ANTIMICROBIAL RESISTANCE PATTERNS OF METHICILLIN RESISTANT STAPHYLOCOCCI IN MEDICAL STUDENTS OF SOME NIGERIAN UNIVERSITIES

BY

ETIM, MARYLENE EFFIONG

(20134871608)

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CERTIFICATION

This is to certify that the research work "Antimicrobial resistance patterns of Methicillin resistant Staphylococci in medical students of some Nigerian universities" was carried out by **Etim, Marylene Effiong (20134871608)** in partial fulfillment for the award of a Master of Science (M. Sc) degree in Medical Microbiology in the Department of Microbiology, School of Biological Sciences, Federal University of Technology, Owerri, Imo state.

Dr. I. E Adieze Supervisor	14/11/2017 **Date**
Dr. (Mrs) E. E. Mike-Anosike Co Supervisor	09/11/17 **Date**
Dr. C. O. Nweke Head of Department	25/10/2017 Date
Prof. J. N. Ogbulie Dean, School of Biological Science	09/11/17 Date
Prof. (Mrs.) Nnenna N. Oti Dean, Postgraduate School	Date
Prof. (Mrs.) C. A. Oyeka External Examiner	25-10-2017 Date

DEDICATION

This work is dedicated to God almighty

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ABSTRACT

This study was undertaken to establish the prevalence of hospital acquired and community acquired methicillin resistant Staphylococci, to determine the antimicrobial resistance patterns and clonal relationships of isolated methicillin resistant Staphylococcifrom medical students of Imo State University, Abia State University and University of Uyo. Four hundred and forty sampleswere collected from palms, nostrils and throats of medical students and screened using standard microbiological techniques. *Staphylococcus* species isolated on the basis of growth on mannitol salt agar were further characterized based on biochemical tests, resistance to oxacillin and other antibiotics. Isolates were classified based on school, class, type of sample, species, age and gender. Data generated were analyzed descriptively and frequencies were expressed in percentages. Polymerase Chain Reaction was used to detect the *Staphylococcus nuc*, *mecA*, and *blaZ*genes. Genetic fingerprinting of 31 different isolates of methicillin resistant Staphylococci from medical students of the three schools sampled was carried out using 2 primers (*blaZ*-F and *blaZ*-R) of Random Amplified Polymorphic DNA to determine if methicillin resistant isolates are clones of each other. A total of 248 *Staphylococcus* spp. were identified as *Staphylococcus aureus* (191) and coagulase-negative Staphylococci (57). The *Staphylococcus aureus* strains were highly resistant to methicillin (oxacillin) and ampicillin (59.7%), penicillin (68.1%) and several non-β-lactams including clindamycin (28.2%), erythromycin (38.3%), trimethoprim (74.3%), and vancomycin (21.8%). The frequency of methicillin resistance among Coagulase negative Staphylococci was 94% with no resistance to nitrofurantoin (0%), moderate resistance to erythromycin (0.8%), vancomycin (4.8%) and clindamycin (13.3%). Out of 193 methicillin resistant isolates, pre-clinical students had higher frequencies with 44% Methicillin resistant *Staphylococcus aureus* compared to their clinical counterparts with 32.6%Methicillin resistant *Staphylococcus aureus* and 11.9% methicillin resistant coagulase negative Staphylococci compared with their clinical counterparts with 11.4% methicillin resistant coagulase negative Staphylococci. The prevalence rate of methicillin resistant Staphylococci for male and female group was 59.7% and 40.3% respectively. Methicillin resistant Staphylococci occurred more in students aged 26-30 years of age. The relationship between age and sex was statistically insignificant ($p>0.05$). A dendrogram was constructed usingthe Unweighted Pair Group Method with Arithmetic Averages analysis with the Random Amplified Polymorphic DNA data generated which clustered thirty-one (31) randomly selected methicillin resistant Staphylococci isolates from these schools into different clusters and sub-clusters based on their genetic relation. However, only two clones were observed in the third sub-cluster of the second cluster. All members of the respective clones are of the same origin and the clonal groupings are in accordance with the geographical distribution indicating possible relationship between host origin and genetic variation among those isolates. Polymerase Chain Reaction based Random Amplified Polymorphic DNA proved to be an easy yet helpful method for molecular typing and identification of clonal relationships. This study has demonstrated high prevalence of methicillin resistant Staphylococci among medical students in Imo State University (21.8%), Abia State University (20.2%) and University of Uyo (58.1%)and underlines the need for periodic surveillance studies of this type. Reassessment of policies on antibiotics use in Nigeria, development and enforcement of measures to prevent the spread of methicillin resistant Staphylococci infections in the community and in the hospitals are recommended.

Keywords: Methicillin, Staphylococci, Coagulase.

CHAPTER ONE

INTRODUCTION

1.1 Background

*Staphylococcus aureus*is a Gram-positive bacterium belonging to the family Staphylococaceae and is often found as a commensal on the skin, skin glands and mucous membranes particularly in the nose of healthy individuals (Shittu *et al.,* 2011). It is a versatile human pathogen causing infections ranging from relatively mild skin and soft tissue infections to life threatening sepsis, pneumonia, osteomyelitis, endocarditis as well as toxin mediated syndromes such as toxic shock syndrome and food poisoning (Shittu *et al.,* 2011).

*Staphylococcus aureus*is usually a harmless colonizer of about one third of healthy humans and is most likely found in the nares. Nasal carriage of *Staphylococcus aureus*has been closely associated with staphylococcal disease (Von Eiff *et al*., 2001). Colonization increases the risk of subsequent infection since those with *Staphylococcus aureus*infections are usually infected with their colonizing strain (Gordon and Lowy, 2008). Infection may occur when there is a breach of the skin or mucosal barrier that allows the organism access to adjoining tissues or the blood stream (Boucher *et al.,* 2010). *Staphylococcus aureus*is able to cause a large diversity of both benign and lethal infections in humans and animals because of a wide range of virulence factors that include various toxins and enzymes (Bal and Gould, 2005). It has emerged as one of the most important human pathogens and has become a leading cause of hospital and community acquired infections (Von Eiff *et al*., 2001).

Bacterial resistance to antibiotics has been recognized since the first antibiotics were introduced for clinical use(Baranovich *et al.,* 2010). Penicillin was first introduced in 1941, when less than 1% of *Staphylococcus aureus*strains were resistant to its action. By 1947, 38% of hospital strains had acquired resistance and currently over 90% of *Staphylococcus aureus*isolates are resistant to penicillin. Prior to the

introduction of penicillin for the treatment of *Staphylococcus aureus*infections in the 1940s, the mortality rate of individuals with Staphylococcal infections was about 80%. However within two years of the introduction of penicillin to medical use, penicillin-resistant strains were discovered (Baranovich *et al.*, 2010).

Increasing resistance to antibiotics is a consequence of selective pressure. By 1960, about 80% of all *Staphylococcus aureus*strains were found to be resistant to penicillin (Deurenberg and Stobberingh, 2008). Methicillin was introduced in 1959 to treat infections caused by penicillin resistant *Staphylococcus aureus*(Enright *et al.,*2002) but by 1961 there were reports of methicillin-resistant *Staphylococcus aureus*(MRSA)from hospitals. Methicillin was the first penicillinase - resistant semisynthetic penicillin to be derived from the penicillin nucleus, 6- aminopenicillanic acid (6-APA). Initially, it was used widely, but because of its toxicity it was gradually replaced with other penicillinase-resistant penicilins such as nafallin, oxacillin etc. The structure of methicillin is as shown in figure 1.1 below.

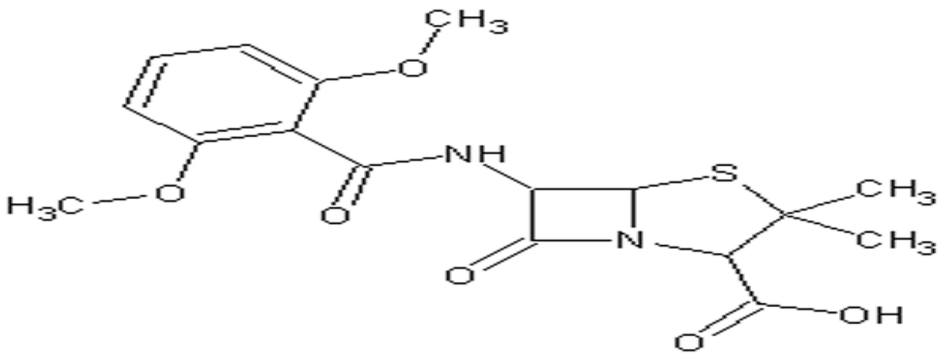

Figure1.1: Structure of Methicillin

Source: (Baranovich *et al.*, 2010).

Methicillin resistant Staphylococcus aureus (MRSA) infections can be classified into two major groups: Hospital-acquired MRSA (HA-MRSA) and Community-acquired MRSA (CA-MRSA). HA-MRSA is responsible for post-operative wound infections, or infections resulting from implanted devices such as

catheters, that are acquired within the healthcare setting (Baranovich *et al.,* 2010. Typically, patients infected with HA-MRSA are immune-compromised and the resulting infections are generally more invasive. CA-MRSA typically manifests itself as skin infections, such as pimples or boils, and is classified as being acquired outside of any type of healthcare setting. These infections are typically more serious than minor skin irritation and affect otherwise healthy individuals. Established risk factors for hospital-acquired methicillin resistant *Staphylococcus aureus*(HA-MRSA) infection include recent hospitalization or surgery, residence in a long-term care facility, dialysis and indwelling percutaneous medical devices and catheters. Cases of MRSA infections have been documented among healthy community-dwelling persons without the established risk factors for MRSA infections. These infections are referred to as community acquired or community associated MRSA infection CA-MRSA. The emergence of CA-MRSA became a cause for concern because it differs from HA-MRSA in that it does not generally belong to the major clonal groups of epidemic MRSA, is susceptible to most non β-lactam antibiotics, contains the type IV staphylococcal cassette chromosome mec and frequently carries genes responsible for the production of Panton-Valentine leukocidin (PVL) (David and Daum, 2010). The PVL toxin is associated with deep skin and soft tissue infection and necrotizing pneumonia. In contrast HA-MRSAs are generally multidrug resistant and contain SCC*mec* types I, II or III.

Studies have shown that the genetic determinants for antibiotic resistance reside on plasmids, chromosomal DNA or on transposable elements. The acquisition or loss of these genetic determinants may contribute to changes in the resistance patterns in a particular environment. The determination of the different antibiotypes of *Staphylococcus aureus* helps in monitoring the antibiotic resistance profile trends which in turn aids in the correct implementation of antibiotic regimens for*Staphylococcus aureus*infections.

Comparative genomics, including comparison at the sequence, transcriptome, and proteome levels, has been an increasingly important approach for scientists to improve knowledge on the pathogenesis and drug resistance of *Staphylococcus aureus*. To effectively develop strategies to control the dissemination of *Staphylococcus aureus*, a thorough knowledge of the spread and the molecular evolution of this bacterium is required. Therefore, several molecular typing methods have been developed during the last decades. The resulting molecular information allows the development of a hypothesis on the evolution of different populations of *Staphylococcus aureus* and also provides epidemiological information which can in turn be used to characterize clones within the hospital environment and the community. MRSA becomes resistant by acquiring a *mecA* gene, usually carried on a larger piece of DNA called a staphylococcal cassette chromosome (SCC) mec. Expression of *mecA* yields PBP 2a, a penicillin binding protein with reduced affinity for β-lactam antibiotic binding (Guiguard *et al.*, 2005).

In Nigeria, high prevalence of methicillin resistant *Staphylococcus aureus*(MRSA) in health care workers is increasingly being reported. However, very little is known regarding antibiotic resistance coagulase-negative Staphylococci (CoNS) strains isolated from health care workers, especially, given their increasing recognition as agents of clinically significant infections and as reservoirs of antimicrobial resistance determinants. In most hospitals in developing countries like Nigeria, there is neither surveillance system or control policy for methicillin resistant Staphylococci. Over the past decade, the emergence of community-associated methicillin-resistant Staphylococci (CA-MRS) has changed the landscape of Staphylococci infections around the globe. Initially recognized for its ability to cause disease in young and healthy individuals in tertiary institutions without healthcare exposures as well as for its distinct genotype and phenotype, this original description no longer fully encompasses the diversity of community-associated methicillin-resistant Staphylococci (CA-MRS) as it continues to expand its niche.

Despite the fact that medical students are exposed to patients with methicillin resistant Staphylococciinfections in the hospital during their training, information on methicillin-resistant Staphylococci among medical students in South-Eastern Nigeria remains to be dearth so as to guide appropriate infection control and preventive measures hence this study.

1.2 Problem Statement

In Nigeria, high prevalence of methicillin resistant *Staphylococcus aureus*(MRSA) in health care workers is increasingly being reported (Olayinka *et al*., 2009, Okwu *et al*., 2012). However, very little is known regarding coagulase- negative Staphylococci (CoNS) strains isolated from health care workers, especially, given their increasing recognition as agents of clinically significant infections and as reservoirs of antimicrobial resistance determinants.

Methicillin Resistant *Staphylococci* hospital acquired and community acquired infections (HA-MRS and CA-MRS) outbreaks and prevalence among various populations is well reported in literature particularly for developed countries. Hospital acquired- methicillin resistant *Staphylococci*(HA-MRS) are known causes of increased hospital stay, cost, morbidity and mortality especially among the critically ill. There is paucity of information on methicillin Resistant Staphylococci in developing nations including the carriage by critical healthcare givers who are potential transmitters. In most hospitals in developing countries like Nigeria, there is neither surveillance system or control policy for methicillin Resistant Staphylococci

Over the past decade, the emergence of community-associated methicillin-resistant Staphylococci (CA-MRS) has changed the landscape of Staphylococci infections around the globe. Initially recognized for its ability to cause disease in young and healthy individuals in tertiary institutions without healthcare exposures as well as for its distinct genotype and phenotype, this original description no longer fully

encompasses the diversity of community-associated methicillin-resistant Staphylococci (CA-MRS) as it continues to expand its niche.

Despite the fact that medical students are constantly being exposed to patients with Methicillin resistant Staphylococci infections in the hospital during their training, information on these infections among medical students in Eastern Nigeria remains to be dearth so as to guide appropriate infection control and preventive measures hence this study.

1.3 Aim and Objectives of Research.

The aim of this study is to determine the antimicrobial resistance patterns of methicillin- resistant Staphylococci isolated from healthy medical students in some Eastern Nigerian tertiary institutions

The Specific Research Objectives are:

1. To isolate and identify *Staphylococcus aureus* and coagulase negative Staphylococci from hands, nose and throat of the healthy medical students.
2. To determine the resistance patterns of isolated Staphylococci to methicillin and other commonly used antibiotics.
3. To find out the percentage of methicillin resistant Staphylococci isolates having constitutive and inducible macrolide-lincosamide-streptogramin B ($cMLS_B$) and $iMLS_B$) phenotype.
4. To evaluate isolates' susceptibility to vancomycin
5. To find out if methicillin resistant isolates are clones of each other and if they possess *mecA*, *nuc* and *blaZ* resistance genes

1.4.Justification of Research

Many studies have characterized the methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin resistant coagulase-negative Staphylococci (MRCONS) isolates from individual hospitals, schools or countries and have identified strains that appear to be well adapted to the hospital

environment, are established in several hospitals within a country,within a school or have spread internationally (epidemic MRSA- EMRSA) (Enright *et al.,* 2002). This has allowed a better understanding of the evolution of both the Staphylococci and methicillin-resistant Staphylococciover time and the ability to compare the genetic variation in different geographic locations. Such studies are important because the epidemiology and resistance patterns of the Staphylococci show large inter-regional variability. The emergence of methicillin resistant Staphylococci strains resistant to glycopeptides, as well as the increasing prevalence in the community highlights the need for worldwide epidemiological studies of this pathogen. The mechanisms for the emergence and spread of Staphylococci clones in Africa are poorly understood. Therefore the study of antimicrobial resistant patterns of isolates, the antibiotic resistance and virulence genes present in the isolates, the clonal relatedness of the Staphylococcispecies mayprovide baseline information needed in establishing effective infection control measures. InNigeria, information on the resistance trends of the Staphylococci and Methicillin resistant Staphylococci both in health-care settings and in the community is limited. There is also inadequate information on the virulence determinants of these strains. The fact that certain virulence factors can be associated with distinct human diseases strengthens the importance of examination of genes encoding pathogenicity factors. This study therefore seeks to investigate the genetic and phenotypic features of the Staphylococciisolated from pre-clinical and clinical students of Imo State University (IMSU), Abia State University (ABSU), and University of Uyo (UNIUYO) in Nigeria. Managing and controlling MRSA outbreaks can have less of a financial impact than if the outbreak is uncontrolled. The results of this study will aid implementation of strategies for the prevention and effective management of methicillin resistant Staphylococcal infections in Nigeria

CHAPTER TWO

LITERATURE REVIEW

2.1 The Staphylococci

The Staphylococci were first seen in pus by Koch in 1878 and were first cultivated in liquid medium by Pasteur in 1880 and named so by Sir Alexandar Ogston in 1881 (Bergdoll and Lee Wong, 2006). The name *Staphylococcus*was derived from Greek words staphyle (bunch of grapes) and kokkos (grain or berry) (Arora, 2006). It is estimated that 20% of human population are long term carriers of *Staphylococcus aureus*(Kluytmans *et al*., 1997) which can be found as part of the normal skin flora and in anterior nares of the nasal passage.

The Staphylococci belong to the family Micrococcaceae and are broadly divided into two main categories of clinical importance: *Staphylococcus aureus*, which are coagulase positive; and a heterogeneous group of Staphylococci that give a negative reaction with coagulase test: the coagulase-negative Staphylococci (CoNS). *Staphylococcus aureus*is characteristically associated with acute pyogenic infections whereas CoNS cause infections in susceptible hosts with certain predisposing conditions. The most common species of CoNS that causes infection is *Staphylococcus epidermidis*. Besides the coagulase test, *Staphylococcus epidermidis* differs from *Staphylococcus aureus* in being negative for mannitol fermentation reaction and deoxyribonuclease test (Shittu*et al*., 2007).

2.1.1. Important Properties of the Staphylococci

Staphylococci are spherical Gram-positive cocci arranged in irregular grapelike clusters. They all produce catalase an important virulence factor which degrades the microbicidal H_2O_2 into O_2 and H_2O. *Staphylococcus aureus*is distinguished from other Staphylococci by the production of coagulase an enzyme that clots plasma. *Staphylococcus aureus*produces a carotenoid pigment that imparts a golden colour to its colonies. This pigment enhances the pathogenicity of the organism by inactivating the

microbicidal effect of superoxides and other reactive oxygen species within neutrophils. *Staphylococcus aureus*haemolyzes red blood cells and is also able to ferment mannitol (Lowy, 1998; Levinson, 2008).

The *Staphylococcus aureus* genome consists of a singular circular chromosome of about 2.7 to 2.9 Mbp containing about 2600 genes composed of core and auxiliary (accessory) genes. It has a G+C content of 33%.

In silico analysis suggests that the core genome makes up about 75% of any *Staphylococcus aureus*genome and is highly conserved between isolates. The majority of genes comprising the coregenome are those associated with central metabolism and other housekeeping functions.

Supplementing these are genes that are associated with common species functions but that are not essential for growth and survival, including virulence genes. Also included are surface binding proteins, toxins, exoenzymes and the capsule biosynthetic cluster. The accessory genome accounts for about 25% of any *Staphylococcus aureus*genome, and mostly consists of mobile genetic elements that transfer horizontally between strains. These elements include bacteriophages, pathogenicity islands, chromosomal cassettes, genomic islands, plasmids and transposons. Accessory genes typically have a different G + C content than those in the core genome, often because they are obtained from other species of bacteria. Many of these genetic elements are known to carry genes associated with virulence, drug and metal resistance, to substrate utilization and miscellaneous metabolism. Therefore, the distribution and horizontal spread of these elements can have important clinical implications. The identification and characterization of these elements provides insights into how *Staphylococcus aureus* cause disease, and their diversity (Shittu *et al.*, 2007). Bacteria obtain genetic information from other cells or the surrounding environment in three ways:

(1) Uptake of free DNA from the environment (transformation),

(2) Bacteriophage transduction, and

(3) Direct contact between bacterial cells (conjugation).

Bacteriophages or bacterial viruses seem to have the greatest impact on staphylococcal diversity and evolution. They are known to transfer genes such as *lukF-PV* and *lukS-PV* that encode the Panton-Valentine leukocidin (PVL) components which is strongly associated with severe forms of pneumonia (necrotic pneumonia) caused by community-acquired *Staphylococcus aureus*strains; the staphylokinase gene (sak) gene, which is a potent plasminogen activator that could facilitate bacterial spreading through its fibrin-specific blood clotting activities and the enterotoxin genes (Shittu *et al*., 2007; Malachowa and DeLeo, 2010). *Staphylococcus aureus*pathogenicity islands (SaPIs) often carry superantigen genes, such as toxic shock syndrome (*tst*) and enterotoxins B and C, implicated in toxic shock and food poisoning. Three families of genomic islands have been discovered in sequenced strains of *Staphylococcus aureus*strains. They carry exotoxin and lipoprotein genes (Shittu *et al*., 2007). All the sequenced *Staphylococcus aureus*strains are known to carry one or more free or integrated plasmids. All types of *Staphylococcus aureus*plasmids frequently carry antibiotic resistance genes, or resistance to heavy metals or antiseptics. Some virulence genes are also known to be carried on plasmids, such as exfoliative toxin B and some superantigens. Other mobile genetic elements found in the *Staphylococcus aureus* chromosome include Staphylococcal cassette chromosomes (SCCs). They encode antibiotic resistance and/or virulence determinants. SCCs encode the methicillin resistance gene (*mecA*) (Shittu *et al*., 2007; Malachowa and DeLeo, 2010).

2.1.2 Biofilm Formation in Staphylococci

The totality of the accumulated bacteria and the extracellular slime on a solid surface is referred to as biofilm. Biofilm consists of multilayered cell clusters embedded in a matrix of extracellular polysaccharide (slime), which facilitates the adherence of microorganisms to biomedical surfaces and protect them from host immune system and antimicrobial therapy. Staphylococci are most often

associated with chronic infections of implanted medical devices. Biofilm formation is regulated by expression of polysaccharide intracellular adhesin (PIA), which mediates cell to cell adhesion and is the gene product of *icaADBC.*The pathogenesis of deviceassociated infections with Staphylococci is mainly characterized by the pathogen's ability to colonize the surfaces of the implanted medical device by the formation of the biofilm which is composed of polysaccharides, proteins, extracellular DNA, and host factors. The use of indwelling medical devices is important in the treatment of critically and chronically ill patients, however bacterial colonization of implanted foreign material can cause major medical and economic sequel. The predominant species isolated in these infections are *Staphylococcusepidermidis* and *Staphylococcus aureus*. In the past two decades, *Staphylococcus aureus*has emerged as one of the most important pathogens causing infections with indwelling medical devices, such as prosthetic joints, prosthetic heart valves, intravascular catheters, and cerebrospinal fluid shunts, which creates an increasing health care problem. For example, prosthetic joint infections occur at a frequency of 1.5–2.5% in primary total hip or total knee arthroplasty with a mortality rate of up to 2.5%. By far the most frequently isolated species from these infections are *Staphylococcus*species, i.e. *Staphylococcus aureus*(22–39%) and coagulase-negative Staphylococci (15–37.5%) (Lentino, 2003). Microorganisms within a biofilm are protected against antimicrobial chemotherapy as well as against the immune system of the host).

2.2*Staphylococcus aureus*

The genus *Staphylococcus*belongs to the family Micrococcaceae. They are spherical cocci about 0.8 to 1.0μm in diameter. They are arranged characteristically in grape-like clusters. They are Gram-positive, non-motile (non-flagellated), non-sporing and non-capsulated (Arora, 2006). The cell walls of Gram positive bacteria exhibit a wide diversity from simple to very complex structures. Staphylococci cell walls have a rather extra ordinary type of structural design and to the most highly cross linked type but

the walls of other Gram positive bacteria exhibit a much lower degree of cross linking and muropeptide fraction of these walls does not contain long oligometric chains. The cell wall envelope functions as a physical barrier that protects the bacteria from their environment and as a rigid exoskeletal element that prevents bacterial rupture in low osmolar environments such as host tissues.

The cell walls of the microorganisms play an important role in the susceptibility to infections and pathogenicity. Structurally, the cell wall of *Staphylococcus aureus*is composed of murein, techoic acid and wall- associated surface proteins. Murein consist of glycan strands that are cross –linked by peptide bridges supplying the structural integrity of the sacculus. It is a distinctive feature of Staphylococci that the observed degree of murein cross-linking which was determined as a ratio of bridged peptides to the total amount of all peptide ends in general is extremely high of the order of 80-90% (Perry*et al*., 2002)

The carbohydrate antigen is a techoic acid which in *Staphylococcus aureus*is a polymer of N-acetylglucosamine and polyribitol phosphate. Teichoic acids function in the specific adherence of Gram-positive bacteria to mucosal surfaces. The protein component of the cell wall includes protein A which reacts with IgG of normal human serum (Lowy, 1998) and it can be released from the bacterial surface by treatment of Staphylococci with lysostaphin, a glycylglycine endopeptidase that cleaves the pentaglycyl cross – bridge of the cell wall. Lysozyme, an N- acetylmuramidase that cuts the glycan strands, release protein A molecules as a spectrum of fragments with varying masses due to the presence of linked peptidoglycan fragments of different sizes. The glycan strands of all bacterial peptidoglycan consist of repeat disaccharide units, N acetylglucosamine-(β1-4)-N-acetylmuramic acid. Glycan chains are cross linked by short cell wall peptides and generate a three dimensional molecular network that maintains the integrity of the bacterium. Finally penicillin binding proteins catalyze the polymerization of lipid II subunits via trans-glycosylation and trans-peptidation reactions, thus generating the crosss-linked peptidoglycan that constitutes the main component of the bacterial cell wall (Perry *et al*., 2002).

2.2.1The CellWall of *Staphylococcus aureus*

The cell wall of *Staphylococcus aureus*is mainly peptidoglycan. The main function of peptidoglycan is to provide a rigid envelope for the cell content. Peptidoglycan consists of alternating polysaccharide subunits of *N*-acetylglucosamine and *N*-acetylmuramic acid with 1,4-β linkages. The peptidoglycan chains are cross-linked by tetrapeptide chains bound to *N*-acetylmuramic acid and by a pentaglycine bridge specific for *Staphylococcus aureus*(Lowy, 1998). Peptidoglycan also has endotoxic properties, and has been reported to cause organ dysfunctions in experimental animals. Most *Staphylococcus aureus*strains produce a slimy, extracellular capsular polysaccharide.

2.2.2. Virulence Determinants of *Staphylococcus aureus*

*Staphylococcus aureus*is a pathogen expressing multiple factors that mediate host colonization, invasion of damaged skin and mucosa, dissemination through the body and evasion of host defence mechanisms (Chanda *et al.*, 2010).The pathogenecity and virulence of *Staphylococcus aureus*infections is associated to various bacterial surface components (e.g., capsular polysaccharide and protein A) including those recognising adhesive matrix molecules e.g.clumping factor (*clf*), Fibronectin Bindng Protein(FBN) and to extracellular proteins e.g., coagulase, haemolysins, enterotoxins Toxic Shock Syndrome toxin, exfoliatins toxin and Panton Valentin leukocidin (Labandeira-Rey *et al*., 2007). Virulence factors can generally be separated into three based on their function:

Adhesins are surface attached proteins that allow the bacteria to attach to a wide variety of human tissues. In *Staphylococcus aureus,*the adhesion genes which include *clf* and *fnb* that encode the fibrinogen and the fibronectin- binding proteins respectively. Fibronectin binding protein (FnBP) A and Fibronectin binding protein (FnBP) B encoded by the *fnb*A and *fnb*B genes respectively, play prominent roles in *Staphylococcus aureus*colonization and attachment of host tissues or implanted biomaterials.

Toxins are secreted proteins that cause tissue damage and generate pus in abscesses which is believed to facilitate transmission between hosts.

Immune dilators are proteins that interfere with host immunity preventing defence against infections.

*Staphylococcus aureus*produces a wide variety of exoproteins that contribute to its ability to colonize and cause disease in mammalian hosts. These factors mediate host colonization invasion of damaged skin and mucosa, dissemination through the body, and evasion of host defense mechanisms. About 50 potential virulence determinants with a wide range of biologic activities have been described in *Staphylococcus aureus*. Staphylococcal infections occur in a stepwise manner, each step involving one or several specific virulence factors (Ferry *et al.,* 2005). Nearly all strains secrete a group of enzymes and cytotoxins which includes four hemolysins (alpha, beta, gamma, and delta), nucleases, proteases, lipases, hyaluronidase, and collagenase. The main function of these proteins may be to convert local host tissues into nutrients required for bacterial growth. Some strains produce one or more additional exoproteins, which include toxic shock syndrome toxin-1 (TSST-1), the staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SEH, and SEI), the exfoliative toxins (ETA and ETB), and leukocidin. Each of these toxins is known to have potent effects on cells of the immune system, but many of them have other biological effects as well. Their primary function in vivo may be to inhibit host immune responses to *Staphylococcus aureus*(Dinges *et al*., 2000).

*Staphylococcus aureus*virulence factors include surface proteins that promote colonization of host tissues, invasion factors that promote bacterial spread in tissues (leukocidin, hyaluronidase), surface factors that inhibit phagocytic engulfment (capsule), biochemical properties that enhance their survival in phagocytes (catalase production), and membrane-damaging toxins that lyse eukaryotic cell membranes (hemolysins, leukotoxin) (Turkyilmaz and Kaya, 2006).

2.2.3. Exfoliative Toxins

Exfoliative toxins also known as “epidermolytic” toxins are specific serine proteases which recognize and cleave desmosomal cadherins in the superficial layers of the skin. They are directly responsible for the clinical manifestation of staphylococcal scalded skin syndrome (Bukowski *et al*., 2010). Human-infecting strains of *Staphylococcus aureus*produce mainly ETA and ETB (1.5% and 0.5% of isolates, respectively, the genes of which are located on the chromosome and plasmid. In Europe, USA, and Africa, ETA is prevalent, and is expressed by more than 80% of toxin-producing strains. Only inJapan, are ETB-producing strains more prevalent than those expressing ETA (Bukowski *et al*., 2010).

2.2.4 Adhesins

*Staphylococcus aureus*possesses various secreted and surface-anchored proteins by which it binds to host extracellular matrix and plasma components. Microbial surface components recognizing adhesive matrix molecules (MSCRAMM) adhesins are generally covalentlynanchored to cell wall peptidoglycan. MSCRAMMs recognize collagen (collagen-binding protein [Cna]), fibronectin (fibronectin-binding proteins [FnBP], such as FnBPA and FnBPB), and fibrinogen (clumping factors [Clf], such as ClfA and ClfB). MSCRAMMs play a major role in adhesion to host tissues. Staphylococcal protein A (*spa*) belongs to the MSCRAMM family and it binds to the von Willebrand factor, a large glycoprotein that mediates platelet adhesin at site of endothelial damage. *spa*displays several additional properties: it interferes with immunoglobulin-mediated opsonization; it mimics a B-cell superantigen; and it binds to tumor necrosis factor (TNF)-R1, a TNF-α receptor (Cheung *et al*., 2002).

2.2.5. Superantigenic Exotoxins

Pyrogenic exotoxin genes are common in *Staphylococcus aureus*, and as many as 73% of *Staphylococcus aureus*isolates carry at least one of the genes encoding a classic pyrogenic exotoxin; however, the distribution among various clonal types differs. Nineteen superantigenic exotoxins have been described

in *Staphylococcus aureus*. They comprise toxic shock syndrome toxin-1 (TSST-1, encoded by the *tst* gene) and staphylococcal enterotoxins (SE) A, B, C, D, E, G, H, I, J, K, L, M, N, O, P, Q, R, and U, encoded by *sea, seb, sec, sed, see, seg, seh, sei, sej, sek,sel, sem, sen, seo, sep, seq, ser,* and *seu*, respectively. These proteins are potent mitogens, are pyrogenic, and augment lipopolysaccharide lethality in animal models. They stimulate large populations of T cells expressing a particular Vβ element in their T-cell receptor, leading to massive T-cell proliferation and uncontrolled release of proinflammatory (Th-1) cytokines such as interferon-γ , TNF-α, interleukin (IL)-1β and IL-6 (Ferry *et al*., 2005).

2.2.6. *Staphylococcus aureus*Enzymes

*Staphylococcus aureus*produces a wide variety of exoproteins, most of them during the post exponential growth phase. These proteins degrade the host tissue to nutrients required for the growth of bacteria, and/or allow the bacteria to penetrate deeper into the host tissue. The majority of strains produce hemolysins, nucleases, proteases, lipases, hyaluronidase, and collagenase. Alpha-hemolysin (or alphatoxin) is dermonecrotic, neurotoxic, and lyses mammalian erythrocytes by forming a pore in the target membrane fatty acids. It is also able to stimulate apoptosis in lymphocytes (Dinges *et al*., 2000). Beta-hemolysin acts as sphingomyelinase, gamma-hemolysin has leucocytolytic activity, and it has been suggested that delta-hemolysin has surfactant or channel forming properties (Dinges *et al*., 2000). Lipases act on the host immune response by inactivating the fatty acids that are intended to disrupt the bacteria. Proteases are involved in the inactivation of host defence peptides and also block antibodies. Staphylokinase is a plasminogen activator (Lahteenmaki *et al*., 2001). Hyaluronidase digests hyaluronic acid present in the skin, bone, umbilical cord, vitreous body of the eye, and synovial fluid. *Staphylococcus aureus*produces an extracellular protein known as coagulase. This protein protects the bacteria from the host defence by clotting fibrin around a focal infection. *Staphylococcus aureus*is known to express a fatty acid modifying enzyme (FAME) which may be associatedwith abscesses where

it could modify antibacterial lipids and prolong bacterial survival. Some *Staphylococcus aureus*strains also produce additional exoproteins, which may havethe evasion of host defence as their major function *in vivo*.

2.2.7. Leukocidins

PVL is a staphylococcal synergohymenotropic exotoxin belonging to the pore-forming toxin family. In vitro, PVL has been shown to induce lysis of host defense cells such as human polymorphonuclear neutrophils (PMNs), monocytes, and macrophages. Recent evidence suggests that PVL may also inactivate mitochondria culminating in apoptosis. Although PVL genes are usually found in only 2% of *Staphylococcus aureus*clinical isolates they have been found in most community associated methicillin resistant *Staphylococcus aureus*(CAMRSA) strains. PVL and leukocidin D-E [LukD-E]) function as superantigens that can manipulate the immune system by hyperstimulating the release of cytokines (SEA, SEB, and TSST)(Ferry *et al*., 2005).

2.2.8. Pathogenesis of *Staphylococcus aureus*

*Staphylococcus aureus*is both a commensal organism and a pathogen. Approximately 30% of healthy individuals are colonized by *Staphylococcus aureus*usually in the anterior nares, vagina and perianal area. Infection occurs when the organism is inoculated into the skin from a site of carriage when host defences are breached whether by shaving, aspiration, insertion of an indwelling catheter or surgery. Colonization increases the risk of infection and allows *Staphylococcus aureus*to be transmitted in both hospital and community settings. The bacterial components and secreted products that affect the pathogenesis of *Staphylococcus aureus*infections are numerous and include surface-associated adhesins, a capsular polysaccharide,exoenzymes, and exotoxins. This constellation of bacterial products allows Staphylococci toadhere to eukaryotic membranes, resist opsonophagocytosis, lyse eukaryotic cells, and triggerthe production of a cascade of host immunomodulating molecules. In establishing an infection,

*Staphylococcus aureus*has numerous surface proteins called ‘microbialsurface components recognizing adhesive matrix molecules’ (MSCRAMMs) that mediateadherence to host tissues (Gordon and Lowy, 2008). The adherence of *Staphylococcus aureus*to host tissueis an important step in pathogenesis as well as in colonization. Surface proteins such asprotein A, clumping factors, fibronectin-binding proteins, and collagen-binding proteins canadhere to extracellular matrix components of the host. The main function of protein A, however, is to bind the IgG Fc-domaine. Once *Staphylococcus aureus*adheres tohost tissues or prosthetic materials, it is able to grow and persist in various ways. *Staphylococcus aureus*can form biofilms (slime) on host and prosthetic surfaces, enabling it to persist by evadinghost defenses and antimicrobials. In vitro studies have shownthat *Staphylococcus aureus*can invade and survive inside epithelial cells, including endothelial cells, whichtheoretically may also allow it to escape host defences, particularly in endocarditis. *Staphylococcus aureus*is able to form small-colony variants (SCVs) andthis may contribute to persistent and recurrent infection. In vitro, SCVs are able to “hide” inhost cells without causing significant host-cell damage and are relatively protected fromantibiotics and host defences. They can later revert to the more virulent wild-type phenotypewhich possibly results in recurrent infection (Gordon and Lowy, 2008). SCVs are slowlygrowing organisms that exhibit a small, non-pigmented, non-haemolytic colony morphotype. They are usually dependent on various substrates (mainly thymidine, haemin and menadione) supplementation for growth and are more resistant to antibiotics such as aminoglycosides and co-trimoxazole (Proctor *et al*., 2006).

*Staphylococcus aureus*has many other characteristics that help it evade the host immune system during an infection. Its main defence is production of an antiphagocytic microcapsule. Most human infections are due to capsular types 5 and 8, with a vast majority of MRSA isolates possessing capsular type 5 (Lowy, 1998, Robbins *et al*., 2004). Together with intercellular polysaccharide adhesins, *Staphylococcus aureus*capsular polysaccharides enhance biofilm constitution by augmenting

adhesiveness.*Staphylococcus aureus*has two main components in its cell wall, namely lipoteichoic acid and peptidoglycan. The hydrophobic domain of lipoteichoic acid plays a role in adherence, whereas peptidoglycan covalently links adhesive proteins (Lowy, 1998, Cheung *et al*., 2002). The zwitterionic capsule is also able to induce abscess formation. The MSCRAMM protein A binds the Fc portion of immunoglobulin and this may prevent opsonization. *Staphylococcus aureus*is able to secrete chemotaxis inhibitory protein of Staphylococci or the extracellular adherence protein, which interfere with neutrophil extravasation and chemotaxis to the site of infection. In addition, *Staphylococcus aureus* produces leukocidins which cause leukocyte destruction by the formation of pores in the cell membrane (Gordon and Lowy, 2008).

During infection, *Staphylococcus aureus*produces numerous enzymes, such as proteases, lipases, and elastases, which enable it to invade and destroy host tissues and metastasize to other sites. The capacity to invade endovascular tissue also favours spread to other tissues. Apart from evasion of host immune defence, bacterial survival within the human host is dependent on successful acquisition of nutrients, particularly iron. During infection, 95% of iron is sequestered within host cells and serum iron is mostly bound to host proteins that are not easily accessed. *Staphylococcus aureus*secretes high affinity iron-binding compounds (aureochelin and staphyloferrin) to make iron available for its use. Once in the blood, the organism spreads widely to peripheral sites in distant organs causing septic shock. Peptidoglycan, lipoteichoic acid, and α-toxin are known to play apart in the initiation of sepsis (Lowy, 1998). Invasion of the bloodstream results in a number of specific staphylococcal infections such as endocarditis, osteomyelitis, renal carbuncle, septic arthritis, or epidural abscess. Production of superantigens results in various toxinoses, such as food poisoning, scalded skin syndrome and toxic shock syndrome. Some strains are able to produce epidermolysins or exfoliative toxins which cause scalded skin syndrome or bullous impetigo (Gordon and Lowy, 2008).

About 35-50% of normal adults carry *Staphylococcus aureus*in the anterior nares; it is also the skin normal flora. Other sites of colonization include the perineum, axillae and vagina (Arora, 2006). *Staphylococcus aureus*carriers are at a higher risk of infection and they are presumed to be an important source of spread of *Staphylococcus aureus*strains among individuals. The primary mode of transmission of *Staphylococcus aureus*is by direct contact: usually skin-skin contact with a colonized or infected individual or contact with contaminated objects and surfaces (Miller and Kaplan, 2009). Various host factors including loss of the normal skin barrier, presence of underlying diseases such as diabetes and acquired immunodeficiency syndrome, or defects in neutrophils function predispose to infection. *Staphylococcus aureus*can cause a range of illness from minor skin infections such as pimples, impetigo, boils (furnicles), cellulitis, folliculitis, carbuncles, scalded skin syndrome and abscesses, to life threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS) bacteremia and sepsis. Most serious of all are the deep seated infections such as osteomyelitis and an infection of the heart valves called endocarditis and toxin mediated diseases such as gastroenteritis, Staphylococcal scalded skin syndrome (Miller and Kaplan 2009).

2.2.9 Nasal Carriage of *Staphylococcus aureus*

*Staphylococcus aureus*colonizes the skin and mucosae of human beings and several animal species. Although multiple animal body sites can be colonized, in human beings the anterior nares of the nose is the most frequent carriage site for *Staphylococcus aureus.*

Approximately 20-30% of the global population is persistently colonized with *Staphylococcus aureus*in the anterior nares (Lamers *et al*., 2011) and 30% are transiently colonized during their lives. The definition of persistent and transient carriage varies according to the study, but generally is described as a single positive culture on a nasal swab (transient) *versus* at least two consecutive positive cultures 1 wk apart (persistent). Colonized patients are the chief source of *Staphylococcus aureus*in hospitals.

Approximately 10 to 40% of people tested as outpatients or on admission are nasal carriers of *Staphylococcus aureus*(Von Eiff *et al*., 2001). A prospective study of 500 surgical ICU patients found that colonization was a risk factor for post operative MRSA infection with all clinical isolates matching isolates from the nares and those with MRSA developed infections almost twice as quickly as those not colonized (Butterly *et al*., 2010). In a study carried out on hospitalized dialysis patients, 1.095% of those with nasal carriage of *Staphylococcus aureus*subsequently developed bacteraemia during a 14-month follow up period and 85.7% of the clinical isolates were identical to the nasal strains. Colonization is also more frequent among younger children and patients with HIV and diabetes. Among 282 recreational drug users in New York City in 1999 to 2000, 46% of HIV-infected subjects and 29% of uninfected subjects were colonized with *Staphylococcus aureus*(Miller *et al*., 2007). Nasal carriage of *Staphylococcus aureus*has been associated with an increased risk of infection in patients after surgery, in patients receiving ambulatory peritoneal dialysis and in patients receiving haemodialysis (Von Eiff *et al*., 2001).

