PLASMID PROFILE, METHICILLIN RESISTANCE DETERMINANTS AND CHARACTERISATION OF STAPHYLOCOCCUS SPECIES ISOLATED FROM CLINICAL AND COMMUNITY ENVIRONMENTS IN IBADAN

BY

CAJETHAN ONYEBUCHI, EZEAMAGU

B.Sc. (Hons) Microbiology (Sokoto), M.Sc. Microbiology (Ibadan)

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DEDICATION

This work is dedicated to my friend and lovely brother, DR. EZEAMAGU, Carriton Sunday who passed on to glory at the peak of his academic career. May his gentle soul rest in the bosom of our Lord Jesus Christ, Amen.

ABSTRACT

Methicillin-resistant s*taphylococcus* (MRS) infections are of global concern in healthcare institutions and community settings with significant morbidity and mortality due to multi-drug resistance challenges. In Nigeria, most methicillin resistance detection was based on phenotypic method with scanty reports on molecular characterisation of MRS. In this study, molecular techniques were used to determine the presence of methicillin resistant gene (*mecA*) with its associated resistance determinants (*vanA* and *blaZ*) and plasmid profile of staphylococci isolated from clinical and community samples.

Staphylococcus species from clinical (55) and community (53) samples were isolated from air, selected waste water drainages and human swabs (eye, semen, ear, high vagina swab, throat, urethra, wound, nostril, skin) in the University of Ibadan and University College Hospital. They were identified using standard microbiological procedures. The isolates identity was confirmed to genus level using 16S-rRNA specific primers and identified to species level by PCR-Restriction Fragment length Polymorphism supplemented with PCR species-specific-primers. The isolates were phenotypically screened for resistance to methicillin and other antibiotics by agar diffusion method. Multiplex PCR was used to assess presence of *mecA* and the resistant determinants while Simplex PCR was used to determine the origin of *mecA* isolates by detecting the presence of Panton-Valentine Leukocidin (PVL). Plasmid profiles of clinical (35) and community (19) isolates with multiple drug resistance were determined using standard procedures. Data was analysed by descriptive statistics.

The organisms were identified as *S. epidermidis* (92.6 %), *S. aureus* (6.5 %) and *S. xylosus* (0.9 %). Phenotypic resistance to methicillin was 72.7 and 62.3 % in clinical and community isolates respectively. In the clinical isolates of *S. epidermidis*, 30.9, 32.7, 34.5,

40.0, 41.8, 60.0, 76.4, and 89.1 % were resistant to Chloramphenicol, Vamcomycin, Streptomycin, Erythromycin, Gentamycin, Tetracycline, Cotrimoxazole and Cloxacillin respectively. Correspondingly, in community isolates of *S. epidermidis,* 28.3, 3.8, 32.1, 50.9, 26.4, 58.5, 90.6 and 92.5 % were resistant to these antibiotics. In the clinical isolates of *S. aureus*, 3.6, 5.5, 5.5, 7.3, 7.3, 7.3, 9.1 and 9.1 % were resistant to Vamcomycin, Erythromycin, Chloramphenicol, Streptomycin, Gentamycin, Tetracycline, Cotrimoxazole and Cloxacillin respectively. In community isolates, 1.9 % *S. aureus* were resistant to Cotrimoxazole, Chloramphenicol, Erythromycin, Gentamycin and Streptomycin while 3.8 % were resistant to Cloxacillin. Among clinical isolates of *S. xylosus*, 1.8 % was resistant to all the antibiotics except Chloramphenicol and Streptomycin. All the strains lacked *vanA* gene, while only clinical isolates (3.6 %) had *mecA* when its specific primers were used and 5.5 % using its regulatory element specific primers in PCR. The *blaZ* gene was found in 16.4 % of clinical and 1.8 % of community isolates. There was no PVL in the isolates with *mecA*. Plasmid size of 23.13kb was found in 94.3 % of clinical and 84.2 % of community isolates.

The detection of *blaZ* gene in community isolates showed that such resistance determinants predominantly found in clinical isolates are also emerging in the community isolates. Hence, setting up antibiotic surveillance system is necessary to minimize this trend.

Keywords: Methicillin resistance, Plasmid profile, *staphylococcus* species, *mecA* gene.

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CERTIFICATION

I certify that this work was carried out by CAJETHAN ONYEBUCHI EZEAMAGU (MATRIC NO: 130958) in the Department of Microbiology, University of Ibadan and at the Nigerian Institute of Medical Research,Yaba, Lagos, Nigeria.

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Supervisor
O.E FAGADE
B.Sc, (Ife) M.Sc, Ph.D (Ibadan)
Prof and Head of Department, Microbiology,
University of Ibadan, Ibadan, Nigeria

ABBREVIATION

CA-MRSA	Community-associated methicillin-resistance *Staphylococcus aureus*
DNA	Deoxyribonucleic Acid
MHA	Mueller-Hinton Agar
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
NA	Nutrient Agar
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
PRSA	Penicillin-resistant *Staphylococcus aureus*
PVL	Panton-Valentine leukocidin
RFLP	Restriction Fragment Length Polymorphism
SCCmec	Staphylococcal chromosomal cassette
TAE	Tris-Acetate- EDTA
TBE	Tris- Boric acid- EDTA
TENS	Tris- EDTA- NaOH- SDS
TSA	Tryptone Soya Agar

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CHAPTER ONE

INTRODUCTION

1.1 Background

Taxonomically, the genus *staphylococcus* is in the bacteria family staphylococcacea. This group of microorganisms when viewed under microscope after staining with Gram reagent, they are spherical bacteria cells arranged in irregular clusters. These clusters take the shape of a bunch of grapes. In Greek the term stapyle refers to a bunch of grapes (Monica, 2002; Sıla 2006). So these bacteria were named staphylococci. In 1884, Rosenbach described staphylococci according to the colony types they had and proposed the appropriate nomenclature: *Staphylococcus aureus* (yellow) and *Staphylococcus albus* (white). The latter species is now named *Staphylococcus epidermidis. Staphylococcus aureus* forms yellowish large colony and *Staphylococcus epidermidis* forms relatively small white colony (Monica, 2002; Sıla 2006).

Generally, s*taphylococcus* species are Gram-positive, non-motile, non-sporing cocci occurring singly, in pair, and irregular clusters; size may be variable (1μm in diameter); colonies are opaque and may be white or creamy and are occasionally yellow or orange (Monica, 2002; Sıla, 2006). The optimum temperature for growth is 30-37^{o}C. They are facultative anaerobes and have fermentative metabolism. *Staphylococcus* species are usually catalase-positive and oxidase-negative. Nitrates are often reduced to nitrites. Some species are susceptible to lysis by lysostaphin, but not by lysozyme and are usually able to grow in 10% Sodium Chloride. Some species produce extra cellular toxins (Jarlov *et al*., 1996). Staphylococci may be identified by production of deoxyribonuclease (Monica, 2002; Sıla 2006).

The genus *staphylococcus* is pathogen of man and animals. Normally, they are grouped into two on the basis of their ability to clot blood plasma: coagulase positive staphylococci and coagulase negative staphylococci (CNS). *Staphylococcus aureus* is a major pathogen which may be associated with severe infections and it is relevant to distinguish it from the opportunistic coagulase negative staphylococci. In routine laboratory practice, the production of coagulase is mostly used as the sole criterion to distinguish *S. aureus* from other staphylococci*;* since other coagulase positive staphylococci such as *S. hyicus*, *S. schleiferi* subspecies *coagulanse* or *intermedius* have been found only occasionally in human carriage or infection (Health Protection Agency, 2007). Teichoic acid composition is also different among the *staphylococcus* species. The cell wall contains ribitol teichoic acid (polysaccharide A) in *Staphylococcus aureus*, glycerol teichoic acid (polysaccharide B) in *S. epidermidis* (Sıla 2006).

Staphylococcus aureus subspecies *anaerobius* is rarely isolated from clinical specimens and grows poorly aerobically. Growth may be CO_2-dependent (Rupp and Archer, 1994) and it is coagulase negative. It may be catalase-negative. Strains may be identified by anaerobic growth and they give a positive coagulase test result. However, because the growth may be poor, the coagulase result may be negative and subspecies isolates could be referred to Reference Laboratory for identification. *Staphylococcus hyicus* may be coagulase positive. *Staphylococcus schleiferi subspecies coagulans* is coagulase negative.

