1827.

Baker, M.D. and Acharya, K.R. (2004). Superantigens: structure function relationships. *International Journal of Medical Microbiology*, **293**:529–537.

Bannerman, T.L. (2003).*Staphylococcus*, *Micrococcus*, and other catalasepositive cocci that grow aerobically. In: Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH, ed. *Manual of clinical microbiology*, ASM Press: Washington DC,**8**:384–404.

Barrett, D., Leimkuhler, C., Chen, L., Walker, D., Kahne, D. and Walker, S. (2005).Kinetic characterization of the glycosyltransferase module of *Staphylococcus aureus* PBP2.*Journal of Bacteriology*,**187**:2215–2217.

Bates, D.M., von Eiff, C., McNamara, P.J., Peters, G., Yeaman, M.R., Bayer, A.S. and Proctor, R.A. (2003).*Staphylococcus aureus* menD and hemB mutants are as infective as the parent strains but the menadione biosynthetic mutant persists within the kidney. *Journal of Infectious Diseases*, **187**:1654–1661.

Ben-David, D., Mermel, L. and Parenteau, S. (2008). Methicillin-resistant *Staphylococcus aureus* transmission: The possible importance of unrecognized health-care worker carriage. *American Journal of Infection Control*, **36**:93-97.

Berger-Bachi, B. and Tschierske, M. (1998). Role of fem factors in methicillin resistance. *Drug Resistance Update*, **1**:325–335.

Bien, J., Sokolova, O. and Bozko, P. (2011).Characterization of virulence factors of *Staphylococcus aureus*: novel function of known virulence factors that are implicated in activation of airway epithelial proinflammatory response. *Journal of Pathogens*, 1-13.

Billot-Klein, D., Gutmann, L., Sable, S., Guittet, E. and van Heijenoort, J. (1994). Modification of peptidoglycan precursors is a common feature of the low level vancomycin-resistant VANB-type *Enterococcus* D366 and of the naturally glycopeptide-resistant species *Lactobacillus casei*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, and *Enterococcus gallinarum*. *Journal of Bacteriology*,**176**:2398–405.

Boisset, S., Geissmann, T., Huntzinger, E., Fechter, P., Bendridi, N., Possedko, M.(2007) *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes Dev*, **21**:1353–1366.

Bokarewa, M.I., Jin, T., and Tarkowski, A. (2006).*Staphylococcus aureus*: Staphylokinase. *The International Journal of Biochemistry and Cell Biology*, **38**(4):504-509.

Borg, M.A., De Kraker, M. and Scicluna, E. (2007).Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in invasive isolates from southern and eastern Mediterranean countries. *Journal of Antimicrobial Chemotherapy*,**60**:1310-1315.

Bouhss, A., Trunkfield, A.E., Bugg, T.D. and Mengin-Lecreulx, D. (2008).The biosynthesis of peptidoglycan lipid-linked intermediates. *FEMS Microbiology Review*,**32**: 208–233.

Breves, A., Aparecida, C., Miranda, C., Flores, C., de Filippis, I., Clementino, M.M. (2015). Methicillin- and vancomycin-resistant *Staphylococcus aureus* in health care workers and medical devices. *Journal of Brazilian Pathology and Medical Laboratory*, **51**(3):143-152.

Bugg, T.D., Wright, G.D., Dutka-Malen, S., Arthur, M., Courvalin, P., Walsh, C.T. (1991). Molecular basis for vancomycin resistance in *Enterococcus faecium*BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and *VanA*. *Biochemistry,* **30**:10408–15.

Carbon, C. (2000).MRSA and MRSE: is there an answer. *Clinical Microbiology and Infection*,**6**(2):17-22.

Centers for Disease Control and Prevention. (1997).*Reduced susceptibility of Staphylococcus aureusto vancomycin—Japan*, 1996. Morbidity and Mortality Weekly Report, **46**:624–6.

Chakraborty, S. P., KarMahapatra, S., Bal, M. and Roy, S. (2011). Isolation and Identification of Vancomycin Resistant *Staphylococcus aureus* from Post Operative Pus Sample. *Al Ameen Journal of Medical Science,* **4**(2):152 -168.

Chakraborty, S.P., Pramanik, P. and Roy, S. (2012). A Review On- Emergence Of Antibiotic Resistant *Staphylococcus aureus* And Role Of Chitosan Nanoparticle In Drug Delivery, *International Journal of Life Science and Pharmaceutical Research*,**2**(1):96-115

Chang, S., Sievert, D.M., Hageman, J.C., Boulton, M.L., Tenover, F.C., Downes, F.P., Shah, S., Rudrik, J.T., Pupp, G.R., Brown, W.J., Cardo, D. and Fridkin, S.K. (2003). Infection with vancomycin-resistant *Staphylococcus aureus* containing the *vanA* resistance gene. *New England Journal of Medicine*, **348**(14):1342-1347.

Cheesbrough, M. (2006): District laboratory practice in tropical countries, Part 2, Second Edition Cambridge University Press, 135–162.

Cheung, A.L. and Zhang, G. (2002).Global regulation of virulence determinants in *Staphylococcus aureus* by the SarA protein family. *Frontiers in Bioscience*, **7**: d1825-d1842.

Cheung, A.L., Bayer, A.S., Zhang, G., Gresham, H. and Xiong, Y.Q. (2004).Regulation of virulence determinants *in vitro* and *in vivo* in *Staphylococcus aureus*. *FEMS Immunology and Medical Microbiology*,**40**:1-9.

Cheung, A.L., Koomey, J.M., Butler, C.A., Projan, S.J. and Fischetti, V.A. (1992).Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (sar) distinct from agr. *Proceedings of the National Academy of Sciences*, **89**(14):6462-6466.

Clements, M.O., Watson, S.P., Poole, R.K. and Foster, S.J. (1999). CtaA of *Staphylococcus aureus* is required for starvation survival recovery cytochrome biosynthesis. *Journal of Bacteriology,* **181**:501–507.

Clinical and Laboratory Standards Institute.(2015). Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Informational Supplement.M100-S25.Volume 35(3).Clinical and Laboratory Standards Institute. Wayne, PA.