A causal relationship between *Staphylococcus aureus*nasal carriage and infection is supported by the fact that nasal *Staphylococcus aureus*strain and the infecting strain share the same phage type of genotype. The proof of principle demonstrating the importance of nasal colonization'srole in the pathogenesis of *Staphylococcus aureus*infection is that, when colonization is eradicated, the riskof clinical infection is lowered. This observation has spurred attempts to eradicate *Staphylococcus aureus*from the nose to prevent infection among high-risk groups (Miller and Diep, 2008).

2.2.10. Colonization of*Staphylococcus aureus*among Health-Care Workers

Health care workers (HCWs) constitute an important reservoir of *Staphylococcus aureus*and serve as a vector of transmission to susceptible patients. Health care workers have also been identified as the source of MRSA in numerous outbreak investigations (Sheretz *et al*., 2001). It has been found that acquisition of MRSA on hands was common after contact with frequently examined skin sites (chest, abdomen,

forearm and hands) of MRSA carriers (Chang *et al*., 2009). Health care workers with dermatitis or other lesions on hands that are colonised with MRSA are most likely to be sources of transmission to patients (Grundmann, 2006). Several studies have shown that improvement in hand-hygiene practices, when coupled with surveillance cultures and contact precautions, have greatly reduced transmission of MRSA. The level of compliance with hand-hygiene needed to stop MRSA transmission is unknown, but one study of intensive care units in which compliance was 59%, predicted that a 12% increase in compliance may be sufficient to prevent transmission (Grundmann, 2002).

2.2.11. The Environment as a Source of Infection

Transmission of *Staphylococcus aureus*in the health care setting occurs through contact with contaminated surfaces in the environment. *Staphylococcus aureus*and MRSA have been shown to persist for weeks on items in the hospital such as patient care equipment, uniforms, computer keyboards, cellular phones and identification badges. Although there is no evidence demonstrating the direct transmission of MRSA from the environment to patients, there is evidence that contamination of the environment with MRSA may be transferred to the gloves of health care workers and this result to transmission to patients. Patients colonized or infected by MRSA have been known to shed MRSA resulting in contamination of their skin, clothing, bedding and environmental surfaces. There is increasing evidence that the environment may play a significant role in the spread of antibiotic-resistant organisms. Staphylococci including MRSA are known to survive in dry conditions and can persist in clinical areas that are inadequately cleaned thereby causing infection (Sexton *et al*., 2006). The ability of MRSA to contaminate a large variety of hospital items such as pens, mattresses, chairs and bed frames has been demonstrated in several studies. Shimori *etal.* (2002) demonstrated that MRSA carried on dust particles could be aerosolized and was present in respirable range. The potential for contaminated environmental surfaces to contribute to transmission of health-care associated pathogens depends on a number of factors

such as the ability of pathogens to remain viable on a variety of dry environmental surfaces, the frequency with which they contaminate surfaces commonly touched by patients and health care workers and whether or not levels of contamination are sufficiently high to result in transmission to patients.

One of the factors responsible for environmental contamination is poor compliance with recommended infection control policies by health care workers. Aggressive control of MRSA strains in the environment in certain countries has contributed to effective strategies to prevent MRSA infection. For example, in The Netherlands, the so-called "search-and-destroy" policy has minimized hospital associated/acquired methicillin resistant *Staphylococcus aureus*(HA-MRSA) infections to such a degree that they have become uncommon, and the rate is far below the rates in neighboring countries (Vriens *et al*., 2002).

2.3. Coagulase-negative Staphylococci

Coagulase negative Staphylococci (CoNS) are normal and abundant colonizers of humans and become pathogenic only in certain situations. They are commonly isolated in clinical specimens and several species are recognized as important agents of nosocomial infections, especially in neonates. Several studies show that most CoNS infections are hospital-acquired or health-care related. Patients with CoNS infections are usually immunocompromised, with indwelling or implanted foreign bodies(Von Eiff *et al*., 2001). Colonization of patients and hospital staff with antibiotic resistant *Staphylococcus epidermidis* precedes infection with these organisms. Some clones are probably endemic in the hospital environment as several studies strongly suggest that they are often caused by strains transmitted among hospitalized patients. CoNS have the ability to survive in the intensive care unit surroundings on medical devices and medical equipment for weeks to months. CoNS are recognized as etiologic agents of a wide variety of infections such as bacteraemia, central nervous system shunt infection, endocarditis, urinary tract infection, surgical site infections, endophthalmitis, conjunctivitis, keratitis, foreign body infections and many other infections. Two main reasons for the increasing rate of CoNS infections are the spreading

antibiotic resistance among CoNS and the increasing use of medical devices in recent years. Insertion or implantation of medical devices has been associated with a risk of bacterial and fungal infections. Medical devices are increasingly used in almost all fields of medicine either for diagnostic or therapeutic procedures and the contamination of such devices occurs most likely by inoculation with few micro-organisms from the patient's skin or mucous membranes during implantation or subsequent manipulations (Von Eiff *et al*., 2001).

There have been difficulties in diagnosing coagulase-negative staphylococcal infections because CoNS are part of the normal skin flora. Therefore, they are the most frequent cause of contamination in blood cultures (Proctor, 2000). Tests based on coagulase production and the thermonuclease reactions are used for differentiation of *Staphylococcus aureus*from other staphylococcal species. Currently, 39 species of CoNS are recognized. Sixteen of these species have been found in specimens of human origin. They are grouped in novobiocin-resistant species (*Staphylococcus cohnii, Staphylococcus saprophyticus, Staphylococcussciuri, Staphylococcus xylosus*) and novobiocin-susceptible species (*Staphylococcus auricularis, Staphylococcus capitis, Staphylococcus caprae, Staphylococcusepidermidis, Staphylococcus haemolyticus, Staphylococcus hominis, Staphylococcus lugdunensis, Staphylococcus pasteuri, Staphylococcus saccharolyticus, Staphylococcusschleiferi, Staphylococcus simulans, Staphylococcus warneri*) (Von Eiff *et al*., 2001).

Staphylococcus epidermidis, Staphylococcus haemolyticus and Staphylococcus hominis are the most frequently encountered CoNS species in clinical samples. *Staphylococcus epidermidis* is the predominant pathogen in intravascular catheter-related infections, nosocomial bacteremia, endocarditis, urinary tract and surgical wounds infections, central nervous system shunt infections, ophthalmologic infections, peritoneal dialysis-related infections and infections of prosthetic joints. *Staphylococcus haemolyticus* has been associated with native valve endocarditis (NVE), septicemia, urinary tract infections, peritonitis and

wound, bone and joint infections. *Staphylococcussaprophyticus* is associated with urinary tract infections in young females. *Staphylococcus lugdunensis* has been implicated in arthritis, catheter infections, bacteremia, urinary tract infections, prosthetic joint infections and endocarditis (Bannerman, 2003). Slime production by CoNS has been identified as an important factor in the pathogenesis of infections. This "slime" or biofilm is the most important virulence factor of *Staphylococcus epidermidis*. The formation of biofilms enables attachment and persistence of the bacteria on foreign materials. Moreover, bacteria organized in biofilms are protected from the action of antibiotics and the immune system. Another important virulence factor of CoNS is the antibiotic resistance. Multi-drug resistance has been detected in more than 80% of CoNS hospital isolates. Rates of methicillin resistance in *Staphylococcus epidermidis* have increased steadily since the early 1960s. Between 1997 and 2002, the rate was about 67% in CoNS isolates from hospital-acquired bacteraemias in England higher than that for *Staphylococcus aureus*. Methicillin resistant strains are known to have high rates of resistance to other classes of antibiotics. Regulation of biofilm formation and methicillin resistance seem to use similar pathways, as insertion of a certain transposon influences both biofilm formation and the expression of methicillin resistance. Methicillin resistance was found to be significantly higher in slime positive isolates (81%) than in slime negative isolates (57%) (Widerström *et al*.,2006; Koksal *et al*., 2007).

Although coagulase-negative Staphylococci present a therapeutic challenge, the infections tend to be less destructive than those caused by *Staphylococcus aureus*(Proctor, 2000).

2.3.1. Differentiation of *Staphylococcus aureus*from Coagulase - negative Staphylococci

The two tests often used to distinguish *Staphylococcus aureus*from other Staphylococci are the coagulase test (coagulation of human or animal plasma) and the thermostable nuclease test (breakdown of deoxyribonucleic acid by nucleases that survive boiling). Generally, *Staphylococcus aureus*is Gram-positive cocci, appearing in clusters; catalase-positive, oxidase-negative. *Staphylococcus aureus*ferments

glucose and lactose to produce acid and gas and also ferments mannitol to produse acids. *Staphylococcus aureus*are coagulase- positive and deoxyribonuclease (DNAse) positive (Cheesbrough, 2012).

2.4The Development of Chemotherapy

The modern era of chemotherapy began with the work of the German physician Paul Ehrlich. Ehrlich was fascinated with dyes that specifically bind to and stain microbial cells. He reasoned that one of the dyes could be a chemical that would selectively destroy pathogens without harming human cells (a magic bullet). By 1904 Ehrlich found that the dye trypan red was active against the trypanosome that causes African sleeping sickness (Wiley *et al*., 2008). In 1927, Gerhard Domagk discovered that prontosil Red, a new dye for staining leather, protected mice completely against pathogenic streptococci and Staphylococci without apparent toxicity. Domagk received the Nobel Prize in physiology or medicine for his discovery of sulfonamides, or sulfa drugs. In the 1920s, Alexander Fleming, a Scottish physician, found that human tears contained a naturally occurring antibacterial substance that he termed lysozyme. This substance unfortunately had little therapeutic value because it could not be isolated in large quantities and was not effective against many micro organisms. However, it prepared Fleming for the discovery of penicillin, the first true antibiotic to be used therapeutically (Wiley *et al*., 2008).

Penicillin was actually discovered in 1896 by a 21-year-old French medical student named Ernest Duchesne. His work was forgotten until Fleming‘s accidental rediscovery of the antibiotic in September 1928. After returning from a weekend vacation, Fleming noticed that a Petri plate of *Staphylococcus*also had a mould growing on it and, like the lysozyme he had discovered years before, there were no *Staphylococcus* colonies surrounding it. It has been suggested that a penicillium notatum spore had made its way onto the petri dish before it had been inoculated with the Staphylococci. The mold apparently grew before the bacteria and produced penicillin. The bacteria nearest the fungus were lysed. Fleming correctly deduced that the mold contaminant produced a diffusible substance, which he called penicillin.

In subsequent studies he showed that this substance could diffuse through agar so that even small amounts of it extracted from both cultures could kill several pathogenic bacteria, including S. *aureus*. Unfortunately, Fleming could not demonstrate that penicillin remained active *in vivo* long enough to destroy pathogens and thus dropped the research (Sykes, 2001; Wiley *et al*., 2008).

In 1939, Howard Florey and Ernest Chain obtained the *Penicillium* culture from Flemming, they then cultured and purified the penicillin. When the purified penicillin was injected into the mice infected with streptococci or Staphylococci, practically all the mice survived. Florey and Chain received the noble prize in 1945 for the discovery and production of penicillin (Wiley *et al.*, 2008). The discovery of penicillin stimulated the search for other antibiotics. Selman Waksman announced in 1944 that he and his associates had found new antibiotic, streptomycin, produced by the actionomycete *Streptomyces griseus*. It was the first drug that could successfully treat tuberculosis. Waksman received the noble prize in 1952, and his success led to a world- wide search for other antibiotic from soil micro organisms. Micro organisms producing chloramphenicol, neomycin, terramycin, and tetracycline were isolated by 1953. The discovery of chemotherapeutic agents and the development of newer, more powerful drugs have transformed modern medicine and greatly alleviated human suffering (Wiley *et al*., 2008).

2.4.1 Antibiotics

2.4.1.1. Definition

An antibiotic is an agent that inhibits bacterial growth or kills bacteria. The term antibiotic is used as a synonym for antibacterials used to treat bacterial infections in both people and animals (WHO, 2011). Any substance of natural, synthetic or semi-synthetic origin which at low concentrations kills or inhibits the growth of micro-organisms but causes little or no host damage (WHO, 2000). Today, however, with increased knowledge of the causative agents of various infectious diseases, antibioticshave come to denote a broader range of antimicrobial compounds, including anti-fungal and other compounds. The

term antibiotic was first used in 1942 by Selman Waksman and his collaborators in journal articles to describe any substance produced by a microorganism that is antagonistic to the growth of other microorganisms in high dilution (Wiley *et al*., 2008).

2.4.1.2 Sources of Antibiotics

There are three major sources from which antibiotics are obtained:

1. Natural (through microorganisms) e.g. bacitracin and polymyxin are obtained from some *Bacillus* species; streptomycin, tetracyclines etc from *Streptomyces* species, gentamicin from *Micromonospora purpurea*; griseofulvin and some penicillins and cephalosporins from certain genera (Penicillium, Acremonium) of the family Aspergillaceae. Most antibiotics in current use have been produced from *Streptomyces* species.

2. Chemical synthesis e.g. chloramphenicol, sulphonamides, quinolones and the oxazolidinones.

3. Semi-synthesis: This means that part of the molecule is produced by a fermentation process and the product is then further modified by a chemical process. Many penicillins (e.g. ampicillin, amoxicillin, methicillin and ticarcillin), cephalosporins and the carbapenems are produced in this way (Von Nussbaum *et al*., 2006).

2.4.1.3` Beta-Lactam Antibiotics

2.4.1.3.1. Penicillins and Mecillinams

The penicillins can be considered as being of the following types:-

1. Naturally occurring, for example those produced by fermentation of moulds such as *Penicillium notatum* and *Penicillium chrysogenum* e.g. benzyl penicillin (penicillin G and phenoxymethyl pencillin G) and phenoxymylpencillin (pencillin V).

2. Semisynthetic: In 1959, the penicillin nucleus: 6- aminopenicillanic acid (6-APA) was isolated in which a thiazolidine is attached to a -lactam ring that carries a free amino group that can be split by

bacterial and other amidases (Russell, 2004; Brooks *et al*., 2004). Acylation of 6-APA with appropriate substances resulted in the production of new penicillins with distinct pharmacological activities. The mecillinams represented by mecillinam and pirmecillinam are essentially 6-- amidino-pencillins with considerable activity against Gram-negative organisms (Russell, 2004).

2.4.1.3.2. Cephalosporins

The cephalosporins are a group of -lactam compounds whose discovery dated back to the 1950‘s when a species of *Cephalosporium* (now *Acremonium*) was isolated near a sewage outfall off the Sardinian coast (Russell, 2004). A study of this isolate revealed the production of these antibiotics:

1. An acidic antibiotic called cephahasporin P, (which is now known to have a steroid- like structure.

2. Another acidic antibiotic, called cephalosporin N which was later shown to be a penicillin since its structure was based on 6-APA.

3. Cephalosporin C, which was obtained during the purification of cephalosporin N, this is a true cephalosporin and from it 7-aminocephalosporanic acid has been obtained for new cephalosporin. Natural cephalosporins have low antibacterial activity but the introduction of various substituents have resulted in cephalosporin with varying pharmacologic properties, antimicrobial spectra and activity (Rusell, 2004, Brooks *et al*., 2004).

There are five generations of cephalosporin now. These include:

a. First generation cephalosporins include cefadroxil, cefazolin cephalothin, cephalaxin

b. Second generation cephahosporin: cephaclor, cephamendole, cefoxitin, cefproxil, cefuroxime.

c. Third generation cephalosporin include cefixime, cefdimir cefotaxime, ceftazidime ceftriaxone.

d. Fourth generation cephalosporin include cefepime.

e. Fifth generation cephalosporin include ceftaroline fosamil and ceftobiprole.

2.4.1.3.3. Clavams

The clavams are structurally similar to the penicillins with two major distinguishing factors. The sulphur in penicillin‘s thiazolidine ring is replaced with oxygen in the clavam oxazolidine ring. Secondly there is no side chain at position 6 (Russell, 2004). Clavulanic acid, a naturally occurring clavam isolated from *Streptomyces clavuligerus* has poor antibacterial activity but is a potent inhibitor of staphylococcal -lactamase and most -lactamase produced by Gram-negative bacteria especially those with a penicillinase rather than cephalosporinase type of enzyme action (Russell, 2004). A formulation of clavulanic acid and amoxicillin (a broad spectrum but -lactamase susceptible penicillin) was introduced into clinical practice in 1981 with remarkable result. Combination of clavulanic acid with another -lactamase susceptible penicillin, ticarcillin extended its spectrum of activity to include *Pseudomonas aeruginosa* (Russell, 2004)

2.4.1.3.4. I-Carbapenems

The I-carbapenems are a family of fused -Lactam antibiotics. They are structural analogues of penicillins or clavams in which the sulphur (penicillin) or oxygen (clavam) is replaced by carbon, examples are imipenem, Doripenem, Meropenem, thienamycin (Russell, 2004).

2.4.1.3.5. Monobactams

The mono bactams are a group of monocyclic -lactam antibiotics produced by various strains of bacteria. The nucleus 3-aminomono bactamic acid (3-AMA) has been produced from naturally occurring monobactams and 6-APA. Several monobactams have been tested for antibacterial activity and aztreonam was found to be highly active against most Gram negative bacteria (Brooks *et al*., 2004) and stable to most types of -lactamases.

2.4.1.3.6. Penicillanic Acid Derivatives

Penicillanic acid derivatives are synthetically produced -lactamase inhibitors. Penicillanic acid sulphine (sodium salt) inhibits staphylococcal -lactamases but not all lactamases produced by Gram negative bacteria being less potent than clavulanic acid. Other examples of penicillanic acid derivatives include -bromopenicillanic acid which inhibits -lactamases; tazobactam which is a penicillinanic acid sulphone derivative with comparable -lactamase inhibitory activity to clavulanic acid. Tazobactam is marketed in combination with piperacillin. Sulbactam is semi-synthetic 6-desamino penicillin sulphone with structural similarity to tazobactam. Sulbactam is a potent inhibitor of -lactamase it also has antibacterial activity against Gram negative organisms (Russell, 2004)

2.4.1.4 Mode of Action of Beta - Lactam Antibiotics

Beta-lactam antibiotics are bacteriocidal, and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity, especially in Gram-positive organisms, being the outermost and primary component of the wall. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by D-alanyl-D-alanine transpeptidases which are penicillin-binding proteins (PBPs). PBPs vary in their affinity for binding penicillin or other β-lactam antibiotics. The amount of PBPs varies among bacterial species.

The initial step in the drug action consists of binding of the drug to cell receptors. After a beta- lactam drug has attached to its receptor, the transpeptidation reaction is inhibited and peptidoglycan synthesis is blocked (Jawetz, 1992). Beta-Lactam antibiotics are analogues of D-alanyl-D-alanine-the terminal amino acid residues on the peptide subunits of the nascent peptidoglycan layer. The structural similarity between β-lactam antibiotics and D-alanyl-D-alanine facilitates their binding to the active site of PBPs. If the synthesis of peptidoglycan is blocked selectively by antibiotic action the bacteria undergo a number of changes in shape and ultimately die following distruption (lysis) of the cells. Mammalian cells do not

possess a cell wall and contain no other macromolecular structures resembling peptidoglycan. Consequently antibiotics which interfere with peptidoglycan have a good selective toxicity (Russell, 2004).

2.4.2. Antistaphylococcal Agents

2.4.2.1 Penicillins

Before the antibiotic era of medicine began some decades ago, the prognosis for patients with severe staphylococcal infections was extremely poor. The introduction of the β-lactam antibiotic penicillin into clinical use in the early 1940s precipitated a dramatic reversal in this situation, with invasive *Staphylococcus aureus*infections being responsive to treatment for the first time. The β-lactam antibiotics or penicillins produce a bactericidal effect by inhibiting the membrane-bound enzymes responsible for catalyzing vital stages in the biosynthesis of the cell wall. Such inhibition is the direct result of the covalent binding of the antibiotic to one or more penicillin-sensitive enzymes, termed penicillin-binding proteins (PBPs) (Jensen and Lyon, 2009).

Currently, 90%–95% of clinical *Staphylococcus aureus*strains throughout the world are resistant to penicillin (Sakoulas and Moellering, 2008). Resistance is due to the production of a penicillinase (or β-lactamase). More than 90% of staphylococcal isolates now produce β-lactamase, which inactivates β-lactam antibiotics by hydrolysis of their blactam ring. *blaZ*encodes β-lactamase and is part of a transposable element on a plasmid, which often also contains genes resistant to other antibiotics, (e.g., gentamicin and erythromycin). An illustration of β-lactamase synthesis and its regulation is depicted in Figure 2.1.overleaf

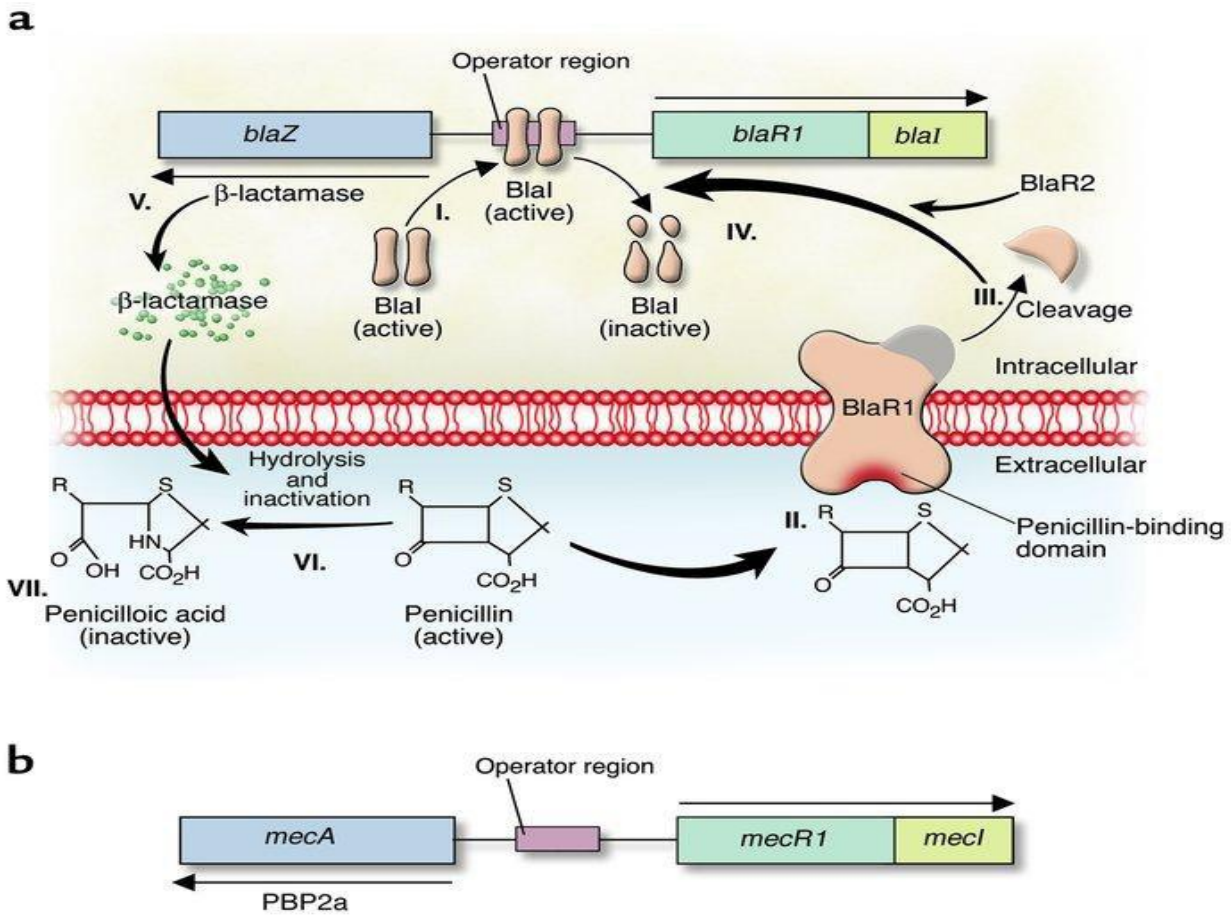

Figure 2.1: Induction of staphylococcal β-lactamase synthesis in the presence of the β-lactam antibiotic penicillin. Source: (Jensen and Lyon, 2009).

(a). I. The DNA-binding protein BlaI binds to the operator region, thus repressing RNA transcription from both *blaZ* and *blaR*1-*blaI*. In the absence of penicillin, β-lactamase is expressed at low levels. II. Binding of penicillin to the transmembrane sensor-transducer BlaR1 stimulates BlaR1 autocatalytic activation. III–IV. Active BlaR1 either directly or indirectly (via a second protein, BlaR2) cleaves BlaI into inactive fragments, allowing transcription of both *blaZ* and *blaR*1-*blaI* to commence. V–VII.β-Lactamase, the extracellular enzyme encoded by *blaZ* (V), hydrolyzes the β-lactam ring of penicillin (VI), thereby rendering it inactive (VII).

(b) Mechanism of S. *aureus* resistance to methicillin. Synthesis of PBP2a proceeds in a fashion similar to that described for β-lactamase. Exposure of MecR1 to a β-lactam antibiotic induces MecR1 synthesis. MecR1 inactivates MecI, allowing synthesis of PBP2a. MecI and BlaI have coregulatory effects on the expression of PBP2a and β-lactamase (Lowy, 2003).

2.4.2.2. Penicillinase-Stable β-lactams

In the early 1960s, the development of antibiotics was directed against *Staphylococcus aureus*. The first cephalosporins- cephalothin and cephaloridine were developed primarily for their stability to staphylococcal penicillinase, but were overtaken by the discovery of how to replace the 6′ phenylacetyl group benzylpenicillin with other acyl substituents. This discovery provided the synthetic route for methicillin, nafcillin and the oxacillins. Despite these successes, the first methicillin-resistant *Staphylococcus aureus*(MRSA) were discovered in the year 1961 just after its introduction. Whereas normal *Staphylococcus aureus*employ three penicillin-binding proteins, PBPs 1, 2, and 3, to catalyse cross-linking ofpeptidoglycan, MRSA have an additional component, PBP 2′ or 2a, which has low affinityfor β-lactams. MRSA consequently are resistant to all β-lactams.

2.4.2.2.1 Genetics of Methicillin Resistance

Methicillin resistance arises due to acquisition of the *mecA* gene which encodes for the 78-kDa penicillin-binding protein (PBP) called PBP2a, which has a very low affinity for methicillin and most other β-lactam drugs. β-lactam antibiotics act by binding to PBPs in the cell wall, resulting in the disruption of synthesis of the peptidoglycan layer and death of the bacterium. Since β-lactam antibiotics cannot bind to PBP2a, synthesis of the peptidoglycan layer and cell wall synthesis are able to continue (Deurenberg *et al.*, 2007).

The *mecA* gene is regulated by the repressor *MecI* and the trans-membrane β-lactam-sensing signal transducer *MecRI*. *mecA*is situated within a particular resistance island called staphylococcal cassette

chromosome *mec* (SCC*mec*). SCC*mec* is an exogenous piece of DNA that may vary between 15 and 60 kb and is absent from methicillin-susceptible Staphylococci. This mobile genetic element also contains regulatory genes, the insertion sequence IS431*mec* and cassette chromosome recombinase (*ccrA*/*ccrB* or *ccrC*) genes that permit intra- and interspecies horizontal transmission of SCC*mec* (Gordon and Lowy, 2008). To date, nine types of SCC*mec* (types I to VIII and VT) have been defined, which can be distinguished by the type of *ccr* gene complex that mediates the site-specific excision and insertion of the SCC*mec* cassette out of or into the bacterial genome and the class of *mec* complex that they bear (David and Daum, 2010). Resistance to penicillins and heavy metals, such as mercury, is encoded by pI258, while tetracycline resistance is encoded by pT181. Transposon Tn554 harbors the *ermA*gene, coding for constitutive and inducible macrolide, lincosamide and streptogramin (MLS) resistance, while ΨTn554 encodes for resistance to cadmium (Gillaspy and Iandolo, 2009). Furthermore, SCC*mec* carries several insertion sequences, such as IS431 and IS1272, as well as the genes responsible for the regulation of the transcription of *mecA*, i.e. Δ*mecR1* (SCC*mec* type I, IV, V, VI an]\

d VII), or *mecR1* and *mecI* (SCC*mec* type II and III) (Deurenberg, 2007). Apart from increasing the range of drug resistance to antibiotics the insertion of these mobile elements provides potential hot spots for recombination, therefore helping remodel the structure of SCC*mec* and giving rise to a greater number of structural variants.

Most hospital MRSA strains harbour SSC*mec* types I, II and III while most strains of community MRSA harbour SCC*mec* types IV, V or VII. The type IV mobile element is smaller than the other types because it contains fewer antibiotic resistant determinants. The small size may enable horizontal transfer of SCC*mec* IV among a bacterial population. CAMRSA carrying SCC*mec* IV has emerged as one of the most virulent strains. Such strains carry additional virulence determinants such as genes for superantigens

(enterotoxin B and C), Panton-Valentine leukocidin (*PVL*), multiple staphylococcal exotoxin *(set*) genes, which are located on the mSab pathogenicity island and exfoliative toxins (Bal and Gould, 2005).

Besides MRSA, methicillin-resistant coagulase negative Staphylococci can harbour SCC*mec*. It has been shown that methicillin-resistant *Staphylococcus epidermidis* isolates from the 1970s harboured SCC*mec* types I–IV .Other studies have found novel SCC*mec* types, or SCC elements without *mecA*, which could be a reservoir for antibiotic resistance islands, in *Staphylococcus aureus*(Deurenberg *et al*., 2007). An illustration of SCC*mec* types I to VIII is shown in Figure 2.2.

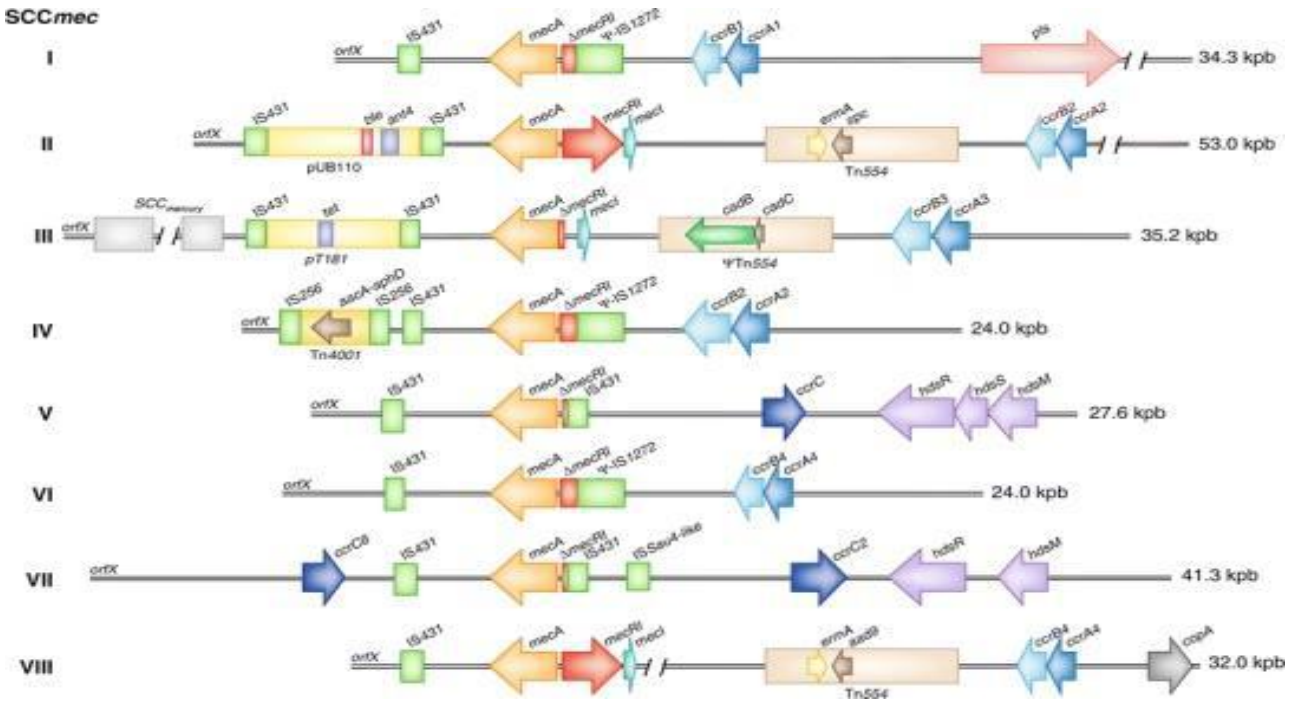

Figure 2.2: Comparison of *Staphylococcus aureus*SCC*mec* types. Class A SCC*mec* contains a complete *mecA* regulon (*mec1-mecR1-mecA*). Class B and class C SCC*mec* contain regulatory genes that are disrupted by IS, IS*1272*-Δ*mecR1-mecA* and IS*431*-Δ*mecR1-mecA*, respectively. Tn*554* encodes erythromycin (*ermA*) and streptomycin/spectinomycin resistance (*aad9* or *spc*); *copA* encodes a putative copper-transport ATPase; *hsdR*, *hsdM*, and *hsdS* encode a partial restriction-modification system (RM)

type I; Tn*4001* encodes an aminoglycoside resistance operon (*aacA-aphD*); plasmid pT181 encodes tetracycline resistance (*tet*); ΨTn*554* encodes cadmium resistance (*cadB, cadC*); and plasmid pUB110 encodes bleomycin (*ble*) and tobramycin resistance (*ant4′*). *pls*Plasmin-sensitive surface protein

Source: (Malachowa and DeLeo, 2010)

2.4.2.3 Macrolides, Lincosamides & Streptogramins

Macrolides, lincosamides and streptogramins were first introduced in 1952 and constitute a group of antibiotics collectively known as MLS. They target the bacterial 50S ribosomal subunit, thereby effectively inhibiting protein synthesis (Schito, 2006). Resistance to the MLS group of antibiotics results from the N6-dimethylation of an adenine residue in the 23S rRNA mediated primarily by *ermA*, *ermB* or *ermC* (found on plasmids or chromosomes), which causes a reduction in affinity with the antibiotic, by enzymatic modification of the antibiotic, or by active transport of the antibiotic out of the cell (Jensen and Lyon, 2009).

2.4.2.4. Fluoroquinolones

Fluoroquinolones were initially introduced for the treatment of Gram-negative bacterial infections but they have also been used to treat bacterial infections caused by pneumococci and Staphylococci (Lowy, 2003). The primary target of quinolones is bacterial DNA gyrase, without which DNA replication is inhibited. Quinolone resistance is as a result of spontaneous chromosomal mutations. These mutations are in the quinoloneresistance- determining region of the enzyme–DNA complex, reducing the affinity of quinolone for its targets (DNA gyrase and topoisomerase IV). An additional mechanism of resistance in *Staphylococcus aureus*is induction of the NorA multidrug resistance efflux pump. Increased expression of this pump in *Staphylococcus aureus*can result in low-level quinolone resistance (Lowy, 2003). In vitro passage of both fluoroquinolone-susceptible MSSA and MRSA in the presence of either ciprofloxacin or levofloxacin is associated with the frequent selection of clones resistant to these antibiotics (Limoncu *et*

al., 2003). Fluoroquinolone use has also been associated with an increased risk of nosocomial acquisition of MRSA (but not of MSSA). The fluoroquinolones with C8 substitutions, such as gatifloxacin and moxifloxacin, appear to be more potent against *Staphylococcus aureus*than are older drugs of this class, and they may be less likely to select resistant mutants, an effect that may be strengthened by the addition of rifampin (Deresinski , 2005).

2.4.2.5 Aminoglycosides

Aminoglycosides were introduced in 1944, and by the 1950s aminoglycoside-resistant strains of *Staphylococcus aureus*had emerged. Aminoglycosides inhibit protein synthesis by binding with the 30S ribosomal subunit and disrupting the translocation of peptidyl-tRNA. Resistance to aminoglycoside antibiotics can be due to mutations that alter the structure of the ribosome so that it no longer binds the antibiotic, or affect the energization of the cellular membrane and thereby diminish the uptake of the aminoglycoside. However, the most widespreadmechanism of resistance to the aminoglycosides is enzymatic modification of the antibiotic. Modified aminoglycosides possess the aminoglycoside-modifying genes *acc, aph* and *ant*, which code for aminoglycoside acetyltransferases, phosphotransferases and adenyltransferases, respectively. The acetylated, phosphorylated or adenylated aminoglycosides are not able to bind with ribosomes and therefore will no longer inhibit bacterial protein synthesis (Schito, 2006; Jensen and Lyon, 2009). The bifunctional enzyme AAC(6′)/APH(2"), encoded by the *aac*(6′)-*aph*(2") gene, inactivates a broad range of clinically useful aminoglycosides such as gentamicin, tobramycin, netilmicin, and amikacin and is the most frequently encountered aminoglycoside resistance mechanism among staphylococcal isolates.

2.4.2.6. Tetracyclines

The tetracyclines are currently not widely used for the treatment of staphylococcal infections. Most strains of MRSA and MSSA are resistant to this group of antibiotics. Contraindications of tetracyclines

are gastrointestinal upset, candidal superinfection, photosensitivity, and, in children, discoloration of teeth (Rayner and Munchkof, 2005). Members of the tetracycline family of antibiotics are actively transported into the bacterial cell by a process which is energized by proton motive force. The attachment of these antibiotics to the 30S ribosomal subunit interferes with the binding of aminoacyl-transfer RNA molecules to the ribosomal acceptor site and the resultant inhibition of protein synthesis causes a bacteriostatic effect. Tetracycline resistance is associated with a decrease in the intracellular accumulation of the antibiotic, and, in *Staphylococcus aureus*, this was originally correlated with decreased uptake. Two mechanisms of tetracycline resistance have been identified in *Staphylococcus*species:

(i) Active efflux resulting from the acquisition of the *tetK* and *tetL* genes located on a plasmid belonging to the Inc3 incompatibility group and

(ii) Ribosomal protection mediated by *tetM* or *tetO* determinants located on either a transposon or the chromosome. A genetic determinant (tet-3490) which mediates resistance to tetracyclinebut not to minocycline has been mapped on the *Staphylococcus aureus*chromosome (Rayner and Munchkof, 2005).

2.4.2.7. Sulfonamides & Trimethoprim

The sulfonamides and trimethoprim are synthetic antibiotics that interfere with different steps in the synthesis of tetrahydrofolic acid, an essential precursor of several amino acids and nucleotides. Sulfonamides are structural analogs of p-aminobenzoic acid and competitively inhibit dihydropteroate synthase, which catalyzes the conversion of p-aminobenzoic acid to dihydropteroic acid. Trimethoprim has high affinity for the enzyme dihydrofolate reductase (DHFR) and therefore competitively inhibits the reduction of dihydrofolic acid to tetrahydrofolic acid, the final step in the pathway (Skold, 2001).

Clinical *Staphylococcus aureus*isolates that are resistant to sulphonamides are known to have chromosomal mutations in the gene that encodes dihydropteroate synthase which most likely reduce its affinity for these drugs. Intermediate-level trimethoprim resistance in *Staphylococcus aureus*is chromosomally mediated via mutations in the *dfrB* gene, which encodes DHFR.The staphylococcal plasmid pSK639, carries the *dfrA* gene which confers resistance to other antimicrobials. In addition to *dfrA*, two other genes that confer resistance to trimethoprim (designated *dfrG* and *dfrK*) have been identified in *Staphylococcus aureus*strains.

2.4.2.8. Glycopeptides

Following the spread of MRSA, Vancomycin and teicoplanin the two glycopeptides have become the mainstay of treatment for MRSA infections (Schito, 2006). The Clinical and Laboratory Standards Institute (CLSI) criteria has it that susceptible strains have a vancomycin minimum inhibitory concentration (MIC) of ≤4 μg/ml and resistant strains have a MIC of ≥32 μg/ml. Strains with MIC between 8 and 16 μg/ml are defined as having intermediate susceptibility (vancomycin intermediate *Staphylococcus aureus*(VISA) (Bal and Gould, 2005). Unlike β-lactam antibiotics, which bind to and interrupt the activity of penicillinbinding proteins (enzymes involved in cell-wall synthesis), vancomycin binds with high affinity to the D-Ala-D-Ala C-terminus of late peptidoglycan precursors and prevents reactions of cell-wall synthesis using these precursors in transglycosylase, transpeptidase, and D,D-carboxypeptidases. Since this mechanism of action is distinct from that of β-lactam antibiotics, vancomycin has been used for the treatment of severe infections caused by methicillin- and β-lactam-resistant Staphylococci (Jensen and Lyon, 2009).

Vancomycin was introduced in 1958, but the first fully vancomycin-resistant *Staphylococcus aureus*(VRSA) clinical isolates were found in 2002. Prior to this, vancomycin-intermediate *Staphylococcus aureus*(VISA) strains were isolated, first in Japan in 1996, and then in many other

countries,including the USA, prompting widespread concern (Schito, 2006). Although vancomycin is still the first choice for treatment of severe multidrug-resistant staphylococcal infections, glycopeptide susceptibility of Staphylococci can no longer be assumed, and vigilance is necessary to monitor the frequency of this emerging problem.

2.4.2.9. Linezolid

Linezolid belongs to a novel class of antibiotics; oxazolidinones. Linezolid has a broad spectrum of activity against gram-positive organisms, including Staphylococci, streptococci, and enterococci. Essentially all strains of *Staphylococcus aureus,* including MRSA, from clinical surveys are inhibited by this agent at or lower than the susceptibility breakpoint of 4μg/mL (Eliopoulos, 2003). It binds to the 50S ribosomal subunit and prevents the formation of the 70S ribosome complex. Its 100% bioavailability following oral administration makes it an attractive therapeutic option for MRSA infections. Resistance to linezolid has been reported in *Staphylococcus aureus*strains. Linezolid resistance in clinical MRSA isolates has been associated with mutations in the central loop of domain V of 23S ribosomal RNA (rRNA) (e.g., G2576T and T2500A) (Meka *et al*., 2004).