Humans are the reservoir for *S. aureus*, and asymptomatic colonization is frequently common than actual infections. Colonization of mucosa membranes or skin, particularly if the subcutaneous barrier is damaged, could be witnessed after birth and may reappear anytime thereafter (Payne *et al.,* 1990). Family members of a colonized infant are at high risk of colonization and this occurs by direct contact with a colonized infant. Carriage rates lie between 25 to 50%. It has been observed that children tend to have higher colonization rates, (Ross *et al*., 1974, Adcock *et al*., 1998). *Staphylococcus aureus* is always considered to be potentially pathogenic bacteria. It is responsible for many human diseases. The clinical manifestations caused by these bacteria can be classified as: cutaneous infections which includes folliculitis, impetigo, wound infections; toxin-mediated infections that includes toxic shock syndrome, food poisoning, scalded skin

syndrome which is seen in children below the age of four, other diseases such as pneumonia, bacteremia, endocarditis, osteomyelitis and septic arthritis are caused by this group of bacteria (Sıla, 2006). The burdens associated with staphylococcal infections are not only because of the painful trauma inflicted on the patients, but also as a result of cost incurred during treatment as most of the drug of choice like beta-lactam antibiotics could no longer effectively produce bactericidal effects on these organisms (Gould, 2006). The reason for this development is attributable to production of beta-lactamase which destroys the functional integrity of the beta-lactam antibiotics. Additionally, the ability of *staphylococcus* species to produce various antibiotic deactivating enzymes has compounded the problems of antimicrobial therapy resulting in multiple resistances to these agents (CME, 2008). The enzymes are carried by plasmids that mediate in resistance to many antimicrobial agents. Plasmid mediated drug resistance in *Staphylococcus aureus* was reported by many workers (Adeleke *et al*., 2002, King *et al*., 2006, Diep *et al*., 2008). Reports on plasmid mediated resistance to chloramphenicol, gentamycin, tobramycin, and kannamycin in *Staphylococcus aureus*. In addition, *Staphylococcus aureus* strains possessing plasmid copy of β- lactamase determinant has been documented (Punithavathi and Krishnaveni, 2012).

The history of staphylococcal resistance has been seen to appear after introduction of new antibiotics. For example, before the widespread use of penicillin in the late 1940s and 1950s, staphylococcal septicemia was associated with an extremely high mortality rate (Smith and Vickers, 1960). Penicillin being the drug of choice dramatically improved the treatment of this infection; however, penicillin-resistant strains were discovered by several investigators within short period after their release for clinical purposes (Fleming 1942, Hobby *et al*., 1942). Between 1950s and 1960s Penicillin Resistant *Staphylococcus aureus* (PRSA) strains were discovered in the community shortly after they were found in hospitals, making hospital control of PRSA essentially unfruitful (Michael 2000). The development of beta-lactamase-resistant penicillins such as methicillin and oxacillin in the early 1960s once again revolutionised the treatment of staphylococcal infections. Within a year of their release, however, methicillin resistant s*taphylococcus aureus* (MRSA) strains were reported (Barber 1961, Jevons 1961) and outbreaks of MRSA infections were reported in several continents within several years (Maranan *et al*., 1997). The problem of

antibiotic resistance has been worsened with the discovery of methicillin resistance gene; *mecA* located on chromosomal cassette (*SCCmec*) which code for penicillin binding protein (PBP2a) with altered affinity to beta-lactam antibiotics (Cohen 1992, Neu 1992).

The increasing infections caused by methicillin-resistance *Staphylococcus aureus* posed serious threat in health care institutions in the United States (Panlilio *et al*., 1992, CDCP 2004). It is one of the most wide spread and virulent nosocomial pathogens and is usually resistant to multiple antibiotics making infections difficult to treat (Cooper *et al*., 2004). Although, the clinical significance of MRSA has been questioned in the past, there is now widespread acknowledgement of its pathogenicity as it has emerged as a significant cause of both nosocomial and community-acquired infections.

Traditionally, MRSA infections have been acquired almost exclusively in hospitals, long-term care facilities or similar institutional settings (Thompson *et al*., 1982), however, Layton *et al.* (1995) revealed that it is also appearing in the community settings and healthy colonized persons/infected patients if undetected/treated are potential reservoirs for transmission which will result in upswing in carrier rates. The emergence of community-associated MRSA (CA-MRSA) infections is of major concern to public health professionals and the earlier reported cases of CA-MRSA infection among Australian aboriginals and Native Americans occurred in children with little or no recognized contact with the hospitals or other health care institutions (Panlilio *et al*., 1992). How these community-acquired strains emerged has been subject of debate. One possibility is that they are feral descendants of hospital isolates. However, direct or indirect exposure to an institutional health-care setting where MRSA is likely to be found together with other risk factors commonly associated with MRSA colonization are totally absent from MRSA acquired from a community reservoir (Michael 2000). The antibiogram patterns observed for these MRSA strains are further evidence of a possible community origin.

The strains of MRSA isolated from children in the community have led to speculation that the epidemiology of *S. aureus* is changing (Boyce 1998, CDC, 1999). The prevalence of *S aureus* infections caused by MRSA as reported by the National Nosocomial Infection Surveillance system in the United States has been steadily increasing, from 2.4% in 1974, 5% in 1981, 29% in 1991 to 43% in 1997 (Haley *et al*., 1982, Panlilio *et al*., 1992,

Michael 2000). MRSA strains now account for more than 50% of *S. aureus* recovered from patients in intensive care units and about 40% of *S. aureus* isolated from non intensive care unit (NFID 2003). The percentage of hospitals managing patients with MRSA infections are also increasing. In a survey of Society for Healthcare Epidemiology of America members in 1990, 97% of the hospitals have managed patients with MRSA. Approximately 478,000 hospitalizations in the U.S. in 2005 were associated with *S. aureus* infections and 58% of those (278,000) were caused by MRSA. MRSA is estimated to cause illness in more than 150,000 persons annually in healthcare facilities in the European Union (Köck *et al*., 2010).

Methicillin resistance was not limited to *S. aureus* only, but also to coagulase negative staphylococci such as *S. epidermidis, S. saprophyticus, S. haemolyticus*. These belong to group of opportunistic pathogens found as normal flora of the skin and mucus membranes in different part of the body (Einsenstein and Schacchter, 1994). For this reason, CNS are often reported without further speciation, assuming that they are contaminating clinical samples but are not involved in the primary infection. However, there is evidence that these bacteria may be responsible for primary infections in patients as a result of increased use of medical in-dwelling plastic devices and compromised or immunodepressed patients (Jarvis and Martone, 1994; Kloos and Bannerman, 1994). In Brazil, a multi centre study showed that 87.7% of CNS isolated from blood cultures were resistant to methicillin (Sader *et al*., 1999). Methicillin resistance among coagulase negative staphylococci is particularly important due to multiple resistance to virtually all β-lactam agents and other antimicrobial classes (Sader *et al*., 1999).

1.2 Statement of the problem

The reported cases of methicillin resistance associated staphylococcal infections in various regions in developing countries like Nigeria are on the increase (Panlilio *et al*.; 1992). *Staphylococcus* species cause both nosocomial and community-acquired infections that are difficult to treat due to their multiple resistance to various antibiotics (Cooper *et al*., 2004). As a result, these infections have been associated with significant morbidity and mortality in health-care institutions. Healthy colonized persons/infected patients if undetected/treated are potential reservoirs for transmission which will result in upswing in

carrier rates especially in community settings. Unfortunately, it has been observed that phenotypic method of methicillin resistance detection are highly dependent on growth conditions (Sinsimer *et al*., 2005) and heterogeneous in nature with the level of resistance varying according to the ß-lactam antibiotic being used (Matthews and Stewart, 1984). The origin of these strains (healthcare or community-acquired) is uncertain and the methcillin resistance determination has been restricted to only *S. aureus*. Beside, the molecular confirmation of methicillin resistance has been based on the detection of ‘’PBP2a’’ but there are reports that when the *mecA* regulatory elements are fully functional, the production of ‘’PBP2a’’ may be repressed leading to a false negative (Ryffel *et al*., 1992, Kuwahara-Arai *et al*., 1996). In addition, the antibiotic vancomycin has been the last weapon against strains of *S. aureus* that are resistant to all other antibiotics, but there has been reported cases of vancomycin resistance first in Japan (1996) and United States (1997) (Hiramatsu *et al*., 1997). Also, whether the resistance in previous strains studied was chromosomal mediated (*mecA*) is unknown (Fagade *et al*., 2010) and the diagnosis of infections associated with staphylococci mostly depends on time-consuming classical microbiological and biochemical methods for speciation and susceptibility testing (Bergeron and Ouellette 1998). Accurate identification of the members of this group at species level is challenging and most of the times, phenotypic identification methods with manual and automated systems are not reliable for correct identification (Layer *et al*., 2006; Kim *et al*., 2008).

1.3 Justification

In developed countries like United States, Canada, several works have been done with respect to methicillin and vancomycin resistance using molecular methods. However, majority of studies on methcillin resistance based on phenotypic methods with few reports using molecular methods have been done in developing countries like Nigeria (Shittu *et al*., 2009, Fadeyi *et al*., 2010). In addition, only few works in the community setting were reported. The detection of methicillin resistance depends extensively on *mecA* gene, however, the indirect detection of *mecA* gene using its regulatory elements and chromosomal cassette may be helpful in assessing correctly methicillin resistance since mutation in *mec* complex can lead to loss of *mecA* gene. Extending detection of methcillin resistance and determining the resistant determinants (*vanA* and *blaZ*) in

coagulase negative staphylococci could help to determine extent of spread of these genes. Likewise, determining the origin of methicillin resistant strains in s*taphylococcus* species will help in treatment options. The identification of staphylococci to species level using polymerase chain reaction - restriction fragment-length polymorphism (PCR-RFLP) supplemented with species-specific primers were considered to be the gold standard methods (Wichelhaus *et al*., 2001).