Cosgrove, S. E., Carroll, K.C., and Perl, T. M., (2004).Susceptibility to vancomycin. *Clinical Infectious Diseases*, **39**:539-545

Costa, A.R., Batistão, D.W.F., Ribas, R.M., Sousa, A.M., Pereira, M.O. and Botelho, C.M.(2013).Microbial pathogens and strategies for combating them: science, technology and education (A. Méndez-Vilas, Ed.) *Staphylococcus aureus* virulence factors and disease, 702-710.

Courvalin, P. (2006). Vancomycin resistance in Gram positive cocci. *Clinical Infectious Diseases,***42**(1):S25-S34.

Cowan, S.T. and Steel, K.J. (2003). Manual for the Identification of Medical Bacteria 3rd ed. / edited and rev. by G.I. Barrow and R.K.A. Feltham.Cambridge University Press. London.

Crossley, K.B. and Archer, G.L. (1997).*The Staphylococci in human disease*. Churchill Livingstone.

Darko, W., Medicis, J.J. and Smith, A. (2003).Mississippi mud no more: cost-effectiveness of pharmacokinetic dosage adjustment of vancomycin to prevent nephrotoxicity. *Pharmacotherapy,* **23**:643–50.

de Haas, C.J.C., Veldkamp, K.E., Peschel, A., Weerkamp, F., van Wamel, W.J.B., Heezius, E.C.J.M., Poppelier, M.J.J.G., van Kessel, K.P.M. and van Strijp, J.A.G. (2004). Chemotaxis Inhibitory Protein of *Staphylococcus aureus*, a Bacterial Antiinflammatory Agent. *The Journal of Experimental Medicine,***199**(5):687-695.

Diekema, D.J., Pfaller, M.A., Schmitz, F.J., Smayevsky, J., Bell, J., Jones, R.N. and Beach, M. (2001). Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, the Western Pacific region for the SENTRY Antimicrobial Surveillance Program 1997–1999. *Clinical Infectious Diseases,* **32**(2):114–132.

Diep, B.A., Gill, S.R., Chang, R.F., Phan, T.H., Chen, J.H., Davidson, M.G. (2006). Complete genome sequence of USA300 an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. *Lancet,* **367**: 731–739.

Dinges, M.M., Orwin, P.M. and Schlievert, P.M. (2000). Exotoxins of *Staphylococcus aureus*. *Clinical Microbiology Reviews,* **13**(1):16-34.

Donabedian, S., Hershberger, E., Thal, L. A., Chow, J. W., Clewell, D. B., Dunn, B. R. and Zervos, M. J.(2000). PCR fragment length polymorphism analysis of vancomycin-resistant *Enterococcus faecium. Journal of Clinical Microbiology,* **38**:2885–2888.

El-Shenawy, M., Tawfeek, M., El-Hosseiny, L., El-Shenawy, M., Farag, A., Baghdadi, H Saleh, O., Mañes, J. and Soriano J M. (2014) Cross Sectional Study of Skin Carriage and Enterotoxigenicity of *Staphylococcus aureus* among Food Handlers. *Open Journal of Medical Microbiology*, **4**:16-22.

Elting, L.S., Rubenstein, E.B., Kurtin, D. (1998). Mississippi mud in the 1990s: risks and outcomes of vancomycin-associated toxicity in general oncology practice. *Cancer,* **83**:2597–607.

File, T.M. (2008). Methicillin-resistant *Staphylococcus aureus* (MRSA): focus on community associated MRSA. *South African Journal of Epidemiology and Infection*, **23**:13-15.

Fitzpatrick, F., Humphreys, H.O. and Gara, J.P. (2005). The genetics of staphylococcal biofilm formation — will a greater understanding of pathogenesis lead to better management of device-related infection? *Clinical Microbiology and Infection,* **11**:967–973.

Foster, T.J. (1996).*Staphylococcus*., in: Baron (Ed.), *Medical Microbiology,* University of Texas Medical Branch at Galveston, Texas.

Foster, T.J. (2005) Immune evasion by staphylococci. *Nature Review Microbiology*,**3**:948-958.

Foster, T.J. and Hook, M. (1998) Surface protein adhesins of *Staphylococcus aureus*. *Trends in Microbiology,* **6**(12):484–488.

Fridkin, S. K., Steward, C. D., and Edwards, J. R. (1999).*Surveillance of antimicrobial use and antimicrobial resistance in United States hospitals*: project ICARE phase 2. Project Intensive Care Antimicrobial Resistance Epidemiology (ICARE) hospitals. *Clinical Infectious Diseases,***29**:245–52.

Gally, D. and Archibald, A.R. (1993). Cell wall assembly in *Staphylococcus aureus*: proposed absence of secondary crosslinking reactions. *Journal of General Microbiology,* **139**:1907–1913.

Georges, H., Leroy, O., Alfandari, S. (1997).Pulmonary disposition of vancomycin in critically ill patients. *European Journal of Clinical Microbiology and Infectious Diseases,* **16**:385-388.

Ghoniem, E.M., El Hendawy, G.R., Abdel Moteleb, T.M., Hassan, H.A. and El Refai Khalil, H.A.(2014).Characterization of Vancomycin Resistant *Staphylococcus aureus* in National Liver Institute. *Menonfia Medical Journal,* **27**:825-832

Gill, S.R., Fouts, D.E., Archer, G.L., Mongodin, E.F., Deboy, R.T, Ravel, J. *et al. (*2005) Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing

methicillin-resistant *Staphylococcus epidermidis* strain. *Journal of Bacteriology,* **187**: 2426–2438.

Gordon, R.J. and Lowy, F.D. (2008).Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. *Clinical Infectious Diseases,* **46(**5):S350–S359.

Grinholc, M., Wegrzyn, G. and Kurlenda, J. (2007). Evaluation of biofilm production and prevalence of the icaD gene in methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains isolated from patients with nosocomial infections and carriers. *FEMS Immunology and Medical Microbiology,* **50**:375–379.

Grundmann, H., Aires-de, S. and Boyce, I. (2006).Emergence and resurgence of methicillin resistant *Staphylococcus aureus* as a public health. *The Lancet,* **368**:874-885.