2.4.2.10. Daptomycin

Daptomycin, a fermentation product of *Streptomyces roseosporus,* is approved for the treatment of enterococcal and staphylococcal infections, including MSSA and MRSA infections, specifically those causing complicated skin and skin-structure infections,*Staphylococcus aureus*bacteraemia, and right-sided endocarditis (Boucher *et al*., 2010). Mutations in thegenes *mprF* (encoding lysylphosphatidylglycerol synthetase), *yycG* (encoding sensorhistidine kinase), and *rpoB* and *rpoC* (encoding β and β subunits of RNA polymerase, respectively) have been found in *Staphylococcus aureus*with daptomycin MICs above the susceptible range (Friedman *et al*., 2006).

2.4.2.11. Tigecycline

Tigecycline a modified tetracycline is a glycylcycline that binds to the 30S ribosomal subunits of a bacterial cell blocking the entry of amino-acyl transfer RNA into the acceptor site. It has a broad spectrum with activity against both Gram-positive (including MRSA and VRE) and Gram-negative (including those producing extended spectrum β-lactamase enzyme) bacteria. The drug is available for parenteral administration only. It is bacteriostatic, has a long half-life, demonstrates a postantibiotic effect and has a good tissue penetration (Ippolito *et al*., 2010; Bal and Gould, 2005). Tigecycline is the first broad-spectrum agent with activity against MRSA. It provides parenteral therapy for complicated skin/skinstructure and intra-abdominal infections. The only prominent adverse effects are associated with tolerability, most notably nausea and vomiting (Casey *et al*., 2007).

2.4.2.12. Quinupristin–dalfopristin

Representatives of the streptogramin A and streptogramin B families of antibiotics occur naturally in combinations that synergistically achieve levels of activity superior to those provided by either antibiotic alone. Quinupristin is a streptogramin B while dalfopristin is a streptogramin A (Eliopoulos, 2003). The compound is bactericidal for *Staphylococcus aureus*. Its mechanism of action involves inhibition of protein synthesis. Each binds to a different site on the 50S ribosomal subunit to form a stable complex. Resistance to streptogramins is due to the modification of the ribosome target site, enzymatic inactivation of the antibiotic or an efflux pump. Methylation of the ribosomal binding site leads to quinupristin resistance, although the susceptibility to dalfopristin can still be retained. Plasmid-mediated resistance to quinupristin is controlled by a specific hydrolase. Quinupristin–dalfopristin can still act as a bacteriostatic agent in face of resistance to a quinupristin component. Dalfopristin resistance occur secondary to an efflux pump mechanism or more commonly due to virginiamycin acetyltransferase-induced acetylation (Bal and Gould, 2005).

2.5. Epidemiology of *Staphylococcus aureus* Infections

Staphylococcus aureus infections are increasingly reported around the world. An increasing number of infections are related to medical developments, such as the use of joint prostheses, immunosuppressants and catheters and such infections are difficult to treat. Infection due to *Staphylococcus aureus* imposes a high and increasing burden on health care resources as well as increasing morbidity and mortality. Recent studies have identified *Staphylococcus aureus* as the main etiological agent of many bacterial infections in sub-Saharan Africa, and a number of investigations have reported that *Staphylococcus aureus* is among the most frequently encountered pathogen in microbiology laboratories in Nigeria (Esan *et al*., 2009; Onipede *et al*., 2009; Shittu *et al*., 2011; Onanuga and Temedie, 2011). *Staphylococcus aureus* is the most frequently occurring bacterial pathogen among clinical isolates from hospital inpatients in the United States and is the second most prevalent bacterial pathogen among clinical isolates from outpatients (Styers *et al*., 2006). An analysis of US inpatients revealed that nearly 400,000 inpatient admissions were reported for *Staphylococcus aureus* infections in 2003 (Noskin *et al*., 2007). According to the Sentry Antimicrobial Surveillance Program, which examined more than 81,000 isolates during the period 1997–2002, *Staphylococcus aureus* was the most common cause of nosocomial bacteremia in North America (26.0%) and Latin America (21.6%) and was the second most common cause of nosocomial bacteremia in Europe (19.5%). Furthermore, *Staphylococcus aureus* was found to be the most common cause of early-onset bacteremia in a study involving 6697 patients with bloodstream infections identified in 59 US hospitals during 2002–2003 (Shorr *et al*., 2006). Infective endocarditis is among the most severe complications of *Staphylococcus aureus* bacteremia, and its incidence has been increasing. *Staphylococcus aureus* is the most common cause of infective endocarditis (IE) diagnosed at major medical centers in the developed world (Miro *et al*., 2005). Many patients are known to acquire *Staphylococcus aureus* infective endocarditis (SAIE) in the hospital, and an intravascular device is often

the source of infection (Naber, 2009). *Staphylococcus aureus*is a frequently isolated pathogen from bronchial secretions of cystic fibrosis (CF) patients. In Europe and the USA, the proportion of MRSA among *Staphylococcus aureus*recovered from CF patients varies from 8% to 23% (Proctor, 2006). In the developing world, mortalityassociated with severe *Staphylococcus aureus*infections far exceeds that in developed countries. Data about the epidemiology and prevalence of staphylococcal infections in Africa are however scarce compared to information about such infections in the rest of the world. Studies have indicated low prevalence of MRSA in Nigeria, Somalia, and Tanzania, but high prevalence in South Africa, Zimbabwe, Kenya, Ethiopia, Egypt, Senegal, and the Ivory Coast have been reported (Okon *et al*., 2009; Adesida *et al*., 2005).

The incidence of nosocomial MRSA has increased greatly in both the United States and Europe. In a US study by the Surveillance Network, annual rates of MRSA were shown to have increased steadily during 1998–2005, with rates of up to 59.2% among *Staphylococcus aureus*isolates in clinical specimens from non-intensive care unit patients. In the same study, MRSAconstituted 49.1% of bloodstream *Staphylococcus aureus*isolates from inpatients and 41.4% of suchisolates from outpatients (Styers *et al*., 2006). MRSA infections account for the death ofabout 19,000 hospitalized American patients annually; this is similar to the number of deathsdue to AIDS, tuberculosis, and viral hepatitis combined. MRSAis the most common multidrug-resistant pathogen causing nosocomial infections in Europe.Estimates indicate that there are approximately 170,000 MRSA infections in European healthcare systems each year, causing more than 5000 fatalities, more than 1 million additional inpatient days, and additional costs of approximately €380 million (Köck *et al*., 2010). A significant increase in methicillin resistance in clinical strains of *Staphylococcus aureus*isolates between 1999 and 2002 was recorded in European countries, particularly Belgium, Germany, Ireland, the Netherlands and the United Kingdom (Tiemersma *et al*., 2004). In a survey of 3051 *Staphylococcus aureus*isolates from 25 university hospitals

(participating in the SENTRY Antimicrobial Surveillance Program) distributed among 15 countries of central and southern Europe, MRSA isolates constituted 25% of all isolates; the highest prevalence was seen in hospitals in Portugal (54%) and Italy (from 43% to 58%), whereas the lowest prevalence was observed in hospitals in Switzerland and The Netherlands (2%). In the European Prevalence of Infection in Intensive Care (EPIC) study, the highest prevalence of MRSA strains was found in Italy (81%) and in France (78%) (Vincent, 2000). The SENTRY program has also noted very high rates of methicillin resistance among *Staphylococcus aureus*isolates from the Asia-Pacific region (160% in Taiwan, Singapore, Japan, and Hong Kong). This conforms to previous reports from these regions which have documented similarly high rates of methicillin resistance. Methicillin-resistant *Staphylococcus aureus*(MRSA) is the most frequently identified antimicrobial drug–resistant pathogen in US hospitals (CDC, 2003). National Nosocomial Infection Surveillance (NNIS) System data reveal a steady increase in the incidence of nosocomial infections caused by methicillin-resistant *Staphylococcus aureus*(MRSA) among ICU patients over time. MRSA now accounts for more than 60% of *Staphylococcus aureus*isolates in US hospital ICUs (NNIS report, 2004). There is evidence that hospital-acquired MRSA infection increases morbidity, the risk of mortality, and costs. The costs accrue either directly as expenses caused by extension of hospital stay, additional diagnostic or therapeutic procedures, and additional antibiotic use or indirectly through the loss of productivity, longterm disability, and excess mortality (Grundmann, 2006). Baseline data from the Pan-European Antimicrobial Resistance Using Local Surveillance (PEARLS) study of 2001 -2002 showed that South Africa had a MRSA prevalence of 33.3% (Bouchillon *et al*., 2004).

Another survey of patients with *Staphylococcus aureus*bacteraemia at two academic hospitals in Johannesburg identified a MRSA rate of 23% (Perovic *et al*., 2006). During the last decade, CA-MRSA has emerged worldwide, not only in the community, but also in healthcare facilities. The first report of

CA-MRSA came in 1993 from Western Australia among aboriginal patients who had never had contact with the health care system. These CA-MRSA strains were isolated from healthy individuals, who had skin and soft tissue infections, but did not possess any known risk factors for MRSA infection. Several CA-MRSA clones have spread worldwide since the 1990s. In the United States, 4 cases of rapidly fatal MRSA infections in children were reported by the Centres for Disease Control and Prevention (CDC) in 1997–1999 (CDC, 1999). However, during recent years, CA-MRSA has started to replace HA-MRSA in healthcare facilities, especially in the USA and Taiwan, where the CA-MRSA prevalence is high (Deurenberg and Stobberingh, 2008). A study performed in a medical centre in Houston, USA, showed that 60% of the nosocomial MRSA isolates were associated with the USA300 clone (Gonzalez *et al*., 2006). In a study, CA-MRSA caused more than 50% of all suppurative skin infections among patients who presented to emergency departments (EDs) in 11 US metropolitan centres (Moran *et al*., 2006). A study by Hota *et al*. (2007) observed that the incidence of CA-MRSA skin and soft tissue infections (SSIs) increased from 24.0 cases per 100 000 people in 2000 to 164.2 cases per 100 000 people in 2005 (risk ratio = 6.84). CA-MRSA infection is transmitted from person to person and as such intrafamilial spread with subsequent onset of clinical infections and outbreaks has been reported (Hota *et al*., 2007). A study carried out in a University hospital in the South western part of Nigeria by Ghebremedhin *et al*. (2009) reported the prevalence of CA-MRSA (47%) which was higher than 29% reported in the study of Taiwo *et al*. (2005). The genes encoding Panton-Valentine leukocidin (PVL) has been traditionally associated with CA-MRSA but there has been a potentially disturbing discovery of considerable numbers of PVL positive MSSA strains from some African countries such as South Africa and Nigeria (Esan *et al*., 2009). A study conducted on MRSA isolates from five African towns, one each in Cameroon, Madagascar, Morocco, Niger and Senegal reported a spread of CA-MRSA carrying the PVL genes especially in Dakar.

2.6. Molecular Typing Methods for *Staphylococcus aureus*

The epidemiology of infectious diseases relies on typing methods as tools for the characterization and discrimination of isolates based on either their genotypic or phenotypic characteristics, which may be used to establish clonal relationships between strains and to trace the geographic dissemination of bacterial clones. Numerous techniques are available to differentiate*Staphylococcus aureus*, and specifically MRSA, isolates. Historically, isolates were distinguished by phenotypic methods, including antibiotic susceptibility testing and bacteriophage typing. Both methods have limitations, as genetically unrelated isolates commonly have the same antibiogram, and many *Staphylococcus aureus*isolates are nontypeable by phage typing (Shopsin and Kreiswirth, 2001). Nowadays, the classification of isolates is mostly based on molecular methods, which usually provide better discriminatory power than phenotypic methods. Initial techniques compared restriction endonuclease patterns of chromosomal or plasmid DNA. The second generation of genotyping methods included Southern blot hybridization using gene-specific probes, ribotyping, polymerase chain reaction (PCR)-based approaches, and pulsed-field gel electrophoresis (PFGE) (Shopsin and Kreiswirth, 2001). Pulsed-field gel electrophoresis (PFGE) is accepted as a "gold standard" for typing several Gram-positive and Gram-negative bacteria and some yeast. Pulsed-field gel electrophoresis is a highly discriminatory and sensitive technique for distinguishing strains of *Staphylococcus aureus*and has been used for microepidemiologic (local or short-term) and macroepidemiologic (national, continental, or long-term) surveys (Çiftci et al.,, 2009). However, it needs expertise and special instrument, and takes time to conduct. PCR-based typing methods provide a feasible alternative tool, which is more rapid and cost-effective than other molecular typing systems. Recently, SCC*mec* typing has been introduced as an additional PCR typing technique for epidemiological investigation (Okuma *et al.*, 2002; Oliviera *et al.*, 2002). Due to the availability and affordability of DNA sequence technology, other sequenced based typing methods have been developed

and are now widely used, such as multilocus sequence typing (MLST) and *spa* typing, which are the most frequently used for *Staphylococcus aureus*. DNA sequence-based typing methods generate unambiguous and portable data,amenable to the creation of central databases, which enable the comparison of local data withdata from previous studies in different geographical locations. Typingmethods are expected to have high and relevant discriminatory power and typeability, goodreproducibility, applicability to all organisms of interest, ease of use, portability (that is, theyshould produce data that can easily be transferred between laboratories or presented in published work), and low cost (Cookson *et al.,* 2007). There are three related reasons for carrying out *Staphylococcus aureus*typing. Firstly, typing allows the detection of transmission events and patterns, from within a single healthcare facility, to between continents, and so is central to the practices of infection control and public health microbiology. Secondly, typing allows the inference of virulence and resistance properties of specific isolates and so can directly influence clinical decisions. The occurrence and spread of methicillin-resistant *Staphylococcus aureus*(MRSA) soon after the introduction of methicillin in clinical practice has led to the appearance of hospital-adapted multiresistant clones, which constitute a constantly growing problem as a major cause of nosocomial infections all over the world. The use of efficient and accurate epidemiological typing methods is a prerequisite for monitoring and for limiting the occurrence and spread of epidemic clones within and between hospitals (Strommenger et al., 2008).

2.6.1. Randomly Amplified Polymorphic DNA (RAPD)

Randomly Amplified Polymorphic DNA (RAPD), a simple PCR based technique, has been extensively used for epidemiological analysis. Moreover, RAPD primers can effectively scans the whole chromosomal DNA for the presence of small inverted repeats and amplifies the intervening DNA segments of variable length that can be used for identifying genetic variation and establishing strain-specific fingerprints. Also the assay can be performed with low concentration of DNA using short

synthetic oligonucleotide primers in length (Idil*et al.*, 2014). Based on their versatility and easy handling, PCR based RAPD is widely used in epidemiological study of MRSA. Moreover, it is very useful to have a precise microbial database linking genetic marker and their clinical outcomes in order to control their spread.

2.6.2. Antibiogram

*Staphylococcus aureus*isolates can always be compared on the basis of their susceptibility to a range of antibiotics. This technique is easy to perform, gives rapid results and, above all, is cheap and readily available in the routine microbiology laboratory. The major disadvantages are poor discriminatory ability and lack of reproducibility. Antibiotic resistance patterns are, to some extent, influenced by the local environment. Thus, the same antibiogram may be produced by unrelated strains as a consequence of the similar selective pressure upon them. It is equally possible that the antibiograms of two isolates from the same clone may differ due to the acquisition or loss of plasmids carrying resistance genes. It has been found in practice that a simple categorization of antibiotics into susceptible and resistant often cannot discriminate between unrelated strains (Weller, 2000). Also the antimicrobial susceptibility pattern of an individual strain may change during treatment, or because of antibiotic selection pressure in hospitals.

2.6.3. Pulsed Field Gel Electrophoresis (PFGE)

PFGE is the most commonly used method for studying local or short-term *Staphylococcus aureus*epidemiology. It has proven invaluable in investigations of nosocomial outbreaks, butdifficulties in reproducibility and interlaboratory reliability have limited its application torelatively small studies (Cookson *et al*., 2007). In PFGE for *Staphylococcus aureus*, the chromosomal DNA is digested with the restriction enzyme SmaI, and the resulting DNA fragments are separated by agarose gel electrophoresis in an electric field with an alternating voltage gradient. The resulting banding patterns are analyzed using a special software package, such as GelCompar II from Applied Maths, using Dice comparison and

unweighted pair groupmatching analysis (UPGMA) settings according to the criteria of Tenover *et al*. (1994). InPFGE, smaI- restricted *Staphylococcus aureus*genomes are compared to determine their geneticrelatedness and also compared against the reference USA genotypes (USA 100, USA 200 etcup to USA 1200) as described by the centers for Disease Control and prevention (CDC)(Shukla *et al.*, 2010). Population structure and genetic diversity of *Staphylococcus aureus*has been extensively studied in the past using PFGE but difficulties in reproducibility andinterlaboratory reliability have limited its application (Lamers *et al*., 2011). This method hasproved very successful for the investigation of nosocomial outbreaks and has also been usedto identify MRSA clones that have a particular ability to cause major outbreaks and to spreadinternationally (epidermic MRSA clones; EMRSA)

2.6.4 Polymerase Chain Reaction (PCR)

PCR is an enzyme-driven process for amplifying short regions of DNA in vitro. The method relies on knowing at least partial sequences of the target DNA and using them to design oligonucleotide primers that hybridise specifically to the target sequences. In PCR, the target DNA is copied by a thermostable DNA polymerase enzyme, in the presence of nucleotides and primers. Through multiple cycles of heating and cooling in a thermocycler to produce rounds of target DNA denaturation, primer hybridisation, and primer extension, the target DNA is amplified exponentially. Theoretically, this method has the potential to generate billions of copies of target DNA from a single copy in less than 1 hour (Yang and Rothman, 2004). There are three main stages in polymerase chain reaction. The first, denaturing, takes place at high temperatures, and is necessary for the separation of the double strand into two single strands. In the second stage, there is annealing of the primers present in the reaction mixture to their complementary region in the template DNA. The annealing temperature used is very important as it determines the specificity (stringency) of the reaction. The third stage, synthesis, is polymerisation by means of a thermostable DNA polymerase (Taq DNA polymerase), extending the primers. Taq DNA polymerase

works in the presence of Mg2+ ions and the four deoxyribonucleotide triphosphates (dNTPs), and results in the duplication of the region of interest *ad libitum*. Each complete cycle takes around five minutes and doubles the quantity of DNA produced in the preceding cycles. For an efficient amplification of DNA, 20 to 40 cycles are necessary. In this way, target DNA is amplified approximately a billion fold. Over the past two decades, PCR has been extensively modified to expand its utility and versatility. Multiplex PCR enables the simultaneous detection of several target sequences by incorporation of multiple sets of primers. PCR has given rise to a variety of techniques with many applications, amongst these, the discrimination between bacterial isolates. Several typing methods based on PCR have been developed for typing *Staphylococcus aureus*. PCR amplification of a tandem repeat region of the extracellular part of staphylococcal coagulase (*coa)*, and subsequent *Alu*I digestion, results in discrimination of strains. The variation in the staphylococcal protein A (*spa)* repeat units is also a target for typing. The ribosomal RNA sequences and the spacer sequence between 16S and 23S rDNA is also a target for PCR-based typing. The 16s rRNA gene has been used extensively for evolutionary studies of bacterial species and the rRNA operon has been used for evolutionary studies of bacterial strains. This sequence varies in length and copy number, so that a single isolate may contain up to 15 different alleles ranging from 906 to 1223 bp (Weller, 2000). It is a major breakthrough since it eliminates the need for laborious post-amplification processing (i.e. gel electrophoresis) conventionally needed for amplicon detection, and allows formeasurement of product simultaneous with DNA synthesis. Amplification products in realtime PCR are measured as they develop by fluorescence resonance emission transfer probes, molecular beacons, or TaqMan probes (Yang and Rothman, 2004; Tenover, 2007).

2.6.5. Coagulase Gene Typing

Amplification of the coagulase gene *(coa)* has been considered a simple and accurate method for typing *Staphylococcus aureus*isolated from distinct sources (da Silva and da Silva, 2005). Production of

coagulase is one of the defining characteristics of *Staphylococcus aureus*and is thought to be an important virulence factor. A typing technique based on the heterogeneity within a specific segment of the coagulase gene has been developed. The 3' region consists of a series of 81 base-pair tandem repeats which vary in number from four to eight. In this method, PCR is used to amplify the 3' region and to produce a primary product, usually a single fragment, but on some occasions two. The size of the fragment is dependent upon the number of repeats within the area amplified. Although this region is quite well conserved, point mutations do occur which affect the number of restriction enzyme binding sites. The amplification product can, therefore, be characterized further by digestion with a restriction enzyme such as *Alu*I, which produces up to four bands of variable length depending on the number of restriction sites present (Weller, 2000).

2.6.6. Staphylococcal Protein A (*spa*) Typing

Another target for PCR based typing of *Staphylococcus aureus*has been the X region, a hypervariable area of the *spa* gene coding for protein A. *spa* typing is a rapid sequence-based approach to characterize MRSA and has high portability, discrimination, and ease of use. It enables the reliable allocation of isolates to the most prevalent epidemic lineages **(**Strommenger *et al*.,2008**).** The main advantage of *spa* typing over MLST is its simplicity, since it involves sequencing of only a single locus. It is therefore less expensive, less laborious and less time consuming (Deurenberg *et al*., 2007). *spa*typing of *Staphylococcus aureus*evaluates sequence polymorphism in the variable X region of the *spa* gene, which encodes staphylococcal surface protein A. The diversity of the *spa* gene, consisting mainly of a number of repeats of 24 bp in length, is attributed to point mutations, as well as to deletions and duplications of the repeats. *spa*typing in contrast to PFGE and MLST, can be used to study both the molecular evolution as well as hospital outbreaks of MRSA (Koreen *et al*., 2004). Another advantage of *spa* typing is the availability of the software package StaphType (Ridom GmbH, Würzburg, Germany) is most widely

used for the analysis of *spa* sequences in Europe but is also used in reference laboratories worldwide to analyze the resulting sequence chromatograms (Deurenberg *et al*., 2007).

2.6.7 Staphylococcal Cassette Chromosome *mec* (SCC*mec*) Typing

The detection of the *mecA* gene by real-time PCR is widely recognized as the gold standard for identification of MRSA. During the past several years, a number of SCC*mec* typing methods based on multiplex PCR (M-PCR) have been developed (Ito *et al*., 2001; Oliveira and de Lencastre, 2002; Zhang *et al*., 2005; Boyce, 2007). The multiplex PCR method developed by Oliveira and de Lencastre, (2002) detects specific genes or motifs located mostly in the J regions of SCCmec type I to IV. The method developed by Ito *et al.* (2001) uses several PCR assays to determine the structure of the *mec* complex and the presence of the different *ccr* genes. Boyce(2007) and Zhang *et al.* (2005) developed an easy-to-use multiplex PCR assay for the determination of the structure of SCC*mec* type I to V. A disadvantage of these methods is that they detect only a single locus on the majority of the SCC*mec* types, and, as a consequence, these two methods have less discriminatory power compared to the methods ofIto *et al*. (2001) and Oliveira and de Lencastre (2002) and SCC*mec* typing is of importance in epidemiological studies to distinguish MRSA strains or to define an MRSA clone in combination with the genotype of methicillin-susceptible *Staphylococcus aureus*(MSSA) strain in which an SCC*mec* element has integrated (Turlej *et al*., 2011).

2.6.8. Multilocus Sequence Typing (MLST)

MLST is the primary means by which *Staphylococcus aureus*strains have been analysed for the past decade. Because of the slow rate of molecular evolution within MLST genes, this methodology is most useful on a global epidemiology scale (Lamers *et al.,* 2011). MLST is a highly discriminatory method of characterizing bacterial isolates on the basis of the sequences of approximately 450-bp internal fragments of seven housekeeping genes i.e. *arcC, aroE, glpF, gmk, pta, tpi* and *yqiL*, each approximately 500-bp in

length. A distinct allele is assigned to each of the different sequences of each housekeeping gene. The alleles of the seven genes define the *Staphylococcus aureus*lineage, resulting in an allelic profile designated sequence type (ST) (Figure 2.3 below).

Isolates are unlikely to have identical allelic profiles by chance and isolates with the same allelic profile are assigned as members of the same clone. Sequence data are readily compared between laboratories and a major advantage of MLST is that results obtainable on different studies can be compared through the internet (Enright *et al*., 2000). The algorithm based upon related sequence types (BURST) is used to define clonal complexes (CCs), and to study the evolutionary events within a *Staphylococcus aureus* population. MLST has a major advantage over PFGE as a reference method due to the unambiguous nature of DNA sequences, which can be stored easily along with corresponding clinical information on each isolate in Internet-linked databases. The *Staphylococcus aureus*MLST website currently contains information on over 1,500 isolates from humans and animals from 40 different countries and represents a useful global resource for the study of the epidemiology of this species and the surveillance of hypervirulent and/or antibiotic-resistant clones (Cookson *et al*., 2007).

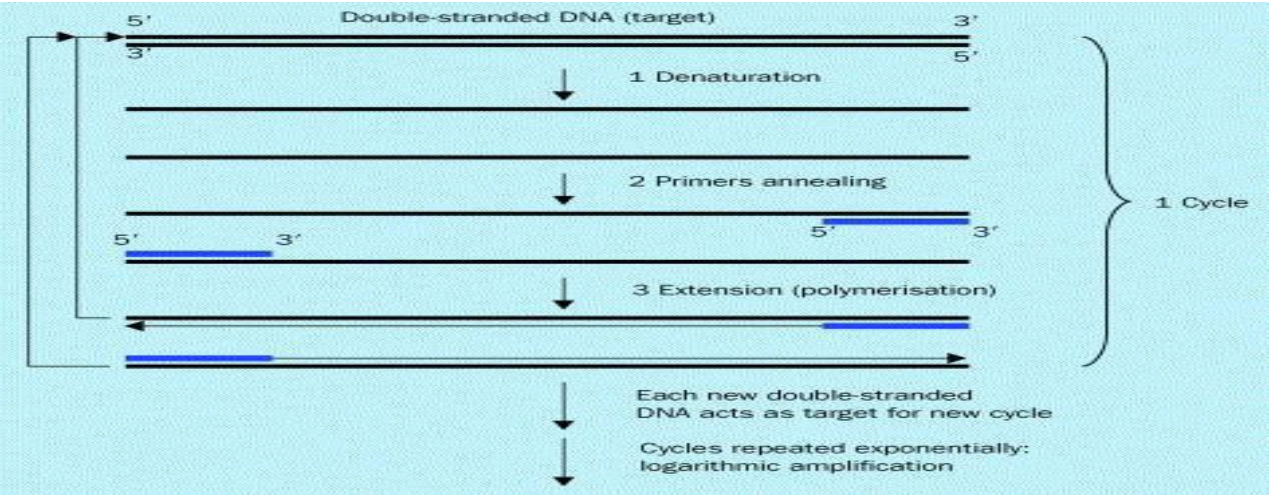

Figure 2.3: Multilocus Sequence Typing (MLST) of *Staphylococcus aureus*

(Source: Lamers *et al.*, 2011)

2.7. Bacterial Drug Resistance

2.7.1 `History of Development of Resistance

Resistant bacteria have always been around and existed long before humans began to use antibiotics therapeutically. What is new in the world of resistance is how quickly new resistant strains arise. The widespread use and misuse of antibiotics contribute to the problem (Saga and Yamaguchi, 2009). The mortality of patients with *Staphylococcus aureus*bacteremia in the pre-antibiotic era exceeded 80%, and over 70% developed metastatic infections. The introduction of penicillin in the early 1940s dramatically improved the prognosis of patients with staphylococcal infection.

However, as early as 1942, penicillin-resistant Staphylococci were recognized, first in hospitals and subsequently in the community. By the late 1960s, more than 80% of both community- and hospital-acquired staphylococcal isolates were resistant to penicillin. This pattern of resistance, first emerging in hospitals and then spreading to the community, is now a well-established pattern that recurs with each new wave of antimicrobial resistance (Chambers, 2001). Today, 80 percent of *Staphylococcus*strains do not respond to penicillin (Appelbaum, 2007). In the 1940s and early 1950s, streptomycin, chloramphenicol, and tetracycline were discovered. By 1953, a strain of *Shigella* was found that resisted these antibiotics and sulfanilamides. By the 1970s, resistant strains of gonorrhea arose. The 1990s saw the development of true superbugs, bacteria that resist all known antibiotics. One antibiotic of last resort is Vancomycin, a powerful antibiotic that attacks bacteria on many fronts. Now there are *Enterococci* strains that resist Vancomycin. Multi-drug resistant tuberculosis strains have arisen. By the 1940s and 1950s, a single antibiotic, such as Streptomycin, no longer cured tuberculosis, as it had in the past. Tuberculosis became the leading cause of death by infectious disease in the world. Increasing resistance to antibiotics is a consequence of selective pressure but the actual incidence of resistance varies between different bacteria species (Jawetz *et al.*, 2007).

The origin of drug resistance may be genetic or non-genetic.

2.7.1.1 Non-genetic Origin

Here the inherent properties of the bacterium are responsible for preventing antibiotic action. There are many antibiotics which are active against Gram positive bacteria which have no effect on Gram negative bacteria and *vice versa*. This intrinsic resistance is thought to be associated with the outer cell layers such as the outer membrane which are absent in Gram positive cells. The Gram negative envelope is effectively impermeable, preventing certain antibiotics from reaching their intracellular target. Active replication of bacteria is usually required for most antibacterial drug actions. However their offspring are fully susceptible e.g. Mycobacteria often survive in tissues for many years after infection yet are restrained by the host‘s defenses and do not multiply. Such persisting organisms are resistant to treatment and cannot be eradicated by drugs. Microorganisms may lose the specific target structure for a drug for several generations and thus be resistant e.g. penicillin susceptible organisms may change to L-forms (protoplasts) during penicillin administration. Lacking most cell wall, they are then resistant to cell wall inhibitor pump e.g. penicillins, cephalosporins and may remain so for several generations in this form as persisters when these organisms revert to their bacterial parent forms by resuming cell wall production, they will then become fully susceptible to penicillin again (Jawetz *et al*., 2007).

2.7.1.2 Genetic Origin

Most drug-resistant microorganisms emerge as a result of genetic change and subsequent selection processes by antimicrobial drugs. Genetic mechanism may be chromosomal or extra-chromosomal.

2.7.1.2.1. Chromosomal Resistance

This develops as a result of spontaneous mutation in a locus on the bacterial chromosome that controls susceptibility to a given antimicrobial. The presence of the drug serves as a selecting mechanism to suppress susceptible strains and promote the growth of drug - resistant mutants. Spontaneous mutation

occurs with a frequency of 10^{-12} to 10^{-7}. This is however an infrequent cause for the emergence of clinical drug resistance within a given patients. Chromosomal mutants are commonly resistant by virtue of a change in a structural receptor for a drug. Mutation may also result in a loss of penicillin receptors in some microbial species making the mutant penicillin resistant.

2.7.1.2.2. Extrachromosomal Resistance

Bacterial may also acquire extrachromosomal genetic elements called Plasmids. Plasmids are circular DNA molecules, have 1-3% of the weight of the bacterial chromosome and may exist free in the bacterial cytoplasm, or may be integrated into the bacterial chromosome. Some carry their own genes for replication and transfer; others rely on genes in other plasmids. There may be 1-40 copies of a particular plasmid present, depending on the type and there may be more than one type of plasmid in each bacterial cell.

Plasmids that carry genes for resistance to antibiotics (r genes) are referred to as R plasmids. Plasmid genes for antimicrobial resistance often control the formation of enzymes capable of destroying antimicrobial drugs. Thus plasmids determine resistance to penicillins and cephalosporin by carrying genes for the formation of β-lactamases. Plasmids code for enzymes that destroy chloramphenicol (acetyltransferase); that acetylate, adenylylate or phosphorylate various aminoglycosides and those that determine the permeability of the cell envelope to tetracyclines.

In most instances, resistance to multiple antimicrobial agents in the Staphylococci is driven by the acquisition of discrete genetic 'accessory' elements comprising plasmids, transposable genetic elements (insertion sequences and transposons) and genomic islands. Such elements incorporate preformed antimicrobial resistance genes and are exchanged via horizontal gene transfer (HGT) between inter-related bacterial strains and even between different species and genera. The conventional microbial HGT

mechanisms of transduction, transformation and conjugation have all been demonstrated in the Staphylococci (Chambers, 2001).

2.7.1.2.3. Vertical Gene Transfer

The spontaneous mutation for antibiotic resistance is on the order of about of about frequency 10-8- 10-9. This means that one in every 108- 109 bacteria in an infection will develop resistance through the process of mutation. In *Escherichia coli,* it has been estimated that streptomycin resistance is acquired at a rate of approximately 10-9 when exposed to high concentrations of streptomycin. Although mutation is a very rare event, the very fast growth rate of bacteria and the absolute number of cells attained means that it doesn't take long before resistance is developed in a population. Once the resistance genes have developed, they are transferred directly to all the bacteria's progeny during DNA replication. This is known as vertical gene transfer or vertical evolution. The process is strictly a matter of Darwinian evolution driven by principles of natural selection: a spontaneous mutation in the bacterial chromosome imparts resistance to a member of the bacterial population. In the selective environment of the antibiotic, the wild types (non mutants) are killed and the resistant mutant is allowed to grow and flourish (Keeling and Palmer, 2008).

2.7.1.2.4. Horizontal Gene Transfer

Another mechanism beyond spontaneous mutation is responsible for the acquisition of antibiotic resistance. Lateral or horizontal gene transfer(HGT) is a process whereby genetic material contained in small packets of DNA can be transferred between individual bacteria of the same species or even between different species (Gyles and Boerlin, 2014).

2.7.1.2.4.1. Mechanisms of Horizontal Gene Transfer

2.7.1.2.4.1.1. Transduction

Transduction is a process by which plasmid DNA is enclosed in a bacterial virus (or phage) and transferred to another bacterium of the same species (Figure 2.4) (Stearns and Hoekstra, 2005). It is a relatively ineffective means of transfer of genetic material but there is evidence that it is clinically important in the transmission of resistance genes between strains of Staphylococci and between strains of Streptococci.

2.7.1.2.4.1.2. Transformation

A bacterium undergoes transformation by taking up naked DNA from its environment and incorporating it into its genome (Stearns and Hoekstra, 2005). It is possible only when the incoming DNA comes from a cell belonging to the same strain as the host bacterium or one that is very closely related.

2.7.1.2.4.1.3. Conjugation

Conjugation involves cell-to-cell contact during which chromosomal or extrachromosomal DNA is transferred from one bacterium to another (Stearns and Hoekstra, 2005). It is the main mechanism for the spread of resistance. The ability to conjugate is encoded in conjugative plasmids; these are plasmids that contain transfer genes which code for the production, by the host bacterium of surface tubules of proteins that connect the two cells-sex pili. The conjugative plasmid then passes from one bacterium to the other which is usually of the same species as shown in figure 2.4 overleaf. Some plasmids can cross the species barrier. Many R plasmids are conjugative. Non conjugative plasmids can make use of sex pili if they coexist in the donor cell with conjugative plasmids (Campbell and Reece, 2002).

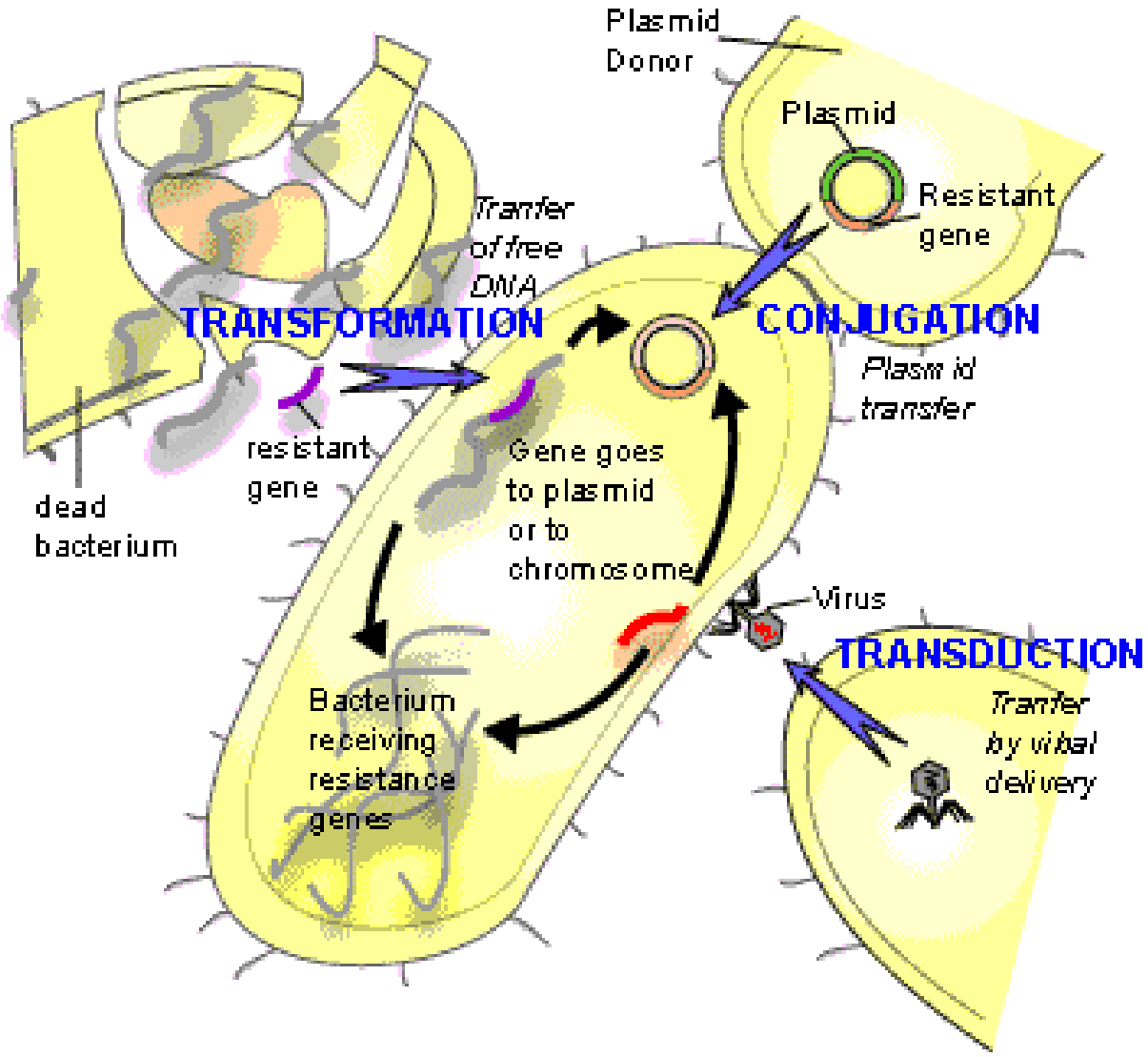

Figure 2.4: Mechanisms of horizontal gene transfer

(Source: Wiley *et al*., 2008)

2.7.2. Mechanism of Resistance to Antibiotics in *Staphylococcus aureus*

*Staphylococcus aureus*can exemplify better than any other human pathogen the adaptive evolution of bacteria in the antibiotic era, as it has demonstrated a unique ability to quickly respond to each new antibiotic with the development of resistance mechanisms (Sofia *et al*., 2013) which include:

a. Enzymatic inactivation of the antibiotic (penicillinase and aminoglycoside-modification enzymes),

b. Alteration of the target with decreased affinity for the antibiotic (notable examples being penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*and D-Ala-D-Lac of peptidoglycan precursors of vancomycin-resistant strains),

c. Trapping of the antibiotic (for vancomycin and possibly daptomycin) and

d. Efflux pumps (fluoroquinolones and tetracycline).

2.7.2.1 Enzymatic Inactivation of the Antibiotic

Mechanism of resistance by enzyme inactivation involves the hyper production of an enzyme that inactivates the antibiotic by an enzymatic cleavage or chemical modification such that they no longer interact with the target site or are no longer taken up by the organism. The most common example of this type of resistance is that mediated by β -lactamases which are widely distributed in both Gram-negative and Gram- positive species (Hawkey, 2000).

The antibiotics concerned are β-lactam ring of penicillins and cephalosporins. Staphylococci are the principal bacteria producing β-lactamase, and the genes which code for the enzymes are on plasmids that are transferred by transduction. In Staphylococci the enzyme is inducible; its synthesis is at a very low level in the absence of the drug. The enzyme may diffuse through the envelope and inactivate antibiotic molecules in the surrounding medium. Gram negative organisms can also produce β-lactamases, which are a significant factor in their resistance to the semisynthetic broad spectrum β-lactam antibiotics. Here the enzyme may be determined by either chromosomal genes or by plasmid genes. The enzymes are

produced constitutively (i.e. they are synthesized even when the substrate is absent) and remain attached to sites in the cell wall preventing access of the drug to the membrane associated target site; they do not inactivate the drug in the surrounding medium. Many of these β-lactamases are encoded by transposons, some of which may also carry resistance determinants to several other antibiotics. Figure 2.5 overleaf illustrates the mechanism of enzymatic inactivation of beta-lactam antibiotics.