1.4 Goals

The goals of this work were to determine the plasmid profile, resistance pattern, resistance determinants and to determine the origin (Healthcare or community-acquired) of methicillin resistant strains of s*taphylococcus* species that were isolated from clinical and environmental samples.

1.5 Aims and objectives

- To isolate s*taphylococcus* species from Clinical specimens and community sources and characterize these isolates using both classical Microbiological and PCR-RFLP (supplemented with PCR-species specific primers) methods.
- To phenotypically screen for methicillin and vancomycin resistance in these isolates as well as to determine the antibiogram of the isolates against commonly use antibiotics.
- To determine the presence of methicillin resistance gene, *mec*A with other resistance determinants (*van*A, and *bla*Z) in these isolates using multiplex PCR.
- To use *mecA* regulatory elements (*mecR1* and *mecI*) and chromosomal cassette to assess methicillin resistance.
- Determine whether the methicillin resistant strain(s) detected were healthcare or community acquired by determining the Panton Valentine Leukocidin status of isolates with *mecA* gene.
- To determine the plasmid profiles of some selected *staphylococcus* species that show multiple resistance to antibiotics.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin of antibiotics

Most antibiotics used clinically are produced by, and isolated from, microorganisms found normally in the soil, and the genus *streptomyces* represents one of the most common of these sources. Molds such as p*enicillium*, the original source of penicillin, are also well known producers of antibiotics. Antibiotics produced by bacteria represent members of a more general class of extracellular substances that kill other bacteria and facilitate competition in the bacterial world. Many of these compounds represent proteins with a large variety of different inhibitory activities, although a number of those that serve as clinically useful antibiotics represent small non-protein molecules. It has been reported that a gram of soil may contain up to 5 thousand or more different species of microbes (Schloss and Handelsman, 2005), hence there are obvious advantages to producing such agents abundantly. Production, however, also requires a means by which organisms can protect themselves from their own "weapons"; and this is done by conferring a self "immunity" or "resistance". Furthermore, soil bacteria that do not necessarily produce extracellular substances often carry multiple "resistance" determinants. This has been shown to be the case for many strains of *streptomyces* (D'Costa *et al*., 2006). Thus the soil is loaded with microbes encoding antibiotics and related resistance, and this has doubtless been the case for many millions of years. When one considers the fact that a single human being carries trillions of bacteria in and on the body, exceeding the number of somatic cells by as much as 10 fold and corresponding to as many as 1000 different species (D'Costa *et al*., 2006), it is highly likely that interaction of at least some of these organisms with resistant strains from the environment is a common occurrence.

Therapeutic exposure to an antibiotic would easily select for increased numbers of such strains.

2.2 Mechanisms of antibiotic action

Most antimicrobial agents used for the treatment of bacterial infections may be classified according to their principal mode of action. There are four major modes of action: interference with cell wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis, and inhibition of a metabolic pathway (Neu, 1992). Antibacterial drugs which work by inhibiting bacterial cell wall synthesis include the β-lactams, such as the penicillins, cephalosporins, carbapenems, and monobactams, and the glycopeptides, including vancomycin and teicoplanin (Neu, 1992). β-Lactam agents inhibit synthesis of the bacterial cell wall by interfering with the enzymes required for the synthesis of the peptidoglycan layer. Vancomycin and teicoplanin also interfere with cell wall synthesis, but achieve this by binding to the terminal D-alanine residues of the nascent peptidoglycan chain, hence preventing the cross-linking steps required for stable cell wall synthesis (Clewell, 1993).

Macrolides, aminoglycosides, tetracyclines, chloramphenicol, streptogramins, and oxazolidinones produce their antibacterial effects by inhibiting protein synthesis (Neu, 1992). Bacterial ribosomes differ in structure from their counterparts in eukaryotic cells. Antibacterial agents take advantage of these differences to selectively inhibit bacterial growth. The bacterial 70S ribosome consists of two subunits, the larger 50S subunit and the smaller 30S subunit. Eukaryotes, on the other hand, have an orthologous, although more complex, 80S ribosome consisting of the larger 60S subunit and the smaller 40S subunit. The majority of inhibitors of protein synthesis in bacteria target the 30S or 50S subunits of the bacterial 70S ribosome. Macrolides, aminoglycosides, and tetracyclines bind to the 30S subunit of the ribosome, whereas chloramphenicol binds to the 50S subunit (Neu, 1992).

Fluoroquinolones exert their antibacterial effects by disrupting DNA synthesis and causing lethal double-strand DNA breaks during DNA replication (Drlica and Zhao 1997) whereas sulfonamides and trimethoprim (TMP) block the pathway for folic acid synthesis,

which ultimately inhibits DNA synthesis (Tenover, 2006). The common antibacterial drug combination of TMP, a folic acid analogue, plus sulfamethoxazole (SMX) (a sulfonamide) inhibits two steps in the enzymatic pathway for bacterial folate synthesis.

Disruption of bacterial structure may be a fifth, although less well characterized, mechanism of action. It is postulated that polymyxins exert their inhibitory effects by increasing bacterial membrane permeability, causing leakage of bacterial contents (Storm *et al*., 1977). The cyclic lipopeptide daptomycin apparently inserts its lipid tail into the bacterial cell membrane causing membrane depolarization and eventual death of the bacterium (Carpenter and Chambers 2004).

2.3 Bacterial drug resistance

Bacterial drug resistance has for long time been recognized as major problem in the chemotherapy of bacterial infections. When new antibiotic is introduced into clinical practice bacteria are not normally resistant to such new drug, until after some months or years of continuous use. The time of emergence and the rate of spread of resistant organisms can be unpredictable and despite the common occurrence of acquired bacterial resistance to many antibiotics, it is not necessarily inevitable. Resistance may be extremely slow to emerge as observed with the resistance of *Staphylococcus aureus* to neomycin, which did not appear until nine years of introducing the antibiotic (Adeleke and Odelola, 1997). In the case of streptomycin, introduced in 1944 for the treatment of tuberculosis (TB; "The Great White Plague"), mutant strains of *Mycobacterium tuberculosis* resistant to therapeutic concentrations of the antibiotic were found to arise during patient treatment. As other antibiotics have been discovered and introduced into clinical practice, a similar course of events has ensued (Davies, and Davies, 2010). Bacterial resistance is observed in vitro whenever the pathogens continue to reproduce at therapeutically attainable concentrations of the antibacterial agent.

Resistance was first noticed in protozoa and later in bacteria with the discovery of sulphonamides in1932 and the introduction of antibiotics in 1938-1940 as antibacterial chemotherapeutic agents (Michael, 1997; Patrick *et al*., 2003). The ever-increasing problem of bacterial drug-resistance since the introduction of this antibacterial agent into clinical practice was highlighted by Oyelese and Oyewo (1995). Among these

antibacterial chemotherapeutic agents were sulphonamides, chloramphenicol, and tetracycline, which were used against strains of *Shigella*, *Escherichia coli* and *Salmonella* (Smith and Halls, 1967). Macrolide antibiotics, such as erythromycin and its successors, were introduced to contend with the problem of methicillin resistance and are widely used for the treatment of Gram positive infections. Not surprisingly, strains resistant due to a number of different mechanisms are now widely disseminated (Davies, and Davies, 2010).

The problem of bacterial resistance has been worsened with the discovery of various drug-inactivating enzymes in bacteria. Notable among these enzymes are ß-lactamases, which act on susceptible antibiotics with cell wall acting activity and the transferases (O-phosphotransferases, O-adenyltransferases and N-acetyltransferases) with activity on certain aminoglycosides. This discovery therefore, necessitated a shift of emphasis from a restrictive form of resistance mediated by extra chromosomal determinant. These extra chromosomal elements are components of genetic elements, plasmids (Adeleke and Odelola, 1997). Not surprisingly, plasmid-mediated transfer of antibiotic resistance has been a major focus of investigation because of its medical and, more recently, practical significance (Norman *et al*., 2009). Plasmid mediated drug resistance in *Staphylococcus aureus* was reported by many workers (Adeleke *et al*., 2002, King *et al*., 2006, Diep *et al*., 2008). Plasmid mediated resistance to chloramphenicol, gentamycin, tobramycin, and kannamycin in *Staphylococcus aureus* strains possessing plasmid copy of β- lactamase determinant has been documented (Punithavathi, and Krishnaveni 2012). It is worth noting that as antimicrobial agents are developed, there is also corresponding increase in the number of beta-lactamases that are emerged (Davies and Davies, 2010).