Hamilton-Miller, J.M. (2002). Vancomycin-resistant *Staphylococcus aureus*: a real and present danger? *Infection,* **30**:118-124.

Hanaki, H., Kuwahara-Arai, K., Boyle-Vavra, S., Daum, R. S, Labischinski, H., and Hiramatsu, K., (1998).Activated cell-wall synthesis is associated with vancomycin resistance in methicillin-resistant *Staphylococcus aureus* clinical strains Mu3 and Mu50.*Journal of Antimicrobials and Chemotherapy*, **42**:199–209.

Handwerger, S. and Skoble, J. (1995).Identification of chromosomal mobile element conferring high-level vancomycin resistance in *Enterococcus faecium. Antimicrobial Agents Chemotherapy,* **39**:2446-2453.

Harraghy, N., Hussain, M., Haggar, A., Chavakis, T., Sinha, B., Herrmann, M. and Flock, J.I. (2003).The adhesive and immunomodulating properties of the multifunctional *Staphylococcus aureus* protein Eap.*Microbiology,***149**(10):2701-2707.

Harris, L., Foster, S. and Richards, R. (2002).An introduction to *Staphylococcus aureus*, and techniques for identifying and quantifying *S. aureus* adhesins in relation to adhesion to biomaterials: review. *European Cells and Materials,***4**:39-60.

Hartleib, J., Kohler, N., Dickinson, R.B., Chhatwal, G.S., Sixma, J.J., Hartford, O.M. *et al.* (2000) Protein A is the von Willebrand factor binding protein on *Staphylococcus aureus*. *Blood,* **96**:2149–2156.

Hauck, C.R. and Ohlsen, K. (2006). Sticky connections: extracellular matrix protein recognition and integrin-mediated cellular invasion by *Staphylococcus aureus*. *Current Opinion Microbiology,* **9**:5–11.

Hemen, J.T., Johnson, J.T., Ambo, E.E., Ekam, V.S., Odey, M.O., and Fila, W.A. (2012): Multi-Antibiotics Resistance of Some Gram negative Bacterial isolates from Poultry Litters of Selected Farms in Benue state. *International Journal of Science and Technology*,**2**(8):543-547.

Heyman, D. (2004). *Control of Communicable Diseases Manual.*18th edn. American Public Health Association, Washington DC.

Hiramatsu, K. (2001). Vancomycin-resistance *Staphylococcus aureus*: a new model of antibiotic resistance. *Lancet Infectious Diseases*, **1**:147-155.

Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T. and Tenover, F.C. (1997).Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. *Journal of Antimicrobial Chemotherapy,* **40**:135-136.

Ho, P.L., Chuang, S.K. and Choi, Y.F. (2008). Community associated methicillin-resistant and methicillin sensitive *Staphylococcus aureus*: skin and soft tissue infections in Hong Kong. *Diagnostic Microbiology and Infectious Disease*, **61**:245-250.

Ikeagwu, I.J., Amadi, E.S., and Iroha, I.R. (2008): Antibiotic sensitivity pattern of *Staphylococcus aureus* in Abakaliki, Nigeria, *Pakistan Journal of Medical Sciences*, **24**: 23-235.

Jun, I.S., Tomoko, F., Katsutoshi, S., Hisami, K., Haruo, N. and Akihiko, K. (2004).Prevalence of erythromycin, tetracycline, and aminoglycoside–resistance genes in methicillin resistant *Staphylococcus aureus* in hospitals in Tokyo and Kumamoto. *Japanese Journal of Infectious Diseases,* **57**:75–7.

Kaneko, J. and Kamio, Y. (2004). Bacterial two-component and hetero-heptameric pore forming cytolytic toxins: structures pore-forming mechanism organization of the genes. *Bioscience, Biotechnology and Biochemistry,* **68**:981–1003.

Klevens, R. M., Morrison, M. A. and Nadle, J. (2007): Invasive methicillin-resistant *Staphylococcus aureus* infections in United States. *Journal of American Medical Association*, **298**:1763-1771.

Kloos, W.E. and Lambe, D.W.J. (1991).*Staphylococcus*., in: Barlows A, Hausler WJ, Hermann KL, Isenberg HD, and Shadomy HJ (Eds.), Manual of Clinical Microbiology, ASM, Washington D.C, Pp. 222-237.

Kluytmans, J., van , B.A. and Verbrugh, H. (1997). Nasal carriage of *Staphylococcus aureus*: epidemiology underlying mechanisms associated risks. *Clinical Microbiology Review*, **10**(3):505-520.

Kotzin, B.L., Leung, D.Y., Kappler, J. and Marrack, P. (1993).Superantigens and their potential role in human disease. *Advances in Immunology*, **54**:99-166.

Kruzel, M.C., Lewis, C.T., welsh, K.J., Lewis, E.M., Dundas, N.E., Mohr, J.F., Armitige, L.Y. and Wanger, A. (2011). Determination of Vancomycin and Daptomycin MICs by Different Testing Methods for Methicillin Resistant *Staphylococcus aureus. Journal of Clinical Microbiology*, **49**(6)2272-2273.

Kuroda, M., Ohta, T., Uchiyama ,I., Baba, T., Yuzawa, H., Kobayashi, I.(2001) Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet,* **357**:1225-1240.

Labischinski, H. (1992). Consequences of the interaction of beta-lactam antibiotics with penicillin binding proteins from sensitive and resistant *Staphylococcus aureus* strains. *Medical Microbiology and Immunology*, **181**:241–265.

Lamer, C., de Beco, V., Soler, P. (1993).Analysis of vancomycin entry into pulmonary lining fluid by bronchoalveolar lavage in critical ill patients. *Antimicrobial Agents and Chemotherapy*,**37**:281–6.

Lannergard, J., von, E.C., Sander, G., Cordes, T., Seggewiss, J., Peters, G. *et al.* (2008).Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, **52**: 4017–4022.

Leclercq, R., Derlot, E., Duval, J., and Courvalin, P., (1988).*Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium*. *New England Journal of Medicine*, **319**:157–161.