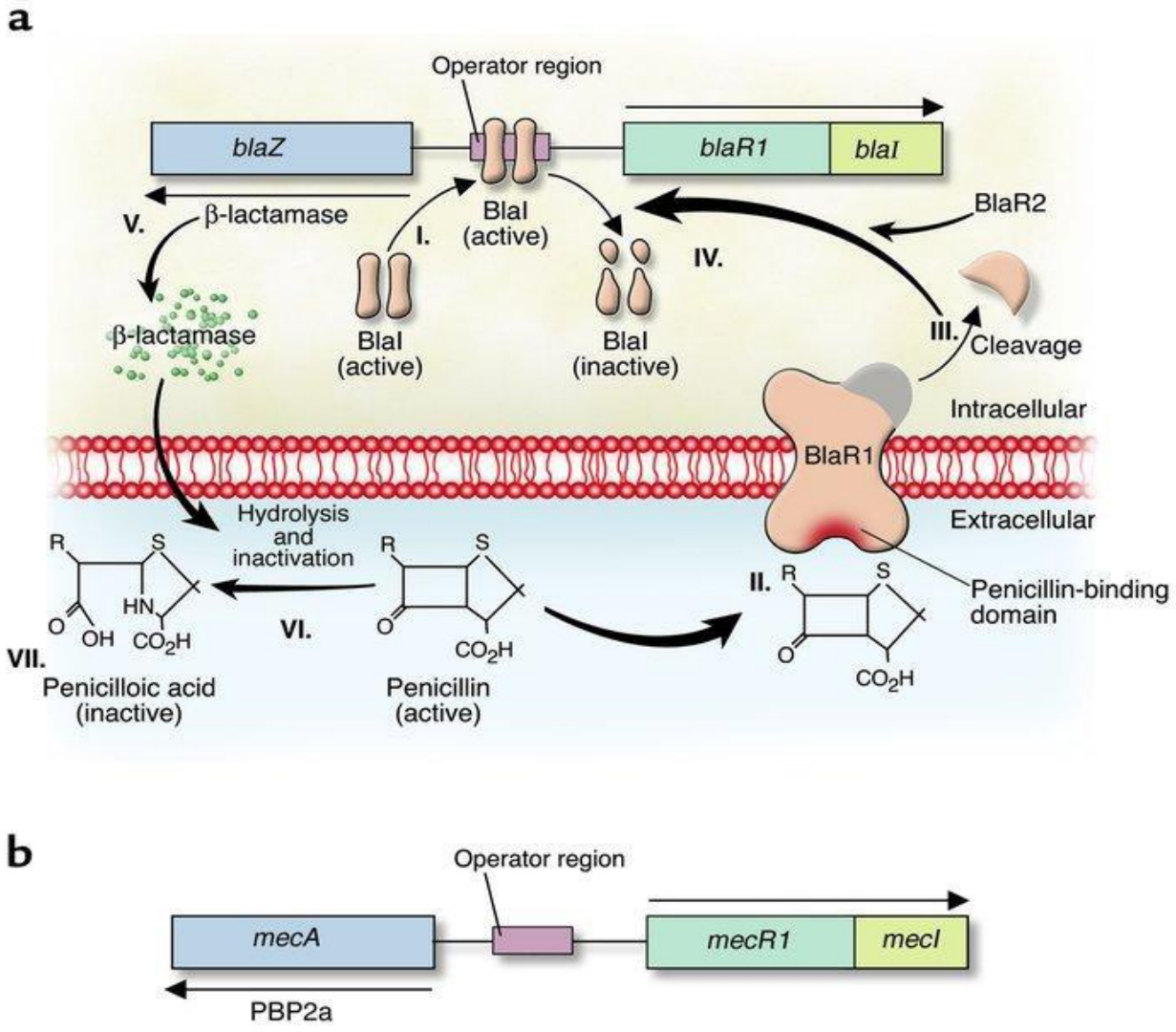

Figure 2.5: Mechanism of enzymatic inactivation of beta- lactam antibiotics

(Source: Lowry, 2003)

2.7.1.1.1 Induction of Staphylococcal β-lactamase Synthesis in the Presence of the β-lactam Antibiotic- Penicillin.

I. The DNA-binding protein BlaI binds to the operator region, thus repressing RNA transcription from both blaZ and blaR1-blaI. In the absence of penicillin, β-lactamase is expressed at low levels.

II. Binding of penicillin to the transmembrane sensor-transducer BlaR1 stimulates BlaR1 autocatalytic activation.

III–IV. Active BlaR1 either directly or indirectly (via a second protein, BlaR2) cleaves BlaI into inactive fragments, allowing transcription of both blaZ and blaR1-blaI to commence.

V–VII.β-Lactamase, the extracellular enzyme encoded by blaZ (V), hydrolyzes the β-lactam ring of penicillin (VI), thereby rendering it inactive (VII) (Lowry,2003)

2.7.1.1.2Mechanism of *Staphylococcus aureus*Resistance to Methicillin.

Synthesis of PBP2a proceeds in a fashion similar to that described for β-lactamase. Exposure of MecR1 to a β-lactam antibiotic induces MecR1 synthesis. MecR1 inactivates MecI, allowing synthesis of PBP2a. MecI and BlaI have coregulatory effects on the expression of PBP2a and β-lactamase.

II. Chloramphenicol inactivation is brought about by chloramphenicol acetyltransferase while inactivation of aminoglycosides may be brought about by phosphorylation, adenylation or acetylation, and the requisite enzymes have been found in both Gram-negative and Gram-positive organisms. The resistance genes are carried on plasmids and transposons (Hawkey, 2000).

2.7.2.2. Alteration of the Target with Decreased Affinity for the Antibiotic

The protein on the 30S subunit of the ribosome, which is the binding site for aminoglycosides, may be altered as a result of a chromosomal mutation. A plasmid mediated alteration of the binding site protein on the 50S subunit underlies resistance to erythromycin. Some Staphylococci carry an altered penicillin binding protein (PBP2a) which is coded for by a mutated chromosomal gene; it has much lower affinity

for penicillins and it confers intrinsic resistance. Vancomycin resistance is another example. Under susceptible conditions, vancomycin prevents cross-linking of peptidoglycan by binding to D-Ala-D-Ala dipeptide of the muramyl peptide. Most Gram positive bacteria acquire vancomycin resistance by changing D-Ala-D-Ala to D-Ala-D-lactate, which vancomycin does not bind to (Sofia *et al*., 2013). Mutations in DNA gyrase A and B subunits in quinolone resistance is another example of an alteration of the drug target. Finally, in Rifampicin resistance, there are mutations in rpoBgene encoding beta-subunit of RNA polymerase.

2.7.2.3 Increased Efflux Activity (Efflux pumps)

Efflux pumps are membrane proteins that have the function of detoxifying cells by expelling noxious molecules. In *Staphylococcus aureus*, several specific efflux pumps have been associated with resistance to antibiotics, such as tetracycline (*TetK*), *TetL*) and macrolides (*MefA*), *MsrA*) (Poole, 2007). Also, several multidrug efflux pumps have been described that are associated with resistance to antibiotics (e.g., fluoroquinolones) (Poole, 2007). In general, specific efflux pumps can be found either in the chromosome or in plasmids, while multidrug efflux pumps are mainly located in the chromosome, (Sofia *et al*., 2013). An important example of decreased drug accumulating is the plasmid-mediated resistance to tetracycline in both gram positive and gram negative bacteria. The resistance genes in the plasmid code for inducible resistance proteins in the membrane which promote energy-dependent efflux of the tetracycline and hence resistance. This has reduced the value of tetracycline in human and veterinary medicine. Tetracycline efflux was discovered in the early 1980s; *tetK* serves as an example for an efflux - mediated tetracycline resistance. Under normal conditions, the efflux gene, *tetK*, is not expressed, due to a suppressor that is bound to the promoter region. However, in the presence of tetracycline, it binds to the repressor, relieves the suppression, and causes transcription and translation of the efflux pump, thereby leading to tetracycline resistance (Krzysztof *et al.*, 2000).

2.7.3 Methicillin

2.7.3.1. History of Methicillin

Before the antibiotic era infections with *Staphylococcus aureus*caused numerous deaths, but the period between 1950 and 1997 represented the golden age for the treatment of staphylococcal infections, as the bacteria were mostly susceptible to penicillins. During the period between 1950 and 1959, approximately 80% of hospital acquired infections caused by *Staphylococcus aureus*became resistant to penicillin due to the production of beta lactamase. In the early 1960, a semi-synthetic, broad spectrum beta-lactam compound called methicillin was introduced, which was active against penicillinase-producing *Staphylococcus aureus*(Chambers, 2009)

2.7.3.2 Methicillin - resistant *Staphylococcus aureus*(MRSA)

Methicillin - resistant *Staphylococcus aureus(*MRSA) is any strain of *Staphylococcus aureus*that has evolved resistance to beta-lactam antibiotic which include the penicillins (Methicillin, dicloxacilin, nafcillin, oxacillin etc) and the cephalosporins. In 1961, the first isolate of MRSA was reported in England. Since then, MRSA has increasingly been isolated in various countries, and at present, it is one of the major causes of nosocomial infection throughout the world, thus it is alternatively called hospital-acquired MRSA (HA-MRSA). In addition, from 1997 to 1999, another class of MRSA has become a major concern worldwide because it has become an emerging pathogen in the community (Deurenberg and Stobberingh, 2008). This new class of MRSA is called community- acquired MRSA (CA-MRSA).

2.7.3.3. Evolution of Methicillin Resistant *Staphylococcus aureus*(MRSA)

Methicillin was introduced in 1959 to treat infections caused by penicillin-resistant *Staphylococcus aureus*. In 1961, the first methicillin-resistant *Staphylococcus aureus*(MRSA) was reported from England. MRSA isolates were soon recovered from otherEuropean countries, and later from Japan, Australia, and the United States. Currently, MRSAis a major cause of nosocomial infections worldwide

and are referred to as hospitalassociated/acquired methicillin resistant *Staphylococcus aureus*(HA-MRSA). The worldwidespread of MRSA is driven by the dissemination of a number of clones with a specific geneticbackground (Enright *et al.*, 2002; Deurenberg *et al.*, 2007). Molecular epidemiologicalstudies have shown the spread of several MRSA clones internationally as well as in thehospital setting. These epidemic hospital-acquired MRSA (HA-MRSA) clones have beenidentified as the Archaic/Iberian, Brazilian/Hungarian, Berlin, New York/Japan, paediatric,EMRSA-15 and EMRSA-16 clones (Baranovich *et al.,* 2010). The Sentry AntimicrobialSurveillance Program investigated the prevalence of MRSA in hospitals worldwide between1997 and 1999; it was observed that the MRSA prevalence was 23% in Australia, 67% inJapan, 35% in Latin America, 40% in South America, 32% in the USA, and 26% in Europe(Diekema *et al*., 2001; Bell and Turnidge, 2002). Infections caused by HA-MRSA have beena problem since the 1970s but in the 1990s, new strains of MRSA referred to as communityassociated methicillin resistant *Staphylococus aureus*(CA-MRSA) appeared in communitydwellers. Individuals infected with CA – MRSA have none of the established risk factorsassociated with HA-MRSA such as recent hospitalization, surgery, dialysis, long term careresidence or indwelling percutaneous medical devices (Shukla *et al*., 2010).The first report of CA-MRSA came in 1993 from Western Australia, and described the observation of CA-MRSA in Aboriginal patients in remote communities.

These CA-MRSA strains were isolated from healthy individuals, who had skin and soft tissue infections, but did not possess any known risk factors for MRSA infection. The Centre for Disease Control and Prevention(CDC)defines CA-MRSA as Methicillin Resistant *Staphylococus aureus* strains isolated in an outpatient setting, or isolated from patients within 48 hours of hospital admission. Furthermore, these patients must have no medical history of MRSA infection or colonization, and no medical history in the past year of either hospitalization (e.g. surgery), admission to a nursing home, or dialysis. Moreover, the

patient should not have permanent indwelling devices, such as catheters or a percutaneous device at the time of culture or previous isolation from the (Naimi *et al*., 2003; Morrison *et al*., 2006). Other criteria used to define CA–MRSA infections relate relevant isolate characteristics such as antimicrobial susceptibility profiles, DNA fragment patterns upon pulsed – field gel electrophoresis, protein A (*spa*) gene typing, carriage of PVL genes; multilocus sequence typing and the type of SCC*mec* element carried (David and Daum, 2010).

CA - MRSA are genetically different from HA – MRSA strains. This suggests that CAMRSA did not originate from HA isolates that escaped from the hospital setting rather; CAMRSA seems to have emerged de novo from established CA-MSSA isolates. CA-MRSA differs from nosocomial MRSA in that it is susceptible to most non-β-lactam antibiotics, contains the type IV SCC*mec* (for staphylococcal cassette chromosome *mec*, the mobile genetic element encoding methicillin resistance), and frequently carries genes responsible for the production of Panton-Valentine leukocidin (PVL). In contrast, nosocomial MRSAs are generally multidrug resistant and contain SCC*mec* types I, II, or III (Diekema *et al*., 2001).

Genotyping tools such as PFGE, Multilocus sequence typing (MLST) and *spa* typing have been used in differentiating the genotypes of CA-MRSA strains from those of other *Staphylococcus aureus*strains. Pulsed-field gel electrophoresis of the sma1 *Staphylococcus aureus*genome shows that CA-MRSAisolates mostly belong to USA300 and USA400 clones and in some cases USA1000 andUSA1100 clones as well. HA-MRSA isolates generally belong to USA100, USA200, andUSA500. More than a thousand MLST allelic profiles for *Staphylococcus aureus*have been identified sofar, of which CA - MRSA strains are primarily represented by sequence type 1 (ST 1) (USA400) and ST8 (USA 300 AND USA 500). The most predominant CA-MRSA spa types aret008 and t128 (Shukla *et al*., 2010). Sequencing of the genome of CA-MRSA strain MW2,which caused fatal sepsis in a 16-month-old girl

from North Dakota, USA identified 19putative virulence genes. These included genes for several superantigens, such asenterotoxins B and C, as well as the amphipathic leukotoxin, the Panton-Valentine leukocidin(PVL) (CDC, 1999). PVL, first described in 1932, is a bicomponent synergohymenotropic(synergistic membrane-tropic) toxin that was present in <5% of unselected *Staphylococcus aureus*isolatesbut is present in the majority of CA-MRSA isolates studied (Deresinski, 2005). PVL is a poreforming protein encoded by the *luk F – PV* and *luk S – PV* genes. While PVL has beenstrongly linked epidemiologically to prevalent CA-MRSA strains, it is not clear certain howthey contribute to virulence (David and Daum, 2010). The PVL toxin has been implicated inmany skin and soft tissue infections and lethal necrotizing pneumonia (Diep *et al*., 2008). Amodel showing the emergence of the PVL toxin in CA-MRSA presented in figure 2.6 below.

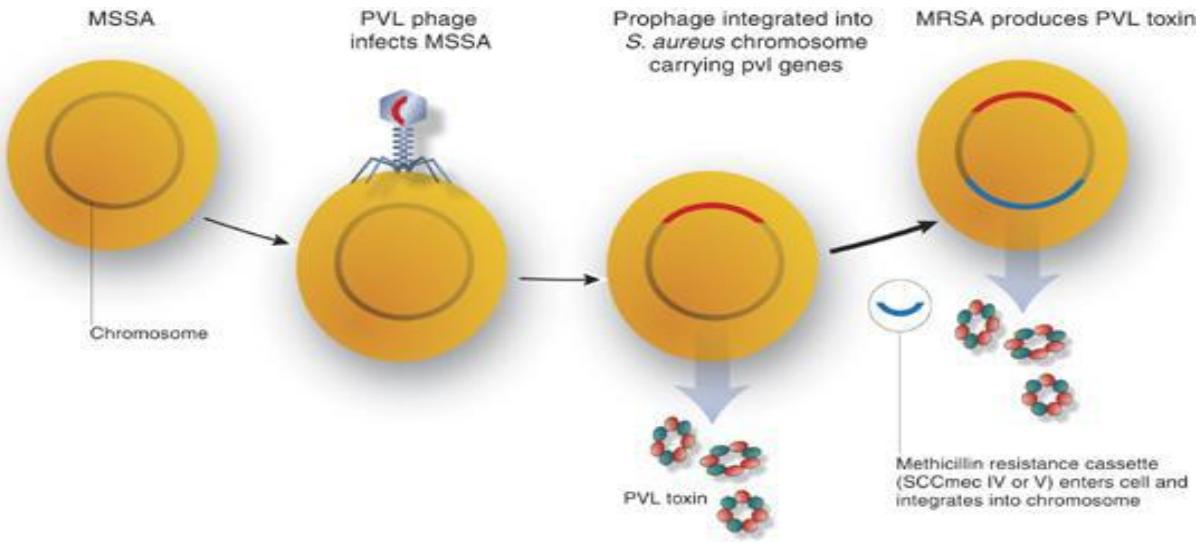

Figure 2.6: Model for the emergence of Panton-Valentine leukocidin (PVL)—producing community-associated methicillin-resistant *Staphylococcus aureus*(MRSA).

(Source: Rehm and Tice, 2010).

A methicillin-susceptible *Staphylococcus aureus*(MSSA) strain is infected and lysogenized by a phage (phiSLT) that harbors the genes encoding the PVL. Then a methicillinresistance cassette (SCC*mec* IV, V, or VT) carrying the *mecA* gene is horizontally transferred into the *pvl*-positive

MSSA strain and integrates into the genome distant from the phiSLT integration site (Rehm and Tice, 2010).

Serious and potentially lethal infections are caused by CA-MRSA. These include necrotizing pneumonia, necrotizing fasciitis, severe sepsis, and Waterhouse-Friderichsen syndrome (characterized by petechial rash, coagulopathy, and cardiovascular collapse). These more serious infections are associated with strains of CA-MRSA found to harbour genes for Panton-Valentine leukocidin and with a higher prevalence of genes for a-toxin and staphylococcal enterotoxin B, compared with that associated with HA-MRSA (Boucher *et al*., 2010). Outbreak reports of CA-MRSA infection in specific groups have suggested that these strains are more aggressive than has been seen previously with *Staphylococcus aureus*outside hospital.

Severe skin and soft tissue infections (perhaps starting with a 'spider bite' lesion), large and/or recurrent abscesses, necrotizing pneumonia or bone and joint infections have been typical. HIV infection has been determined to be an independent risk factor for CA-MRSA infection and colonization in some settings, particularly after 2001. A study carried out at an HIV clinic in Dallas Texas, in 2003 to 2004 revealed that 93% of isolates from SSTIs were MRSA (Skiest *et al*., 2006). Outbreaks of CA-MRSA infection have been reported among athletes and may be spread through repeated skin-skin contact, especially contact between broken skin, which occurs in games and practices. CA-MRSA infection has also been associated with exposures to various contaminated fomites, including whirlpools, shared razors, and shared towels, benches, body suits worn by fencers, and even a bar of soap (CDC, 2003; Nguyen *et al*.,

2005). In non-outbreak settings, close contact with a person who has a skin infection has been associated with CA-MRSA infections. This is particularly common in households where objects, such as toilet handles, doorknobs, and kitchen sinks serve as reservoirs for CA-MRSA strains and play an important role in recurrent infections (Miller and Diep, 2008). The Centres for Disease Control and Prevention has developed a model called the "Five Cs of CA-MRSA Transmission" (Ragan, 2006). The model suggests that MRSA infection results from the following constellation of risks:

(1) Contact (direct skin-skin contact); (2) cleanliness; (3) compromised skin integrity; (4) contaminated objects, surfaces, and items; and (5) crowded living conditions.

Resistant Staphylococci pose a problem for clinicians and their patients. Due to the limited therapeutic options, infections caused by these strains are usually difficult to treat. Secondly, invasive infection with MRSA is associated with increased mortality, although this has not been substantiated in all studies. Thirdly, by establishing an ecological niche in the hospital, the resistant strains have the potential to spread and colonise patients. Reasons for the increased mortality associated with MRSA infections are likely to be multifactorial and may not be completely understood. Potential explanations include increased virulence of the pathogen, clinical differences between patients infected with MRSA and those infected with MSSA (eg, MRSA infects older persons and immunocompromised patients), decreased efficacy of MRSA therapy relative to MSSA therapy (specifically, vancomycin, compared with β-lactam antibiotics), and delay in initiation of appropriate therapy (Boucher *et al*., 2010).

Traditionally, because of the universal resistance of MRSA to β-lactams and because of the lack of other effective alternatives, the glycopeptide vancomycin became the mainstay of treatment, because it provides in vitro activity against all Staphylococci and demonstrates clinical response against MRSA infection. However, in vitro susceptibility of MRSA to vancomycin is no longer universal (Tverdek *et al*., 2008).

2.7.3.4 Hospital - Acquired MRSA (HA- MRSA) and Community - Acquired (CA- MRSA) MRSA

HA-MRSA is typically defined as MRSA isolated from in - patients that had been MRSA-negative at the begining of hospitalization or MRSA isolated from in patients 48 hours or more after hospitalization (Naimi *et al.,* 2003). Epidemiologically, CA-MRSA are isolates from out patients with no history of hospitalization within the previous year and who presented no other established risk factors for MRSA infection such as surgery, residence in a long term care facility, dialysis or in-dwelling percutaneous medical device or catheters.

2.7.3.5 Clinical Implications of Methicillin Resistant *Staphylococcus aureus*

MRSA is a life-threatening, mullti - drug resistant bacterium, changing its resistance patterns by acquiring resistance to each new antimicrobial agent. This has made MRSA infection more difficult to treat with standard classes of antibiotics and thus more dangerous. MRSA strains are a persistent and increasing cause of nosocomially acquired infection in the world (Narezkina *et al*., 2006)

It has been difficult to quantify the degree of morbidity attributable to MRSA. In-patients with *Staphylococcus aureus*infection had, on average, 3 times the length of hospital stay, 3 times the total charges and 5 times the risk of in-hospital death than in - patients without this infection. They affect patients in high dependency units such as intensive care units, burns units and cardiothoracic units. There are also several epidermic strains circulating in milk. Clinical implications of CA-MRSA include skin and soft tissue infection (in form of skin abscess, carbuncle, furuncle impetigo); pneumonia, osteomyelitis, arthritis, endocarditis. CA-MRSA can also cause wound infections, (Demlin and Water house, 2007) CA-MRSA has also been detected from surgical site infection, urinary tract infection (Baba-Moussa *et al.,* 2008), Infection of the eye and orbit, meningitis and sinusitis (Munckhof *et al*., 2008).

2.7.3.6 Methicillin Resistant *Staphylococcus aureus* Clones

MRSA is typed based on genetic characteristics such as

a. multilocus sequence type (MLST) (Enright *et al*., 2002),

b. protein A gene (*spa*) type (Korean *et al*., 2004),

c. *agr* type (Strommenger *et al.,* 2004),

d. coagulase type (Kinoshita *et al.,* 2008), and

e. SCC*mec* type (Oliveira *et al*., 2002; Zhang *et al*., 2005; IWG-SCC 2009).

Generally, sequence typing (ST) and analysis of the clonal complex (cc) to which each ST type belongs is a standard for genotyping.

2.7.3.7 Mechanism of Methicillin Resistance

Methicillin resistance in clinical isolates has been reported to arise from expression of a methicillin-hydrolysing β-lactamase and through the expression of an altered form of PBP2 that has a lower penicillin-binding affinity and higher rates of release of the bound drug compared to the normal PBP2. However, the main mechanism of methicillin resistance in *Staphylococcus aureus*is through the expression of a foreign PBP, PBP2a (not to be confused with PBP2), that is resistant to the action of methicillin but which can take over the transpeptidation (cross-linking) reactions of the host PBPs. Synthesis of PBP2a is regulated and normally kept at low level, but the level of synthesis can be enhanced if mutations occur in the regulatory genes(Naimi *et al.,* 2003).

2.7.3.7.1 Altered Penicillin Binding Protein (PBP2a)

MRSA differ genetically from methicillin-sensitive *Staphylococcus aureus*isolates by the presence, in the chromosome, of a large stretch of foreign DNA (40-60 Kb), referred to as the *mec* element, and the presence of the *mecA* gene that encodes the 76 KDa penicillin-binding protein, PBP2a (also referred to as PBP2′). The *mecA* gene has been proposed to originate from *Staphylococcus sciuri*. Although the

mechanism of gene acquisition from this specie is not known, two genes, *ccrA* and *ccrB*, present on the *mec* element from one isolate, have been shown to code for recombinase proteins that are capable of excising and integrating the *mec* element into the chromosome. In common with other PBPs, PBP2a has the common structural motifs that are associated with penicillin binding yet its affinity for β-lactam antibiotics is greatly reduced. PBP2a is not able to completely compensate for the other PBPs since cells grown in the presence of methicillin exhibit a marked reduction in the degree of cross-linking. However, the limited degree of cross-linking is enough to ensure survival of the cell (Zhang*et al*., 2005).

2.7.3.7.2 Regulation of PBP2a Expression

Adjacent to *mecA* on the staphylococcal chromosome are two genes, *mecR1* and *mecI*, that are co-transcribed divergently from *mecA*. The *mecR1* gene encodes a membrane-bound signal transduction protein (MecR1) while *mecI* encodes a transcriptional regulator (MecI). Between *mecA* and *mecR1* are the promoters for these genes and an operator region that encompasses the −10 sequence of *mecA* and the -35 sequence of *mecR1*. MecR1 and MecI have high protein sequence homology with the proteins, BlaR1 and BlaI, respectively that are involved in the inducible expression of the plasmid-mediated staphylococcal β-lactamase gene, *blaZ*. The arrangement of the genes coding for *BlaR1* and *BlaI* resembles the *mecA* system suggesting that *mecA* may have acquired the regulatory genes from the *blaZ* system sometime in the past. The operator regions are similar enough to allow BlaI to regulate PBP2a expression. Consequently, the presence of a plasmid carrying the *blaZ* regulatory genes can render PBP2a expression inducible under the control of BlaR1 and BlaI, a situation that commonly occurs in clinical isolates of MRSA (Zhang*et al*., 2005). Figure 2.7 overleaf shows aschematic representation of the *mecA-mecR-mecI* coding region, repression of *blaZ* and *blaR1-blaI* transcription by *BlaI* in the absence on an inducer and induction.

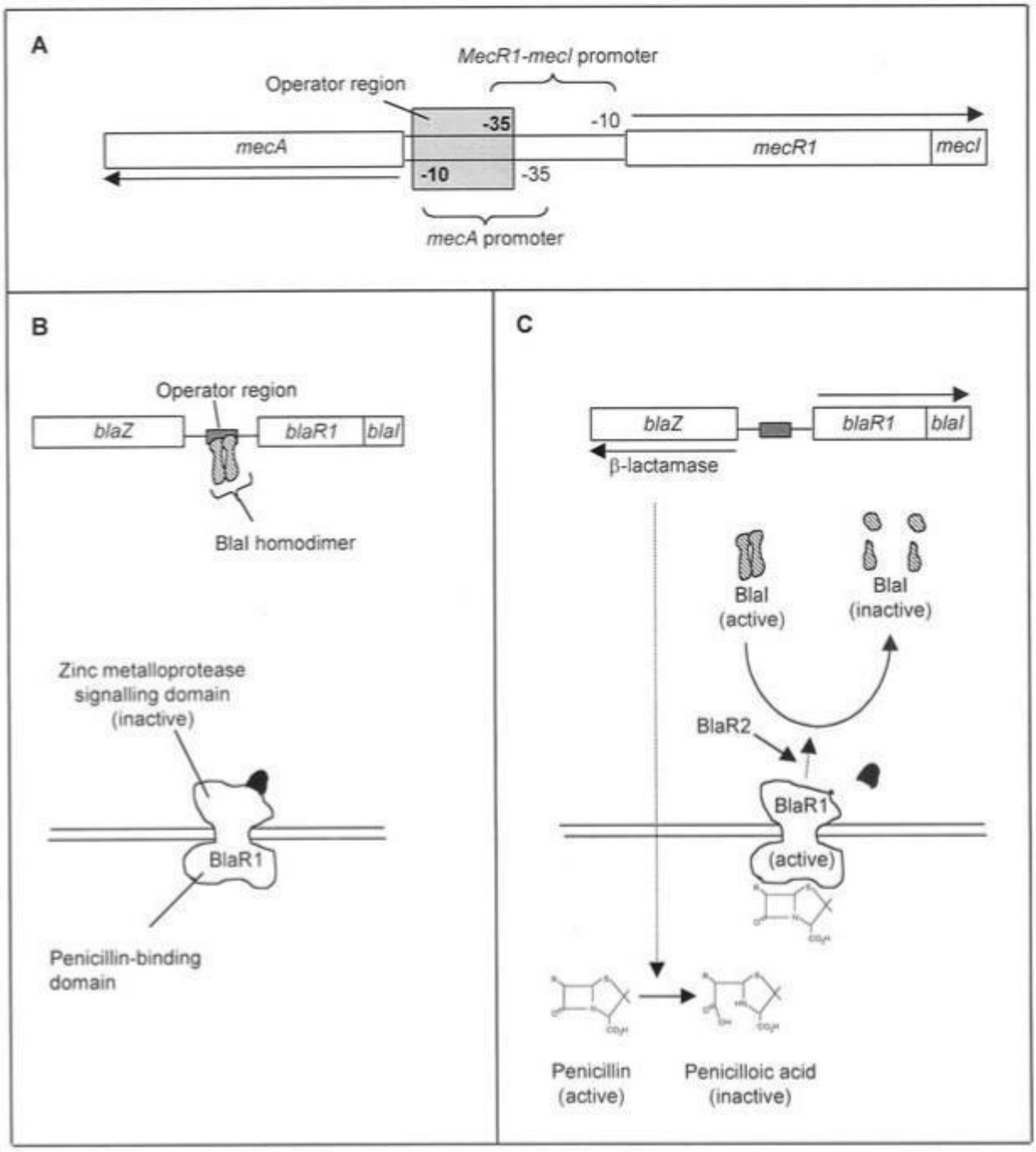

Figure 2.7: A, Schematic representation of the *mecA-mecR-mecI* coding region. Arrows indicate the relative directions of transcription of the mecA and mecR1-mecI genes. **B**, Repression of *blaZ* and *blaR1-blaI* transcription by *BlaI* in the absence on an inducer. **C**, Induction .

(Source: Zhang*et al*., 2005).

The nature of the signalling system for inducible β-lactamase expression has been elucidated (Zhang *et al*., 2005). *BlaI*, a DNA-binding protein, binds to the operator region as a homodimer and represses RNA transcription from both *blaZ* and *blaR1-blaI* (Figure 2.7). Consequently, in the absence of a β-lactam antibiotic, β-lactamase is expressed at low levels. BlaR1, present in the cytoplasmic membrane, detects the presence of the β-lactam by means of an extracellular penicillin-binding domain and transmits the signal via a second intracellular zinc metalloprotease signalling domain (Figure 2.7). Binding of a β-lactam to BlaR1 stimulates the autocatalytic conversion of the intracellular zinc metalloprotease domain of BlaR1 from an inactive proenzyme to an active protease (Zhang *et al*., 2005). The activated form of BlaR1 is thought to directly or indirectly cleave BlaI resulting in fragments that are incapable of forming dimers and binding DNA. Without BlaI bound to the operator site, transcription of both *blaZ* and *blaR1-blaI* can commence and β-lactam resistance can be conferred through β-lactamase synthesis (Figure 2.7). An additional gene product, BlaR2, also regulates β-lactamase synthesis, although the role of this protein has not been elucidated. Whether there are other proteins involved in the signalling system also remains to be determined.

Unlike β-lactamase synthesis, expression of PBP2a is not strongly inducible in isolates carrying the normal regulatory genes (*mecA* and *mecR1-mecI*) and induction is much slower (15 minutes for β-lactamase expression compared to up to 48 hours for PBP2a synthesis). This is because MecI is a tight regulator of *mecA* transcription and most β-lactam antibiotics do not efficiently activate MecR1. Consequently, some isolates, referred to as pre-MRSA, are methicillin-sensitive despite carrying the *mecA* gene. However, selective pressure through antibiotic usage has promoted *Staphylococcus aureus*isolates that have mutations or deletions in *mecI* or the *mecA* promoter/ operator region giving rise to an inactive repressor and constitutive PBP2a expression.

2.7.3.7.3 Internal and External Factors Affecting Methicillin Resistance

Since PBP2a is essential in conferring methicillin resistance, any factor that interferes with the expression of the *mecA* gene or with the activity of PBP2a will affect methicillin resistance. Genetic and biochemical studies have established that PBP2a has strict substrate requirements. Consequently, any factors that influence formation of the substrate have the potential to perturb or modulate methicillin resistance. Studies have shown that PBP2a requires: Glycan chains to be of certain lengths (Pinho *et al*., 2001), the stem peptide to have the normal peptide configuration and the pentaglycine cross-bridge to be intact.

External factors that affect methicillin resistance include among others, salt concentration, pH, medium composition, osmolarity and temperature. Some of these external influences are exploited in the clinical laboratory to enhance the detection of strains exhibiting heterogeneous methicillin resistance; isolates are grown in the presence of high sodium chloride concentrations (4%) and at lower temperatures (30-35°C) (Levinson, 2008).

2.7.4 Clinical Treatments for Methicillin resistant Staphylococus aureus (MRSA) Infections

2.7.4.1 Current Clinical Treatments

The difficulty in identifying new compounds with suitable antibacterial activity is one of the major problems faced in the fight against resistant organisms (Payne *et al*., 2007; Wright and Sutherland, 2007). Penicillin was the first class of the -lactams for the treatment of bacterial infections. With the development of resistance, however, many of these -lactams became ineffective against a significant proportion of *Staphylococcus aureus*clinical strains (Guignard *et al.,* 2005). The glycopepides belong to a different class of antibiotics effective against Gram-positive organisms. Vancomycin and teicoplanin are the preeminent members of this class of antibiotics (Loffler and Macdougall, 2007), and vancomycin is the antibiotic most commonly used to treat MRSA infections, the inhibition of cell wall biosynthesis by the glycopetides results from their stable noncovalent binding to the D-Ala-D-Ala terminal of

peptidoglycan precursors. However some MRSA strains have evolved resistance to vanvomycin (Sakoulas and Moellering, 2008). Linezoid, quinupristin-dalfopristin, daptomycin, and tigecycline represent newer agents for the treatment of *Staphylococcus aureus*infections, including those caused by non-vancomycin susceptible MRSA. Linezolid is a synthetic oxazolidinone antimicrobial agent that blocks the formation of protein synthesis initiation complexes. Quinupristin-dilfopristin is a mixture of semisyntheric streptogramin derivatives that bind to different sites of the 50S ribosomal subunit, resulting in the irreversible inhibition of bacterial protein synthesis (Llarrull *et al*., 2009). Daptomycin is a cyclic lipopeptide that forms a calcium ion complex in the bacterial cytoplasmic membrane causing the loss of the transmembrane electrical potential gradient.

2.7.4.1.1 New Weapons in the Pipeline: -lactam Antibiotics that Inhibit PBP 2a

Four -lactams antibiotics are being evaluated for the treatment of MRSA associated infections by targeted inhibition of PBP 2a transpeptidase activity. In a review published in 2005, Guignard *et al*., (2005) described 16 novel anti MRSA -lactam antibiotics, only 1 of which (ceftobiprole) remains in clinical development. In a later review, Page (2006) reported on a new cephalosporin (ceftaroline) also active against MRSA, two additional -lactams antibiotics ME1036 and PZ-601, have been added to the anti MRSA pipeline.

2.7.4.1.1.1. Ceftobiprole Medocaril

Ceftobiprole medocaril, a water-soluble cephalosporin prodrug, belongs to a new class of cephem antibiotics with activity against a wide range of Gram-positive organisms, including MRSA and penicillin- resistant *Streptococcus pneumoniae*, and Gram negative pathogenic bacteria (Bush *et al*., 2007). Rapid cleavage of ceftobiprole medocaril in plasma produces the active drug, ceftobiprole. Ceftobiprole inactivates all four *Staphylococcus aureus*PBPs and PBP 2a, as indicated by competition assays against a fluorescent -lactam. Ceftobiprole medocaril, the prodrug, is converted rapidly and almost

completely by type A esterases to active ceftobiprole. The pharmacokinetics and pharmacodynamic of ceftobiprole describe a drug that should be appropriate for the early emprirical hospital treatment of patients with infections (Moisan *et al*., 2008). In another phase 3 study, the cure rates for patient with MRSA infections were 92% (56/61) with ceftobiprole treatment and 90% (54/60) with vancomycin monotherapy. Ceftobiprole monotherapy was as effective as vancomycin monotherapy or vancomycin plus ceftazidime.

2.7.4.1.1.2. Ceftaroline Fosamil

Ceftaroline fosamil, a water soluble N-phosphono type cephalosporin prodrug, is a member of a new class of cephem antibiotics having antibacterial activity against a wide range of species including the resistant Gram positive pathogens MRSA and multidrug-resistant *Streptococcus pneumoniae*, as well as common Gram-negative pathogenic bacteria. Ceftaroline fosamil, discovered by Takeda Chemical Industries (Osaka, Japan), is currently being developed by Forest Laboratories (New York, NY) for the treatment of infections, including community acquired pneumonia. Ceftaroline, the active form of ceftaroline fosamil, is a potent inhibitor of PBP 2a of MRSA (IC50= 0.16 to 0.18g/ml) (Moisan *et al*., 2008), which translates into a high level of inhibitory activity (MIC for MRSA =0.25 to 0.5g/ml) (Moisan *et al*., 2008) Ceftaroline fosamil the prodrug, undergoes rapid conversion by plama phosphates to active ceftaroline. Two phase 3 clinical trials with patients with cSSSIs have been completed, and two phase 3 clinical trials with patients with community-acquired pneumonia are currently on going in United States. In a randomized, double blinded study of the efficacy and safety of ceftaroline versus those of vancomycin plus aztreonam in patients with cSSSIs, ceftaroline monotherapy (intravenous) was as effective and well tolerated as vancomycin plus aztreonam combination therapy for the treatment of patients infected with both Gram positive and Gram-negative pathogens (Corey *et al*., 2008). Girish and Balakrishnan, (2011) reported that the United States Food and Drug Administration has granted approval

for ceftaroline fosamil (teflaro) on October 2010, to treat adults with community acquired bacterial pneumonia and acute bacterial skin and skin structure infections, including MRSA. The dose of ceftaroline fosamil recommended is 600 mg intravenously, every 12 h for patients with normal renal function or mild renal dysfunction. The drug formulation of ceftaroline fosamil available is in a powder form for intravenous administration as 400 mg and 600 mg vials (FDA, 2011).

2.7.4.1.1.3. ME1036.

ME1036 is a broad spectrum carbepenem that binds with a high affinity to PBP 2a of MRSA (IC50=0.13 to 0.73g/ml) (Kurazono *et al*., 2004) and that exhibits potent in vitro inhibitory activity against MRSA. The inhibitory effect of ME1036 on the enzymatic activity of PBP 2a also appears to coincide with facilitated opening of the active site by allosteric interactions. ME1036 has activity against MRSA and multidrug-resistant streptococci, in addition to broad-spectrum activity against organisms that include extended-spectrum -lactamase-producing Enterobacteriaceae and common anaerobes (Kurazono *et al.*, 2004)

2.7.4.1.1.4. PZ-601 (Razupenem).

PZ-601 is a new carbapenem being developed by Protez pharmaceuticals (now Novartis) that has demonstrated a high degree of potency against MRSA. PZ-601 has in vivo efficacy against VISA and is in phase 2 clinical trials for the treatment of cSSSIs. In a study carried out to determine the safety and multiple dose pharmacokinetics of PZ-601 in health male volunteers PZ-601 did not cause any serious adverse events (Lo *et al*., 2008) The -Lactam antibiotics that inhibit PBP2a is a great progress and offers a glimmer of hope for the future treatment of MRSA infections including those caused by vancomycin-resistant and intermediate strains.

2.7.4.2. Detection of Methicillin Resistance

The Clinical Laboratory Standards Institute (CLSI) recommended the following for detection of oxacillin/methicillin resistance.

2.7.4.2.1. Minimum Inhibitory Concentration (MIC) Susceptibility Method

This involves direct colony inoculums where isolated colonies from an 18 to 24 hour nonselective agar plate are used to prepare a direct inoculum equivalent to a 0.5 McFarland Standard. Here oxacillin is the preferred agent for detection of methicillin resistance. For supplementation of test medium, the addition of 2% NaCl to broth dilution is recommended to enhance detection of MRSA. Incubation time and temperature are 24 hours and 35°C respectively. The interpretation of results are as follows: Oxacillin-Susceptible *Staphylococcus aureus*MIC ≤2 µg/ml while Oxacillin-Resistant *Staphylococcus aureus*MIC ≥4 µg/ml (CLSI, 2012).

2.7.4.2.2. Disk diffusion Susceptibility Method

This involves a direct colony inoculum preparation; Mueller Hinton agar and 1 µg oxacillin disk are used. Incubation is for 24 hrs at 35° C. Interpretation of results: Resistant (MRSA):<10 mm zone size of inhibition Confirm with Oxacillin Screening Agar: 11-12 mm zone size of inhibition Susceptible (No MRSA): >13 mm zone size of inhibition (CLSI, 2012).

2.7.4.2.3. Oxacillin Agar Screening Test and Cefoxitin Disk Test.

When performed correctly, both disk diffusion and MIC tests accurately detect MRSA. The oxacillin screening plate can be used in addition to or as a backup method. Mueller Hinton agar with 4% NaCl and 6 µg/ml of oxacillin is used. Direct colony inoculum preparation is done, swab is dipped into inoculum, excess fluid is expressed and quadrant of the agar is streaked or spot inoculated 1 to10 µl of the suspension to a quadrant of the plate. Incubation is done for 24 hours at 35° C. > 1 colony or light film of growth = oxacillin /methicillin-resistant (CLSI, 2012).

The cefoxitin disk test was first approved by the Clinical and Laboratory Standards Institute (CLSI, 2012) for predicting *mecA* mediated resistance in *Staphylococcus*spp. It is a disk diffusion test with specific breakpoints for both *Staphylococcus aureus*(≤21mm for resistant and ≥ 22mm for susceptible) and coagulase negative Staphylococci (CoNS). (≤ 24mm for resistant and ≥25mm for susceptible) (CLSI, 2013). The test was approved for two reasons:

a) The test is highly sensitive and specific for the presence of *mecA* mediated resistance especially for CoNS. The cefoxitin disc test is more accurate than oxacillin susceptibility testing for detection of *mecA* mediated resistance.

b) The cefoxitin disc test is easier to read than the oxacillin disk diffusion test. The zone around an oxacillin test must be read with close scrutiny and transmitted light. The cefoxitin disk test produces clearer zones and can be read with reflected light.

Oxacillin and cefoxitin are tested instead of methicillin because methicillin is no longer commercially available and that oxacillin maintains its activity during storage better than methicillin and is more likely to detect heteroresistant strains.

2.7.4.3 Additional Tests to Detect Methicillin Resistant *Staphylococcus aureus* infections.

i. Amplification tests based on polymerase chain reaction (PCR) are available to detect the *mecA* gene. However, *mecA* PCR tests will not detect novel resistance mechanisms such as *mecC* or uncommon phenotypes such as borderline-resistant oxacillin resistance.

ii. Latex agglutination methods are available for detection of the penicillin-binding protein 2a (PBP2a)(CLSI, 2013).

2.8. Strategies for Control of StaphylococciInfections

Antimicrobial-resistant microorganisms have caused clinical problems since anti-infectives were introduced into medical practice. During the past decade, the incidence of multidrugresistant organisms

has reached unprecedented levels, affecting the practice of medicine in virtually all specialties and settings. Health care-associated infections with multi-drugresistant organisms affect patient care by increasing morbidity, mortality, and costs, which derive from increased durations of hospitalization and use of more-expensive antimicrobial agents (Strausbaugh *et al.,* 2006). Infection transmission risks are present in all hospital settings. However, certain hospital settings and patient populations have unique conditions that predispose patients to infection. Transmission of MRSA within and between healthcare facilities has been well documented using molecular typing techniques, such as pulsed-field gel electrophoresis (PFGE). Outbreaks involving clonal spread within single facilities have also been frequently reported.