2.4 Non-genetic origin of drug resistance

The origin of drug resistance may be genetic or non-genetic. Active replication of bacteria is usually required for most antibacterial drug actions. Consequently, microorganisms that are metabolically inactive (non-multiplying) may be resistant to drugs (Blumberge, and Strominger, 1974). Example: Mycobacteria often survive in tissues for many years after infection yet are restrained by the host' defenses and do not multiply. Such "persisting" organisms are resistant to treatment and cannot be eradicated by drugs. However, if they start to multiply (e g. following corticosteroid treatment of the patient), they are fully

susceptible to the same drug. Also, microorganisms may lose the specific target structure for a drug (Blumberg and Strominger, 1974) for several generations and thus be resistant. Example: Penicilin susceptible organisms may change to L-forms (protoplasts) during penicillin administration. Lacking most cell wall-inhibitor drugs (Penicillins, Cephalosporins) and may remain so for several generations as "persisters". When these organisms revert to their bacterial parent forms by resuming cell wall production, they are again fully susceptible to penicillin (Buchanan and Strominger, 1976).

2.5 Genetic origin of drug resistance

The vast majority of drug resistant microbes have emerged as a result of genetic changes and subsequent selection processes. Genetic changes may be chromosomal or extra chromosomal (Davies and Courvalin, 1977). Bacteria contain chromosomes made of double-stranded circular molecule of DNA. Through genetic exchange mechanisms, many bacteria have become resistant to multiple classes of antibacterial agents, and these bacteria with multidrug resistance have become a cause for serious concern, particularly in hospitals and other healthcare institutions where they tend to occur most commonly. These chromosomes are supper coiled and folded within the cell to allow for orderly segregation into the daughter cells. Bacterial chromosomes replicate semi conservatively and sequentially. In some bacteria, replication is known to precede bidirectional (Davies and Courvalin, 1977).

2.6 Chromosomal resistance

This develops as a result of spontaneous mutation in a locus that controls susceptibility to a given antimicrobial. The presence of the drug serves as selective mechanism to suppress susceptibility and promote the growth of drug-resistant mutants. Spontaneous mutation occurs with a frequency of 10^{-12} to 10^{-7} and thus is an infrequent cause for the emergency of clinical drug resistance within a given patient. However, chromosomal mutants for resistance to Rifampin occur in many bacteria with a high frequency of 10^{-7} to 10^{-5}. Consequently, treatment of bacterial infection with Rifampin as the sole drug generally fails (Davies and Courvalin, 1977). Chromosomal mutants are commonly resistant by virtue of a change of structural receptor for a drug. Thus, the P 12 protein on the 30S subunit of the bacterial ribosome serves as a receptor for streptomycin resistance

attachment. Mutation in the gene that controls that structural protein results in streptomycin resistance. A narrow region of the bacterial chromosome contains structural genes that code for a number of drug receptors, including those for erythromycin tetracycline, Lincomycin, amino glycosides. Mutation may be also result in loss of penicillin receptors in some microbial species, making the mutant penicillin-resistant. Also, susceptible bacteria can acquire resistance to an antimicrobial agent via new mutations (Fernando and Jose, 2013). Such spontaneous mutations may cause resistance by altering the target protein to which the antibacterial agent binds by modifying or eliminating the binding site (e.g., change in penicillin-binding protein 2b in pneumococci, which results in penicillin resistance), upregulating the production of enzymes that inactivate the antimicrobial agent (e.g., erythromycin ribosomal methylase in staphylococci), downregulating or altering an outer membrane protein channel that the drug requires for cell entry (e.g., OmpF in *E. coli*), or upregulating pumps that expel the drug from the cell (efflux of fluoroquinolones in *S. aureus* (Fernando and Jose, 2013). In all of these cases, strains of bacteria carrying resistance-conferring mutations are selected by antimicrobial use, which kills the susceptible strains but allows the newly resistant strains to survive and grow. Acquired resistance that develops due to chromosomal mutation and selection is termed vertical evolution.

2.7 Extra chromosomal resistance

Bacteria also contain extra chromosomal genetic elements called plasmids. Plasmids are circular DNA molecules with 1-3% of the weight of the bacterial chromosome, may exist freely 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. R factors are a class of plasmids that carry genes for resistance to one and often several antimicrobial drugs and heavy metals. Plasmid genes for antimicrobial resistance often control the formation of enzymes capable of destroying antimicrobial drugs. Thus, plasmids determine resistance to penicillins and cephalosporins by carrying genes for the formation of ß-lactamses. Plasmids code for enzymes that destroy chloramphenicol (acetyl transferase); enzymes that acetylate, adenylate, or phosphorylate various aminoglycosides, enzymes that determine the permeability of the cell envelope to tetracyclines and others (Tipper, 1979).

2.8 Mechanism of bacterial drug resistance

Bacteria may manifest resistance to antibacterial drugs through a variety of mechanisms. Some species of bacteria are innately resistant to more than one class of antimicrobial agents. In such cases, all strains of that bacterial species are likewise resistant to all the members of those antibacterial classes. Of greater concern are cases of acquired resistance, where initially susceptible populations of bacteria become resistant to an antibacterial agent and proliferate and spread under the selective pressure of use of that agent (Tenover, 2006).

Several mechanisms of antimicrobial resistance are readily spread to a variety of bacterial genera. First, the organism may acquire genes encoding enzymes, such as β-lactamases, that destroy the antibacterial agent before it can have an effect. Second, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect. Third, bacteria may acquire several genes for a metabolic pathway which ultimately produces altered bacterial cell walls that no longer contain the binding site of the antimicrobial agent, or bacteria may acquire mutations that limit access of antimicrobial agents to the intracellular target site via downregulation of porin genes (Tenover, 2006). Thus, normally susceptible populations of bacteria may become resistant to antimicrobial agents through mutation and selection, or by acquiring from other bacteria the genetic information that encodes resistance. The last event may occur through one of several genetic mechanisms, including transformation, conjugation, or transduction.

2.9 The genus *staphylococcus*

Taxonomically, the genus s*taphylococcus* is in the bacterial family staphylococcacea. When these microorganisms are viewed under microscope after staining with Gram stain, they are spherical bacterial cells arranged in irregular clusters. These clusters take a shape of bunch of grapes. In Greek the term *stapyle* refers to a bunch of grapes. So these bacteria were named staphylococci. The genus *staphylococcus* is pathogen of man and animals. Normally, they are grouped into two on the basis of their ability to clot blood plasma; coagulase positive staphylococci and coagulase negative staphylococci.

2.10 Coagulase negative *staphylococcus* species

Coagulase-negative staphylococci (CNS), represent the majority of species, have been considered to be saprophytic or rarely pathogenic (Kloos and Schleifer, 1975). Over the last decade, however, CNS have been recognized as the etiological agents of a series of infectious processes, representing the microorganisms most commonly isolated from blood cultures (Huebner and Goldmann, 1999). About half of CNS species naturally colonize humans, and at present they are considered essentially opportunistic etiological agents, that prevail in numerous organic situations to produce severe infections (Bannerman, 2003).

The emergence of CNS as pathogens of different infections can be the result of the increasing use of invasive procedures such as intravascular catheters and prostheses in patients undergoing intensive treatment, immunocompromised patients, premature children, patients with neoplasias, and transplant patients (Kloos and Bannerman, 1994). The species that most frequently cause diseases in humans are *S. epidermidis* (bacteremia, infections due to implanted medical devices such as prostheses and catheters, infection of surgical wounds, peritonitis in patients on continuous peritoneal dialysis, osteomyelitis, endophthalmitis), *S. haemolyticus* (endocarditis, peritonitis, septicemia, and infections of the urinary tract, wounds, bone, and joints), and *S. saprophyticus* (urinary infections and septicemic processes). Other significant opportunistic pathogens include *S. hominis*, *S. warneri*, *S. capitis*, *S. simulans*, *S. cohnii*, *S. xylosus*, and *S. saccharolyticus* (Bannerman 2003). *S. lugdunensis* seems to be associated with endocarditis after implantation of prosthetic valves, with peritonitis, with soft tissue infection, and with vertebral osteomyelitis (Osmon *et al*., 2000).

2.11 Coagulase positive *staphylococcus* species

Coagulase positive staphylococci include *S. hyicus*, *S. schleiferi* subspecies *coagulanse* or *intermedius* and *S. aureus*. With exception of *S. aureus*, other coagulase positive species are rarely isolated from clinical specimens and therefore, less important in this current study. *Staphylococcus aureus* is a bacterium that belong to the Kingdom: Eubacteria, Phylum: Firmicutes, Class: Bacilli, Order: Bacillales and Family: staphylococcaceae, It is frequently found in the human respiratory tract and on the skin. Although *S. aureus* is not

always pathogenic, it is a common cause of skin infections (e.g. boils), respiratory disease (e.g. sinusitis), and food poisoning. Disease-associated strains often promote infections by producing potent protein toxins, and expressing cell-surface proteins that bind and inactivate antibodies.