Leclercq, R., Dutka-Malen, S., Duval, J. and Courvalin, P. (1992).Vancomycin resistance gene *vanC* is specific to *Enterococcus gallinarum. Antimicrobial Agents and Chemotherapy*, **36**:2005–2008.

Lee, L.Y.L., Höök, M., Haviland, D., Wetsel, R.A., Yonter, E.O., Syribeys, P., Vernachio, J. and Brown, E.L. (20004).Inhibition of Complement Activation by a Secreted *Staphylococcus aureus* Protein. *Journal of Infectious Diseases,* **190**(3):571-579.

Lee, L.Y.L., Liang, X., Höök, M. and Brown, E.L. (2004).Identification and characterization of the C3 binding domain of the *Staphylococcus aureus* extracellular fibrinogen-binding protein (Efb).*Journal of Biological Chemistry*,**279**(49):50710-50716.

Levine, D. (2006). Vancomycin: A History. *Clinical Infectious Diseases*,**42**(1):S5-12.

Lin, Y.C. and Peterson, M.L. (2010).New insights into the prevention of staphylococcal infections and toxic shock syndrome. *Expert Review of Clinical Pharmacology*, **3**(6):753-767.

Lindsay, J.A. and Holden, M.T. (2004).*Staphylococcus aureus*: superbug super genome? *Trends in Microbiology*, **12**:378–385.

Lindsay, J.A., Ruzin, A., Ross, H.F., Kurepina, N. and Novick, R.P. (1998). The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. *Molecular Microbiology,* **29**:527–543.

Liu, G.Y., Essex, A., Buchanan, J.T., Datta, V., Hoffman, H.M., Bastian, J.F., Fierer, J. and Nizet, V. (2005). *Staphylococcus aureus* golden pigment impairs neutrophil killing and

promotes virulence through its antioxidant activity. *The Journal of Experimental Medicine*, **202**(2):209-215.

Livermore, D.M. (2001). Antibiotic resistance in staphylococci. *International Journal of Antimicrobial Agents*, **16**:3-10.

Lodise, T.P., Lomaestro, B. and Graves, J. (2008). Larger vancomycin doses (≥4 grams/day) are associated with an increased incidence of nephrotoxicity. *Antimicrobial Agents and Chemotherapy*, **52**:1330–1336.

Lowy, F.D. (1998).*Staphylococcus aureus* infections. *New England Journal of Medicine*,**339**(8):520-532.

Lujan, S.A., Guogas, L.M., Ragonese, H., Matson, S.W. and Redinbo, M.R. (2007)."Disrupting antibiotic resistance propagation by inhibiting the conjugative DNA relaxase".*PNAS*, **104**(30):12282–12287

Menestrina, G., Serra, M.D. and Prevost, G. (2001).Mode of action of beta-barrel pore-forming toxins of the staphylococcal alpha-hemolysin family. *Toxicon*, **39**:1661–1672.

Moellering, R.C. Jr (2006). Vancomycin: A 50-Year Reassessment. *Clinical Infectious Diseases*, **42**(1):3-4.

Mohammedi, I., Descloux, E. and Argaud, L. (2006). Loading dose of vancomycin in critically ill patients: 15 mg/kg is a better choice than 500 mg. *International Journal of Antimicrobial Agents*, **27**:259–262.

Morfeldt, E., Taylor, D., von Gabain, A. and Arvidson, S. (1995). Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAIII. *EMBO Journal*, **14**:4569–4577.

Moses, A., Uchenna, U and Nworie, O. (2013).Epidemiology of Vancomycin Resistant *Staphyloccus aureus* among Clinical Isolates in a Tertiary Hospital in Abakaliki, Nigeria."*American Journal of Epidemiology and Infectious Disease*,**1**(3):24-26.

Murray, B.E. and Nannini, E.C. (2005).Glycopeptides (vancomycin and teicoplanin), streptogramins (quinupristin-dalfopristin), and lipopeptides (daptomycin). In: Mandell, G.L., Bennett, J.E., Dolin, R., eds. Mandell, Douglas and Bennett's principles and practice of infectious diseases. 6th ed. Oxford: Churchill Livingstone, Pp. 417–40.

Naing, L., Winn, T. and Rusli, B.N. (2006). Practical issues in calculating the sample size for prevalence studies. *Archives of Orofacial Sciences*, **1:**9-14.

Navarro, F. and Courvalin, P. (1994).Analysis of genes encoding d-alanine-d-alanine ligase-related enzymes in *Enterococcus casseliflavus* and *Enterococcus flavescens. Antimicrobial Agents Chemotherapy*, **38**:1788–93.

Neu, H.C. (1994). Emerging trends in antimicrobial resistance in surgical infections.A review.*The European journal of surgery*, **573**:7-18.

Noble, W.C., Virani, Z. and Cree, R.G. (1992).Cotransfer of vancomycin and other resistant genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*. *FEMS Microbiology Letter,***72**:195–198.

Novick, R.P. (2003). Mobile genetic elements and bacterial toxinoses: the superantigen-encoding pathogenicity islands of *Staphylococcus aureus*. *Plasmid*,**49**: 93–105.

Novick, R.P. and Geisinger, E. (2008).Quorum Sensing in Staphylococci.*Annual Review of Genetics*, **42**(1), 541-564.

Novick, R.P. and Jiang, D. (2003).The staphylococcal saeRS system coordinates environmental signals with agr quorum sensing. Microbiology **149**(10): 2709-2717.

Novick, R.P., Ross, H.F., Projan, S.J., Kornblum, J. and Kreiswirth.(1993). Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO Journal*,**12**(10): 3967-3975.

Oliveira, G. A., Dell'Aquila, A. M., and Masiero, R.L. (2001).*Isolation in Brazil of nosocomial Staphylococcus aureus with reduced susceptibility to vancomycin*. *Infection Control and Hospital Epidemiology*, **22**:443–8.

Okolie, C.E., Essien, U.C. and Idoko, J. (2015). Genetic and Phenotypic Identification of Vancomycin-Resistant *Staphylococcus aureus* Isolates from Retail Poultry Carcasses in Omu-Aran, North-Central Nigeria, *British Biotechnology Journal,* **6**(2):87-92.