Colonised and infected patients represent the most important reservoir of MRSA in healthcare facilities. Active surveillance and decolonisation are the two main targeted control measures for reducing the transmission of MRSA. Active surveillance culture involves performing culture of specimens typically obtained from patients' nares at hospital admission to detect MRSA carriers and, thus, to institute isolation precautions to reduce the likelihood of transmission to other patients. Isolation interrupts cross infection through physical or behavioural barriers such as disposable gloves and aprons (contact precautions) or the placement of patients in isolation wards, single rooms, or cohort groups, with or without nursing staff designated for the exclusive care of MRSA infected patients. Some infection control experts, policy makers, legislators, and consumer groups suggest that active surveillance cultures should be performed routinely and call for universal screening of all persons admitted to the hospital (Boucher *et al.*, 2010; Robotham *et al.,* 2011). Success in controlling MRSA has been greatest in countries that adhere to rigorous transmission-based control policies that include active surveillance cultures to identify colonized patients and strict application of barrier precautions for patients colonized or infected with MRSA.

Decolonization strategies are based on robust literature involving this strategy to prevent health care-associated MRSA (HA-MRSA) infection, a context in which the model of pathogenesis is based on the tenet that infection is preceded by colonization (Miller and Diep, 2008). Decolonisation attempts to eliminate or suppress MRSA using topical antimicrobials such as chlorhexidine and intranasal mupirocin, thereby reducing the bacterial load available to cause endogenous infection and transmission to other patients (Robotham *et al.,* 2011).

Suppression of carriage, eradication of carriage, or both has been used at times to help control the spread of MRSA. Because health care workerss can become colonized and spread MRSA to patients, control has sometimes necessarily involved eradication of MRSA colonization in health care workers. Treating colonized or infected health care workers who were epidemiologically implicated in outbreaks has also helped control outbreaks. In the Netherlands, hospitals routinely give decolonisation therapy to patients and health-care workers who are colonized (Grundmann *et al.,* 2006). Other strategies for control of infection include the underlisted.

2.8.1. Hand Washing

Hand hygiene has been cited frequently as the single most important practice to reduce the transmission of infectious agents in healthcare settings and is an essential element of Standard Precautions. The term "hand hygiene" includes both hand washing with either plain or antiseptic-containing soap and water, and use of alcohol-based products (gels, rinses, foams) that do not require the use of water (Siegel *et al*., 2007). Transient contamination of health-care workers' hands has been documented on many occasions and is widely believed to be the predominant method by which MRSA is transmitted to patients. Several studies have shown that improvement in hand-hygiene practices, when coupled with surveillance cultures and contact precautions, have greatly reduced transmission of MRSA.

2.8.2. Screening of Staff

Health-care workers who are nasal carriers can serve as sources of MRSA transmission, although they are not nearly as important a reservoir as are colonised or infected patients. Howerver, failure to identify health-care workers who are persistently colonised or infected can lead to continuing transmission despite implementation of barrier precautions and hand hygiene (Grundmann *et al*., 2006).

2.8.3. Environmental Cleaning

The hospital environment has been known to be a reservoir for infection therefore cleaning and disinfecting non-critical surfaces in patient-care areas are part of standard precautions. The US Centres for Disease Control and Prevention has recommended that hospitals have adequate procedures for routine care, cleaning, and disinfection of environmental surfaces, beds, bedrails, bedside equipment, and other frequently touched surfaces (Siegel *et al*., 2007).

An important effort to control the spread of MRSA includes antibiotic stewardship which involves avoidance of inappropriate or excessive antibiotic prophylaxis and therapy as well as ensuring correct dosage and duration of antibiotic therapy. Education and training of health care workers and the general public on preventing the transmission of infectious agents is also a very important aspect of controlling the spread of MRSA. MRSA control programs are multifaceted because no one measure has been proved to be completely effective.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study Area and Population

The study was conducted with samples obtained from college of medicine, Abia State University,Uturu and Aba campuses, Imo State University School of medicine, Owerri and Orlu campuses, and University of Uyo College of Medicine, University of Uyo permanent site and Teaching Hospital campuses. Samples were analysed at New Concept Laboratories, Obinze, Owerri, Imo State and Ever Oak Laboratories, Itam, Uyo, Akwa Ibom State. Molecular studies were carried out at Nigerian Institute of Medical Research (NIMR), Lagos State. Four hundred and twenty medical students (420) made up of one hundred (100) medical students from Imo State University, eighty (80) from Abia State University and two hundred and forty (240) from University of Uyo participated in the study.

3.2. Sampling

Sample size was determined by simple random sampling. Medical students that consented to participate in this study were divided into two groups. The first group consisted of pre-clinical students (Students in Year One and Year Two) who have not been attached to any hospital. Isolates obtained from these, constituted Community acquired Staphylococci. The second group consists of clinical students (Students in Year Three to Year Five) who are currently attached to medical, surgical, emergency and intensive care units of their respective Teaching Hospitals. Isolates from these candidates, constituted Hospital acquired Staphylococci.

3.3. Collection of Samples and Pre-treatment

Swab samples were collected from the nose, hands, and throat of the candidates by inserting and swabbing with sterile cotton swab stick in the nostrils three times, between the fingers and throats of the candidatesfollowing the methods of Cheesbrough (2012). Sterile cotton swabs pre-wetted with sterile

saline was used for the sample collection. The samples were labeled, packaged and transported on ice packs to the Microbiology laboratory in Owerri and Uyo as the case may be and cultured within 3 hours of collection.

A total number of 440 samples were collected from nasal, hands, and throatswabs of pre-clinical and clinical medical students of Imo State University, Abia State University and University of Uyo over a period of 3 months (July to September, 2015).

Questionnaireswere issued to the students prior to sample collection and information concerning their demographics (sex, age), hygienic practices and medical history data were obtained alongside signed informed consent from all participants. The exclusion criteria were as follows: history of hospitalization, history of upper respiratory infection, smoking, and antibiotic therapy in the past one month.

3.4 Isolation and Preliminary Identification of Staphylococcal Isolates

3.4.1. Growth on Selective media

Each swab sample was inoculated onto mannitol salt agar and the plates were incubated aerobically at 37°C for 24-72 hours as described by Cheesbrough (2012). Isolates that produced colonies exhibiting characteristic raised, deep golden yellow colouration and pink colourations were selected and further examined by microbiological standard tests, including colonial morphology on blood agar, Gram staining, catalase and coagulase tests. Upon confirmation, the isolates were streaked on nutrient agar slants and stored in the refrigerator until required for further tests.

3.4.2 Gram Staining

The method described by Cheesbrough (2012) was carried out. A smear of the sample was made on a clean glass slide and heat-fixed. The smear was stained with crystal violet and fixed with Lugol‘s iodine and decolorized rapidly with acetone after which it was counterstained with safranin solution. On

examination microscopically, isolates that appear as violet cocci, predominantly clusters were selected for further identification.

3.4.3 Biochemical Tests

3.4.3.1 Catalase Test

Catalase test was carried out as described by Cheesbrough (2012). The ability to produce the enzyme catalase by the test organisms was assayed by the addition of about 1 ml of a 3% hydrogen peroxide solution on a 24 hour nutrient agarculture of the isolate. The test organisms were emulsified in three drops of distilled water on a clean glass slide placed in a Petri dish. Two drops of hydrogen peroxide H_20_2 (3%) was then added to the emulsified colonies. Immediate generation of gas bubbles indicate a positive reaction. This was carried out for all the isolates and immediate evolution of gas was noted and recorded.

3.4.3.2.Coagulase Test

The method described by Cheesbrough (2012) was used to differentiate *Staphylococcus aureus*which produces the enzyme coagulase from Coagulase-negative Staphylococci (CoNS)which does not produce the enzyme coagulase.To detect bound coagulase, a drop of physiological saline was placed on each end of a slide. A colony of the test organisms was emulsified in each drop to make two separate thick suspensions. A drop of plasma was then added to one of the suspensions and mixed gently and the ability (positive) or inability to form a clumping within 10 seconds was noted and recorded for each of the isolates tested.

3.5. Antimicrobial Susceptibility Testing

Disk diffusion technique (Bauer *et al.*, 1986) were performed for each of the isolates previously identified as *Staphylococcus aureus*orCoagulase-negative Staphylococci using Mueller Hinton agar (Oxoid, UK). Discrete colonies of isolates on nutrient agar plates were emulsified in 3 – 4 ml of sterile

physiological saline and the turbidity adjusted to 0.5 McFarland standards (approximately a cell density of 1.5×10^8cfu/ml). Using sterile swab sticks, the surface of Mueller Hinton agar (MHA) in a 90 mm diameter plate was inoculated with the standardized bacterial suspension by streaking the surface of the agar in three directions, rotating the plate approximately 60 degree to ensure even distribution. The inoculated plates were allowed to dry for 10 minutes before the antibiotic discs were applied aseptically to the surface of the agar. After 30 minutes of applying the discs, the plates were inverted, and incubated at 37 ℃ for 24 hours. The following antibiotics from Oxoid, UK were used for the susceptibility test: Oxacillin(1µg), Penicillin(10 units), Ampicillin(10 µg), Erythromycin (15µg), Vancomycin (30µg), Ciprofloxacin (5µg), Nitrofurantoin (300 µg), Trimethoprim (5µg), Clindamycin (2µg).

3.6. Detection of Methicillin Resistance

Staphylococcusisolates were innoculated on Mueller-Hinton agar from a 0.5 Mc Farland standard suspension. Oxacillin (1µg) discs were placed on the already inoculated plates.The plates were incubated at 37°C for 24 hours as recommended by the Clinical Laboratory Standards Institute (CLSI, 2013). The isolates that showed no zones of inhibition around oxacillin discs were considered methicillin resistant. Zones of inhibition which were equal to or more than 13 mm were susceptible to Oxacillin and the Staphylococciisolate producing it was reported as Methicillin susceptible/sensitive Staphylococci and those isolates which produced a zone of inhibition which were less than or equal to 10 mm were considered as Methicillin Resistant Staphylococci (CLSI, 2013).

3.7. Determination of Inducible Clindamycin Resistance

The method described by Deresinski (2005) and Yilmaz *et al.* (2007) was carried out. Resistance to clindamycin induced by another member of the macrolide-lincosamide-streptogramin B (MLS) family, erythromycin was determined by the double-disc diffusion, D-test. A 0.5 McFarland equivalent suspension from overnight culture of the organisms was inoculated onto Muller Hinton agar plates by

swabbing and allowing the plates to dry. Clindamycin (2 μg) and erythromycin (15 μg) discs were applied on the Muller Hinton agar plate, 15 mm apart. Plates were incubated at 37°C for 18 hours after which their zones of inhibition were analyzed. A D-shaped (blunted clindamycin zone) between the discs is indicative of erythromycin (*erm*)-mediated inducible resistance to clindamycin (positive D-test), while the absence of D-shaped and clindamycin growth inhibition zone diameter ≤14 is indicative of constitutive clindamycin resistance.

3.8. Determination of Minimum Inhibitory Concentration (MIC) of Vancomycin

Minimum Inhibitory Concentration test using vancomycin E-test strips was conducted for Staphylococci isolates that showed susceptibility to Vancomycin 30 μg discs in order to verify their susceptibility (CLSI, 2012). These isolates include Nasal 57, Palm 202, Nasal 101, Palm 201, Palm 204, Palm 11, Palm 208, Palm 215, Palm 205, Nasal 16, Palm F, Nasal 10, Nasal R, Palm D, Nasal 13, Palm Q, Nasal 97, Nasal 77, Nasal 91, Palm 94, Palm 63, Palm 78, Palm D, Nasal 10 and Nasal 17 from Imo State University; Nasal U30, Throat U4, Palm U20, Palm U9, Palm U26, Palm U25, Nasal U1, Palm U33, Palm A2, Palm A7, Palm A12, Palm A25, Palm A23, Palm A28, Palm A22, Palm A6 and Palm A26 from Abia State University; Nasal UY3,Nasal UY1, Nasal UY4, Nasal UY2, Palm UY3, Nasal UY9, Palm UY115, Nasal UY5, Palm UYCL 71, Nasal UYCL 27, Nasal UYCL 1, Palm UYCL 12, Palm UYCL 8,Palm UYC 1, Nasal UYC4, Palm UYCL 5, Palm UY 103, Nasal UY109, Nasal UY116, Nasal UY111, Nasal UY77, Nasal UY65, Nasal UY8, Nasal UY7, Nasal UYCL 8, Nasal UYCL 21, Palm UYCL 72, Throat UYCL 76, Nasal UYCL 36, Throat UYCL 8 and Nasal UYCL 35 from University of Uyo. Discrete colonies of isolates on nutrient agar plates were emulsified in 3 – 4 ml of sterile physiological saline and the turbidity adjusted to 0.5 McFarland standards. Using sterile swab sticks, the surface of Mueller Hinton agar (MHA) in a 90 mm diameter plate was inoculated with the standardized bacterial suspension by streaking the surface of agar in three directions, rotating the plate approximately

60° to ensure even distribution. The inoculated plates were allowed to dry for 10 minutes before the vancomycin E-test strip was applied aseptically to the surface of the agar with the MIC scale facing upward and code of the strip to the outside of the plate; pressing it with a sterile forceps to the surface of the agar and ensuring that whole length of the antibiotic gradient was in complete contact with the agar surface. The strip displays MIC scale ranging from 256mg/ml to 0.015 mg/ml. The plate was incubated in an inverted position for 37°C for 18 hours (CLSI, 2013).

3.9. Molecular Analysis on the Isolated Staphylococci

3.9.1. Plasmid/Genomic DNA Extraction

Extraction of the plasmid DNA from thirty-one (31) isolates was carried out using alkaline lysis method described by Sambrook *et al.* (1989) with a few modifications.DNA extraction was carried out directly from the samples by boiling as follows. Organisms in nutrient broth (1.5 ml) were centrifuged at 10,000 revolutions per minute (r.p.m) for 5 minutes after which the supernatant was discarded and the pellets washed twice with sterile water. After this, 200µl of sterile water was added to the pellets, the pellets were vortexed to homogenize and boiled in a water bath at 100°C for 10 minutes. This was followed by vortexing and centrifugation at 12,000rpm for 5 minutes. The supernatant containing the DNA were transferred to another tube and stored at -20°C. The concentration and purity of the extracted DNA was estimated using a spectrophotometric measurement of the absorption at 260nm and multiplying by the dilution factor and using the relationship that an A_{260} of 1.0 = 50µg/ml pure dsDNA.

Concentration (µg/ml) =A_{260} reading-A_{230} reading) x dilution factor x 50µg/ml

Total yield was obtained by multiplying the DNA concentration by the final total purified sample volume as follows:

DNA yield (µg) = DNA concentration x total sample volume (ml).

3.9.2. Polymerase Chain Reaction (PCR)

The method described by Casey *et al*., (2007) was used. PCR amplifications were performed on genomic DNA a total volume of 25 µL made up of the genomic DNA, PCR H_2O, primers and Red Taq Mastermix (Sigma-Aldrich, USA). The Red Taq mastermix contained Taq DNA polymerase, antibodies to Taq DNA polymerase, 32mM $(NH_4)_2SO_4$, 130mM Tris HCl, 0.02% Tween 20, 2mM MgCl2 and dNTPs (dATP, dCTP, dGTP, dTTP). The protocol for amplification was set in the PCR thermocycler (Bio-Rad, Germany) according to the primers used. After the amplification, the PCR products were separated by agarose gel electrophoresis.

3.9.3 Agarose Gel Electrophoresis

The method described by Casey *et al*., (2007) was used. Gel electrophoresis is used to separate DNA on the basis of their sizes by applying an electric field to move the DNA through an agarose matrix. Agarose powder (Segenetic, Germany) was used in preparing the agarose gels used in this study at different concentrations. The agarose was dissolved in 0.5 × TBE: Tris-Borate-EDTA and microwaved to dissolve the agarose. The molten agarose was placed on a stirrer and allowed to cool down. Casting of the gel was done by placing a comb into the cast and then pouring the agarose gently into the cast. The gel was allowed to solidify after which the comb was removed. The electrophoresis chamber was filled with the running buffer (0.5 × TBE) and casting tray was placed in the chamber. The gel was totally submerged in the buffer. A tracking dye was added to the PCR products that were colourless to make them visible. The amplicon (10µl) was loaded into each well. A 1kb molecular weight marker- *Hind* III Lambda DNA ladder (New England Biolabs, Germany) was loaded into the first and the last well as a standard for estimating the size of the resulting DNA fragment. The electrophoresis chamber was connected to the power source and the DNA was run at 160V for 30-45 min. The separated DNA fragments were visualized by staining the gel with ethidium bromide for 15 min and then de-stained in water for 15 min.

The DNA bands were viewed by illumination with UV light and images recorded by Imager photography.

3.9.4. Identification of *Staphylococcus aureus* by detection of the *nuc* gene.

*Staphylococcus aureus*strains produce an extracellular thermostable nuclease which is coded for by the *nuc*gene. Amplification of the *nuc* gene was carried out to verify the identity of the *Staphylococcus aureus*strains isolated in this studyfollowing the method described by Brakstad *et al*. (1992).

The *nuc* gene was amplified with primer set *nuc1* (GCGATTGATGGTGATACGGTT) and *nuc2 (*5' AGCCAAGCCTTGACGAACTAAAGC 3'). PCR was performed in 25 µl of a reaction mixture containing DNA (10-200ng), and 1X Master mix (Thermo Scientific Dream Taq Green PCR Master mix). Thermal cycling was conducted in an Eppendorf (Nexus Series) thermal cycler for an initial denaturation at 95°C for 5 minutes followed by 30 amplification cycles of 30 seconds at 95ºC for 30 seconds; at 53ºC for 30 seconds and at 72ºC for 1 minute. This was followed by a final extension step at 72ºC for 5 minutes. The amplification product was separated on 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. DNA ladder 100bp was used as DNA molecular weight standards. A band with a molecular weight of about 300bp signifies the presence of the *nucA* gene. MRSA (ATCC 49230) was used as the Positive control while *Staphylococcus epidermidis (*ATCC 12228) was used as the negative control.

3.9.5. Detection of Antibiotic Resistant Genes

The antibiotic resistance genes present in the Staphylococcistrains were detected by polymerase Chain Reaction (PCR)

3.9.5.1. Detection of the *mecA* Gene

Methicillin resistance was determined by *mecA* gene amplification byPCR as described by Murakami *et al.* (1991). The *mecA* gene was amplified with primer set *mecA*1 (AAAATCGATGGTAAAGGTTGGC)

and *mecA*2 (5' AGTTCTGCAGTACCGGATTTTGC 3') as described by Del Vechio *et al.*(1995). PCR was performed in 25 µl of a reaction mixture containing DNA (10-200 mg/ml), and 1X Master Mix (Thermo Scientific Dream Taq Green PCR Master mix). Additional Taq DNA polymerase was incorporated to make the final concentration of Taq DNA polymerase as 2.5U, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in an Eppendorf (Nexus Series) thermal cycler for an initial denaturation at 95°C for 5 minutes followed by 30 amplification cycles at 95ºC for 30 seconds; at 55ºC for 30 seconds and at 72ºC for 1 minute. The amplification product was separated on 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. DNA ladder (Solis Biodyne)100bp was used as DNA molecular weight standards. A band with a molecular weight of 533 bp signified the presence of the *mecA* gene.

3.9.5.2 Detection of the beta-lactamase Gene (*blaZ* gene)

The method described by Casey *et al*., (2007) was used. The *blaZ* gene was amplified with the primer set *blaZ*-F (AAG AGA TTT GCC TAT GCT TC) and blaZ-R (GCT TGA CCA CTT TTA TCA GC). The PCR was performed in 25 µl of a reaction mixture containing DNA (10-200ng), and 1X Master Mix (Thermo Scientific Dream Taq Green PCR Master mix), and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in an Eppendorf (Nexus Series) thermal cycler for an initial denaturation at 95°C for 5 minutes followed by 30 amplification cycles at 95ºC for 30 seconds; at 52ºC for 30 seconds and at 72ºC for 1 minute. The amplification product was separated on 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. DNA ladder (Solis Biodyne)100bp was used as DNA molecular weight standards. A band with a molecular weight of 517bp signified the presence of the *blaZ* gene.

3.9.6. Random Amplified Polymorphic DNA (RAPD-PCR) Fingerprinting and Analysis

The procedure for RAPD typing was performed following the method described by Casey *et al*. (2007) with few modifications. RAPD-PCR was carried out to detect the clonal relatedness of tested Staphylococci. It was carried out on the extracted DNA sample using the RAPD primer OPC -10 (5'-TGTCTGGGTG-3'). The PCR reaction was carried out in a 25 µl reaction mixture containing 1X PCR buffer (Solis Biodyne), 2.5mM Magnesium Chloride, 0.2mM of each dNTP, 40 pMol of primer, 1U Taq DNA polymerase, 10-200ng of DNA, and sterile deionized water was used to make up the reaction mixture. Amplification was carried out in an Eppendorf Nexus thermal cycler using the following cycling conditions; an initial denaturation at 95ºC for 5 minutes which was followed by 40 consecutive cycles at 95ºC for 1 minute, at 30ºC for 1 minute and at 72ºC for 2 minutes. This was followed by a final extension at 72ºC for 10 minutes.

The PCR products were separated on a 1.5% Agarose gel and DNA ladder (Solis Biodyne)100 bp was used as DNA molecular weight standard. RAPD fingerprints was analysed using the Sequentix - Digital DNA Processing (Germany).Cluster analysis of the binary matrix distance based on RAPD data sets were transferred into dendrogram using Pearson coefficient method. A dendrogram based the unweighted pair group method with arithmetic averages (UPGMA)analysis using the RAPD data generated by primer set *blaZ*-F (AAG AGA TTT GCC TAT GCT TC) and *blaZ*-R (GCT TGA CCA CTT TTA TCA GC) is shown in figure 4.13

3.10 Statistical analysis

Frequencies and percentages were calculated for the studied variables. All data were analysed using SPSS Statistics, version 17.0. The ages and sexes of medical students of Imo State University, Abia State University and University of Uyo were compared using Analysis of variance (ANOVA) test. *P* values of <0.05 were considered statistically significant.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS

A total of four hundred and twenty (420) medical students made up of two hundred and twenty (220) malesand two hundred (200) females were screened for methicillin resistant Staphylococci (MRS). Out of these 248(59.0%) were positive for methicillin resistant Staphylococci (MRS). 148(59.7%) males and 100(40.3%) females were positive for methicillin resistant Staphylococci (MRS). Analysis of data according to age showed that the higest number of positive cases occurred in age group 26 -30 with 125(50.4%) and age group 21-25 with 94(37.9%). The least prevalence occurred in students aged less than 21 with 11(4.4%). The samples collected from the palms, throats and noses of medical students are shown in appendix 7.

4.1.1Biochemical and Confirmatory Tests

All the isolates that were Gram positive cocci, catalase positive, and coagulase positive with visible mannitol fermentation on mannitol salt agar were characterized as *Staphylococcus aureus*and all the isolates that were Gram positive cocci, catalase positive, and coagulase negative without mannitol fermentation were characterized as coagulase-negative Staphylococciand used for subsequent tests. Plate 1 overleaf shows fermentation of mannitol characterized by colour change of pink mannitol salt agar plate to yellow confirmative of *Staphylococcus aureus* and non-fermentation of mannitol characterized by pink colonies and no colour change of pink mannitol salt agar plate confirmative of coagulase-negative *Staphylococcus* spp.

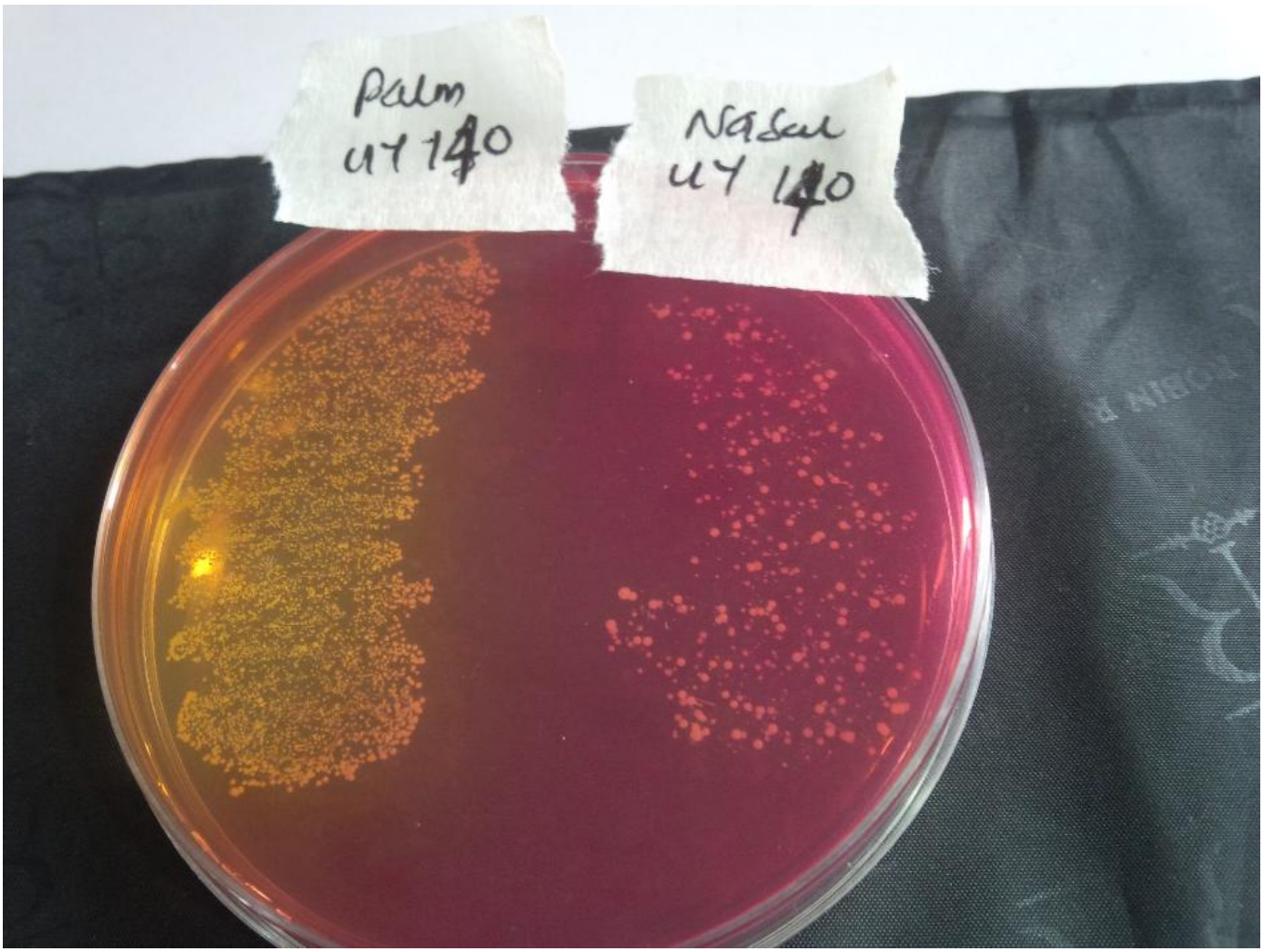

Plate 1: ***Staphylococcus aureus*** **(yellow colonies) and coagulase-negative** ***Staphylococcus*** **spp. (pink colonies) on a mannitol salt agar plate from pre-clinical students of University of Uyo.**

4.1.2. Classification of isolated Staphylococci

4.1.2.1. Isolated Staphylococci classified according to Type of Sample.

Four hundred and twenty (420) students were screened, 440 samples received and 248 (56.3%) Staphylococci were isolated from them. Of the 248 isolated Staphylococci, 122(58.7%) were from palm swabs, 115(73.7%) were from nasal swabs and 11(14.5%) were from throat swabs. However, the result shows that 73.7% of all nasal swabs were positive for Staphylococci, same for 58.7% of all palm swabs and 15.5% of all throat swabs. The summary of the result is presented in Table 4.1.

From the Table 4.1, the medical students harboured the Staphylococci most in their nostrils as seen with a percentage occurrence of 73.7%. The least occurrence of Staphylococci was from the throats with a percentage occurrence of 14.5%.

From the respective institutions, 144 Staphylococci was isolated from University of Uyo, 54 from Imo State University and 50 from Abia State University. As shown in table 4.2, 134 Staphylococci were isolated from pre-clinical students and 114 Staphylococci were isolated from the clinical students screened.

From table 4.2, Staphylococci were the most isolated from palm swabs of clinical students of Abia State University (100%) and pre-clinical students of Imo State University (66.7%). However, in University of Uyo, nasal swabs were the most colonized with Staphylococci with a percentage occurrence of 63.2% in pre-clinical students and 55.2% in clinical students.

From the Table 4.3, 134 Staphylococci were isolated from pre-clinical students of all three institutions and 114 were isolated from clinical students of all three institutions.

Table 4.1. Staphylococci isolated from Palm, throat and nasal swab samples of medical students

Swab samples	Samples screened	Staphylococci isolated	Percentage positive
Palm swabs	208	122	58.7 %
Nasal swabs	156	115	73.7%
Throat swabs	76	11	14.5%
Total	440	248	

Table 4.2.PercentageStaphylococci isolated from the nostrils, palms and throats of candidates from the respective institutions

	Isolates from medical students of Imo State University, n=54(%)			Isolates from medical students of Abia State University, n=50(%)			Isolates from medical students of University of Uyo, n=144(%)		
	Pre-clinical students	Clinical students	IMSU Total	Pre-clinical students	Clinical students	ABSUTotal	Pre-clinical students,	Clinical students,	UNIUYO Total
Palm swab	24(66.7)	8(44.4)	32(59.3)	18 (60)	20(100)	38(76)	21(30.9)	31(40.8)	52(36.1)
Throat swab	-	-	-	4 (13.3)	-	4(8)	4(5.8)	3(3.9)	7(4.9)
Nasal swab	12(33.3)	10(55.6)	22(40.7)	8(26.7)	-	8(16)	43 (63.2)	42(55.2)	85(59)
Total	36(100)	18(100)	54(100)	30 (100)	20(100)	50(100)	68(100)	76(100)	144(100)

Key: - , Sample not obtained, IMSU-Imo State University, ABSU- Abia State University, and UNIUYO-University of Uyo.

Table 4.3. Total Staphylococci isolated from pre-clinical and clinical medical students

Swab samples	Staphylococci isolated from Pre-clinical students; n=134(%)	Staphylococci isolated from Clinical Students; n=114(%)
Palm swabs	63(47.0)	59(51.8)
Throat swabs	8(6.0)	3(2.6)
Nasal swabs	63(47.0)	52(45.6)
Total	134(100)	114(100)

4.1.2.2. Isolated Staphylococci classified according to gender.

The distribution of Staphylococci isolated from each sample among male and female pre-clinical and clinical students in all screened institutions is presented in table 4.4. From this table, males harbor more Staphylococci in their noses and throats with percentage occurrence of 51(65.4%) and 7(9%) respectively in the pre-clinical class as compared with the females in the same class with a percentage of occurrence in noses and throats of 12 (21.4%) and 1(1.8%) respectively while the females harbour more Staphylococci in their palms with a frequency of occurrence of 43(76.8%) when compared with their male counterparts with a frequency of occurrence of 20(25.6%) in their palms also in the pre-clinical class. From Table 4.4, palm swabs from female students in the clinical class are the most colonized with a percentage occurrence of 84.1% while throat swabs from female students in the pre-clinical class are the least colonized with a percentage occurrence of 1.8%. However nasal carriage is higher in males in both the pre-clinical class (65.4%) and the clinical class (64.3%) than the females in the pre-clinical and clinical classes with 21.4% and 15.9% respectively.

From table 4.5, it is observed that out of 109 males screened in the pre-clincal class, 78 (71.6%) are colonized with Staphylococci in all three schools and 70 (63.1%) in the clinical class. In females, out of 111 screened in the pre-clinical class, 56 (50.4%) harbor Staphylococci and out of 89 screened in the clinical class, 44 (49.4%) female students harbour Staphylococci with a frequency of 148 (59.7%) more than the females with a frequency of 100 (40.3%).

From Table 4.5, 71.6% males harbor the Staphylococci and 50.4% female students harbor the Staphylococci in the pre-clincal class while in the clinical class, out of 114 female students screened, 63.15 male students are colonized with Staphylococci and 49.4% female students are colonized with the Staphylococci.

Table 4.4. Distribution of isolated Staphylococci according to gender from screened institutions

Samples	Staphylococci isolates from medical students					
	Isolates from pre-clinical students			Isolates from clinical students		
	Male	Female	Total	Male	Female	Total
	n=78	n= 56	n=134	n= 70	n= 44	n=114
Palm swabs	20(25.6)	43 (76.8)	63(47.0)	22(31.4)	37(84.1)	59(51.8)
Throat swabs	7(9.0)	1(1.8)	8(5.9)	3(4.3)	-	3(2.6)
Nasal swabs	51(65.4)	12(21.4)	63(47.0)	45(64.3)	7(15.9)	52(45.6)
Total	78 (100)	56(100)	134(100)	70(100)	44(100)	114(100)

Key: - , Sample not obtained

Table 4.5. Distribution of *Staphylococcus* spp. isolatesaccording to gender in allthe pre-clinical and clinical classes.

	Isolates from pre-clinical students			Isolates from clinical students		
Gender	Screened Male students	Males that tested positive	Percentage positive	Screened Female students	Females that tested positive	Percentage positive
Male	109	78	71.6%	111	70	63.1%
Female	111	56	50.4%	89	44	49.4%
Total	220	134	60.9%	200	114	57.0%

4.1.2.3. Isolated Staphylococciclassified according to Species.

Table 4.6 shows *Staphylococcus aureus* isolates and coagulase–negative *Staphylococcus* species isolated from medical students in the pre-clinical and clinical classes of all screened institutions. *Staphylococcus aureus* occurred more in palm swabs with a percentage occurene of 56.8% in clinical students and 46.6% in pre-clinical students. On the other hand, nasal swabs carried more coagulase negative Staphylococci than palm swabs with a Percentage occurrence of 65.4% in clinical students and 51.6% in pre-clinical students. All throat swabs recorded nil occurrences for coagulase-negative Staphylococci.

From Table 4.6, *Staphylococcus aureus* was isolated more in palm swabs of both pre-clinical students (46.6%) and clinical students (56.8%) followed by nasal swabs in both the pre-clinical class (41.7%) and clinical class (39.8%). Coagulase negative Staphylococci were mostly isolated from palm swabs of both pre-clinical and clinical students. From Table 4.7, out of 248 Staphylococci isolates, 191 were identified as *Staphylococcus aureus* and 57 were identified as Coagulase-negative Staphylococci.

4.1.2.4. Staphylococciisolated from medical students classified according to age distribution in all screened institutions.

Table 4.8 shows the distribution of isolated staphylocci from medical students according to age in all screened institutions. From the table, out of 248 isolated Staphylococci, 50.4% of students aged 26-30 were the most colonized with Staphylococci, 37.9% of students aged 21-25 were colonized with Staphylococci, 7.3% of students aged greater than 31 were colonized with Staphylococci and 4.4% of students aged less than 21 were colonized with Staphylococci.

Comparing between classes, 47.6% and 51.6% of students aged 21-25 were the most colonized with *Staphylococcus aureus* and coagulase-negative Staphylococci respectively in the pre-clinical class while in the clinical class, 58% and 61.5% of students aged 26-30 were the most colonized with *Staphylococcus aureus* and coagulase-negative Staphylococci respectively.

Table 4.6. Distribution of Staphylococci isolatesaccording to species from swab samples received.

	Isolates from pre-clinical students		Isolates from clinical students	
	SA	CONS	SA	CONS
	n= 103(%)	n= 31(%)	n= 88(%)	n=26(%)
Palm swabs	48(46.6)	15 (48.4)	50(56.8)	9 (34.6)
Nasal swabs	43(41.7)	16 (51.6)	35(39.8)	17 (65.4)
Throat swabs	12(11.7)	-	3(3.4)	0 (0.0)
Total	103 (100)	31(100)	88(100)	26 (100)

-: no sample received. SA: *Staphylococcus aureus*, CONS- Coagulase negative Staphylococci

Table 4.7. Staphylococci isolates classified according to class.

Staphylococci isolates	Isolates from pre-clinical students; n=134(%)	Isolates from clinical Students; n=114(%)	Total N=248
Staphylococcus aureus	103(76.9)	88(77.2)	191(77.0)
Coagulase-negative Staphylococci	31(23.1)	26(22.8)	57(23.0)
Total	134(100)	114(100)	248 (100)

Table 4.8. Distribution of the isolated Staphylococci according to age in all screened institutions

Ages	Staphylococci isolates from medical students				
	Isolates from pre- clinical students		Isolates from clinical students		Total
	SA	CONS	SA	CONS	N=248
	n=103	n= 31	n= 88	n=26	
<21	11(10.7)	0(0.0)	0(0.0)	0(0.0)	11(4.4)
21-25	49(47.6)	16(51.6)	29(33.0)	0(0.0)	94(37.9)
26-30	43(41.7)	15(48.4)	51(58.0)	16(61.5)	125(50.4)
>31	0(0.0)	0(0.0)	8(9.0)	10(38.5)	18(7.3)
Total	103(100)	31(100)	88(100)	26(100)	248 (100)

Key: SA- *Staphylococcus aureus*; CoNS- Coagulase-negative Staphylococci; 0(0.0) - no organism isolated**.**

4.1.3 Antibiotic Resistance Testing

Antibiotic resistance test was carried out for the 248 Staphylococciisolates and the zones of inhibition obtained were classified based on Clinical and Laboratory Standards Institute (2012) interpretative chart for antimicrobial sensitivity testing.Table 4.9 shows the antibiotic resistance profile of *Staphylococcus aureus* in pre-clinical and clinical students. From this table, in both the pre-clinical and clinical class, the organism shows the highest resistance of 98.1% and 94.3% to trimethoprim respectively. This is followed by penicillin, oxacillin and ampicillin with the organism shows resistance of 87.4%, 82.5% and 79.6% respectively in the pre-clinical class. The most susceptible drug is nitrofurantoin in both the pre-clinical class and the clinical class with 4% and 4.5% resistance respectively.

Table 4.10 shows the antibiotic resistance profile of isolated *Staphylococcus aureus* from Imo State University, Abia State University and University of Uyo.

Comparing the total resistance in the three schools: the resistance test of the *Staphylococcus aureus*isolates showed that trimethoprim had the highest resistance of 100% in University of Uyo, 95.4% in Imo State University followed by penicillin with 89.9% in University of Uyo and then penicillin and oxacillin with 87.5% in Abia State University.Comparing the resistance between classes, in table 4.19, the beta-lactams penicillin and oxacillin showed the very high resistances of 100% and 96.7% respectively in pre-clinical students of Imo State University. Trimethoprim also had a resistance of 100% in pre-clinical students of Imo State Universityfollowed by ampicillin with a resistance of 93.3% in pre-clinical students of Imo State University. The least resistance of 7.0% is seen in nitrofurantoin in pre-clinical students of University of Uyo followed by 7.1% recorded still in nitrofurantoin in clinical students of University of Uyo and 10% in erythromycin in pre-clinical students of Imo State University. The Antibiotic resistance profile of coagulase-negative Staphylococci isolated from pre-clinical and clinical students is shown in table 4.11.From the Table 4.11, in both the pre-

clinical and clinical class, the organism shows the highest resistance of 100% to trimethoprim respectively. This is followed by penicillin, oxacillin and ampicillin with the organism shows resistance of 70.9%, 70.9%, 70.9% respectively in the pre-clinical class and 88.5%, 88.5% 88.5% respectively in the clinical class. The most effective drug is nitrofurantoin in both classes with 0% resistance and erythromycin in the clinical class with 0% resistance.

Table 4.12 shows the antibiotic resistance profile of isolated coagulase-negative Staphylococci from Imo State University, Abia State University and University of Uyo.Comparing the total resistance in the three schools from Table 4.12, the resistance test of the coagulase-negative Staphylococciisolates showed that penicillin, oxacillin and ampicillin had the highest resistance (100%) in Imo State University and Abia State University (ABSU). Trimethoprim also had a 100%resistance in all three institutions. Comparing the resistance between classes, penicillin, oxacillin, ampicillin and trimethoprim showed the highest resistances of 100% in clinical students of Abia State University (ABSU) and in pre-clinical students of Imo State University. Nitrofurantoin records nil resistance across all classes in all three schools.Erythromycin recorded 33.3% resistance in pre-clinical students of Imo State University. Vancomycin has a completely nil resistance in Imo State University. Ciprofloxacin is also completely susceptible in pre-clinical and clinical students of Imo State University (IMSU) and Abia State University (ABSU).

Table 4.9.Antibiotic resistance profile of *Staphylococcus aureus* in pre-clinical and clinical students.