2.12 *Staphylococcus aureus* and human food-borne intoxication

Staphylococcus aureus is a foodborne pathogen with ability to produce heat-stable enterotoxins during growth on different types of foods, such as meat and poultry products, eggs, cream-filled pastries, potatoes, and some salads (Doyle *et al*., 2011). Several staphylococcal associated enterotoxins have been known which are responsible for rapid onset of nausea and vomiting within few hours after ingestion. Fewer than 200 ng toxin is adequate to cause symptoms (Evenson *et al*., 1988). Although symptoms may be severe, they usually resolve within a day and serious complications, hospitalization, and death are rare, afflicting primarily the very young, the elderly, the chronically ill and those who have consumed a large amount of contaminated food (Doyle *et al*., 2011). There are fewer citations on vegetables as agent for transfer of *S. aureus*, yet, two outbreaks in restaurants in the U.S. in 2003 and 2005 were traced to carrots, green peppers, and leeks. Moreover, a survey of processed vegetables and sprouts in Korea reported that close to 11% were contaminated with *S. aureus* (Seo *et al*., 2010).

Staphylococcal enterotoxins cause foodborne illness in about 241,000 persons in the U.S. yearly (Griffin *et al*., 2011). Twenty-one outbreaks in the U.S. in 2007 (and 14 in 2008) and 291 outbreaks in Europe in 2008 (EFSA, 2010) were attributed to staphylococcal enterotoxin poisoning. Data from Centers for Disease Control and Prevention (CDC) indicate that nearly half of the 542 outbreaks occurring in 1998–2008 were associated with some type of meat. Seafood, potatoes/rice/noodles, vegetables/salads, combination foods, and dairy products were also reported as food vehicles (ECDC, 2010). Reported annual outbreaks during this 10-year period peaked in 2002 and then declined. Approximately 53% of reported outbreaks affected only 2 to 4 people, whereas only 6.7% of outbreaks involved more than 50 cases (Doyle *et al*., 2011).

In some occasions, ingestion of staphylococci can cause enteritis (enterocolitis). This occurs occasionally in infants, immunocompromised patients and others receiving large doses of antibiotics (Doyle *et al*., 2011). When normal human intestinal flora is depleted, *S. aureus* cells may grow in the intestines and produce enterotoxins that cause profuse diarrhea (Lin *et al*., 2010). *Staphylococcus aureus* has also been a problem for caterers and others involved in food preparation. According to several studies, *Staphylococcus aureus* is present in nasal passages or skin of about 50% of people and in intestines of about 20% of people in the general population (Acton *et al*., 2009). Thus, asymptomatic food handlers may harbour *S. aureus* and can contaminate food during preparation (Todd *et al*., 2008). If contaminated foods, for example salads or some desserts at a picnic, are left out at room temperature for extended periods, *S. aureus* may multiply and produce enterotoxins.

Staphylococcal food poisoning is poised to be greatly under reported (by about 25-fold) and under diagnosed (by about 29-fold) (Doyle *et al*., 2011). The short duration of illness and infrequent complications always bring it to the attention of health care professionals. *Staphylococcus aureus* has also been a food safety concern for meat producers and food processors for decades because it is abundant in the environment and often detected in air, dust, water, raw milk, other foods, and on environmental surfaces (Doyle *et al*., 2011). *Staphylococcus aureus* cells are killed by heat, if however, enterotoxins have already been produced in a food, the toxins will survive approved doses of irradiation and some thermal processes, including pasteurization (Rose *et al*., 1988; Genigeorgis, 1989).

2.13 *Staphylococcus aureus* and Non-foodborne human illness

Approximately 50% of healthy adults carry *S. aureus* in their nasal passages or on skin; about half of those persons are persistent carriers and the remainders are intermittent carriers (Frank *et al*., 2010). Some data indicate that host genetic factors (Ruimy *et al*., 2010) and competing microflora (Doyle *et al*., 2011) may affect persistence of colonization by *S. aureus*. A review of published data revealed that, overall, nasal, inguinal or axillary colonization with *S. aureus* was associated with a four-fold increase in serious infections (Safdar and Bradley, 2008). Asymptomatic carriage or colonization of individuals with *S. aureus* may be a risk factor for person-to-person transmission of these

bacteria and for contamination of food. Virtually all *S. aureus* isolates are coagulase positive, i.e. they produce an enzyme that causes clotting of blood plasma and produce many other virulence factors (besides enterotoxins) such as exfoliative toxins, toxic shock syndrome toxin, and leukocidins which are responsible for a variety of mild to severe skin and soft tissue infections and numerous serious infections, including endocarditis, endophthalmitis, osteomyelitis, meningitis, bacteraemia, pneumonia, and toxic shock syndrome (Doyle *et al*., 2011).

2.14 *Staphylococcus aureus* and Animal infections

Whether animals can be persistent carriers of *S. aureus* in a manner similar to humans is yet unknown. However, animals can intermittently harbour *S. aureus*. A recent study found that 10% of healthy dogs visiting a clinic for regular vaccinations harboured *S. aureus* (Rubin and Chirino-Trejo, 2010). Infections due to *S. aureus* have been reported in many mammal species as well as for wild and domestic birds and in some reptiles. Some animals are asymptomatic while others suffer respiratory, gastrointestinal, or skin and soft tissue infections. *Staphylococcus aureus* is a significant cause of mastitis in cows and small ruminants (Vanderhaeghen *et al*., 2010). Molecular analyses of isolates from different animals have revealed that there are some strains that appear to be host-adapted to a particular animal species (horses, cattle, pigs, sheep, chickens, or humans) and other strains can colonize multiple species of animals (Stegger *et al*., 2012). *Staphylococcus aureus* can be transferred between humans and animals, and frequently infections in companion animals can be traced back to their human caretakers (Rutland *et al*., 2009).

2.15 Routes of infection of *Staphylococcus aureus*

Staphylococci are spread among humans and animals and between species either by direct physical contact or indirectly through clothing, towels, equipment, food, air, or surfaces contaminated by infected or colonized persons or animals (Doyle *et al*., 2011).

2.15.1 Human route

Methicilin-Resistant *Staphylococcus aureus* was transmitted from a source, most commonly a patient's skin, to other patient skin areas, furniture, or note pads by the hands of healthcare workers in 22 of 24 cases (Stiefel *et al*., 2011). In one case a doctor entering an intensive care unit with MRSA on his/her hands contaminated a notes trolley near a

patient (Ludlam *et al*., 2010). MRSA transmission in a UK hospital was audited by swabbing patients' skin and their environment and also the hands of healthcare workers. Another study found that the frequency of transfer of MRSA from the skin of a colonized patient to a gloved hand was 40% (Stiefel *et al*., 2011). Hospital outbreaks of MRSA have been traced to lax hygiene practices among healthcare workers, and MRSA outbreaks in the community often occur in groups of people living in close quarters where they may transmit MRSA through direct physical contact (Doyle *et al*., 2011). A cluster of CA-MRSA cases (strain USA300) in the Netherlands occurred in a beautician, her customers, family members, and contacts. Skin treatments (waxing) performed by the beautician were identified as the likely mode of transmission (Huijsdens *et al*., 2008).

2.15.2 Airborne transmission

Methicilin-Resistant *Staphylococcus aureus* was detected in all of 57 samples taken of the air in pig fattening facilities. MRSA constituted about 0.1% of mesophilic bacteria detected in the air samples (Schulz and Hartung, 2009). MRSA is present in the nose and on the skin and is shed into the environment by infected or colonized people and animals, indicating that airborne transmission is a possible route for infection. MRSA strains, identical to clinical isolates from patients, were detected in the air of hospital rooms (Gehanno *et al*., 2009).

2.15.3 Animal contact

Methicilin-resistant *Staphylococcus aureus* (LA-MRSA ST398) was first described in pigs in the Netherlands in 2003 (De Neeling *et al*., 2007). Subsequent studies reported detection of this strain among farmers, and a survey indicated that human carriers of ST398 were 12.2–19.7 times more likely to be pig or cattle farmers than to work at other jobs (Van Loo *et al*., 2007). Whereas the overall number of MRSA infections in the Netherlands appears to have stabilized, an increasing percentage of MRSA infections in the country are caused by this livestock-associated strain, even among people without known exposure to pigs or veal calves. Total MRSA isolates submitted to the national laboratory in the Netherlands in 2008 numbered 2693. Of these, 42% were identified as the LAMRSA ST398 strain as compared to 30% in 2007 and 14% in 2006. Only 29% of people surveyed indicated contact with live pigs or veal calves (Haenen *et al*., 2010). A

recent study in Germany found that 86% of farmers and 45% of veterinarians exposed to pigs with ST398 also carried this strain. However, it was not readily transmitted from the workers to others as only 4–9% of family members and other close contacts tested positive for ST398 (Stegger *et al*., 2012). Horses may be colonized or infected with an uncommon horse-adapted MRSA strain, CMRSA-5, and several studies have reported this strain in horse farmers and veterinarians (Abbott *et al*., 2010). Companion animals may also be carriers of MRSA. MRSA was first detected in a companion animal in a ward cat in a geriatric rehabilitation unit in England. The cat was apparently infected by a resident and then served as a reservoir spreading the infection to other human residents (Scott *et al*., 1988). HA-MRSA strains have been detected in therapy dogs and cats visiting human long-term care facilities and may be a souce of infection to residents (Lefebvre and Weese, 2009). Similar MRSA strains have been detected in dogs and their owners, but surveys of dogs or humans colonized with MRSA have demonstrated that only a small number of human-dog pairs are infected with the same MRSA strain (Faires *et al*., 2009). Evidence indicates that pets can acquire MRSA from humans and that the reverse is also true.