Onanuga, A. and Temedie, T.C. (2011).Multidrug-resistant, intestinal *Staphylococcus aureus*in self-medicated, healthy adults in Amassoma, south-south, Nigeria. *Journal of Health Population and Nutrition*,**29**(5):446-53.

Otalu, O. Jr., Kabir, J., Okolocha, E.C. and Umoh, V.J. (2011). Multi-drug Resistant Coagulase Positive *Staphylococcus aureus* from Live and Slaughtered Chickens in Zaria, Nigeria. *International Journal of Poultry Science,* **10**(11):871-875.

Patti, J.M., Bremell, T., Krajewska-Pietrasik, D., Abdelnour, A., Tarkowski, A., Ryden, C. and Hook, M. (1994). The *Staphylococcus aureus* collagen adhesin is a virulence determinant in experimental septic arthritis. *Infectious Immunology*, **62**:152–161.

Peacock, S.J., De Silva, I. and Lowry, F.D. (2001). What determines nasal carriage of *Staphylococcus aureus*? *Trends in Microbiology*,**9**(12):605-610.

Percival SL, Chalmers RM, Embrey M, Hunter PR, Sellwood J, Wyn-Jones P (2004). Microbiology of Waterborne Diseases. Elsevier Academic Press, San Diego, USA.

Perichon, B. and Courvalin, P. (2006).Synergism between beta-lactams and glycopeptides against *vanA* type methicillin-resistant *Staphylococcus aureus* and heterologous expression of the *vanA* operon. *Antimicrobial Agents and Chemotherapy*, **50**:3622-3630.

Plata, K., Rosato, A.E. and Węgrzyn, G. (2009).*Staphylococcus aureus* as an infectious agent: overview of biochemistry and molecular genetics of its pathogenicity. *Acta Biochimica Polomica*, **56**(4):597–612.

Plata, K., Rosato, A.E. and Węgrzyn, G. (2009) *Staphylococcus aureus* as an infectious agent: overview of biochemistry and molecular genetics of its pathogenicity, **56**(4):597–612.

Prakash, M., Karthikeyan, V., Karmegam, K.S. (2006).*Journal of Ecotoxicology and Environmental Monitoring*, **16**:387-389

Prasad.M.P. (2014).Molecular characterization of *Van A* gene from vancomycin resistant *Staphylococcus aureus* isolated from clinical samples. *European Journal of Biotechnology and Bioscience*,**2**(3):43-47.

Prat, C., Bestebroer, J, de Haas, C.J.C., van Strijp, J.A.G. and van Kessel, K.P.M. (2006).A new staphylococcal anti-inflammatory protein that antagonizes the formyl peptide receptor-like 1.*The Journal of Immunology*,**177**(11):8017-8026.

Primo, M.G., Guilarde A.O., Martelli, C.M., Batista, L.J. and Turchi, M.D. (2012). Healthcare-associated *Staphylococcus aureus* bloodstream infection: Length of stay, attributable mortality and additional direct costs. *Brazilian Journal of Infectious Diseases*, **16**: 503-509.

Proctor, R.A., van Langevelde, P., Kristjansson, M., Maslow, J.N. and Arbeit, R.D. (1995). Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. *Clinical Infectious Diseases*, **20**:95–102.

Proctor, R.A., von Eiff, C., Kahl, B.C., Becker, K., McNamara, P., Herrmann, M. and Peters, G. (2006). Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nature Review Microbiology*, **4**:295–305.

Rajaduraipandi, K., Mani, K.R., Panneerselvam, K., Mani, M. and Bhaskar, M. Manikandan P. (2006). Prevalence and antimicrobial susceptibility pattern of methicillin resistant *Staphylococcus aureus*: A multicentre study. *Indian Journal Medical Microbiology*, **24**(1):34-38.

Ray, C. and Ryan, K.J. (2003). Sherris Medical Microbiology : An Introduction to Infectious Diseases.

Reynolds, P.E. (1998). Control of peptidoglycan synthesis in vancomycin-resistant enterococci: D,D-peptidases and D,D-carboxypeptidases. *Cell Molecular and Life Sciences*, **54**:325–31.

Reynolds, P.E. (1998). Structure, Biochemistry and Mechanism of Action of glycopeptides antibiotics. *European Journal Clinical Microbiology Infectious Diseases,* **8**(11):943-950.

Reynolds, P.E. (2005). Courvalin P. Vancomycin resistance by synthesis of precursors terminating in d-alanyl-d-alanine. *Antimicrobial Agents Chemotherapy,***49**:21–25.

Reynolds, P.E., Arias, C.A. and Courvalin, P. (1999).Gene *van XYC* encodes D,D-dipeptidase (VanX) and D,D-carboxypeptidase (VanY) activities in vancomycin-resistant *Enterococcus gallinarum* BM4174. *Molecular Microbiology*, **34**:341–9.

Reynolds, P.E., Depardieu, F., Dutka-Malen, S., Arthur, M. and Courvalin, P. (1994).Glycopeptide resistance mediated by enterococcal transposon Tn1546 requires production of VanX for hydrolysis of d-alanyl-d-alanine. *Molecular Microbiology*, **13**:1065–70.

Rohde, H., Knobloch, J.K., Horstkotte, M.A. and Mack, D. (2001).Correlation of *Staphylococcus aureus* icaADBC genotype and biofilm expression phenotype. *Journal of Clinical Microbiology*, **39**:4595–4596.

Rohrer, S., Maki, H. and Berger-Bachi, B. (2003). What makes resistance to methicillin heterogeneous? *Journal of Medical Microbiology,* **52**:605–607.

Rooijakkers, S.H.M., Ruyken, M., Roos, A., Daha, M.R., Presanis, J.S., Sim, R.B., van Wamel, W.J.B., van Kessel, K.P.M. and van Strijp, J.A.G. (2005). Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. *Nature Immunology,* **6**(9):920-927.