Antibiotics	Isolates from pre- clinical students(%) n=103	Isolates from clinical students (%) n= 88
Penicillin	90 (87.4)	69 (78.4)
Oxaciliin	85 (82.5)	63 (71.5)
Ampicillin	82 (79.6)	66 (75.0)
Nitrofurantoin	9 (8.7)	4 (4.5)
Erythromycin	43 (41.7)	52 (59)
Trimethoprim	101 (98.1)	83 (94.3)
Ciprofloxacin	39 (37.9)	28 (31.8)
Vancomycin	36 (34.9)	18 (20.5)
Clindamycin	39 (37.9)	31 (35.2)

Table 4.10. Antibiotic Resistance Profile of *Staphylococcus aureus* in medical students

Antibiotics	Isolates of medical students from IMSU, n=44(%)			Isolates of medical students from ABSU, n=47(%)			Isolates of medical students from UNIUYO, n=99(%)		
	Pre-clinical students, n=30 (%)	Clinical students, n=14(%)	Total Resistance, n=44 (%)	Pre-clinical students, n=30(%)	Clinical students, n=18(%)	Total Resistance, n=47 (%)	Pre-clinical students, n=43 (%)	Clinical students, n=56(%)	Total Resistance, n=99 (%)
Penicillin	30 (100)	8 (57.1)	38(86.4)	24(80)	18(100)	42(87.5)	36(83.7)	43(76.8)	89(89.9)
Oxacillin	29(96.7)	8 (57.1)	37 (84.1)	24(80)	18(100)	42(87.5)	32(74.4)	37(66.1)	69(69.7)
Ampicillin	28 (93.3)	8 (57.1)	36 (81.8)	21(70)	18(100)	39(81.3)	33(76.7)	40(71.4)	73(73.7)
Nitrofurantoin	6 (20)	0(0.0)	6(13.6)	0(0.0)	0(0.0)	0(0.0)	3(7.0)	4(7.1)	7(7.07)
Erythromycin	3(10)	8(57.1)	11(25)	10(33.3)	12(66.7)	22(45.8)	30(69.8)	32(57.1)	62(62.6)
Trimethoprim	30(100)	12 (85.7)	42 (95.4)	28(93.3)	15(83.3)	43(89.6)	43(100)	56(100)	99(100)
Ciprofloxacin	13 (43.3)	0(0.0)	13 (29.5)	13(43.3)	16(88.9)	19(39.6)	13(30.2)	12(21.4)	25(25.3)
Vancomycin	8(26.7)	3(21.4)	11 (25)	9(30)	7(38.9)	16(39.6)	19(44.2)	8(14.3)	27(27.3)
Clindamycin	8 (26.7)	3(21.4)	11 (25)	13(43.3)	10(55.6)	23(47.9)	18(41.9)	18(32.1)	36(36.4)

Table 4.11.Antibiotic resistance profile of coagulase-negative Staphylococciin pre-clinical and clinical students.

Antibiotics	Isolates from pre- clinical students(%) n=31	Isolates from clinical students (%) n= 26
Penicillin	22 (70.9)	23 (88.5)
Oxaciliin	22 (70.9)	23 (88.5)
Ampicillin	22 (70.9)	23 (88.5)
Nitrofurantoin	0 (0.0)	0 (0.0)
Erythromycin	2 (6.5)	0 (0.0)
Trimethoprim	31 (100)	26 (100)
Ciprofloxacin	18 (58.1)	15 (57.7)
Vancomycin	5 (16.1)	10 (38.5)
Clindamycin	9 (29.0)	11 (42.3)

Table 4.12. Antibiotic Resistance Profile of coagulase- negative Staphylococci in medical students

Antibiotics	Isolates of medical students from IMSU			Isolates of medical students from ABSU			Isolates of medical students from UNIUYO		
	Pre-clinical students, n=6 (%)	Clinical students ,n=4(%)	Total Resistance, n=10 (%)	Pre-clinical students, n=0(%)	Clinical students, n=2(%)	Total Resistance, n=2 (%)	Pre-clinical students, n=25 (%)	Clinical students, n=20(%)	Total Resistance, n=45 (%)
Penicillin	6 (100)	4(100)	10(100)	0(0.0)	2(100)	3(100)	16(64)	17(85)	33(37.8)
Oxacillin	6(100)	4(100)	10 (100)	0(0.0)	2(100)	2(100)	16(64)	17(85)	33(37.8)
Ampicillin	6 (100)	4(100)	10 (100)	0 (0.0)	2(100)	2(10 0)	16(64)	17(85)	33(37.8)
Nitrofurantoin	0 (0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Erythromycin	2(33.3)	0(0.0)	2(20)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Trimethoprim	6 (100)	4(100)	10(100)	0 (0.0)	3 (100)	2 (100)	25 (100)	20 (100)	45(100)
Ciprofloxacin	0 (0.0)	1(25)	0 (0.0)	0(0.0)	0 (0.0)	0 (0.0)	18(72)	14(70)	32(71.1)
Vancomycin	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2(100)	2(100)	5(20)	7(35)	12(26.7)
Clindamycin	2(33.3)	0(0.0)	2(20)	0 (0.0)	2(100)	2(100)	7(28)	8 (40)	15 (33.3)

KEY: 0.0- nil resistance, IMSU-Imo State University, ABSU- Abia State University, UNIUYO- University of Uyo

4.1.4 Comparison of Methicillin Resistance among Staphylococci Isolates

Out of the 248 isolated Staphylococci, 191(77.0%) were *Staphylococcus aureus*and 57(22.9%) were Coagulase-negative Staphylococci. Of the 191 *Staphylococcus aureus*isolated, 148(77.5%) were Methicillin Resistant *Staphylococcus aureus* (MRSA), 36 (18.8%) were Methicillin Susceptible *Staphylococcus aureus* (MSSA) and 7(3.7%) were Methicillin Intermediate *Staphylococcus aureus*(MISA). Of the 57 isolated Coagulase-negative Staphylococci, 45(79.0%) were Methicillin Resistant Coagulase-negative Staphylococci (MRCONS), 3(5.3%) were Methicillin Susceptible Coagulase-negative Staphylococci (MSCONS)and 9 (15.7%) were Methicillin Intermediate Coagulase-negative Staphylococci(MICONS) . This is as represented in table 4.13.

From this table, in all three schools, the percentages of methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin resistant coagulase-negative Staphylococci recorded were higher when compared with methicillin susceptible and methicillin intermediate Staphylococci isolates.

4.1.5. Inducible Clindamycin Resistance

Of the 248 Staphylococciisolates screened, 39(15.7%) tested positive to the inducible Clindamycin D-test. only 4(1.6%) of the Staphylococciisolates were both resistant to erythromycin and susceptible to clindamycin but with no D-zone, showing that erythromycin resistance observed did not have any inductive effect on the clindamycin (showing a D-Test negative result). 181 (73%) Staphylococci isolates were resistant to both erythromycin and clindamycin which implies that the Clindamycin resistance observed is constitutive. The result is presented in Table 4.14 and the pictoral view in Plates 2, 3, 4 and 5 on pages 122, 123, 124 and 125 respectively.

Table 4.13. Methicillin susceptibility profile of Staphylococciisolated from medical students in all three institutions.

Isolates from medical students						
Medical students	*Staphylococcus aureus*, n=191(%)			Coagulase- negative *Staphylococcus*, n=57(%)		
	MISA	MSSA	MRSA	MICONS	MSCONS	MRCONS
Pre-clinical	3(1.6)	14(7.3)	85 (44.5)	7(12.2)	2(3.5)	23(40.4)
Clinical	4 (2.1)	22 (11.5)	63 (33.0)	2(3.5)	1(1.8)	22(38.6)
Total	7(3.7)	36(18.8)	148(77.5)	9(15.7)	3(5.3)	45(79.0)

Legend: MISA: Methicillin Intermediate *Staphylococcus aureus*, MSSA: Methicillin Susceptible *Staphylococcus aureus*, MRSA: Methicillin Resistant *Staphylococcus aureus*, MRCONS: Methicillin Resistant coagulase negative *Staphylococcus*, MSCONS: Methicillin Susceptible coagulase negative *Staphylococcus*, MICONS: Methicillin Intermediate coagulase negative *Staphylococcus*

Table 4.14. Susceptibility to erythromycin and clindamycin for all Staphylococci isolates

Susceptibility pattern (phenotype)	**Number of Isolates**				Total
	Isolates from Pre-clinical students; n=134	Percentages	Isolates from clinical students n= 114	percentages	N=248(%)
Ery S clin S	2	1.5%	22	19.3	24(9.7)
Ery R clin R (constitutive MLS_B)	104	77.6%	77	67.5	181(73.0)
Ery R clin S(D- test positive i MLS_B)	28	20.9%	11	9.6	39(15.7)
Ery R clin S (D-test negative MS)	Nil	0%	4	3.5	4(1.6)
Total	134	100%	114	100%	248(100)

Legend: Ery: Erythromycin, Clin: clindamycin, S: sensitive, R: resistant, constitutive MLS_B: consititutive MLS_B phenotype, i MLS_B: inducible MLS_B phenotype, MS: MS phenotype, MSSA: Methicillin Susceptible *Staphylococcus aureus*, MRSA: Methicillin Resistant *Staphylococcus aureus*, MLS_B: Macrolide-Lincosamide- Streptogramin B. Figures in parenthesis are in percentage

Plate 2: Sensitivity test plate showing Erythromycin Resistant, Clindamycin Sensitive (D-test positive) Staphylococci isolate

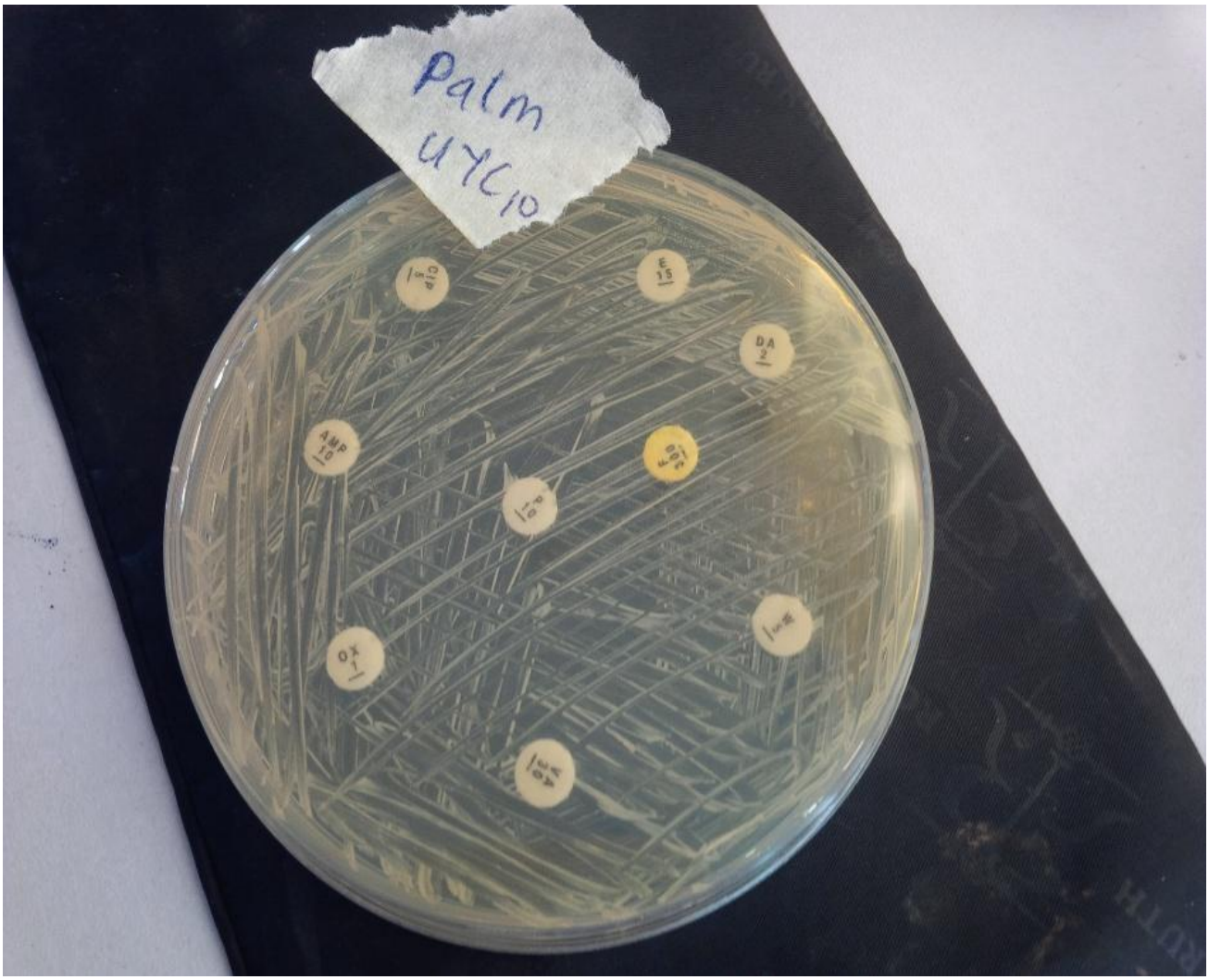

Plate 3: Sensitivity test plate showing Erythromycin Resistant, Clindamycin Resistant Staphylococci isolate

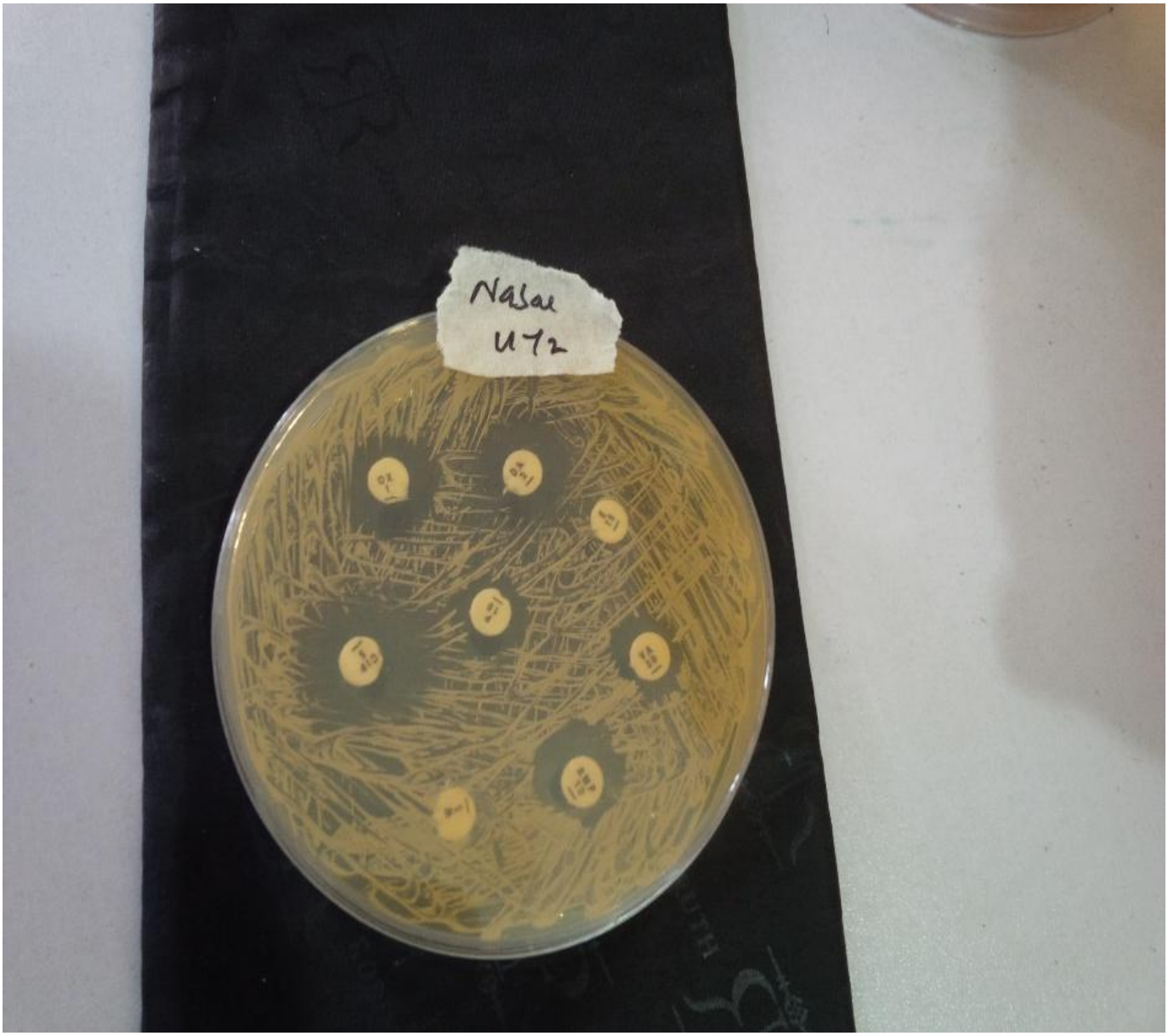

Plate 4: Sensitivity test plate showing Erythromycin Resistant, Clindamycin Sensitive (circular zone) Staphylococci isolate

Plate 5: Sensitivity test plate showing Erythromycin Sensitive, Clindamycin Sensitive Staphylococci isolate

4.1.6 Comparing Clindamycin Susceptibility with Methicillin Resistance in all screened institutions

From table 4.15, 19.2% of Methicillin resistant *Staphylococcus aureus (*MRSA)and 9.1% of Methicillin susceptible *Staphylococcus aureus*(MSSA) isolates tested positive to D-test in the pre-clinical classes and in the clinical classes, 10.6% of Methicillin resistant *Staphylococcus aureus* (MRSA) and 2.4% of Methicillin susceptible *Staphylococcus aureus*(MSSA) tested positive to the D-test for inducible clindamycin resistance. Constititive resistance was also higher in Methicillin resistant *Staphylococcus aureus* (MRSA) in both pre-clinical and clinical classes (43.4% and 51.8%) respectively than in their clinical counterparts with 4.0% in the pre-clinical class and 5.9% in the clinical class.

Of the 148 isolated MRSA, 4(2.7%) tested negative to the D-test and 28(18.9%) tested positive to D-test.

Table 4.15.Inducible clindamycin resistance in Methicillin resistant and Methicillin susceptible *Staphylococcus aureus* isolates (MRSA and MSSA) in all screened institutions.

Clindamycin susceptibility	**Methicillin resistance**					
	Isolates of medical students					
	Pre-clinical students, n=99			Clinical students, n=85		
	MRSA	MSSA	Total	MRSA	MSSA	Total
Ery S clin S	23(23.2)	1(1)	24(24.2)	6(7.1)	6(7.1)	12(14.1)
Ery R clin R (constitutive MLS_B)	43(43.4)	4(4.0)	47(47.5)	44(51.8)	5(5.9)	49(57.6)
Ery Rclin S(D- test positive i MLS_B)	19(19.2)	9(9.1)	28(28.3)	9(10.6)	2(2.4)	11(12.9)
Ery R clin S (D-test negative MS)	0(0.0)	0(0.0)	(0.0)	4(4.7)	9(10.6)	13(15.3)
Total	85(85.9)	14(14.1)	99(100)	63(74.1)	22(25.9)	85(100)

Legend: MISA: Methicillin Intermediate *Staphylococcus aureus*, MSSA: Methicillin Susceptible *Staphylococcus aureus*, MRSA: Methicillin Resistant *Staphylococcus aureus*, MRCONS: Methicillin Resistant coagulase negative *Staphylococcus*, MSCONS: Methicillin Susceptible coagulase negative *Staphylococcus*, MICONS: Methicillin Intermediate coagulase negative *Staphylococcus*, Ery: Erythromycin, Clin: clindamycin, S: sensitive, R: resistant, constitutive MLS_B: consititutive MLS_B phenotype, i MLS_B: inducible MLS_B phenotype, MS: MS phenotype, MSSA: Methicillin Susceptible *Staphylococcus aureus*,MRSA: Methicillin Resistant *Staphylococcus aureus*.MLS_B: Macrolide-Lincosamide- Streptogramin B. Figures in parenthesis are in percentage

4.1.7.MinimumInhibitory Concentration (MIC) of Vancomycin

Tables 4.16 and table 4.17 respectively give details of isolates that were susceptible to vancomycin.

From Table 4.16, out of 148 methicillinresistant *Staphylococcus aureus*(MRSA)isolated, 49 (33.1%) were susceptible to vancomycin. In Imo State University (IMSU), 16(43.2%) methicillin resistant *Staphylococcus aureus*(MRSA)isolates were susceptible to vancomycin. In Abia State University (ABSU), 15(35.7%) methicillin resistant *Staphylococcus aureus*(MRSA)isolates were susceptible to vancomycin. In University of Uyo (UNIUYO), 18(26%) methicillin resistant *Staphylococcus aureus*(MRSA)isolates were susceptible to vancomycin.

From Table 4.17, out of 45methicillin resistant coagulase-negative Staphylococci (MRCONS)isolated, 26(57.8%) were susceptible to vancomycin. 9(90%) from Imo State University, 2(100%) from Abia State University and 15(45.5%) from University of Uyo.

EUCAST interpretation of vancomycin MIC breakpoint describes isolates with MIC $\leq$ 2 μg/ml as being susceptible, 4-8 μg/ml intermediate and $\geq$16 μg/ml resistant. With this, only nasal 10 and nasal 17 coagulase-negative Staphylococci isolates from Imo State University with MICs 8 and 4 have intermediate resistance to vancomycin. Also nasal 97 from a pre-clinical student of Imo State University had MIC of 16 μg/ml being resistant to vancomycin. Also, in University of Uyo, Throat UYCL 76 and Throat UYCL 8 from clinical students had MICs 8 μg/ml being intermediate resistant and 16 μg/ml being resistant to vancomycin. All other vancomycin susceptible isolates were confirmed as susceptible with the MIC determination using the vancomycin E-test strips. Tables 4.18, 4.19 and 4.20 show the summary of minimum inhibitory concentration of vancomycin susceptible Staphylococciisolates in pre-clinical and clinical students of Imo State University, Abia State University and University of Uyo.

Table 4.16. Susceptibility to vancomycin in methicillin resistant *Staphylococcus aureus*isolates

	Medical students from IMSU			**Medical students from ABSU**			**Medical students from UNIUYO**			**Total**
	Pre-clinical, n=29	Clinical, n=8	Total ,n=37	Pre-clinical, n=24	Clinical, n=18	Total ,n=42	Pre-clinical, n=32	Clinical, n=37	Total , n=69	148
vancomycin	10(34.5)	6 (75)	16(43.2)	8 (33.3)	7(38.9)	15(35.7)	8(25)	10(27)	18(26)	49(33.1)

Abbreviations- IMSU: Imo State University, ABSU- Abia State University, UNIUYO-University of Uyo

Table 4.17 Susceptibility to vancomycin in methicillin resistant coagulase-negative Staphylococci isolates

	Isolates of medical students from IMSU			**Isolates of medical students from ABSU**			**Isolates of medical students from UNIUYO**			**Total**
	Pre-clinical, n=7	Clinical, n=3	Total, n=10	Pre-clinical, n=nil	Clinical, n=2	Total, n=2	Pre-clinical, n=16	Clinical, n=17	Total, n=33	45
vancomycin	6(85.7)	3(100)	9(90)	0(0%)	2(100)	2(100)	8(50)	7(41.2)	15(45.5)	26(57.8)

Abbreviations- IMSU: Imo State University, ABSU- Abia State University, UNIUYO-University of Uyo

Table 4.18. Minimum inhibitory concentration of vancomycin-susceptible *Staphylococcus aureus* andCoagulase- negative Staphylococciisolates from pre-clinical and clinical medical students in Imo State University (IMSU)

***Staphylococus aureus* isolates from pre-clinical students of Imo State University**		***Staphylococus aureus* isolates from clinical students of Imo State University**		**Coagulase- negative Staphylococci isolates from pre-clinical students of Imo State University**		**Coagulase- negative Staphylococci isolates from Clinical students of Imo State University**	
Isolates	**MIC (µg/ml)**	**Isolates**	**MIC (µg/ml)**	**Isolates**	**MIC (µg/ml)**	**Isolates**	**MIC (µg/ml)**
Nasal 57	0.5	Palm F	0.015	Nasal 97	16	Palm D	2
Palm 202	0.015	Nasal 10	0.25	Nasal 77	0.25	Nasal 10	8
Nasal 101	0.25	Nasal R	0.5	Nasal 91	0.03	Nasal 17	4
Palm 201	0.0625	Palm D	0.0625	Palm 94	0.125		
Palm 204	0.125	Nasal 13	0.03	Palm 63	0.0625		
Palm 11	0.03	Palm Q	0.125	Palm 78	0.015		
Palm 208	0.015						
Palm 215	0.5						
Palm 205	0.25						
Nasal 16	0.5						

Table 4.19. Minimum inhibitory concentration of vancomycin-susceptible *Staphylococcus aureus* andCoagulase- negative Staphylococciisolates from pre-clinical and clinical medical students in Abia State University (ABSU)

***Staphylococus aureus* isolates from pre-clinical students of Abia State University**		***Staphylococus aureus* isolates from clinical students of Abia State University**		**Coagulase- negative Staphylococci isolates from Clinical students of Abia State University**	
Isolates	**MIC (µg/ml)**	**Isolates**	**MIC (µg/ml)**	**Isolates**	**MIC (µg/ml)**
Nasal U30	0.03	Palm A2	0.03	Palm A6	0.25
Throat U4	0.0625	Palm A7	1	Palm A26	0.125
Palm U20	2	Palm A12	2		
Palm U9	0.125	Palm A25	0.015		
Palm U26	1	Palm A23	0.0625		
Palm U25	0.25	Palm A28	0.0125		
Nasal UI	0.015	Palm A22	0.25		
Palm U33	2				

Table 4.20.Minimum inhibitory concentration of vancomycin-susceptible *Staphylococcus aureus* andCoagulase- negative Staphylococciisolates from pre-clinical and clinical medical students in University of Uyo

***Staphylococus aureus* isolates from pre-clinical students of University of Uyo**		***Staphylococus aureus* isolates from clinical students of University of Uyo**		**Coagulase- negative Staphylococci isolates from pre-clinical students of University of Uyo**		**Coagulase- negative Staphylococci isolates from Clinical students University of Uyo**	
Isolates	**MIC (µg/ml)**	**Isolates**	**MIC (µg/ml)**	**Isolates**	**MIC (µg/ml)**	**Isolates**	**MIC (µg/ml)**
Nasal UY3	0.03	Palm UYCL 71	2	Palm UY 103	0.5	Nasal UYCL 8	0.03
Nasal UY1	0.5	Nasal UYCL 27	0.5	Nasal UY 109	0.25	Nasal UYCL 21	0.0625
Nasal UY4	2	Nasal UYCL 1	0.0125	Nasal UY 116	0.03	PalmUYCL 72	0.125
Nasal UY2	0.125	Palm UYCL 12	0.0625	Nasal UY 111	0.125	ThroatUYCL 76	8
Palm UY3	0.0625	Nasal UYCL 30	0.25	Nasal UY 77	0.0625	Nasal UYCL 36	0.015
Nasal UY9	0.025	Palm UYCL 5	0.03	Nasal UY 65	0.03	ThroatUYCL 8	16
PalmUY115	0.015	Palm UYCL 8	0.125	Nasal UY 8	0.5	Nasal UYCL 35	0.25
Nasal UY5	2	Palm UYC 1	2	Nasal UY 7	0.5		
		Nasal UYC 4	0.15				
		Palm UYCL 5	0.5				

4.1.8.Plasmid Extraction of Methicillin resistant Staphylococciisolates

The plasmid extraction carried out with Plasmid MiniPrep Kit (Norgen Biotek Cooporation) for thirteen (13) methicillin resistant *Staphylococcus aureus*(MRSA) isolates and eighteen (18) methicillin resistant coagulase-negative Staphylococci (MRCONS) isolates had the following results. Out of thirty-one (31) isolates tested, the following twenty-one (21) isolates had plasmid bands: Palm A11, Palm A1, Palm A17, Palm U22, Palm A21, Nasal UY131, Nasal A3, Nasal 18, Nasal UYCL4, Nasal R, Nasal UYCL99, Nasal 101, Nasal 77, Nasal i, Nasal U30, Palm UY1, Nasal U7, Nasal U1, Palm X, Palm UYCL200 and Palm UY103 (Figures 4.1, 4.2, 4.3 below). Palm A11, Palm A17, Nasal 18, and Nasal UYCL4 showed five bands each suggesting carriage of five plasmids each. These isolates with plasmids represent 13 (41.9%) of phenotypic methicillin resistant *Staphylococcus aureus*(MRSA) tested and 8(25.8%) of the methicillin resistant coagulase-negative Staphylococci (MRCONS) isolates tested. The summary of the plasmid profiles of the *Staphylococcus aureus*isolates are presented in Table 4.21. Out of the thirty-one (31) Staphylococci isolates tested, only twenty-one (21) had plasmid bands with molecular size range between 1000 and 10,000 base pairs. The resistance pattern of plasmid DNA- containing isolates are presented in Table 4.22. The resistance pattern of the plasmid - containing isolates showed 21 (100%) of isolates to be resistant to three or more classes of antibiotics which is an indication of multi-drug resistance. These multi-drug resistant isolates are all methicillin resistant *Staphylococcus aureus*and coagulase-negative Staphylococci.

Table 4.21. Identification of Staphylococci isolates and their plasmid profiles

Isolates	Number of plasmids	Methicillin resistance identity
Palm A11	5	Methicillin resistant *Staphylococcus aureus* (MRSA)
Palm A1	8	Methicillin resistant coagulase-negative Staphylococci (MRCONS)
Palm A17	5	Methicillin resistant coagulase-negative Staphylococci (MRCONS)
Palm U22	7	Methicillin resistant *Staphylococcus aureus* (MRSA)
Palm A21	9	Methicillin resistant *Staphylococcus aureus* (MRSA)
Nasal UY131	12	Methicillin resistant *Staphylococcus aureus* (MRSA)
Nasal A3	8	Methicillin resistant coagulase-negative Staphylococci (MRCONS)
Nasal 18	5	Methicillin resistant coagulase-negative Staphylococci (MRCONS)
Nasal UYCL4	5	Methicillin resistant *Staphylococcus aureus* (MRSA)
Nasal R	2	Methicillin resistant *Staphylococcus aureus* (MRSA)
Nasal UYCL99	2	Methicillin resistant *Staphylococcus aureus* (MRSA)
Nasal 101	6	Methicillin resistant coagulase-negative Staphylococci (MRCONS)
Nasal 77	4	Methicillin resistant coagulase-negative Staphylococci (MRCONS)
Nasal i	2	Methicillin resistant *Staphylococcus aureus* (MRSA)
Nasal U30	1	Methicillin resistant *Staphylococcus aureus* (MRSA)
Palm UY1	1	Methicillin resistant coagulase-negative Staphylococci (MRCONS)
Nasal U7	1	Methicillin resistant *Staphylococcus aureus* (MRSA)
Nasal U1	1	Methicillin resistant *Staphylococcus aureus* (MRSA)
Palm X	1	Methicillin resistant *Staphylococcus aureus* (MRSA)
Palm UYCL200	1	Methicillin resistant coagulase-negative Staphylococci (MRCONS)
Palm UY103	3	Methicillin resistant *Staphylococcus aureus* (MRSA)

Table 4.22Antibiotic Resistance pattern of plasmid DNA- containing Staphylococciisolates

S/No	Laboratory no.	Resistance pattern	Number of antibiotics
1	Palm A11	OX, P, AMP, CIP, CLIN, W, E.	7
2	Palm A1	VAN, F, OX, AMP, P. CIP, CLIN, W, E	9
3	Palm A17	OX, P, AMP, VAN, W, CIP, CLN	7
4	Palm U22	OX, AMP, P, F, VAN, CLIN, W, E, CIP	9
5	Palm A21	OX, AMP, P, E, W, CIP	6
6	Nasal UY131	OX, P, AMP, E, W, CIP, VAN	7
7	Nasal A3	CLIN, E, W, OX, AMP, P	6
8	Nasal 18	W, CIP, F, OX, AMP, P	6
9	Nasal UYCL4	OX, AMP, P. E, VAN, W, F	7
10	Nasal R	OX, AMP, P, VAN	4
11	Nasal UYCL99	OX, P, AMP, W, CIP, VAN	6
12	Nasal 101	CIP, VAN, W, E	4
13	Nasal 77	OX, AMP, P, W, CLIN, CIP, E	7
14	Nasal i	OX, P, AMP, VAN	4
15	Nasal U30	OX, P, AMP, W, E	5
16	Palm UY1	P, AMP, OX, CLIN, F, VAN. E, CIP, W	9
17	Nasal U7	P, AMP, W, OX	4
18	Nasal U1	OX, AMP, P, W, CLIN, CIP	6
19	Palm X	OX, AMP, P, CIP, E	5
20	Palm UYCL200	CIP, OX, AMP, P, VAN, F, W, CLIN, E	9
21	Palm UY103	AMP, OX, P, F, E, CLIN, CIP, W	8

OX- Oxacillin CIP- Ciprofloxacin P- Penicillin F- Nitrofurantoin E- Erythromycin
AMP- Ampicillin VAN- Vancomycin W- Trimethoprim CLIN- Clindamycin

4.1.9. Genomic DNA Extraction of StaphylococciIsolates

The genomic DNA extraction of thirty-one (31) Staphylococciisolates was carried out using Genomic DNA isolation kit by Norgen Biotek Cooporation and the presentation of the DNA bands of the Staphylococci isolates on 1.5% electrophoresis gel is shown in Figures 4.1, 4.2, and 4.3. The DNA bands are of the molecular weight 200 – 1031 base pairs in comparism with molecular weight marker.

4.1.10. Polymerase Chain Reaction (PCR) for Detection of Staphylococcal *nuc*, *mecA* Gene and *blaZ* Genes

The Results of Polymerase Chain Reaction (PCR)products of nuc, *mecA, and blaZ* on 1.5% agarose gel using 2x PCR control master mix is shown in figures 4.1, 4.2, and 4.3 respectively. The size of the amplicon fornuc, *mecA* and *blaZ* gene cosrrespond to 300bp, 533bp and 517bp, respectively, as repersented by MassRuler DNA ladder. Out of the thirty-one (31) isolates tested, 2 (6.5%) amplified nucat 300bp (Palm 94 and nasal 77) indicating that 2/31 were confirmed as having the *Staphylococcus* nucleus (nuc) gene. Amplification of the 533bp indicates presence of *mecA* gene coding for methicillin resistance and only 5 isolates (Palm U22, Nasal UY131, Nasal U7, Nasal Ui, and Palm X) representing 16.1% of the 31 Staphylococciamplified *mecA* gene. The PCR result showed that 2/39 (Palm U16 and Nasal 101) representing 6.5% amplified *bla Z* the gene coding for the presence of beta lactamase at 517bp. Details are shown in figures 4.1 and 4.2.

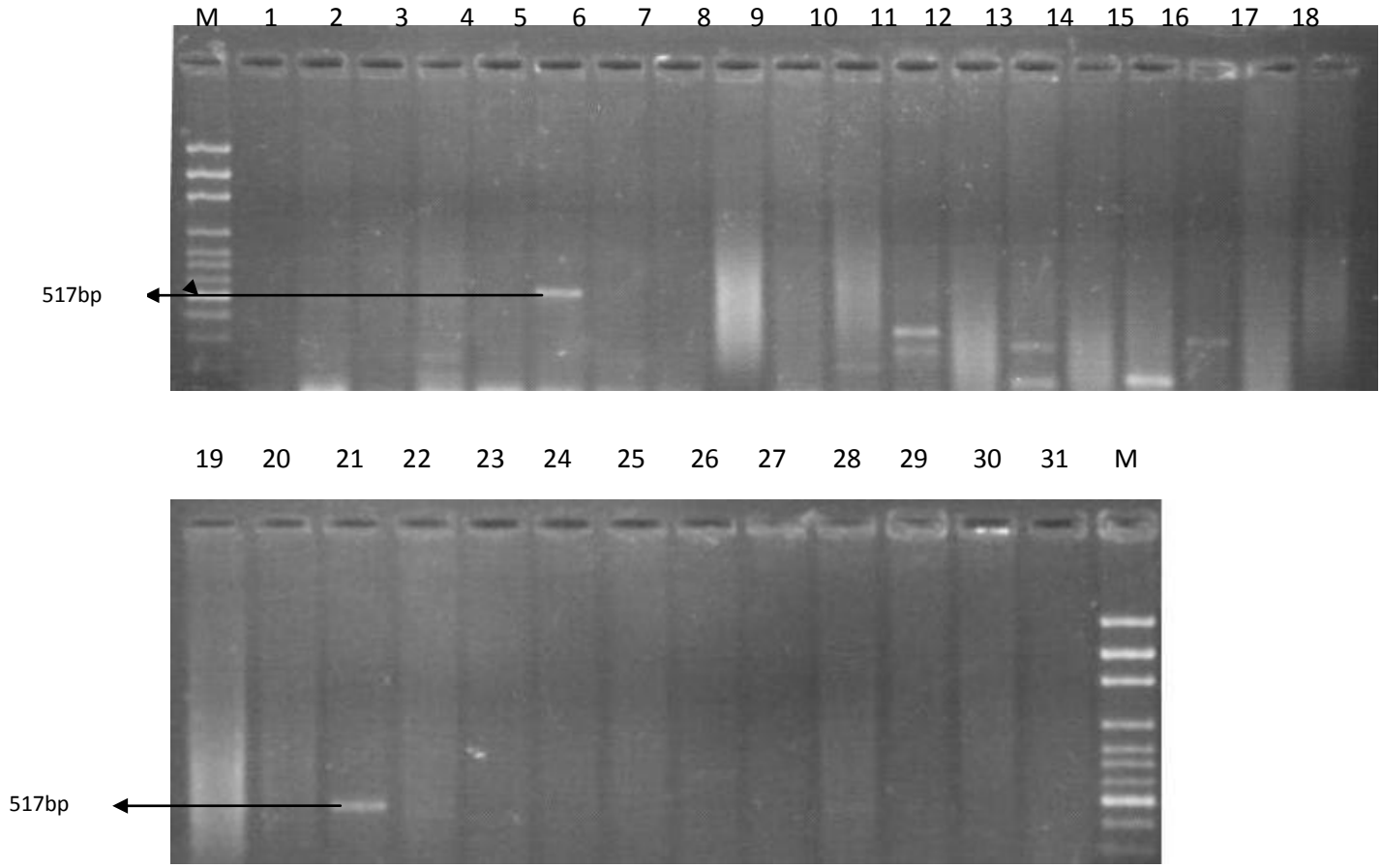

Figure 4.1: Polymerase Chain Reaction (PCR) for detection of *BlaZ* gene from Staphylococci isolates from pre-clinical and clinical medical students from Imo State University (IMSU), Abia State University (ABSU) and University of Uyo (UNIUYO). Each lane represents the PCR profile of the isolate of which the number is indicated above:1-31. The lane for ladder contains the molecular length markers. Lane M=Standard molecular weight maker (Lamda DNA)

Lanes 1-31= Staphylococci isolates.

1= Palm 2(IMSU) 11= Palm U 22(ABSU) 21= Nasal 101 (IMSU) 31= Palm X (IMSU)
2= Palm UycL200 (UNIUYO) 12= Palm A 21(ABSU)22= Nasal 77(IMSU)
3= Palm 94(IMSU)13= Nasal Uy 131(UNIUYO) 23= Nasal I (IMSU)
4= Palm U 16(ABSU) 14= Nasal A3(ABSU)24= Nasal U 30 (ABSU)
5= Palm Uy 1 (UNIUYO)15= Nasal 18 (IMSU) 25= Nasal UycL 83 (UNIUYO)
6= Palm A11(ABSU) 16= Nasal UycL 4(UNIUYO)26= Nasal Uy 136(UNIUYO)
7= Palm Uy 103 (UNIUYO) 17= Nasal Uy 115(UNIUYO)27= Nasal U 7 (ABSU)
8= Palm A(ABSU) 18= Nasal R (IMSU)28= Nasal 25(IMSU)
9= Palm A1(ABSU) 19= Nasal UycL 22 (UNIUYO)29= Nasal U I (ABSU)
10= Palm A 17(ABSU) 20= Nasal Uy CL 99 (UNIUYO) 30= Nasal 57(IMSU)

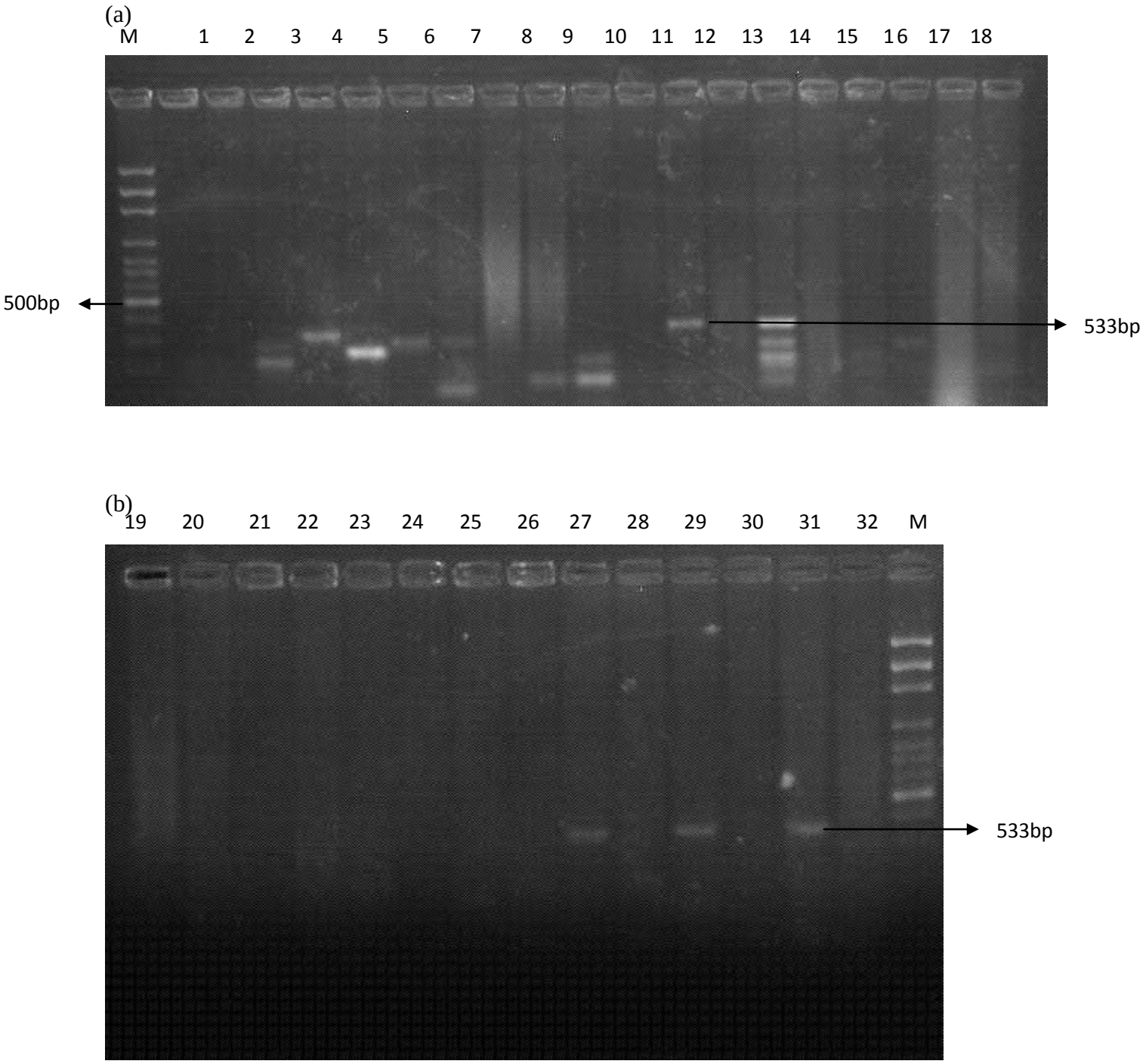

Figure 4.2: Polymerase Chain Reaction (PCR) for detection of *mecA* gene from Staphylococci isolates from pre-clinical and clinical medical students from Imo State University (IMSU), Abia State University (ABSU) and University of Uyo (UNIUYO). Each lane represents the PCR profile of the isolate of which

the number is indicated above: (a) isolates 1 – 18 and (b) isolates 19 - 31. The lane for ladder contains the molecular length markers. Lane M=Standard molecular weight maker (Lamda DNA).