2.15.4 Contaminated equipment and surfaces

Transmission of MRSA in healthcare facilities can occur by touching contaminated surfaces. Experiments have shown that gloved hands can pick up MRSA from bedrails, call buttons, tables, and phones at a frequency of 45% (Stiefel *et al*., 2011). Community-associated MRSA strains on contaminated needles have been transmitted among illicit drug users. Numerous reports have detailed outbreaks in high school and collegiate athletes where MRSA was detected on equipment and surfaces in athletic facilities as well as on towels and clothing (Bowers *et al*., 2008, Buss *et al*., 2009). Patients with end-stage renal disease (ESRD) are particularly vulnerable to invasive *S. aureus* infections because their blood must be treated using dialysis machines at least three times per week to remove toxins. These patients are frequently hospitalized; receive long courses of antibiotic treatment, and 14% die annually as a result of infections. Incidence of invasive MRSA was estimated at 45.2 cases/1000 population among dialysis patients, the highest for any patient population and about 100 times greater than incidence in the general population (Collins *et al*., 2007). An increasing proportion of MRSA infections in ESRD patients are

due to community-associated MRSA strains (Johnson *et al*., 2006). Surfaces in both homes and healthcare facilities may harbor MRSA (Scott *et al*., 2008). For example, MRSA was detected on surfaces in 7 of 25 ambulances tested (Semmons *et al*., 2010). A recent study of persons with MRSA in veterans' hospitals revealed that illicit drug users were more likely to be infected with USA300 (CA-MRSA) than non-drug users (Kreisel *et al*., 2010). Non-sterile equipment was cited as the cause of CA-MRSA infections in tattoo recipients in several states (Loeffler *et al*., 2010).

2.15.5 Contaminated food

Methicilin-resistant *Staphylococcus aureus* does not appear to be transferred readily from meat to meat handlers. It was not detected on hands or in noses of 89 persons working in cold meat processing facilities or institutional kitchens in the Netherlands even though 14% of samples of meat (veal, pork, chicken) that they worked with did contain MRSA. Most of the MRSA isolates were identified as ST398, livestock-associated MRSA (Doyle *et al*., 2011). However, MRSA strains have been detected in meat and may also be present in a variety of other foods. The origin of these contaminants has been traced to infected / colonized food handlers in some outbreaks (Jones *et al*., 2002). Studies have demonstrated that meat can also become contaminated during slaughter and processing of animals carrying MRSA (Beneke *et al*., 2011). To date, there have been only two reported outbreaks associated with MRSA-contaminated food (Doyle *et al*., 2011). The first was a community outbreak of foodborne illness caused by CA-MRSA occurred in Tennessee in 2000 (Jones *et al*., 2002). Identical MRSA isolates were recovered from 3 ill persons, the coleslaw they purchased from a convenience store deli, and the nose of a food handler at the convenience store. This strain produced enterotoxin C. The second reported outbreak of MRSA occurred in a Dutch hospital and affected 27 patients and 14 healthcare workers from 1992 to 1993, resulting in five deaths. Epidemiological investigations indicated that a colonized food handler apparently contaminated food (a peeled banana tested positive for MRSA) served to hospital patients, and some nurses may have inadvertently spread the bacteria to different wards (Kluytmans *et al*., 1995). In some surveys, MRSA detected on meat was identified as the livestock- associated strain, ST398 (Doyle *et al*., 2011).

2.16 Origin of methicillin resistance

Methicillin-resistant strains of *S. aureus* are clonal descendents from the few ancestral strains that acquired *mecA* (Kreiswirth *et al*., 1993). How *mecA* was acquired by Methicillin-resistant staphylococci is not known, but transposition is a plausible mechanism. The presence of one or more copies of the IS*431* element (also known as IS*257*, a putative mobile element often associated with genes encoding a variety of resistance determinants) within *mec*, inverted repeats at the ends of *mec*, and identification of two opening reading frames within *mec* that may encode recombinases all suggest that *mecA* and its associated DNA are mobile elements (Gillespie *et al*., 1987; Skinner *et al*., 1988; Archer *et al*., 1994; Stewart and Holt, 1963; Hiramatsu *et al*., 1996). The ß-lactamase plasmid may provide a temporary insertion site for the *mec*-containing transposon. There is one report, somewhat controversial because it has never been confirmed, of a *mecA* containing transposon, Tn*4291*, residing in an insertion site on the ß-lactamase plasmid pI524 (Trees and Iandolo, 1988).

2.17 History of staphylococcal resistance

S*taphylococcus aureus* has historically been a major human pathogen and continues to be one of the most commonly implicated bacteria causing human disease throughout the world. Before the widespread use of penicillin in the late 1940s and 1950s, staphylococcal septicemia was associated with an extremely high mortality rate (Smith and Vickers, 1960). Penicillin dramatically improved the treatment of this infection; however, penicillin-resistant strains were noticed by several investigators shortly after their detection (Fleming, 1942, Hobby *et al*., 1942). Penicillin-resistant *S. aureus* (PRSA) rose to prominence in the hospital setting in the 1950s and 1960s. PRSA strains were discovered in the community shortly after they were found in hospitals, making hospital control of PRSA essentially meaningless within two decades of the strains' appearance (Michael, 2000). Within the past 20 years, over 90% of North American community and hospital isolates of *S aureus* have been found to be penicillin resistant. The development of beta-lactamase-resistant Penicillins such as Methicillin and Oxacillin in the early 1960s once again revolutionized the treatment of staphylococcal infections. Within a year of their release, however, resistant *S aureus* strains were reported (Barber, 1961; Jevons, 1961) and outbreaks of MRSA infections were described on several continents within

several years (Maranan *et al*., 1997).Over the next 30 years, MRSA emerged as a near ubiquitous nosocomial pathogen.

The prevalence of *S. aureus* infections being caused by MRSA as reported by the National Nosocomial Infection Surveillance system in the United States has been steadily increasing, from 2.4% in 1974, 5% in 1981, 29% in 1991 to 43% in 1997 (Haley *et al*., 1982, Panlilio *et al*., 1992). Furthermore, the percentage of hospitals treating patients with MRSA infections is also increasing. In a survey of Society for Healthcare Epidemiology of America members in 1990, 97% reported having managed patients with MRSA in their hospitals. While the prevalence of MRSA is increasing, it is increasing at a slower rate than did PRSA in the 1950s and 1960s. It is tempting to predict that MRSA will follow a course similar to that of PRSA, namely that rapid and widespread colonization of people outside of the hospital milieu will result in MRSA becoming the predominant phenotype causing human disease. Such an outcome would obviously have a profound effect on hospital infection control practice and on the empirical use of Vancomycin therapy for community-acquired staphylococcal infections. The resulting increased use of Vancomycin would in turn have grave implications for the selection of other multi-drug-resistant organisms such as vancomycin-resistant enterococci and vancomycin intermediate *S. aureus*, both of which are selected for by vancomycin use (Eliopoulos, 1997; Fridkin, 2001). Before making such grave predictions, however, it is important to question whether the epidemiology of PRSA and MRSA are truly comparable and examine critically the evidence suggesting that MRSA may be becoming a pathogen in the community.

2.18 Heterogeneous resistance

A distinctive feature of methicillin resistance is its heterogeneous nature (Matthews and Stewart, 1984; Hartman and Tomasz 1986), with the level of resistance varying according to the culture conditions and ß-lactam antibiotic being used. The majority of cells in heterogeneous strains (typically 99.9% or more) are susceptible to low concentrations of ß-lactam antibiotic, e.g., 1 to 5 mg/ml of methicillin, with only a small proportion of cells (e.g., 1 in 106) growing at methicillin concentrations of 50 mg/ml or greater. Most clinical isolates exhibit this heterogeneous pattern of resistance under routine growth conditions.