Ruzin, A., Lindsay, J. and Novick, R.P. (2001). Molecular genetics of SaPI1 — a mobile pathogenicity island in *Staphylococcus aureus*. *Molecular Microbiology,* **41**:365–377.

Ryan, K.J. and Ray, C.G. (2004).*Sherris Medical Microbiology: An Introduction to Infectious Diseases*. 4th edn. McGraw Hill Publishers.

Rybak, M.J. (2006). The pharmacokinetic and pharmacodynamics properties of vancomycin. *Clinical Infectious Diseases,* **42**(1):S35–S39.

Saha, B., Saha, D., Niyogi, S. and Bal, M. (1989). A new method of plasmid DNA preparation by sucrose-mediated detergent lysis from *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive). *Annals of Biochemistry*, **176**:344–349.

Saha, B., Singh, A.K., Ghosh, A. and Bal, M. (2008). Identification and characterization of a vancomycin resistant *Staphylococcus aureus* isolated from Kolkata (South Asia). *Journal of Medical Microbiology*, **57**:72-79.

Salgado, C. D., Farr, B. M., and Calfee, D. P. (2003).Community-acquired methicillin-resistant *Staphylococcus aureus:* a meta-analysis of prevalence and risk factors. *Clinical Infectious Diseases,* **36**:131–9.

Scheffers, D.J. and Pinho, M.G. (2005). Bacterial cell wall synthesis: new insights from localization studies. *Microbiology and Molecular Biology Reviews,* **69**:585–607.

Schito, G.C. (2006) The importance of the development of antibiotic resistance in *Staphylococcus aureus*. *Clinical Microbiology and Infection,* **12(**1):3–8.

Sievert, D.M., Boulton, M.L., Stoltman, G., Johnson, D., Stobierski, M.G., Downes, F.P., Somsel, P.A., Rudrik, J.T., Brown, W., Hafeez, W., Lundstrom, T., Flanagan, E., Johnson, R., Mitchell, J. and Chang, S. (2002). *Staphylococcus aureus* resistant to vancomycin—United States, *Morbidity and Mortality Weekly Reports,* **51**:565–567.

Skhirtladze, K., Hutschala, D. and Fleck, T. (2006).Impaired target site penetration of vancomycin in diabetic patients following cardiac surgery. *Antimicrobial Agents and Chemotherapy*,**50**:1372–5.

Srinivasan, A., Dick, J.D. and Perl, T.M. (2002).Vancomycin resistance in staphylococci. *Clinical Microbiology Review*, **15**:430–438.

Stevens, D.L. (2006). The role of vancomycin in the treatment paradigm. *Clinical and Infectious Diseases,* **42**(1):S51–7.

Switalski, L.M., Patti, J.M., Butcher, W., Gristina, A.G., Speziale, P. and Hook, M. (1993). A collagen receptor on *Staphylococcus aureus* strains isolated from patients with septic arthritis mediates adhesion to cartilage. *Molecular Microbiology,* **7**:99–107.

Talaro, K. P. and Talaro, A. (2002): Foundations in Microbiology. 4 Ed. McGraw Hill, New York, 544–552.

Tenover, F.C., Lancaster, M.V., Hill, B.C. (1998). Characterization of staphylococci with reduced susceptibility to vancomycin and other glycopeptides. *Journal of Clinical Microbiology*, **36**:1020-1027.

Tenover, F.C., Weigel, L.M., Appelbaum, P.C., McDougal, L.K., Chaitram, J., McAllister, S., Clark, N., Killgore, G., O'Hara, C.M., Jevitt, L., Patel, J.B. and Bozdogan, B. (2004) Vancomycin-resistant *Staphylococcus aureus* isolate from a patient in Pennsylvania. *Antimicrobial Agents and Chemotherapy*, **48**(1):275-280.

Thati, V., Shivannavar, C.T. and Gaddad, S.M. (2011).Vancomycin resistance among methicillin resistant *Staphylococcus aureus* isolates from intensive care units of tertiary care hospitals in Hyderabad. *Indian Journal of Medical Research,* **134**(5):704–708.

Tiwari, H.K and Sen, M.R. (2006).Emergence of vancomycin resistant *Staphylococcus aureus* (VRSA) from a tertiary hospital from northern part of India. *BMC Infectious Diseases,* **6**:156-161.

Tiwari, H.K., Sapkota, D., and Sen M. R. (2008).Infection and Drug Resistance.**1**:57–61

Torres, V.J., Attia, A.S., Mason, W.J., Hood, M.I., Corbin, B.D., Beasley, F.C., Anderson, K.L., Stauff, D.L., McDonald, W.H., Zimmerman, L.J., Friedman, D.B., Heinrichs, D.E., Dunmsn, P.M., and Skaar, E.P. (2010). *Staphylococcus aureus* fur regulates the expression of virulence factors that contribute to the pathogenesis of pneumonia. *Infection and Immunity,* **78**(4):1618-1628.

Ubeda, C., Maiques, E., Knecht, E., Lasa, I., Novick, R.P. and Penades, J.R. (2005). Antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci. *Molecular Microbiology,* **56**:836–844.

Uttley, A. H., Collins, C. H., and Naidoo, J., (1988).Vancomycin-resistant enterococci. *Lancet*, **1**:57–8.

Virgincar, N. and MacGowan, A. (2004).Glycopeptides (dalabavancin, oritavancin, teicoplanin, vancomycin). In: Yu, V.L., Edwards, G., McKinnon, P.S.*et al*., eds. Antimicrobial therapy and vaccines. Volume II: antimicrobial agents. 2nd ed. Pittsburgh: ESun Technologies, Pp181–99.

von Eiff, C. (2008). *Staphylococcus aureus* small colony variants: a challenge to microbiologists and clinicians. *International Journal of Antimicrobial Agents,* **31**: 507–510.

Wang, J.T., Chang, S.T. and Ko, W.J. (2001). A hospital acquired outbreak of methicillin-resistant *Staphylococcus aureus* infection initiated by a surgeon carrier. *Journal of Hospital Infection*, **47**:104-109.