Lanes 1-31= Staphylococci isolates.

1= Palm 2(IMSU) 11= Palm U 22 (ABSU) 21= Nasal 101(IMSU) 31= Palm X(IMSU)

2= Palm UycL 200(UNIUYO) 12= Palm A 21(ABSU) 22= Nasal 77(IMSU)

3= Palm 94 (IMSU)13= Nasal Uy131(UNIUYO)23= Nasal I (IMSU)

4= Palm U 16 (ABSU) 14= Nasal A3(ABSU)24= Nasal U 30(ABSU)

5= Palm Uy 1(UNIUYO) 15= Nasal 18(IMSU) 25= Nasal UycL 83 (UNIUYO)

6= Palm A11(ABSU) 16= Nasal UycL 4(UNIUYO) 26= Nasal Uy 136(UNIUYO)

7= Palm Uy 103(UNIUYO) 17= Nasal Uy 115(UNIUYO) 27= Nasal U 7(ABSU)

8= Palm A(ABSU) 18= Nasal R(IMSU)28= Nasal 25(IMSU)

9= Palm A1(ABSU) 19= Nasal UycL 22(UNIUYO) 29= Nasal U i (ABSU)

10= Palm A 17(ABSU) 20= Nasal Uy CL 99(UNIUYO) 30= Nasal 57(IMSU)

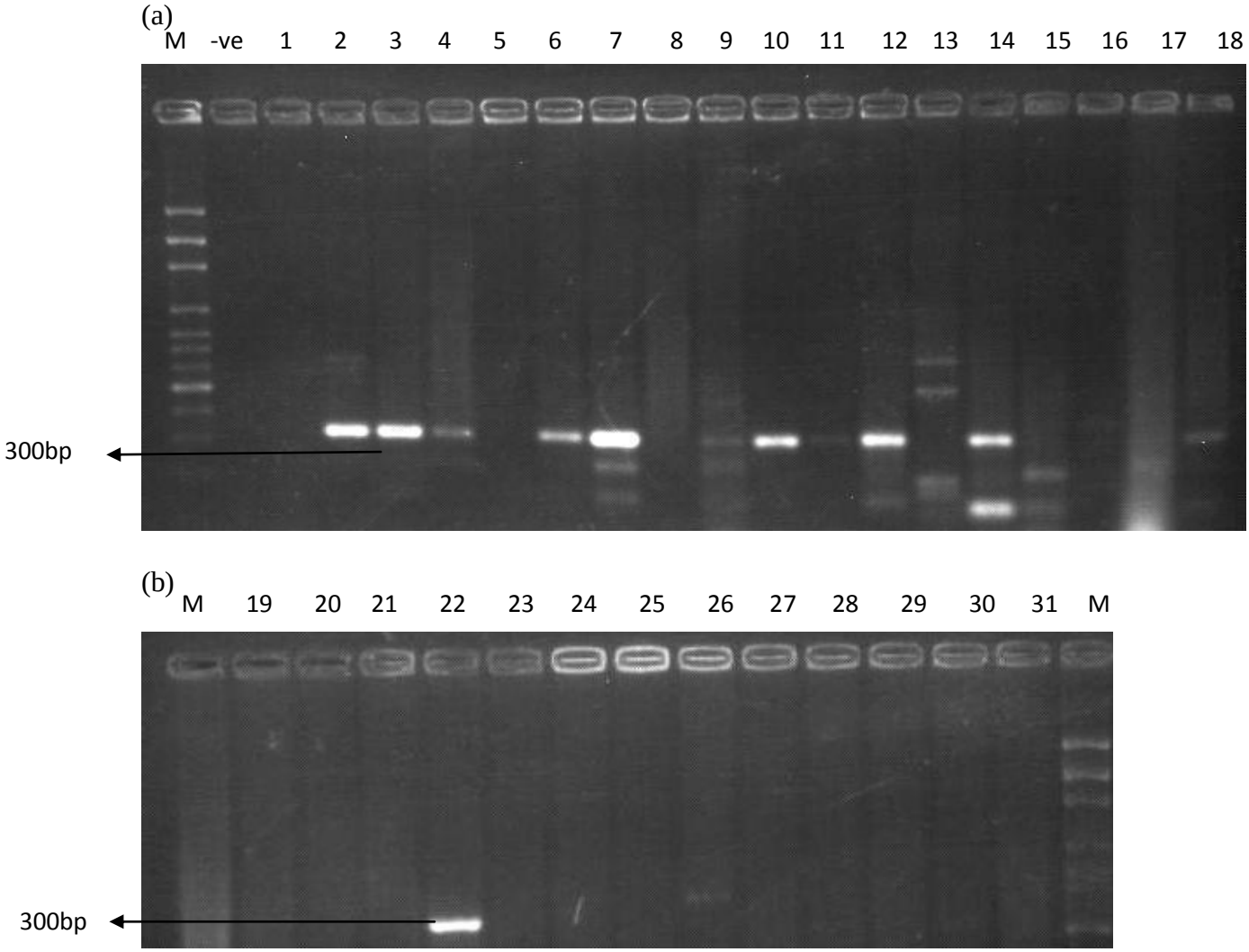

Figure 4.3: Polymerase Chain Reaction (PCR) for detection of *nuc* gene from Staphylococci isolates from pre-clinical and clinical medical students from Imo State University (IMSU), Abia State University (ABSU) and University of Uyo (UNIUYO). Each lane represents the PCR profile of the isolate of which the number is indicated above: (a) isolates 1 – 18 and (b) isolates 19 - 31. The lane for ladder contains the molecular length markers. Lane M=Standard molecular weight maker (Lamda DNA)

Lanes 1-31= Staphylococci isolates.

1= Palm 2(IMSU) 11= Palm U 22(ABSU) 21= Nasal 101 (IMSU) 31= Palm X (IMSU)
2= Palm UycL200 (UNIUYO) 12= Palm A 21(ABSU)22= Nasal 77(IMSU)
3= Palm 94(IMSU)13= Nasal Uy 131(UNIUYO) 23= Nasal I (IMSU)
4= Palm U 16(ABSU) 14= Nasal A3(ABSU)24= Nasal U 30 (ABSU)
5= Palm Uy 1 (UNIUYO)15= Nasal 18 (IMSU) 25= Nasal UycL 83 (UNIUYO)
6= Palm A11(ABSU) 16= Nasal UycL 4(UNIUYO)26= Nasal Uy 136(UNIUYO)
7= Palm Uy 103 (UNIUYO) 17= Nasal Uy 115(UNIUYO)27= Nasal U 7 (ABSU)
8= Palm A(ABSU) 18= Nasal R (IMSU)28= Nasal 25(IMSU)
9= Palm A1(ABSU) 19= Nasal UycL 22 (UNIUYO)29= Nasal U I (ABSU)
10= Palm A 17(ABSU) 20= Nasal Uy CL 99 (UNIUYO) 30= Nasal 57(IMSU)

4.1.11 Random Amplified Polymorphic DNA (RAPD) Analysis

A total of thirty-one (31) Methicillin resistant Staphylococci isolates obtained from pre-clinical and clinical medical students from Imo State University (IMSU), Abia State University (ABSU) and University of Uyo (UNIUYO) were fingerprinted by the RAPD technique. RAPD analysis using two primers yielded 1– 7 distinct bands per isolate, revealing DNA markers ranging from 100 to 7000 bp. The banding patterns of all the isolates used in this study are presented in Figure 4.4. RAPD analysis was performed using Sequentix-DNA Digital Processing (Germany). The lanes of the gel images have been extracted using GelQuest 3.05 (Sequentix, Germany) and were transformed into a trace data curve. Using the same software, a binary matrix was created taking into consideration these parameters: base sizes, peak heights, areas and area-to-height ratio. For clusteranalysis, the Jaccard-distance measure was used. The distance matrix was analyzedusing the Neighbor-Joining (NJ) algorithm phylogram (Figure 4.5). A dendrogram representation (Figure 4.6) using unweighted pair group method with arithmetic averages (UPGMA) was used in order to visualize the tree structure and the real distances between the samples. The dendrogram obtained using a two primer sets resulted to two main groups, groups I and II with two main clusters. The first cluster consisted of 12 isolates (lanes 25, 17, 31, 30, 12, 10, 23, 19, 6, 26, and 16 representing the following isolates: NASAL UYCL 83, NASAL UY 99, PALM X, NASAL 57, PALM A21, PALM A 17, NASAL I, NASAL UYCL 22, PALM Y, PALM A 11, NASAL UY 136 and NASAL UYCL 4 respectively), all from pre-clinical and clinical students of all three schools sampled. The second cluster has 3 sub-clusters. The first sub-cluster contained 3 of the isolates (lanes 22, 8, 24 representing isolates: NASAL 77, PALM A28, and NASAL U30 respectively), from a pre-clinical student of IMSU, a clinical student of ABSU and a pre-clinical student of ABSU respectively. The second sub-cluster contained 5 isolates (lanes 14, 4, 5, 2 and 15 representing isolates: NASAL A3, PALM U16, PALM UY1, PALM UYCL 200 and NASAL 18 respectively) from a pre-clinical student of IMSU, a pre-clinical

student of ABSU, a pre-clinical student of UNIUYO, a clinical student of UNIUYO and a clinical student of IMSU respectively. The third sub-cluster contained 5 isolates (lanes 11, 7, 27 and 13 representing isolates: PALM U22, PALM UY103, NASAL U7 and NASAL UY131) from a pre-clinical student of ABSU, a pre-clinical student of UNIUYO, a pre-clinical student of ABSU and a pre-clinical student of UNIUYO. This third sub-cluster shows that the samples, PALM U22 and NASAL U7 from pre-clinical students of ABSU are genetically related and the samples, PALM UY103 and NASAL UY131 from pre-clinical students of UNIUYO are also genetically related. Also PALM U22 and NASAL U7 contained the *MecA* gene as well as NASAL UY131. The dendrogram obtained showed that the isolatesare widely disseminated. Only the third sub cluster of the second cluster with a small population (4) showedsimilarities in all parameters which include the source of sample and expression of *MecA*gene.By using RAPD-PCR assay, 28 major banding patterns were recovered from 31 isolates. The neighbor – joining algorithm in figure 4.5 shows exactly the same genetic relation as shown in the dendrogram by UPGMA in figure 4.6.

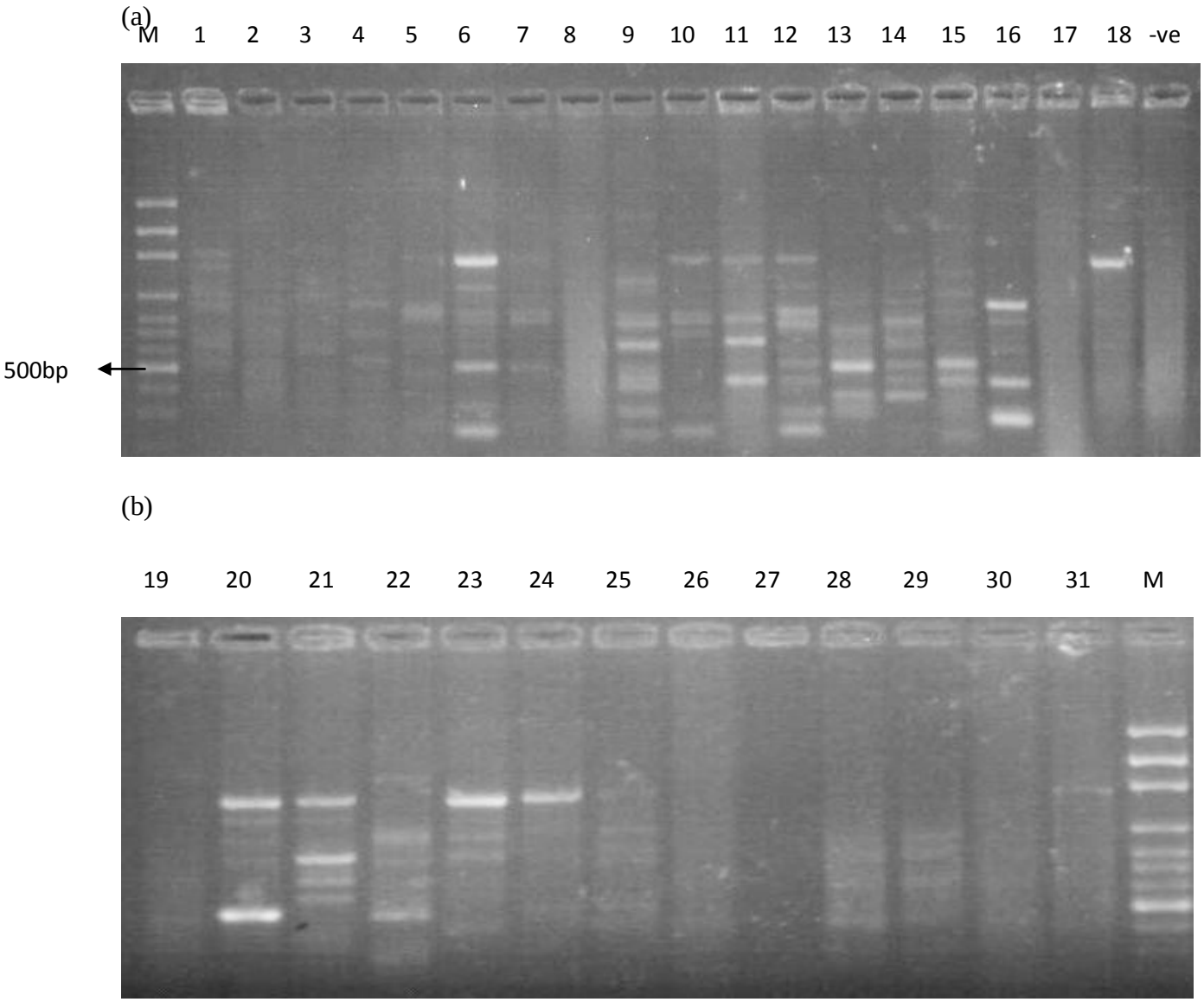

Figure 4.4: Randomly amplified polymorphic DNA-polymerase chain reaction fingerprint patterns of Methicillin resistant Staphylococci isolates from from pre-clinical and clinical medical students from Imo State University (IMSU), Abia State University (ABSU) and University of Uyo (UNIUYO). DNA sizing and quantifications were analyzed using a capillary electrophoresis system (Agilent 2100 bioanalyzer - Agilent Technologists,USA). RAPD fingerprints were analysed using the Sequentix - Digital DNAProcessing (Germany). Each lane represents the RAPD profile of the isolate of

which the number is indicated above: (a) isolates 1 – 18 and (b) isolates 19 - 31. The lane for ladder contains the molecular length markers. Lane M=Standard molecular weight maker (Lamda DNA) Lanes 1-31= Staphylococci isolates.

1= Palm 2(IMSU) 11= Palm U 22 (ABSU) 21= Nasal 101(IMSU) 31= Palm X(IMSU)

2= Palm UycL 200(UNIUYO) 12= Palm A 21(ABSU) 22= Nasal 77(IMSU)

3= Palm 94 (IMSU)13= Nasal Uy131(UNIUYO)23= Nasal I (IMSU)

4= Palm U 16 (ABSU) 14= Nasal A3(ABSU)24= Nasal U 30(ABSU)

5= Palm Uy 1(UNIUYO) 15= Nasal 18(IMSU) 25= Nasal UycL 83 (UNIUYO)

6= Palm A11(ABSU) 16= Nasal UycL 4(UNIUYO) 26= Nasal Uy 136(UNIUYO)

7= Palm Uy 103(UNIUYO) 17= Nasal Uy 115(UNIUYO) 27= Nasal U 7(ABSU)

8= Palm A(ABSU) 18= Nasal R(IMSU)28= Nasal 25(IMSU)

9= Palm A1(ABSU) 19= Nasal UycL 22(UNIUYO) 29= Nasal U i (ABSU)

10= Palm A 17(ABSU) 20= Nasal Uy CL 99(UNIUYO) 30= Nasal 57(IMSU)

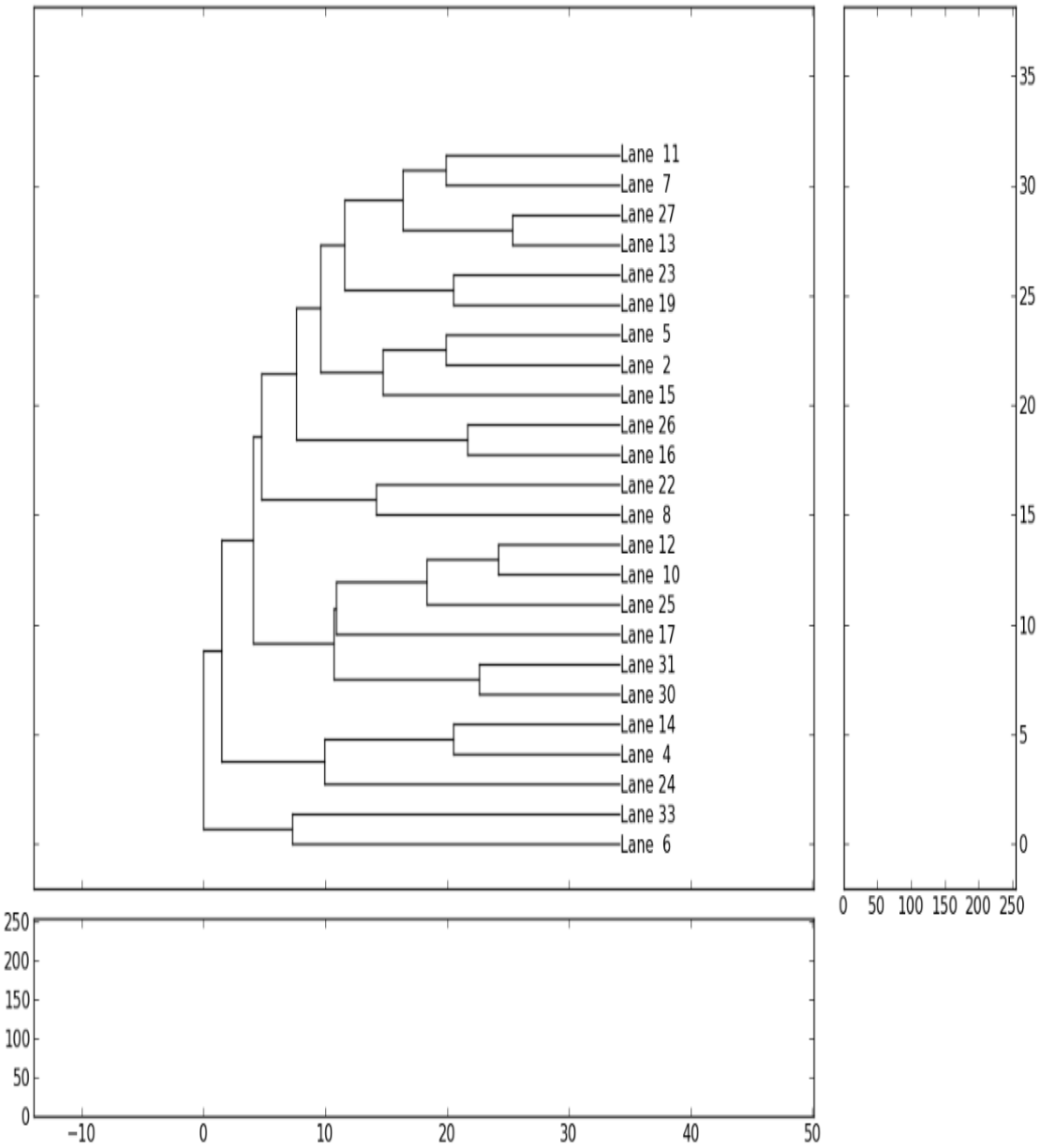

Figure 4.5: Phylogram showing Neighbour-Joining algorithm of 31 Methicillin resistant Staphylococci isolates

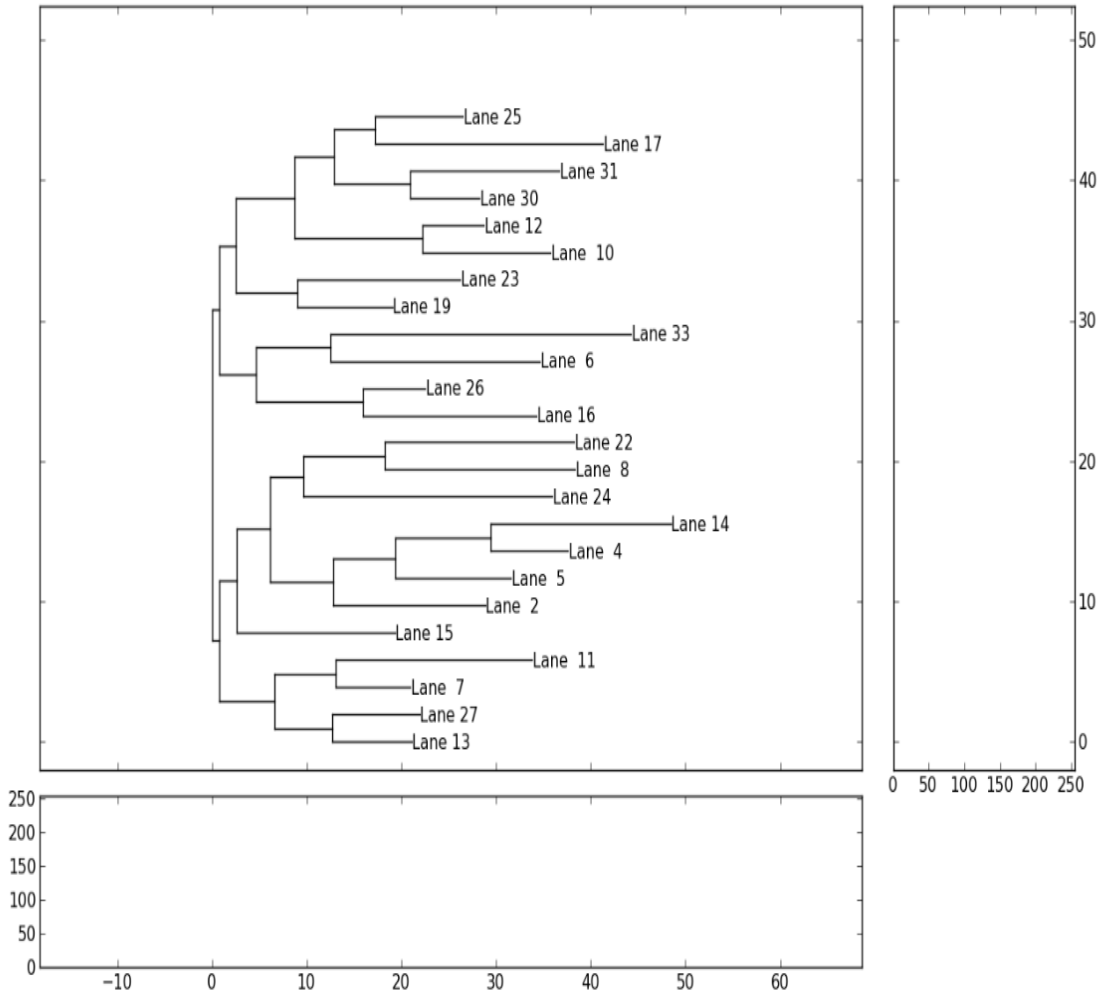

Figure 4.6: Dendrogram showingGenetic relationship between 31 Methicillin Resistant Staphylococciisolates, from six locations, as estimated by clustering analysis of RAPD profiles obtained with the primersset *blaZ*-F (AAG AGA TTT GCC TAT GCT TC) and *blaZ*-R (GCT TGA CCA CTT TTA TCA GC).The dendrogram was generated by the unweighted pair group method with arithmetic averages (UPGMA).

4.2. DISCUSSION

Mis-identification of bacterial pathogens has dire consequences on the patient along with increased medical cost. Molecular techniques with high throughput and specificity as obtained in this study will reduce labour, time and cost of investigation which will subsequently impact on high quality health delivery. All Staphylococcistrains in this study were identified by Gram stain, coagulase, catalase, and were confirmed to be *Staphylococcus aureus*by molecular techniques by amplification of the *nuc* gene. Out of the 248 staphylococcal isolates evaluated in this study, 191 (77.0%) *Staphylococccus aureus*strains and 57(23.0%) coagulase negative Staphylococci (CoNS) were identified by the various methods.

Highly automated identification systems are nowadays widely distributed in many clinical microbiology laboratories and these systems improve the quality of patient care and enable more-cost-effective management of the same by enabling clinical microbiologists to identify medically relevant bacteria more rapidly and accurately (Funke and Funke-Kissling, 2005). For molecular identification, *Staphylococcus aureus*strains produce an extracellular thermostable nuclease and its gene, the *nuc* gene is used in many laboratories for the identification of *Staphylococcus aureus*isolates by the polymerase chain reaction. This method secures a rapid diagnosis of *Staphylococcus aureus*infection (Brakstad *et al.*, 1992). In this study, out of the 31 Staphylococci isolates subjected to molecular identification with PCR, only 2(6.5%) had the *nuc* gene and were confirmed to be *Staphylococcus aureus* by PCR. Nasal 77 and palm 94 were thus confirmed to be *Staphylococcus aureus*.

For the sample type, of the 440 samples received, 115(73.7%) of nasal swab samples tested positive, 122(58.7%) of palm swabs tested positive and 11(14.5%) of throat swabs tested positive. From 191 Staphylococcus aureus isolated, 78 (40.8%) were from anterior nares, 98(51.3%) were from the hands and 15(7.9%) were from the throats of medical students. The real incidence rate of *Staphylococcus aureus*from

the throat might be higher than in the present data because sampling from the throat is more difficult than from the nose.

Pant and Rai's (2007)findings revealed higher *Staphylococcus aureus* nasal colonization rate (43.8%) in staffs of teaching hospital in Nepal. Also, in Abia state of Nigeria, Chigbu and Ezeronye (2003)reported 50% nasal colonization in both hospital and non-hospital subjects. Chatterjee et al. (2009)showed that the overall prevalence of *Staphylococcus aureus* nasal colonization was 52.3%. Whereas Onanuga and Temedie (2011)showed that 33.3% *Staphylococcus aureus* isolates were obtained from 120 nares specimens screened. Whilst, Adesida et al. (2007)reported a much lower (14.0%) nasal colonization in medical students in Lagos, Nigeria. These variations may be attributed to the characteristics of the population under study. A population that is on antibiotics at the time of sampling may yield a much lower prevalence of *Staphylococcus aureus* while a population from hospital settings may yield a much higher prevalence because of the high prevalence of infectious patients in that environment. Other factors that can cause variations may be sampling and culture techniques.

For gender, Inthisstudy,malestudentshad a highercolonization ofStaphylococci (65.4%)nasalcarriage than females with 21.4% in the pre-clinical students and 64.3% and 15.9% respectively in the clinical students.Thisisinagreementwith Cheng *et al.*, (2012) thatfoundthatStaphylococcinasalcarriagerateishigherinmalesthanfemales.However, in disagreement with Shakya *et al.*, (2010),they stated thattheprevalenceofmethicillin resistant Staphylococciwasmorecommonin femalesthanmales. Also in both pre-clinical and clinical students, males harbour more Staphylococci with percentages of 71.6% in pre-clinical and 63.1% in clinical compared to the females with 50.4% in the pre-clinical students and 49.4% in the clinical students. A similarresultwasobtainedbyBraga*etal.*, (2014) and Okwu *et al.*, (2012) whoshowedthattheprevalenceofmethicillin resistant Staphylococciare more common in males andthan females. Also, in this study, it was observed that females harboured more Staphylococci

in their hands with a frequency of 80 (80%) than males who recorded a frequency of 42(28.3%) Itisobservedthat although femalesharbour a greaterdiversityofbacteriaontheirhandsthanmales,butitisnotobviouswhetherthisisduetophysiologicalfactorsordifferencesinhygieneandcosmeticusage(Fierer *et al*., 2008).Furthermore,themicrobialdifferencesbetweenmaleandfemalecouldbeduetothephysiologicalandanatomicaldifferencesbetweenthe genderscutaneousenvironmentssuchassweat,sebumandhormoneproduction (Giacomoni *et al.*, 2009).

The occurrence of MRSA within the genders indicated that males are at a significantly higher risk of harbouring or being infected with MRSA strains in palm and nasal swab samples. Personal hygiene and genetic aspects may explain this finding. This is in disagreement with the reports of Okwu, *et al*., (2012).

The exact reason for this disparity in MRSA isolation is not clearly known. It is however, possible that the dressing style of females that encourages more flowing garments may facilitate contamination and transference of MRSA to palms or anterior nares. However, there was no statistical significant difference in staphylococcal carriage between ages, sexes and the students classes or schools ($p>0.05$). This is in agreement with the reports of Ankur, *et al*., (2008); Khanna, *et al*.(2008) and Okwu, *et al*., (2012).

For the age distribution, the prevalence rate of methicillin resistant Staphylococci were higher in students aged 21-25 years in some clinical classes. This is in agreement with reports of Adesida *et al*.,(2007)whose highest prevalencewas established among the subjects in the age group 21-25. On the whole, students aged 26-30 are more colonized with methicillin resistant Staphylococci than others. This disagrees with Edem *et al*. (2013) which isolated more methicillin resistant Staphylococci from students aged 30-39 than from those aged 20-29.

The preponderance of OX AMP P W E ; OX P AMP W E CIP CLIN ; OX P AMP E CIP W CLIN F and OX P AMP W E biotypes in this study showed cross-resistance between first generation penicillins and the macrolides and/or trimethoprim. The clinical importance of this is that these drugs should not constitute a first line therapeutic regimen in every hospital setting. The high level of resistance to the beta lactam antibiotics (penicillin and ampicillin) in this study in addition to β-lactamase activity in over 80% of the isolates can be associated with the pattern of use of these antibiotics in Nigeria. They are over the counter drugs and can be purchased without prescription documents. The antimicrobial resistance profile also revealed that the levels of resistance to trimethoprim,erythromycin and ciprofloxacin were remarkable among the *Staphylococcus aureus*isolates; 98.1%, 41.7% and 37.9% respectively in pre- clinical students; 94.3% , 59% and 31.8% in clinical students. Previous studies have shown a significant number of *Staphylococcus aureus*isolates from South West Nigeria were resistant to erythromycin, ciprofloxacin and trimethoprim (Shittu *et al.*, 2007; Ghebremedhin *et al.,* 2009). It has been reported that trimethoprim is no longer recommended for Nigerian MRSA strains in the University College Hospital, Ibadan, because its rate of resistance is approximately 53% (Ghebremedhin *et al*., 2009). A study conducted in Cameroun revealed antimicrobial resistance patterns of twenty-one antibiotypes of which the most prevalent was trimethoprim/sulfamethoxazole, gentamicin, ampicillin and penicillin (STXT GEN AMP P) which accounted for 34.1% of the isolates (Nkwelang *et al*., 2009). However it is noteworthy that most of the *Staphylococcus aureus*in this study were susceptible to nitrofurantoin, some to vancomycin and clindamycin. A study by Shittu *et al.* (2011) had reported all methicillin susceptible *Staphylococcus aureus*isolates studied to be sensitive to teicoplanin, vancomycin, phosphomycin, fusidic acid, rifampicin, daptomycin, mupirocin, linezolid and tigecycline. However, resistance was reported for erythromycin, clindamycin in MRSA strains. Nitrofurantoin is widely used for the treatment of urinary tract infections caused by Staphylococci and for the prevention of nasal carriage of MRSA. Therefore the 100%

susceptibility to the drug recorded in this study is encouraging because of our high rate of nasal carriage. Another study by Cennet *et al*. (2016) had reported low level nitrofurantoin resistance in MRSA isolated in Turkey. Their study agrees with our study.We recommend that drugs to which susceptibilities are still high be administered judiciously to prevent resistance to such drugs.

MRSA isolates were resistant to three or more groups of antimicrobial in 77.5% of the cases. The mis-use and mis-application of many antimicrobial agents in many parts of Nigeria may contribute to the high MRSA rate in IMSU, ABSU and UNIUYO. This poses a significant difficulty in antimicrobial agent choice for patients with this variety of infections. The third-generation cephalosporins, quinolones and improved macrolides indicated for serious infections may have substantially lost their place in treatment of many MRSA infections in IMSU, ABSU and UNIUYO. This may indirectly increase cost of treatment and additional difficulty in control (Tiwari *et al*., 2008)

After nitrofurantoin, the lowest resistance of 21.4% was observed in clinical students of IMSU in vancomycin and clindamycin, the lowest resistance of 38.9 % in ABSU was in clinical students with vancomycin, the lowest resistance of 14.3% in UNIUYO was in vancomycin too. This may suggest that these agents remain important in the management of MRSA in these schools.

The highest level of antimicrobial resistance showed by *Staphylococcus aureus*isolates in this study was observed in oxacillin, penicillin and trimethoprim(100%) which are in agreement with the reports of Okwu, *et al*., 2012. Most of the isolates were resistant to the other antibiotics used and their resistance profiles are similar to the profiles reported by Okwu, *et al*., (2012). This is probably because the strains of *Staphylococcus aureus*isolated in this study might be similar to the strains isolated by Okwu, *et al*., (2012). The similarity of strains was however not investigated in this study. However, this is in conformity with previous observations that most isolates of *Staphylococcus aureus*were resistant to a large number of commonly prescribed antibiotics.

Most of the Coagulase negative Staphylococci isolated from this study is highly resistant to the beta-lactams (penicillin, oxacillin and ampicillin) in both classes. A resistant pattern of P, OX,AMP, W, VAN, CIP is about 80%. Multi drug resistance is present in about 90% of these Staphylococci. The results of this study showed that certain CoNS species tended to be more resistant to antibiotics than *Staphylococcus aureus*. 45(79%) out of 57 isolated Staphylococci are methicillin resistant.Multidrugresistance in CoNS has significant implications especially as regards the dissemination of resistance genes to susceptible *S. aureus.* The recent findings of genomic research strongly suggest that coagulase-negative staphylococci represents the gene pool for the ongoing generation of novel SCC types from which methicillin resistance in *Staphylococcus aureus* might originate. In this respect, it is necessary to seriously consider multidrug resistant CoNS as reservoirs for the spread of resistance genes within microbial communities. They should be controlled by appropriate hygiene measures in a manner similar to that for MRSA (Ziebuhr *et al.*, 2006).

The results of this study showed that certain CoNS species tended to be more resistant to antibiotics than *Staphylococcus aureus*. CoNS traditionally have been considered low-virulence pathogens. However, since the 1980s, CoNS have been increasingly recognized as a prevalent cause of hospital-acquired infections. The widespread use of antibiotics for prophylaxis or therapy has provided a reservoir of antibiotic-resistant strains in hospitals, which show wide variability in their pattern of resistance to antimicrobial agents (Mulder *et al*., 1997; Cimolia and Carter, 2002). The majority of the CoNS isolates are known to be highly resistant to multiple antibiotics. About 37 (65%) of the CoNS isolated in this study were resistant to at least 5 antibiotics, 8(14%) to at least 7 antibiotics and 5(8.8%) were resistant to all 9 antibiotics.The resistance pattern of CONS is very significant as 3 strains were methicillin susceptible but

were resistant to clindamycin, vancomycin and nitrofurantoin. The high level of resistance to the glycopeptides and lincosamides among these species calls for concern.

Forty-five out of the fifty-seven CoNS isolates were methicillin resistant. Resistance to oxacillin in CoNS was 79.0% and most isolates were also resistant to all beta lactams, beta-lactam/ beta-lactamase inhibitor combinations, and cephalosporins. The high resistance rates observed in CoNS isolates supports previous reports about these Staphylococcal species. *Staphylococcus haemolyticus* has been reported to possess the highest level of antimicrobial resistance among all CoNS species, and heteroresistance to glycopeptides is common. About 70% of *Staphylococcus epidermidis* strains circulating in the hospital environment have been reported to be resistant to methicillin and most of these strains are also resistant to other antimicrobial agents (Miragaia *et al*., 2002). The resistance to methicillin may be due to the expression of *mecA* gene or as a result of the thickening of the cell wall of the organisms. Resistance to the other antibacterial agents may be driven by the acquisition of discrete genetic accessory elements comprising plasmids, transposable genetic elements (insertion sequences and transposons) and genomic islands (Owolabi *et al*., 2015).

Multidrug resistance in Coagulase negative Staphylococci (CONS) has significant implications especially as regards the dissemination of resistance genes to susceptible *Staphylococcus aureus.* The recent findings of genomic research strongly suggest that coagulase-negative Staphylococci represent the gene pool for the ongoing generation of novel SCC types from which methicillin resistance in *Staphylococcus aureus*might originate. In this respect, it is necessary to seriously consider multiresistant CoNS as reservoirs for the spread of resistance genes within microbial communities. As a consequence, they should be controlled by appropriate hygiene measures in a manner similar to that for MRSA (Ziebuhr *et al.,* 2006). The likely transfer of a type V SCC*mec* from methicillin resistant *Staphylococcus haemolyticus* to *Staphylococcus aureus*in a neonatal intensive care unit has been described (Berglund and Söderquist,

2008). The establishment of MRCoNS in the hospital environment is worrisome since these strains, predominantly involved in nosocomial infections, can accumulate resistance determinants to practically all classes of antimicrobials, and these are potentially transferable to *Staphylococcus aureus*and other microorganisms. The level of multi-drug resistance shown by CA-MRSA isolates in this study is of great concern. They were resistant from five to nine antibacterial agents and the resistance rate is higher than the values reported in different studies. The increased resistance could be due to self-medication, inappropriate prescription and indiscriminate use of antibiotics (Owolabi *et al.,* 2015).

In this study, Staphylococci isolated from pre-clinical students constitutes community-acquired infections because these students were not attached yet to hospital wards for their rounds while those isolated from clinical students constituted hospital- acquired because students from this class were attached to the hospital wards for their practicals. Of the 248 isolated Staphylococci, 193 constitute methicillin resistant Staphylococci. Of the 193 methicillin resistant Staphylococci, 108(56%) are from the pre-clinical students and therefore constitute community acquired methicillin resistant Staphylococci and 85(44%) are from clinical students and therefore constitute hospital- acquired methicillin resistant Staphylococci. From 148 methicillin resistant *Staphylococcus aureus* isolated, 85(57.4%) are from the pre-clinical students and therefore constitute community acquired methicillin resistant *Staphylococcus aureus*(CA-MRSA) and 63(42.6%) are from clinical students and therefore constitute hospital- acquired methicillin resistant *Staphylococcus aureus*(HA-MRSA). From 45 methicillin resistant CONS isolated, 23(51.1%) are from the pre-clinical students and therefore constitute community acquired methicillin resistant CONS (CA-MRCONS) and 22(48.9%) are from clinical students and therefore constitute hospital- acquired methicillin resistant CONS (HA-MRCONS). The observed prevalence of CA-MRSA in this study is higher than that previously reported in a student community in the United States where 29% of volunteers

carried CA-MRSA.The prevalence of CA-MRSA carriers was 57.4% in this study which is higher than the 11.1% reported by Ankur, *et al*., 2008. The variation in values is in agreement with the reports of Ankur, *et al*., 2008 and Okwu, *et al*., 2012 who stated that the carriage rates vary in different communities.

However, comparing between the three schools studied here, Imo State University (IMSU), Abia State University (ABSU) and University of Uyo (UNIUYO) recorded prevalence rates of 34.1%, 28.2% and 37.6% respectively for community acquired methicilllin resistant *Staphylococcus aureus* (CA-MRSA) and recorded 12.7%, 28.6% and 58.7% respectively for hospital acquired methicillin resistant *Staphylococcus aureus* (HA-MRSA). The difference in prevalence rates of MRSA in the three schools could be associated with the size of the study population.

Comparing between schools, Imo State University, Abia State University and University of Uyo recorded prevalence rates of 30.4%, nil and 69.6% respectively for community acquired methicillin resistant coagulase negative Staphylococci (CA-MRCONS) and recorded 13.6%, 9.09% and 77.3% respectively for hospital acquired methicillin resistant coagulase negative Staphylococci (HA-MRCONS). University of Uyo recorded a higher rate of methicillin resistant coagulase negative Staphylococci (MRCONS) and methicillin resistant *Staphylococcus aureus* (MRSA). However, the prevalence rate (58.7%) of community acquired methicilllin resistant *Staphylococcus aureus*(CA-MRSA) in University of Uyo was higher than the prevalence rate (37.6%) of hospital acquired methicillin resistant *Staphylococcus aureus* (HA-MRSA). This higher prevalence of CA-MRSA in Uyo is supported by previous studies by Edem *et al*., 2013. Also, the prevalence rate (58.7%) of hospital acquired methicilllin resistant coagulase negative Staphylococci (HA-MRCONS) in University of Uyo was higher than the prevalence rate (69.6%) of community acquired methicilllin resistant coagulase negative Staphylococci (CA-MRCONS). This is slightly higher than the prevalence of HA-MRCONS of 56.4% recorded by Owolabi *et al*., 2015. The results of this study is consistent with the findings of Diekema *et al*., 2001 that the CONS are one of the

most important agents of nosocomial infections worldwide which are complicated by the high level of methicillin resitance frequently observed among them.