Heterogeneous strains can, however, appear homogeneous (i.e., 1% or more of cells grow at 50 mg of methicillin per ml) under certain culture conditions, such as growth in hypertonic culture medium supplemented with NaCl or sucrose or incubation at 30°C (Sabath and Wallace, 1974). Addition of EDTA (pH 5.2) or incubation at 37 to 43°C favors a heterogeneous pattern and may suppress resistance entirely. These changes in expression of resistance with different culture conditions are transient and entirely phenotypic. Passage of a heterogeneous strain in the presence of ß-lactam antibiotic alters the resistance phenotype by selecting for highly resistant mutant clones (Sabath and Wallace, 1974; Chambers and Hackbarth, 1987). These clones produce a homogeneous population of highly resistant cells that can grow at methicillin concentrations of 50 to 100 mg/ml. The trait tends to be unstable in these laboratory-selected clones. With repeated subculture in antibiotic-free medium, the proportion of highly resistant cells gradually diminishes and the original heterogeneous pattern reemerges. There are rare clinical isolates, with the COL strain of *S. aureus* being most extensively studied, and some laboratory-derived mutants that consistently are homogeneous despite repeated subculture.

2.19 Borderline resistance

Another type of methicillin resistance is that exhibited by so-called borderline (or low-level) Methicillin -resistant strains. These strains are characterized by methicillin MICs at or just above the susceptibility breakpoint (e.g., Oxacillin MICs of 4 to 8 mg/ml). Borderline strains may be divided into two categories on the basis of whether *mecA* is present. Borderline strains that contain *mecA* are extremely heterogeneous (Gerberding *et al*., 1991). These strains have a resistant subpopulation of cells, although it may be quite small, that can grow at high drug concentrations (Gerberding *et al*., 1991).

Borderline strains that do not contain *mecA* can be differentiated phenotypically from extremely heterogeneous *mecA*-positive strains in that highly resistant clones do not occur. Borderline resistance in *mecA*-negative strains has been hypothesized to result from modification of normal PBP genes or overproduction of staphylococcal ß-lactamase (Tomasz *et al*., 1989; McMurray *et al*., 1990). There is good evidence that modified PBPs can produce borderline resistance. Tomasz *et a*l. (1989) reported alterations of penicillin binding by PBPs 1, 2, and 4 in b-lactamase-negative, *mecA*-negative borderline clinical

isolates. Berger-Ba¨chi *et al*. (1986) found alterations of PBPs 2 and 4 in resistant mutants selected by passage in antibiotic. Kinetic assays demonstrated that penicillin binding by altered PBPs occurred more slowly (i.e., a reduced rate of acylation) and that release of bound penicillin occurred more rapidly (i.e., increased rate of deacylation) in resistant than susceptible strains (Chambers *et al*., 1994). These binding alterations are the result of point mutations in the penicillin-binding domain (Hackbarth *et al*., 1995). Over expression of PBPs (PBP 4 in particular) also can produce low-level resistance (Henze and Berger-Ba¨chi B 1996). The net effect of the changes in binding kinetics and PBP over expression is that more enzyme is unbound and free for cell wall synthesis in resistant than in susceptible cells when ß-lactam antibiotic is present.

The role of ß-lactamase overproduction in borderline resistance is less clear, although the mechanism is plausible. Because even ß-lactamase -stable ß-lactam antibiotics may be slowly hydrolyzable by staphylococcal ß-lactamase (Kernodle *et al*., 1989), overproduction of b-lactamase could result in borderline MICs. Culture conditions used to enhance Methicillin resistance also favor overproduction of ß-lactamase (Coles and Gross, 1967, Chambers, 1997). Borderline strains that are ß-lactamase hyperproducers are *mecA* negative, show relatively high levels of ß-lactamase activity in biochemical assays, and exhibit a lowering of MICs into the susceptible range upon addition of ß-lactamase inhibitors, such as clavulanate or sulbactam, or upon elimination of the ß-lactamase plasmid (McMurray *et al*., 1990). ß-lactamase hyperproducers belong almost exclusively to phage group 94/96 and possess a common 17.2-kb ß-lactamase plasmid that encodes a type A staphylococcal ß-lactamase ((McMurray *et al*., 1990). However, whether this ß-lactamase actually accounts for the observed resistance is not clear. Transformants with ß-lactamase plasmid from a borderline, overproducing strain can remain susceptible (Barg *et al*., 1991). Methicillinase activity distinct from plasmid-encoded staphylococcal ß-lactamase also has been reported in borderline ß-lactamase hyperproducers, suggesting that a novel ß-lactamase, rather than overproduction per se, may be important (Massida *et al*., 1992). However, the putative methicillinase gene has yet to be identified (Massida *et al*., 1992). Finally, PBP 2 sequence analysis of some ß-lactamase-hyperproducing borderline strains has revealed mutations identical to some of those found in a ß-lactamase-negative borderline strain that has modified PBPs (Cantoni *et al*., 1989,

Hackbarth *et al*., 1995). No clinical data suggest that the level of resistance expressed by *mecA*-negative borderline strains leads to treatment failure. Data from animal studies show that the semi synthetic penicillinase-resistant Penicillins are effective for infections caused by *mecA*-negative borderline strains (Chambers *et al*., 1989). Hence, other than the difficulty in differentiating *mecA*-negative from *mecA*-positive strains expressing borderline resistance, the phenomenon is probably of little, if any, clinical significance.

2.20 Heterogeneous and Homogeneous Resistance

The phenomenon of heterogeneous versus homogeneous resistance in wild-type strains is completely unexplained. The cell wall from heterogeneous strains is indistinguishable by high-pressure liquid chromatography assay from the cell wall of susceptible strains, further substantiating the conclusion that the known factors are not deficient in such strains (Chambers, 1997). Stably homogeneous clones selected after a single passage of a heterogeneous strain in a ß-lactam antibiotic have mutations at non-*fem*, non-*mec* loci, designated *chr**, indicating that high-level resistance can be achieved through multiple pathways (Ryffel *et al*., 1994). Introduction of *mecA* cloned from a heterogeneous strain into a *chr** or naturally occurring homogeneous background in which the resident *mecA* has been inactivated reproduces the homogeneous phenotype (Murakami and Tomasz, 1989, Ryffel *et al*., 1994). Cloning of a chromosomal element that confers homogeneous, high-level resistance to an Imipenem-selected mutant has been reported, and its characterization promises to shed light on this intriguing property (Chambers, 1997).

Although the *fem*, *aux*, and other factors appear not to explain heterogeneous or homogeneous resistance in wild-type strains (i.e., those that have been adequately characterized are present and functioning normally regardless of whether the phenotype is susceptible, heterogeneous, or homogeneous), a working model of resistance can be proposed based on an understanding of these factors. Heterogeneous strains may be deficient in a factor or lack a critical modification in a biochemical pathway, possibly for cell wall synthesis, that is important to the functions of PBP 2a, which, after all, is a "foreign" PBP. Homogeneous strains then arise from heterogeneous strains by ß-lactam antibiotic selective pressure favoring mutants whose genetic background allows for a fully functional PBP 2a (Hiramatsu, 1995, Suzuki *et al*., 1993).

2.21 Mechanisms of resistance and historical epidemiology of PRSA and MRSA

Beta-lactam antibiotics achieve bacterial killing by binding to penicillin-binding proteins (PBP), thus inhibiting the cross linking of the bacterial cell wall. The mechanism of penicillin resistance in staphylococci is well known and involves the production of beta-lactamase(s), which hydrolyzes the cyclic amide bond of the beta-lactam ring. It is clear that beta-lactamase-producing strains of *S. aureus* existed before the discovery of penicillin. Parker and Lapage, (1957) reported that the majority of isolates responsible for outbreaks of staphylococcal food poisoning before 1940 were, in retrospect, found to be penicillin resistant. The ultimate origin of staphylococcal beta-lactamase is unclear; however, it is believed that it may have an as yet unknown role in cell wall synthesis. The production of beta-lactamase in most strains is inducible by the presence of beta-lactam antibiotics; however, rare strains that constitutively produce beta-lactamase have been reported (Rosdahl and Rosendal, 1983).

The staphylococcal beta-lactamase is encoded by the *blaZ* gene, which is controlled by both a repressor gene (*blaI*) and an antirepressor gene (*blaR1*) (Rowland and Dyke, 1990; Wang *et al*.; 1991). These regulatory elements allow for the inducible expression of beta-lactamase should a beta-lactam antibiotic be present in the environment. In most *S. aureus* strains, the beta-lactamase gene and regulatory elements are located on an easily transferable plasmid (Chambers *et al*., 1995). Weber and Goering (1988) have reported a beta-lactamase transposable element, *Tn4201*, which is capable of movement between plasmid and chromosomal sites, and is equally well expressed in either insertion orientation. The percentage of *S aureus* strains that produce beta-lactamase by this mechanism is not known.

The mechanism of staphylococcal resistance to methicillin and other beta-lactam antibiotics is considerably different. MRSA strains produce a unique penicillin-binding protein, PBP2a, which has a much lower affinity for beta-lactam antibiotics (Hartman and Tomasz, 1984). The gene encoding for PBP2a has been named *mecA* and is incorporated into the chromosome of MRSA strains as part of a conserved 30 kb region termed *mec*. The origin of the *mecA* gene and *mec* DNA has also been the subject of intense investigation. It has been suggested that MRSA arose as a result of horizontal transfer of

mec-encoding DNA between *S. aureus* and coagulase-negative staphylococci at some point(s) in the past (Mussuer and Kapur, 1992, Archer *et al*., 1994). Other potential sources of the *mec* locus have recently been discovered: The MRSA PBP2a has a high degree of homology with PBP molecules produced by *Staphylococcus sciuri* (Wu *et al*., 1996, and Couto *et al*., 1996) and *Enterococcus hiriae* (Piras *et al*., 1993).