Wang, Q.M., Peery, R.B., Johnson, R.B., Alborn, W.E., Yeh, W.K. and Skatrud, P.L. (2001).Identification and characterization of a monofunctional glycosyltransferase from *Staphylococcus aureus*. *Journal of Bacteriology,* **183**:4779–4785.

Warren, R. (2012). *Staphylococcus aureus* infection and antibiotic resistance on older adult. *Clinical Infectious Diseases,* **34**:211-216.

Wayne, P. A., (2007). National Committee for Clinical Laboratory Standards. *Methods for antimicrobial susceptibility tests for bacteria that grow aerobically. 5th ed*. Approved standard, M7-A5. National Committee for Clinical Laboratory Standards.

Wenzel, R.P., and Edmond, M. B. (1998).*Vancomycin-resistant Staphylococcus aureus: infection control considerations*. *Clinical Infectious Diseases,* **27**:245–51.

Wertheim, H.F., Melles, D.C., Vos, M.C., van Leeuwen, W., van Belkum, A., Verbrugh, H.A. and Nouwen, J.L. (2005).The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infectious Dieasess*,**5**:751–762.

Wertheim, H.F.L., Melles, D.C., Vos, M.C., van Leeuwen, W., van BelKum, A, Verbrugh, H.A. and Nouwen, J.L. (2005). The role of nasal carriage in *Staphylococcus aureus* infections. *The Lancet Infectious Diseases,* **5**(12):751-762.

Whitener, C. J., Park, S. Y., and Browne, F.A., (2004).*Vancomycin- resistant Staphylococcus aureus in the absence of vancomycin exposure*. *Clinical Infectious Diseases,* **38**:1049-1055.

Wilhelm, M.P. and Estes, L. (1999). Symposium on antimicrobial agents—part XII. *Vancomycin. Mayo Clinic Proc.*, **74**:928–935.

Wilkinson, B.J. (1997). Biology in: Crossley, K.B. and Archer, G.L. (Eds.), The Staphylococci in Human Diseases. Churchill Livingston, London, Pp. 1-38.

Winn Washington, A.S., Janda. W., Koneman, E., Procop, G., Schreckenberger, P., Woods G.(2006).*Koneman's Color Atlas and Textbook of Diagnostic Microbiology*. LippincottWilliams & Wilkins, Philadelphia.

Wu, S., de Lencastre, H. and Tomasz, A. (1996).Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing. *Journal of Bacteriology*, **178**(20):6036-6042.

APPENDICES

MINISTRY OF HEALTH, KADUNA STATE

All Communication to be addressed to:
THE HON. COMMISSIONER
Quoting Reference and Date
Telephone: 234-248048
Website: ttp://www/moh.kd.gov.ng.
Email: info@moh.kd.gov.ng.

Independence Way,
P.M/;B 2014
Kaduna.
Kaduna State, Nigeria.

MOH/ADM/744/VOL.I/302

11th June, 2015

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NOTICE OF APPROVAL AFTER FULL COMMITTEE REVIEW

RE: PHENOTYPIC AND GENOTYPIS CHARACTERIZATION OF VANCOMYCIN RESISTANCE STAPHYLOCOCCUS AUREUS ISOLATED FROM PATIENTS ATTENDING SOME SELECTED HOSPITAL IN KADUNA METROPOLIS, KADUNA STATE NIGERIA

Name of Principal Investigator	-	Usman Aisha
Address of Principal Investigator	-	Dept. of Microbiology, Faculty of Sciences, Ahmadu Bello University, Zaria.
Date of Receipt of Application	-	5th May, 2015
Date of Ethical Approval	-	28th May, 2015

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

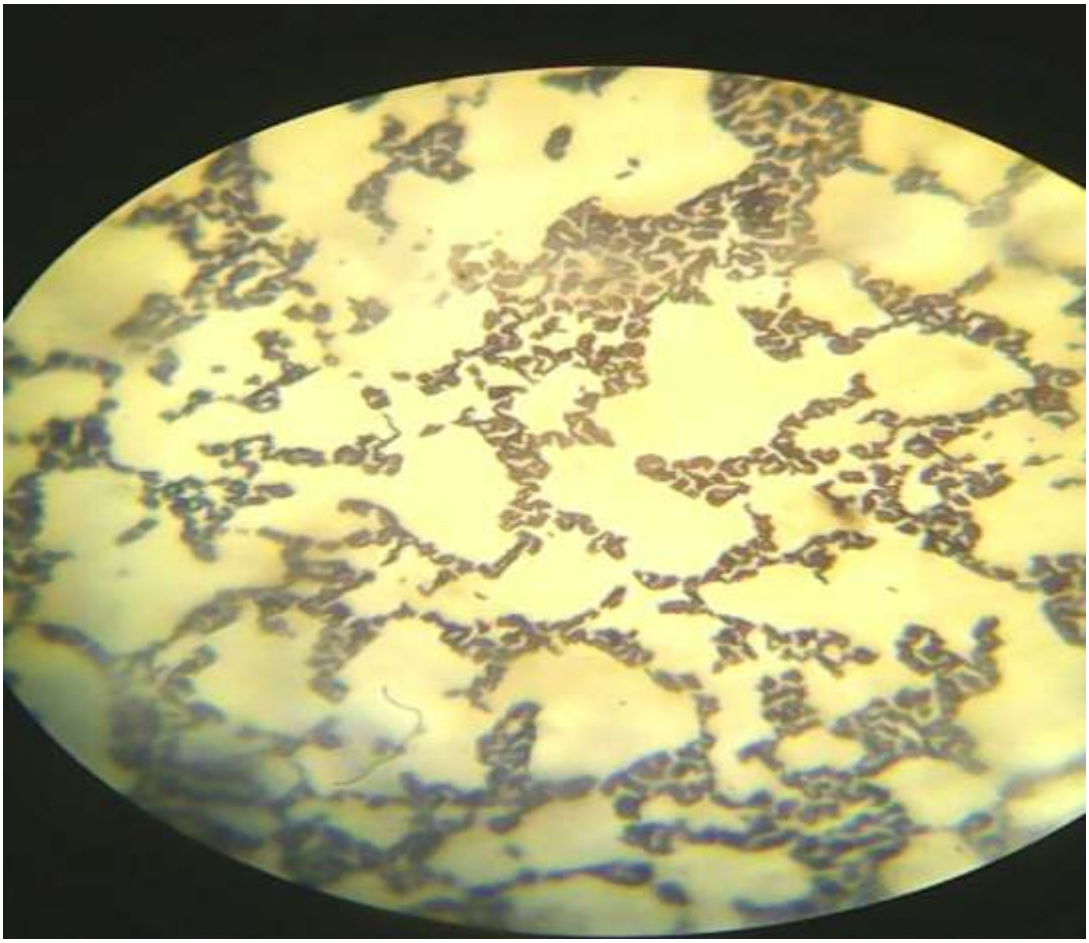
If there is delay in starting the research, inform the HREC so that the dates of approval can be adjusted accordingly.