A positive D-test indicates the ability of MRSA strains to become resistant to clindamycin during antibiotic therapy. A negative D-test indicates the effectiveness of clindamycin in treating patients with MRSA.Of the 148 isolated MRSA, 4(2.7%) tested negative to the D-test and 28(18.9%) tested positive to D-test. For many years clindamycin was the preferable antibiotic to be used in treating MRSA infections. This study shows that clindamycin might not be effective in treating MRSA and should not be used to treat MRSA that are acquired during a hospital stay

The resistance to erythromycin (macrolide) observed in this study had an inductive effect on the clindamycin. There are two primary mechanisms of resistance to macrolide antibiotics. The first involves macrolide efflux and is relatively common in *Staphylococcus aureus*in some geographic areas. A specific efflux pump is encoded by the gene *msr*(A) in Staphylococci (Ross *et al*., 1990). This energy-dependent pump effectively expels macrolides from the bacterial cell before they can bind to their target site on the ribosome. Notably, this mechanism of resistance does not create resistance to lincosamides (e.g., clindamycin and lincomycin), but only to macrolides, azalides (e.g., azithromycin), and group B streptogramins (e.g., quinupristin) (Ross *et al*, 1990; Roberts *et al*; 1999). The second mechanism of resistance to macrolides in Staphylococci involves modification of the drug-binding site on the ribosome. This results in resistance to macrolides (and azalides), lincosamides, and group B streptogramins and is commonly referred to as MLSB resistance (Roberts *et al*., 1999). An *erm* gene, usually *erm*(C) or *erm*(A), encodes methylation of the 23S rRNA binding site that is shared by these 3 drug classes. Phenotypically, resistance can be expressed constitutively (the MLSBc phenotype) or only when induced into production (the MLSBi phenotype). When an *erm* gene is present, resistance to macrolides arises through binding of a

macrolide to upstream translational attenuator sequences. This binding subsequently leads to alteration of the mRNA secondary structure, exposure of the ribosomal binding site, and translation of the *erm* methylase. For constitutive resistance (MLSBc) to be present, additional changes in these 5′ upstream sequences are required. These modifications can include deletions, duplications, or other mutations, and they result in constitutive expression of the methylase gene with obvious resistance to MLSB drugs. Clindamycin represents a useful option for therapy for various CA-MRSA infections, including musculoskeletal infections, skin and soft-tissue infections, and even pneumonia with empyema (Martinez-Aguilar *et al*., 2003). Many CA-MRSA isolates are resistant to macrolides because of *msrA*-mediated efflux, and they are thus susceptible to clindamycin (Martinez-Aguilar *et al*., 2003), this was the case with the macrolide resistant and clindamycin sensitive isolate observed in this study, 75% of the MRSA isolates are erythromycin susceptible and clindamycin susceptible. However, the use of clindamycin for these infections has been somewhat hampered by concern over possible inducible resistance to clindamycin and its impact on clinical outcomes and this inducible resistance was observed in this study.

The accurate detection of beta-lactam and *mecA*-mediated resistance in *Staphylococcus aureus*is essential for the treatment of overt infections and the implementation of infection control practices. Resistance to penicillin in *Staphylococcus aureus*is mediated by production of a penicillinase, encoded by *blaZ* gene. About 6.5 % of the *Staphylococcus aureus*strains in this study harboured the *blaZ* gene. Reliable detection of penicillinase production is important because penicillin is considered to be superior to oxacillin against isolates that do not produce a penicillinase. An erroneus report of penicillin susceptibility could result in potentially inadequate therapy of *Staphylococcus aureus*infections (Kaase *et al.,* 2008).

Infections caused by methicillin resistant Staphylococci strains have become one of the most commonly acquired types of nosocomial infections, resulting in increased morbidity, mortality, length of hospital

stay, and health care costs (Begier *et al*., 2004). Consequently, there is a need for rapid, reliable, and cost-effective methods for the detection of MRSA. Because many of the MRSA clones exhibit a heteroresistance phenotype, with only a few staphylococcal cells of the population expressing methicillin resistance (Berger-Bächi and Rohrer, 2002), detection of the *mecA* gene by molecular methods has become the reference method for confirmation of MRSA strains. There are strains of *Staphylococcus aureus*that hyper produce beta lactamase known as borderline oxacillin resistant *Staphylococcus aureus*(BORSA) and while they appear oxacillin resistant, they do not possess the usual genetic mechanism for such resistance. There are also strains of *Staphylococcus aureus* known as modified *Staphylococcus aureus*(MODSA) which possess a modification of existing penicillin binding proteins rather than the acquisition of a new PBP as is the mechanism for classical MRSA. Neither BORSA nor MODSA possess the *mecA* gene and therefore are not reported as MRSA (Mathews *et al.*, 2010). Of the 13 strains of *Staphylococcus aureus*tested, 5 of them were found to be *mecA* positive representing 38.5% of the strains. This result is not in agreement with the phenotypic tests for antimicrobial susceptibility which identified the MRSA strains in this study with resistance to oxacillin. The gold standard for detection of MRSA is the detection of *mecA* gene by polymerase chain reaction. Expression of *mecA* gene yields an altered penicillin binding protein, PBP2a with reduced affinity for β- lactam antibiotic binding. In this study 5(16.1%) out of the 31 Staphylococciisolates were confirmed as methicillin resistant *Staphylococcus aureus*by the detection of *mecA* gene. In the North-western part of Nigeria where this study was carried out, there has not been any reported case of detection of *mecA* gene in clinical isolates to the best of our knowledge. Olayinka *et al*., (2009) reported the absence of *mecA* gene in MRSA isolates obtained from clinical isolates from Medical Microbiology laboratory of Ahmadu Bello University Teaching Hospital, Zaria. Similar report on absence of *mecA* gene in MRSA isolates from non- hospital sources in Zaria were given by Olonitola *et al*., (2007). However there are reported cases of detection of

mecA gene in MRSA isolates from other parts of this country, Nigeria which include the following: In a research in Benin city, Nigeria, 4 isolates representing 11% were confirmed to carry *mecA* gene according to molecular technique (Obasuyi, 2013); Another research by Esan*et al.*, (2009) confirmed only one MRSA isolate from health care institutions from Ekiti and Ondo states. In another research carried out by Shittu *et al.*, (2011), two MRSA isolaes with *mecA* gene were detected in Ile-Ife, one from Lagos and two from Ibadan (all in South western Nigeria). In the same study, five MRSA isolates with *mecA* gene were detected in Maiduguri (North eastern Nigeria). Okon *et al*., (2013) reported the detection of 12.5% MRSA from clinical specimens from six tertiary hospitals in North-eastern Nigeria. However Olowe *et al.*, (2013) reported a higher prevalence of 19.2% MRSA from clinical isolates in Medical Microbiology Laboratory of University Teaching Hospital, Ado-Ekiti.

In this study, comparison was made between the use of oxacillin disk diffusion test and PCR for *mecA* gene for MRSA detection. PCR for *mecA* gene was positive for 5(16.1%) isolates; these 5 isolates were detected by oxacillin in disk diffusion test (palm U222, Nasal UY 131, Nasal U7, Nasal Ui and palmX). However, oxacillinn disc diffusion test detected resistance in 148(77.5%) out of 190*Staphylococcus aureus* isolates without the amplification of *mecA* gene. The question is what was responsible for the resistance observed in the other phenotypic MRSA isolates which were *mecA* negative as observed in this study? There are two types of methicillin resistance in Staphylococci: intrinsic high level resistance (*mecA* positive) and intermediate resistance (borderline resistance) (Khorvash *et al,* 2008). Intrinsic high level resistance in MRSA is mediated by PBP2a encoded by chromosomal *mecA* (Hackbarth and Chambers, 1998). PBP2a increases resistance to all beta lactam antibiotics including penicillin, cephalosporins, cephamycins and carbapenems by decreasing affinity for binding these antibiotics. Also, some strains of *Staphylococcus aureus*produce large amounts of penicillinase that hydrolyze the penicillinase resistant penicillins as was observed in this study. Susceptibility tests to oxacillin or methicillin in these strains may

show reduction or border line in susceptibility and they are named as Border line oxacillin resistant *Staphylococcus aureus*(BORSA). The mechanism of resistance of these *mecA* negative strains may be as a result of the production of modified PBPs 1 and 2 with reduced affinities for beta lactamase, production of a new beta lactamase, over production of PBP4 or increased beta lactamase production (Khovash *et al*., 2008). The result of the β- lactamae test in this study showed that 64% produced β- lactamase while 15% of them were hyper producers, this may be a possible reason for the phenotypic MRSA observed.

In addition, there was a report by Van Griethysen *et al*., (2005) about the loss of *mecA* gene during storage of 36/250 (14.4%) confirmed MRSA strains at -80°C with the MicroBank system (Pro-Lab Diagnostics, Canada). Also in a study on loss of the *mec*A gene during storage of methicillin-resistant *Staphylococcus aureus*isolates in North western Nigeria by Kumurya, (2013), it was reported that *mec*A gene was lost in 95.0% of 100 MRSA isolates after 2 years of storage at −80°C with the Micro bank system (Pro-lab Diagnostics, Austin, Tex.). Considering the time interval between the preliminary characterization, storing and sub-culturing over a considerable period of time before the final molecular characterization, and the inconsistent power supply in this environment it is therefore not impossible that some *mecA*-containing isolates might have lost the gene on prolong storage at temperature much higher than -80°C.

In this study, the encountered plasmids sizes were between 9.2 and 13.3 kilobase. This is similar to the reports of Diep *et al.,* (2006), Uchechi and Erinma, (2007), Adeleke *et al*., (2010), Akinjogunla and Enabulele, (2010) and Tula *et al*., 2013). The molecular weight of the plasmids observed in this study falls into the category of small multicopy plasmids that carry single resistance. They can also be described as mobilizable resistant plasmids which are relatively small (often less than 10kb in size) encoding only a handful of genes including the resistance genes (Esimone *et al*., 2010).

The resistance gene that was tested for in this study was *blaZ* which codes for β- lactamase and it was borne on the plasmids. It can therefore be suggested that the plasmids observed in this study were

plasmids coding for *blaZ* gene eventhough they may also be coding for other resistant genes which were not tested for since some of the isolates were multi drug resistant. All the phenotypic MRSA tested were found to have plasmids out of which 39/148 (26.4%) were multi- drug resistant. Multidrug resistant organisms are defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Nikaido, 2009; Magiorakos *et al*., 2012; CDC, 2013; WHO, 2014). Plasmids are small, double-stranded DNA molecules that can exist independently of the chromosome. Some plasmids are able to integrate into the chromosome and are thus replicated with the chromosome (Wiley *et al*., 2008).

Plasmid profile has been reported to be useful in tracing the epidemiology of antibiotic resistance (Olowe *et al*., (2007). It is suggested that more than 90% of MRSA strains carry plasmids while numerous studies have supported the important role plasmids play in staphylococcal multi-drug resistance (Paulsen *et al*., 1998; O'Brien *et al.*, 2002). Individual plasmid mediated resistance in MRSA isolates has been reported by Al-Mohana *et al.,* (2012) and plasmid carried by resistant isolates have been studied (Dar *et al*., 2006). High level of resistance to antibiotics has been associated in most instances with the presence of plasmids (Bhaka *et al*., 2003; Daini *et al.,* 2006; Diep *et al*., 2006).

The prevalence of MRSA in this study was high compared to a previous report of 20.23% in Southwestern Nigeria (Ghebremehin *et al*., 2009). This study has revealed that *Staphylococcus aureus* is the most predominant microorganism from palm, nasal, and throat swabs (Bell and Turnidge, 2002). The resistance of *Staphylococcus aureus* to many groups of antimicrobial agents represents a serious concern in therapeutic option available to the clinician in managing such infections. Methicillin resistance, the marker of multi-drug resistance showed a high prevalence (77.5%) among isolates of *Staphylococcus aureus*. A previous study in Nigerian women recorded 71.2% (Onanuga *et al.,* 2005). The high rate from this study is not unexpected because it has been reported that MRSA prevalence is forever increasing (Voss, 2006). However, an earlier multicenter study in Southwestern Nigeria reported 1.4% (Adesida *et al*., 2005). The

difference in prevalence of MRSA in the three studies could be associated with the size of the study population; with Ghebremehin *et* al., (2009), the study population was significantly higher than that of Adesida *et al*., (2005) and the present study. Despite the high MRSA prevalence rate reported in this study, it highlights the occurrence of multiresistant MRSA in Eastern Nigeria.

Methicillin-resistant Staphylococci are resistant to all other penicillins, carbapenems, cephems and beta-lactam/beta-lactamase inhibitor combinations (Clinical and Laboratory Standards Institute., 2006). It is therefore advisable that these antibiotics should not be used for treating of methicillin-resistant Staphylococci infections. The antimicrobial susceptibility pattern showed that all the MRSA strains were resistant to at least five antibiotics including penicillins, penicillin/beta-lactamase inhibitor combinations, oxacillin, cephalosporins and carbapenem. These points to an emerging resistance to these drugs. It is therefore important that this resistance be monitored to prevent the spread. Previous studies have noted the existence of MRSA strains with low-level resistance that contain the *mecA* gene but that have oxacillin MICs below the CLSI breakpoint (≤2 μg/ml and sometimes as low as 0.5 μg/ml) (Felten *et al*., 2002). Parenteral glycopeptides (vancomycin) are the mainstay of therapy for MRSA infections but there has been concern about the development of vancomycin resistance in multidrug-resistant strains of *Staphylococcus aureus* (Ward *et al*., 2001).

Typing of Methicillin resistant Staphylococci isolates within the context of epidemiological investigation of nosocomial and community-acquired infections is of great significance for considering the connection and similarities of isolates, for establishing the series of infections and accordingly, for the application of suitable infection control and preventive measures.

Clonal relatedness can only be deduced from molecular studies comparing isolates from Methicillin resistant Staphylococci strains. The main goal of this investigation was to probe the molecular diversity of

Methicillin resistant Staphylococci isolates obtained from pre-clinical and clinical students of IMSU, ABSU and UNIUYO in Eastern Nigeria by the RAPD molecular typing method. From the results acquired from this investigation, it was established that diverse banding patterns of the amplified products formed by using a two primers had permitted the genotyping of the Methicillin resistant Staphylococci isolates. The fingerprints created by these primers disclosed distinctive profiles for every strain in terms of number and location of RAPD bands. The dendrogram (Figure 4.6) and the neighbor joining algorithm phylogram (figure 4.5) attained in the present investigation conceded with 31 isolates showing distinct clustering of these isolates. The RAPD analysis in this study, however, was not able to establish to a large extent the correlation of the characteristics of the isolates based on the clinical specimen source, and the incidence of the *mecA* gene. As shown in the results in the sub-clusters, there was a clustering of isolates from pre-clinical and clinical students of IMSU, ABSU and UNIUYO and presence and absence of *MecA* gene and a combination of both *Staphylococcus aureus* and coagulase-negative Staphylococci in the same cluster. This may be due to chance accrual of neutral mutations or to alteration of the predominant strain to adapt to constant environmental changes. Bacterial clones are genetically duplicate cells derived from a sole common ancestor. However, over time, members of a sole clone may discriminate through point mutations, recombination and the gaining or deletion of mobile genetic elements. This distinction provides further revenues for the procurement of pathogenic features, namely antibiotic resistance and thus, genetic distinction offers rise to broad genomic and phenotypic array (Noriega *et al*., 2010). However, 4 out of the 31 isolates showed genetic relatedness as shown by the similarity cluster analysis by UPGMA. 2 were from pre-clinical students from ABSU and 2 were from pre-clinical students of UNIUYO. All 4 isolates are methicillin –resistant *Staphylococcus aureus* isolates and were all resistant to more than 5 antibiotics. These isolates are therefore from the same source. RAPD markers revealed possible relationship between host origin, mutation and genetic variation among *Staphylococcus aureus* isolates, and this demonstrated

its fingerprinting and diagnostic potential. Obviously, for these DNA bands patterns to have a practical meaning in the areas of medicine, population biology and epidemiology, specific DNA bands must be related to host origins, mutation and virulence genes. This could be accomplished by a systematic comparison of DNA band patterns among bacteria contrasting for the different host origins, mutation and virulence genes present. A Similar approach has been used to differentiate aggressive from non-aggressive isolates of the oilseed rape pathogen Phoma lingam (Schafer and Wostmeyer, 1992). The DNA fingerprint defined for each race of *Staphylococcus aureus* should be useful for epidemiological surveys, medical diagnoses, and in the identification of new virulent strains and isolates and their origin.

There is also a growing proof that virulence genes can be moved between hospital-acquired (HA) and community-acquired (CA) -MRSA strains. For instance, SCC*mec* type IV traits, usually linked with CA-MRSA, have been seen in nosocomial MRSA strains with susceptibility to four or more antimicrobials signifying that CAMRSA clones have been presented into the hospital and now circulating as nosocomial pathogens (Van Leeuwen *et al*., 2001). Likewise, clones initiating in hospitals have also been found to be dispersing in the community. Molecular epidemiological investigations have emphasized the persistent worldwide evolution and distribution of MRSA clones with greater resistance to antimicrobial drugs and higher virulence (Noriega *et al*., 2010). The reasons causing the dissemination of MRSA clones are only vaguely studied and understood, but may comprise the rapid movement of human populations, unsuccessful methods to regulate the spread of methicillin resistant Staphylococci from infected patients and treatment approaches, including the incorrect usage and selection of antibiotics.

The dendrogram indicated that the strains are from six different locations within in the city and placed in the different groups based on their genetic similarities. The reason for this situation especially in developing countries might be due to the factors such as antibiotic misuse, shortfalls in infection control and moreover urban migration might increase the chance of dissemination of resistant strains in to the

health care setup as well as in the community. Recent studies in Nigeria and Nepal have demonstrated that urban residents are more likely to harbour resistant bacteria than people residing in rural or provincial areas (Onasanya*et al.,* 2003)

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Methicillin-resistant Staphylococci infections remain a most important menace to public health due to the fast dissemination and diversification of Methicillin-resistant *Staphylococcus aureus* (MRSA) and Methicillin-resistant coagulase-negativeStaphylococci (MRCONS) clones with greater virulence and antimicrobial resistance. In Nigeria, Methicillin-resistant *Staphylococcus aureus* (MRSA) and Methicillin-resistant coagulase-negativeStaphylococci (MRCONS)are principal sources of nosocomial infections and the prevalence ofMethicillin-resistant *Staphylococcus aureus* (MRSA) and Methicillin-resistant coagulase-negativeStaphylococci (MRCONS) in community- acquired infections is mounting. Categorization of these clones issignificant if suitable local treatment plans are to be established. A comprehensiveinvestigation of clones disseminating within an area may be used to evaluate theassociation between clonal types, disease symptoms, antibiotic choice and clinicaloutcomes. The application of the PCR based Random Amplified Polymorphic DNA (RAPD-PCR) to Staphylococciisolates from medical students in three different institutions has provided important information on theirclonal relationships and this might also strengthen our understanding of the Staphylococcipopulation circulating in Nigeria. There is the need for consistent on-going antimicrobialresistance surveillance for important and commonly isolated clinically significant pathogensof staphylococcal species to form the basis for developing and implementing measures thatcan reduce the burden of antimicrobial resistance and prevent a probable impending publichealth problem.

Methicillin resistance in *Staphylococcus aureus* and coagulase-negativeStaphylococci is usually accompanied by resistance to other groups ofantimicrobial agents, so therapeutic options are limited. Therefore, surveillance of theantimicrobial susceptibility patterns of *Staphylococcus aureus* and

coagulase-negativeStaphylococci is of utmost importance in understandingnew and emerging resistance trends and in the management of both hospital and community-acquiredinfections. Knowledge about the nature and number of Methicillin-resistant Staphylococci clones that aredisseminating is required to implement any strategies to control the transmission of methicillin resistant Staphylococci infections,either within hospitals or the community. The knowledge about the evolution of methicillin resistant Staphylococci infections could be increased through investigations of historic strain collections to find the molecular eventsthat have led to the origin of Methicillin resistance *Staphylococcus aureus* (MRSA) clones, both in healthcare facilities and in thecommunity. Because of the emergence of vancomycin-resistant strains also recorded in this study, newantimicrobials are needed as alternative agents against these multiply resistant strains. Themost important measures to ensure control of emergence and dissemination of antibiotic resistancegenes are an adequate antibiotic usage without causing the selective pressures but perhapsmore important is the application of infection-control practices to prevent transmission ofresistant organisms. This is necessary because of the multi-resistant isolates obtained from theenvironment in this study. The importance of local, national, and international surveillanceprograms cannot be overemphasized as data generated from these programs can assist in thedesign of appropriate measures for controlling the emergence and spread of antimicrobialresistance in an attempt to limit the scope of this disturbing worldwide problem. Moleculartechniques are also important in the identification of multiresistant pathogens. Thecombination of different virulence factors such as the exotoxins, enterotoxins and the Panton Valentine Leukocidin encoding genes are known to contribute to the incidence and severity of Staphylococciinfection.It is therefore that these virulence factors be routinely investigated in the laboratory so as toidentify toxin-associated diseases as this will lead to proper management of such diseases.

This study has described the antibiotic resistance profile ofmethicillin resistant Staphylococci infections isolated from palm, nasal and throat swabs of pre-clinical and clinical medical students inImo State University (IMSU), Abia State University (ABSU) and University of Uyo obtained from their palms, nasal and throat swabs within Imo, Abia and Akwa Ibom states of Nigeria. This information is helpful in establishing effective infection control measures in health care settings in Nigeria.Information on antibiotic susceptibility pattern and virulence determinants present in Staphylococci*isolates* from palms, nasal and throat swabs could be used as genetic markers to investigate outbreaks of Staphylococciinfections. This information is necessary for global epidemiological studies.The data provided on methicillin resistant Staphylococci strains can serve as a baseline for future methicillin resistant Staphylococci surveillance to study the evolution of MRSA clonal types.

This study gives us a clear indication that RAPD analysis is a rapid, accurate and a highly tool that can give the genetic relatedness among Staphylococci strains and can group the isolates according to the geographical area and determine genetic diversity of strains that are otherwise impossible by biochemical analysis. RAPD improves the understanding of the epidemiology of Staphylococci isolates and thus, aides the formulation of effective control measures. As RAPD analysis can correctly type Staphylococci isolates, this technique would be of great use in preventing nosocomial Staphylococci infections and thus be applied in hospitals.

5.2. Recommendations

Resistance to vancomycin - a reserved drug for methicillin resistant *Staphylococcus aureus* (MRSA) observed in this study highlights the magnitude of the burden of methicillin resistant *Staphylococcus aureus* (MRSA) in the community. A renewed effort must therefore be put in place at control measures that should include a renewed awareness, isolation of methicillin resistant *Staphylococcus aureus* (MRSA) infected patients in hospitals and multidrug resistance surveillance and enforcement of empiric use of antimicrobial agents to stem the tide of methicillin resistant *Staphylococcus aureus* (MRSA).

This study has further established that *Staphylococcus aureus*and coagulase-negative Staphylococciis a major causative agent for both community and hospital acquired infections, therefore it is recommended that proper infection control measures be put in place in our hospitals, in primary and tertiary institutions especially in Imo State University (IMSU), Abia State University (ABSU) and University of Uyo (UNIUYO)

Now that *mecA*and *blaZ*genes mediated methicillin resistant Staphylococci infections has been detected in Eastern Nigerian Instututions, there is need for the various institutions and the government to develop a means of combating its spread either in the hospital or within the community before it becomes a major health problem in Nigeria. Part of the measures to be taken should include:

i. Hospital workers are to be screened regularly for methicillin resistant Staphylococciinfections.
ii. Decolonization may be beneficial in preventing surgical site infections in patients undergoing certain orthopaedic surgery.
iii. Adjust antibiotics based on results of culture and sensitivity testing.
iv. Monitor response to therapy.
v. Patient education: Provide education on infection control and wound care to patients and care givers.

vi. Proper hygiene and cleanliness should be maintained in our hospitals, patients beddings should be washed and changed regularly. Transmission from patients to patients should be prevented by health workers‘ washing their hands with disinfectant before and after touching each patient

vii. Contact precaution should be used for all patients with known methicillin resistant *Staphylococcus aureus* (MRSA) infections for patients with skin or soft tissue infection compatible with a diagnosis of a staphylococcal infection until susceptibilities are known and for all patients with maintained body secretion or wound drainage.

viii. There is the need for our research institutes to be properly funded and the provision of standard laboratories made available in different part of the country for detailed molecular study on typing and classification of MRSA for epidemiological control. Further study is needed to define the optimum use of ciprofloxacin and gentamicin as single agents or in combination therapy for MRSA colonization and infection.

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Appendix 1

WRITTEN CONSENT FORM ISSUED TO SCREENED STUDENTS UPON REQUEST

RESEARCH TOPIC: ANTIMICROBIAL RESISTANCE PATTERN OF METHICILLIN RESISTANT *Staphylococcus aureus*and coagulase-negative Staphylococci IN PRE-CLINICAL AND CLINICAL STUDENTS IN SOME EASTERN NIGERIAN TERTIARY INSTITUTIONS.

Investigators: Miss Marylene Etim

Study Coordinator: Dr. IfechiAdieze

Introduction:

My name is Miss Marylene Etim , I am a postgraduate student of department of microbiology, Federal university of Technology, Owerri, Imo state. I am conducting a study onAntimicrobial Resistance Pattern Of Methicillin Resistant *Staphylococcus aureus*and coagulase-negative Staphylococciin Pre-Clinical And Clinical Students in some Eastern Nigerian Tertiary Institutions.The study aims at determining theAntimicrobial Resistance Pattern Of Methicillin Resistant *Staphylococcus aureus*and coagulase-negative Staphylococci isolated from hands, noses and throats of pre-clinical and clinical medical students in some Eastern Nigerian Tertiary Institutions**.**

You are being asked to participate in a research study. Please take your time to review this consent form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this study and you may discuss it with your friends, family or (if applicable), your doctor before you make your decision. This consent may contain words that you do not understand. Please ask the study staff to explain any words or information that you do not clearly understand.

Purpose of Study

This study is being conducted to learn about what situations are risk factors putting people living in the community and clinical students at risk of becoming infected with the antibiotic resistant bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA). Antibiotic resistant bacteria are those that can survive in the presence of antibiotics. Many of these bacteria are found in hospitals, nursing homes and other healthcare facilities. Sometimes, however, antibiotic resistant bacteria may affect people living in the community who have not had any contact with healthcare facilities. This study is about the bacteria, *Staphylococcus aureus.* *Staphylococcus aureus*can live in the nose, on the skin, or in the bowel without making people sick. It can, however, cause serious infections. The goal of this study is to learn more about a certain type of *Staphylococcus aureus* that is resistant to many of the antibiotics that are normally used to treat infections caused by it. This special type of *Staphylococcus aureus* is known as "methicillin-resistant *Staphylococcus aureus"* or MRSA for short, while the other type of *Staphylococcus aureus* is methicillin susceptible and is therefore known as methicillin-susceptible *Staphylococcus aureus* or MSSA for short. Three groups of people will be asked to participate in this study, those infected or colonized with MRSA (cases) and those infected or colonized with MSSA (controls) and a group that is Non-Infected (controls).

Study Procedures

You are being approached to participate in this project as you have been identified as having either (a) been infected or colonized with MRSA, (b) been infected or colonized with MSSA, or (c) not infected or colonized with MRSA or MSSA. If you agree to participate in this study the researchers may get medical information from you. A telephone and/or personal interview will also be performed by the researcher working on this study. You will be asked about your health and about the ways that you might have come in contact with this bacterium. This study will give the researchers important information on how people come in contact and either become colonized or infected with these bacteria.

Participation in this study will only be during the time when the questions are being asked. You can stop participating at any time.

Risks and Discomforts

There is no anticipated harm from participating in this study. There may be a slight inconvenience of answering questions over the telephone or in person about how you may have come in contact with MRSA/MSSA or other medical problems which could put you at risk of coming in contact with MRSA/MSSA such as age, years, year of study, gender, recent antibiotics consumption, smoking status, hospitalization in previous 3 years, gym or sports team, antibiotics use ongoing in the previous 3 months, dermatitis, sinusitis, rhinitis, external otitis, cystic fibrosis, other chronic condition, history of MRSA colonization or infection, and frequent visits to a sports centre.

Benefits

There is no direct benefit for you to participate in this study other than you will be helping the researchers learn more about the risk factors for coming in contact and becoming colonized or infected with MRSA.

Alternative Procedures: The alternative procedure is to not participate.

Costs

All the procedures which will be performed as part of the study are provided at no cost to you. You will receive no payment or reimbursement for any expenses related to taking part in this study.

Confidentiality

Information gathered in this research study may be published or presented in public forums, however, your name and other identifying information will not be used or revealed. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Data may be returned to the researcher in a way that potentially identifies the participant. When Investigation Forms are sent by fax, they will only be sent on a secure confidential line to your school. When Investigation Forms are sent

by mail, the originals will be sent by registered mail and a signature will be required on receipt. Your personal health information may be disclosed if required by law. Medical records that contain your identity will be treated as confidential in accordance to the Personal Health Information Acts of Nigeria. Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as representatives from the Federal University of technology Research Ethics Committee. We will keep the things we learn confidential. You will not be identified by these records. The files and computerized information will be stored as confidential. The computerized database will have a secret code that only the investigators will know.

Voluntary Participation/Withdrawal from the Study

Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. During interractions, you may refuse to answer individual questions. Your decision not to participate or to withdraw from the study will not affect you. After your interview, and prior to the data being included in the final report, you will be given the opportunity to review the Investigation Form of your interview, and to add, alter, or delete information from the form as you see fit. If you choose to withdraw from the study, your data will be deleted from the study and destroyed. If the study staff feel that it is in your best interest to withdraw you from the study, they will remove you without your consent.

Dissemination of Results

Results of the study will be disseminated to the study participants, the Medical Officer of Health, and will be shared with the Educational Subcommittee of this project to develop documents to curb the spread of CA-MRSA in the communities and HA-MRSA in the hospital. In addition, findings will be published in reviewed microbiology journals with the approval of study participants.

Significant New Findings

Any significant new findings that develop during the course of the study which may affect your willingness to continue in the research will be provided to you by the study group.

Debriefing and Feedback Procedures

Following participation in the research study, you will be given the opportunity to read and revise your Case-Control Investigation Form and acknowledge its accurate portrayal of what had been said during the interraction with the research group. If you indicate on this consent form that you would like to receive a copy of the final results of this study, these will be sent to you directly upon completion of the research.

Questions

You are free to ask any questions that you may have about this research study and your rights as a research participant. If any questions come up during or after the study, or if you have a research-related injury, contact the study investigators and the study staff: Miss Marylene Etim, dept of microbiology, federal university of technology, Owerri : 07064216262, Dr. Adieze , same address: 08035490116

For questions about your rights as a research participant, you may contact the same people.

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all your questions.

Legal Rights

You are not waiving any of your legal rights by signing this consent form nor releasing the investigator(s) from their legal or professional responsibility.

Statement of Consent

I have read this consent form or it has been read to me. I have had the opportunity to discuss this research study with Dr. Adieze and/or her research student. I have had my questions answered by them in a

language I understand. The risks and benefits have been explained to me. I understand that I will be given a copy of this consent form after signing it. I understand that my participation in this study is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study. I understand that information regarding my personal identity will be kept confidential, but that confidentiality is not guaranteed. I authorize the inspection of any of my recordsthat relate to this study by the medical schools, Abia State University, Imo State University and University of Uyo.

By signing this consent form, I have not waived any of the legal rights that I have as a participant in this study.

Please indicate whether you would like a copy of the final results of this study:

Yes…….. No…….

I consent to the following as part of this study:

a) Allowing the research staff to review my health records Yes……. No…..

b) My family physician/hospital/nursing station releasing my health information to the study staff Yes….. No…

c) The research staff asking me questions about my health Yes….. No…

e) Having my data included in medical journal articles, conference presentations, etc. however my name and other identifying information will not be used or revealed.

Yes…….. No……

_____ _____

Name and Signature of Participant Date

_____ _____

Name and Signature of Person Explaining Consent Date

_____ _____

Name and Signature of Legal Guardian Date

_____ _____

Name and Signature of Witness Date

Translation Assistance: If the understanding of this consent process has been aided with the assistance of a translator in a language other than English, please indicate language:

_____

Language Translated

_____ _____

Name and Signature of Translator Date

Appendix 2

Etim, Marylene Effiong
Department of Microbiology
School of Science
Federal University of Technology,
Owerri, Imo state.
9th August 2015

The Provost
College of Medicine and Health Sciences
University of Uyo
Uyo, Akwa Ibom State

Sir,

Request for Permission to collect Samples

I, Etim, Marylene Effiong, an M.Sc. student of medical microbiology, department of microbiology of Federal University of Technology, Owerri hereby request for your permission to collect palm, nasal and throat swab samples from pre-clinical and clinical students from medical students in your institution. This is to enable me carry out my research work on the topic: Antimicrobial Resistant Patterns of Methicillin resistant Staphylococci from medical students of some Nigerian Universities. Attached herewith is a copy of my research proposal for your perusal. I will seek oral and written (if requested) consent from the students before collection. I will be grateful if my request is granted and approval is issued. Thanks in anticipation.

Approved
18/8/15

Yours faithfully,

Etim, Marylene Effiong

PROVOST COLLEGE OF HEALTH SCIENCES
UNIVERSITY OF UYO
SIGN
DATE

Appendix 3

A QUESTIONNAIRE FOR THE RESEARCH TOPIC: ANTIMICROBIAL RESISTANCE PATTERN OF METHICILLIN RESISTANT STAPHYLOCOCCI IN MEDICAL STUDENTS OF SOME SOUTH-EASTERN UNIVERSITIES.

AUGUST 2015

Investigator: Miss Marylene Etim

Study Coordinator: Dr Ifechi Adieze

My name is Marylene Etim, a post-graduate student of microbiology from Federal University of Technology, Owerri, Imo State. I am conducting a study on Antimicrobial resistance patterns of methicillin resistance Staphylococciin pre-clinical and clinical medical students. The study aims at assessing the prevalence of methicillin resistant Staphylococci among clinical and pre-clinical medical students and at determining their antimicrobial resistance pattern.

QUESTIONS:

Have you ever received health care survey on the above named topic: YES…….NO……..

Place of Birth…….. Year of study (1^{st}, 2^{nd}, 3^{rd} etc)……...

Class: pre-clinical…….Clinical……..……

Gender: Male……...Female……..

Duration of Hospital Exposure

No exposure……..12 hours per week……

24 hours per week……More than 24 hours a week…….

Address: Medical Hostel…….. Off Campus……..

Hospitalizations within the previous year: YES……NO…….

A history of caring for inpatients within the past year: YES……...NO……

Outpatient clinic visits within the past year: YES……..NO…….

The use of antibiotics within the last 3 months: YES…….NO……

A history of skin and soft tissue infection within the last month: YES……NO…….

Smoking status: YES……..NO……..

History of cold and fever in the past 2 weeks: YES……..NO……..

Unhealed wound: YES……NO…….

Often touch nose: YES……NO……

Often wash hands after touching nose: YES……..NO……

Current Exposure to Cigarette Smoke (Operationalized as either being a current smoker or living in a house with a current smoker): YES……NO…….

Thank you for agreeing to take this survey. All of the answers you provide in this survey will be kept confidential. No identifying information will be given out. The survey data will be reported in a summary fashion only and will not identify any individual person. This survey will take about 10 minutes or less to complete.

……..

Initials and signature.

Appendix 4

Profiles of medical students screened.

Variables		Medical students from Imo State University		Medical students from Abia State University		Medical students from University of Uyo		Total
		Pre-clinical students, n=50 (%)	Clinical Students, n=50(%)	Pre-clinical students, n=50(%)	Clinical Students, n=30(%)	Pre-clinical students, n=120(%)	Clinical Students, n=120(%)	N=420(%)
Gender	Male	13 (26)	21 (42)	27 (54)	18 (60)	69 (57.5)	72 (60)	220(52.3)
	Female	37 (74)	29 (58)	23(46)	12 (40)	51(42.5)	48(40)	200(47.6)
Age	≤21	3 (6)	-	5(10)	-	15(12.5)	8 (6.7)	31(7.4)
	21-25	35(70)	19 (38)	29 (58)	9 (30)	71(59.2)	49(40.8)	212(50.4)
	26-30	12 (24)	27 (54)	19 (38)	18 (60)	34(28.3)	50 (41.7)	160(38.0)
	≥31	-	4 (8)	-	3(10)	-	10 (8.3)	17(4.0)
Subjectswith history of cold and fever in the past weeks		3 (6)	18 (36)	25 (50)	12 (40)	56(46.7)	21(17.5)	135(32.1)
Subjectswith outpatient clinic visits in the past month		15(30)	27 (54)	38 (76)	30 (100)	104(86.7)	120(100)	334(79.5)
Subjectswithhistory of skin and soft tissue infections within the last one year		-	-	2 (4)	-	1(0.83)	-	3(0.7)
Subjectswith unhealed wound		-	2 (4)	3 (6)	-	-	-	5(1.2)
Subjects that often touch nose		50 (100)	50 (100)	50 (100)	28 (93.3)	118 (98.3)	110(91.7)	406(96.6)
Often wash hands after touching nose		28 (56)	44 (88)	21 (42)	12 (20)	34(28.3)	76(63.3)	215(51.2)
Subjectsthat have any underlying diseases		7 (14)	-	-	-	-	-	7(1.7)
Subjects that often visit hospital		9 (18)	50 (100)	26 (52)	30(100)	73(60.8)	120(100)	308(73.3)

Appendix 5

Table 4.2. Palm, throat and nasal swab samples received from medical students

Swab samples	Pre-clinical students	Clinical students	Total
Palm swabs	105	103	208
Throat swabs	53	23	76
Nasal swabs	77	79	156
Total	235	205	440

Appendix 6

Palm, throat and nasal swab samples obtained frommedical students per institution

Swab samples	**Students from IMSU; n=107**			**Students from ABSU; n=83**			**Students from University of Uyo; n= 250**			**Total number of Samples from all Institutions; n=440**
	Pre-clinical students, n=54	Clinical students, n=53	Total, n=107	Pre-clinical students, n=53	Clinical students, n=30	Total, n=83	Pre-clinical students, n=128	Clinical students, n=122	Total, n=250	
Palm swabs	37	36	73	31	30	61	37	37	74	208(47.3)
Throat swabs	-	-	-	14	-	14	39	23	62	76(17.3)
Nasal swabs	17	17	34	8	-	8	52	62	114	156(35.5)
Total	54	53	107	53	30	83	128	122	250	440(100)

Key: - , No sample obtained; IMSU- Imo State university; ABSU- Abia State University

Appendix 7

Percentage*Staphylococcus aureus* and Coagulase–negative *Staphylococcus* species isolated from multiple source (Palm/Throat/Nasal) in individual medical students of Imo State University

Samples	Isolates of medical students from Imo State University n=54(%)					
	Pre- clinical students			Clinical students		
	SA	CoNS	Total	SA	CoNS	Total
Palm/Nasal swabs	10(18.5)	0 (0.0)	10(18.5)	4 (7.4)	0 (0.0)	4(7.4)
Palm swabs only	13(24.1)	6(11.1)	19(35.2)	6(11.1)	0 (0.0)	6(11.1)
Nasal swabs only	7(12.9)	0 (0.0)	7(12.9)	4 (7.4)	4 (7.4)	8 (14.8)

Key: SA- *Staphylococcus aureus*; CoNS- Coagulase-negative Staphylococci

Appendix 8

Percentage *Staphylococcus aureus* and Coagulase–negative *Staphylococcus* species isolated from multiple source (Palm/Throat/Nasal) in individual medical students in Abia State University (ABSU)

Samples	Isolates of medical students from Abia State University, n=50(%)					
	Pre- clinical students			Clinical students		
	SA	CONS	Total	SA	CONS	Total
Palm /nasal /throat swabs	10(20)	-	10(20)	-	-	-
Palm/Nasal swabs	5(10)	-	5(10)	-	-	-
Palm/Throat swabs	2(4)	-	2(4)	-	-	-
Nasal/throat swabs	2(4)	-	2(4)	-	-	-
Palm swabs only	7(14)	-	7(14)	18(36)	2(4)	20(40)
Nasal swabs only	4(8)	-	4(8)	-	-	-
Throat swabs only	-	-	-	-	-	-

Key: SA- *Staphylococcus aureus*; CoNS- Coagulase-negative Staphylococci

Appendix 9

Percentage *Staphylococcus aureus* and Coagulase–negative *Staphylococcus* species isolated from multiple source (Palm/Throat/Nasal) in individual medical studentsin University of Uyo

Samples	Isolates of medical students from University of Uyo, n=144(%)					
	Pre- clinical students			Clinical students		
	SA	CONS	Total	SA	CONS	Total
Palm /nasal /throat swabs	9(6.25)	5 (3.47)	14(9.72)	11(7.64)	4(2.78)	15(10.42)
Palm/Nasal swabs	5(3.47)	4(2.78)	9(6.25)	6(4.17)	1(0.69)	7(4.86)
Palm/Throat swabs	3(2.08)	2 (1.39)	5(2.8%)	3(3.47)	1(0.69)	4(2.78)
Nasal/throat swabs	4(2.78)	2(1.39)	6(4.17)	3(3.47)	1(0.69)	4(2.78)
Palm swabs only	2(1.39)	2(1.39)	4(2.78)	12(8.33)	1(0.69)	13(9.03)
Nasal swabs only	20(13.89)	10(6.94)	30(20.83)	21(14.58)	12(8.33)	33(22.92)
Throat swabs only	-	-	-	-	-	-

Key: SA- *Staphylococcus aureus*; CoNS- Coagulase-negative Staphylococci

Appendix 10

CLSI (2012) Breakpoints

Penicillin: ≤28mm (Resistant), ≥29mm (Susceptible)

Oxacillin: ≤10mm (Resistant), ≥13mm (Susceptible)

Ampicillin: ≤28 mm (Resistant), ≥29 mm (Susceptible)

Erythromycin: ≤13 mm (Resistant), 14 mm-22 mm (Intermediate), ≥23 mm (Susceptible)

Vancomycin: ≤6 mm (Resistant), ≥7 mm (Susceptible)

Ciprofloxacin: ≤15mm (Resistant), 16mm-20mm (Intermediate), ≥21mm (Susceptible)

Nitrofurantoin: ≤14mm (Resistant), 15mm-16mm (Intermediate), ≥17mm (Susceptible)

Trimethoprim: ≤10mm (Resistant), 11mm-15mm (Intermediate), ≥16mm (Susceptible)

Clindamycin: ≤14mm (Resistant), 15mm-20mm (Intermediate), ≥21mm (Susceptible)