However, researcher is kindly requested to submit a copy of his/her findings to the state Ministry of Health, please.

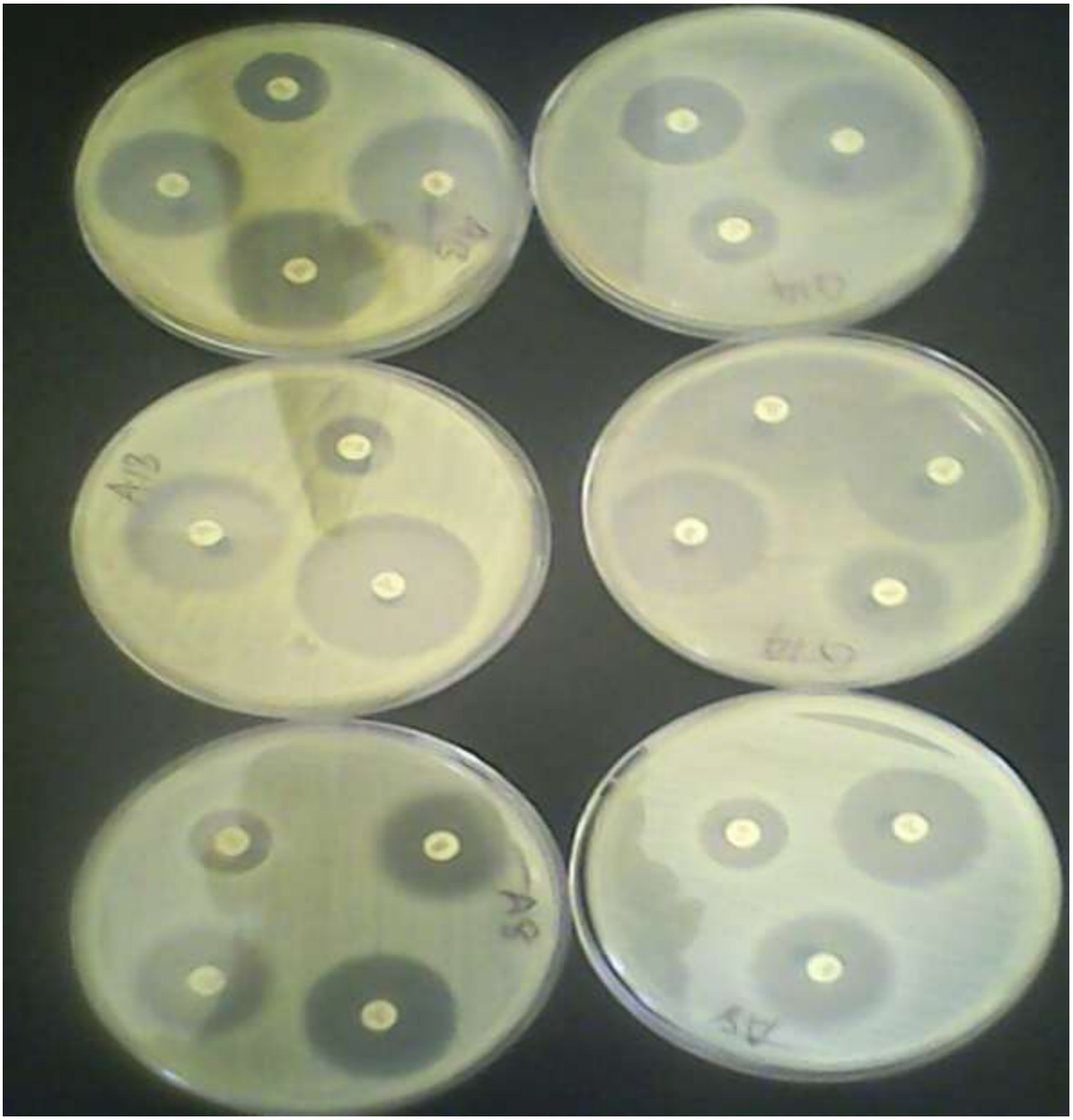
DR. B. M. JATAU
Chairman
Research Ethics Committee

KADUNA STATE MINISTRY OF HEALTH
OPEN REGISTRY
DESPATCHER
SIGN. ... DATE 11/6/15

Appendix I: Ethical approval for the study from Ministry of Health, Kaduna State.



Appendix II: Microscopic view of a Gram-stained isolate: Gram positive cocci in bunches.



Appendix III: Result of the antibiotic susceptibility test.

Appendix IV: Phenotypic characterization of the *S. aureus* isolates

Test	Reaction of *S. aureus*	Number of isolates		
		Screened	positive	percentage (%)
Colonial morphology on:				
Mannitol Salt Agar	yellow colonies	300	77	25.67
Blood Agar	creamy white colonies	300	77	25.67
Microscopic identification:				
Gram staining	Gram positive cocci in bunch.	77	68	88.31
Biochemical identification:				
Catalase test	bubbles formation.	68	61	89.71
Coagulase test	coagulation	61	53	86.89
DNase test	clear zone around the colonies	53	52	98.11
Sugar fermentation tests				
Sucrose	positive reaction (red)	53	53	100.00
Mannitol	positive reaction (red)	53	52	98.11
Arabinose	negative reaction (yellow)	53	52	98.11
Rafffinose	negative reaction (yellow)	53	52	98.11