2.22 Methicilin-resistant *Staphylococcus aureus* in the hospital setting

Risk factors for the hospital acquisition of MRSA include prolonged hospitalization, stay in an intensive care unit, chronic diseases such as chronic renal failure and malignancy, prior exposure to antibiotics, surgery and contact with a patient known to be colonized or infected with MRSA (Boyce, 1998). MRSA infection is typically preceded by colonization of the anterior nares and skin. Other sites of potential colonization include the urine of patients with indwelling urinary catheters, the implantation sites of invasive devices and postoperative wounds. Once acquired, MRSA carriage, such as that of wild type *S. aureus*, is typically difficult to eradicate and carriage is often long term. One study involving patients associated with a tertiary care teaching hospital in the United States documented a median duration of MRSA colonization of more than 3.5 years after acquisition (Sanford *et al*., 1994). It is a common practice to discharge MRSA colonized patients who were isolated while hospitalized to their homes or long care facilities where contact precautions may not be practiced. Transmission of MRSA to close contacts when colonized patients are discharged home has been well documented but has rarely been associated with invasive disease (Reboli *et al*., 1990, Hicks *et al*., 1991, Michael, 2000). Similar transmission has been reported to occur in nursing homes (Muder *et al*., 1991, Bradley *et al*., 1991). The extent to which nosocomial strains of MRSA are then further transmitted into the community is essentially unknown.

2.23 Methicilin-resistant *Staphylococcus aureus* in the community setting

Defining true cases of community-acquired MRSA can be problematic because of the ambiguity associated with the definition of community-acquired infections. A community acquired infection has been traditionally defined as one that occurs within 48 to 72 h of hospitalization, unless it is clear that it was acquired during a previous hospitalization (Brachman, 1998). This definition has been used by hospital epidemiologists to classify

and study patients with nosocomial infections (i.e., those who acquired their infection at least 72 h after hospitalization) rather than to study community-acquired disease (Michael, 2000). There are several problems with this definition. Diseases such as HIV, hepatitis C and hepatitis B, which have long incubation periods, may not be taken into account and thus may be misclassified. In addition, the use of this definition to classify community-acquired MRSA cases is complicated by the fact that MRSA carriage can continue for years after acquisition (Michael, 2000).

A widely used, yet arbitrary, definition for prior hospitalization refers to hospitalization within six months to one year of the current admission. This may misclassify a substantial proportion of true nosocomial MRSA patients who have been colonized with MRSA for a prolonged period after a previous remote hospitalization. Thus, even remote hospitalization is an important variable that must be taken into account (Michael, 2000). Finally, many institutions classify MRSA colonization acquired in a nursing home or long term care facility as community acquired, which is misleading because such institutions and their patients are not representative of the general population. Defining a case as community acquired can be greatly aided by molecular epidemiological techniques such as ribotyping and pulsed field gel electrophoresis (PFGE). Should an MRSA isolate yield a restriction pattern distinct from known nosocomial isolates, this argues against nosocomial acquisition (Michael, 2000). With the above discussion in mind, there have been multiple reports of community-acquired MRSA infections from several countries published over the past two decades. Most of the earlier studies did not adequately control for prior hospitalization as a risk factor for MRSA colonization, hence 'community acquired' strains may in fact be misclassified nosocomial strains.

2.24 Global distribution of MRSA

The first report of community-acquired MRSA in the United States was published in 1982. Saravolatz *et al.* (1982) reported a case-control study of community-acquired MRSA infections in 24 intravenous drug abusers from Detroit who had been self administering Cephalosporins as prophylaxis against skin infections. The community-acquired MRSA isolates were shown to be the same phage type and have similar antimicrobial sensitivity patterns as the known predominant nosocomial isolate,

suggesting the outbreak was caused by a nosocomial strain. In the same year, another Detroit hospital reported 24 cases of MRSA endocarditis in intravenous drug abusers (Levine *et al*., 1982). In 1986, another outbreak of MRSA infections in intravenous drug abusers was reported, this time in Boston (Craven *et al*., 1986). Seven patients were described as having community acquired disease, but this was neither defined specifically nor was information provided regarding past hospitalizations. Moreno *et al*, (1995) reported that 58% of MRSA strains isolated from patients in Texas over a 21-month period were from the community, defined as MRSA isolated within 48 h of admission Moreno *et al*.; 1995). A case control study failed to reveal significant risk factors for the community acquisition of MRSA compared with Methicillin sensitive *S. aureus*.

Community-acquired MRSA was first reported in Canada in 1990. Taylor *et al.* (1990) reported a multi strain cluster of MRSA from a native community in Alberta. All admissions from the native community were screened upon admission to the hospital over two year and 5% of those screened were found to be positive for MRSA. However, 91% of positive patients had been hospitalized within the preceding 12 months. Embil *et al.* (1994) retrospectively studied all known cases of MRSA reported by five tertiary care teaching hospitals located on the Canadian prairies between the years 1990 and 1992. They noted that 85 (62%) of the isolates were found on admission screening and were classified as community-acquired using the traditional definition.

In Australia, MRSA has been known to be present in the eastern states since the mid-1970s (Pavillard *et al*., 1982) and an eastern MRSA strain was reported to cause outbreak in Western Australia in the early 1980s (Pearman *et al*., 1985). Following this outbreak, Western Australian hospitals remained remarkably free of MRSA until Udo *et al.* (1993) reported the emergence of a community-based strain of MRSA in Western Australia. May *et al.* (1993) described 62 cases of invasive MRSA infections from 15 French hospitals over a one-year period. Less than 10% of cases were considered to be community acquired when community acquired was defined as isolation of the organism within 48 h of admission. Simpson *et al.* (1995) reported a family outbreak of MRSA in England following the development of an MRSA otitis media by a child hospitalized for the insertion of tympanic tubes. Both parents were injection drug users, and both developed

MRSA endocarditis with strains of the same susceptibility pattern and phage type as the child and the known nosocomial strain.

2.25 Methicilin-resistant *Staphylococcus aureus* in Africa

The report of MRSA epidemiology in African countries is scanty as compared to developed countries. Nevertheless, one of the first cases reported in the continent was in S. Africa in 1978 (Scragg *et al*., 1978) and a study at Khartoun hospital, Sudan, had prevalence of 11% (Musa *et al*., 1999). The surveillance study by Kesah *et al*. (2003) in part of African countries including Nigeria, Cameroon, Kenya and Algeria between 1996 and 1997 revealed rates in the range of 21-30% prevalence. The prevalence rates in North Africa (e.g Algeria) and Malta have below 10% (Kesah *et al*., 2003), but as of 2011, Algeria had 45% (Antri *et al*., 2011). The results of similar studies conducted in different locations in Nigeria; Ilorin (34.7%), Calabar (36.4%) and Jos (43%) revealed varying results in the same country (Taiwo *et al*., 2004, Azeez *et al*., 2008, Ikeh, 2003). In Abidjan, the rate was 25% among inpatients (Akoua-Koffi *et al*., 2004) against 31.5% in Uganda (Ojulong *et al*., 2009).

2.26 Epidemiology of MRSA in Nigeria

The first report of MRSA in Lagos, Nigeria was documented by Rotimi *et al*. (1987) where 50.6% of *S. aureus* were resistant to methicillin. Several reports of MRSA from some tertiary health institutions in different regions of Nigeria such as Ibadan (Okesola *et al*., 1999), Jos (Ikeh, 2003), Zaria (Olayinka *et al*., 2003), Ilorin (Taiwo *et al*., 2004), Osogbo (Taiwo *et al*., 2008). The study by Adesida *et al*. (2005) in 10 hospitals in the north-central part during 1998–2002, revealed a prevalence of MRSA of 9%. Onanuga *et al*. (2005) reported MRSA prevalence (71.7%) in urine samples collected from healthy women volunteers in Abuja. In another study, Taiwo *et al*. (2005) in south-west, the prevalence of MRSA was 32% while another study was conducted during 2006–2007 in 2 hospitals in the south-west part of the country, assessing mostly wound samples from adult, pediatric, and neonatology patients, the prevalence of MRSA was 20%. Forty-seven percent and 53% of the isolates were community-and hospital-acquired, respectively. All MRSA isolates reported by these researchers were sensitive to vancomycin (disk diffusion with 30μg vancomycin or vancomycin broth dilution) with the exception of the studies by

Olayinka *et al*. (2005) and Onolitola *et al*. (2007) who reported 57.7% and 86% vancomycin intermediate resistance rates respectively among staphylococcal isolates from symptomatic and healthy adults in northern region of Nigeria. Over the last 20 years the incidence of both community-acquired (CA) and hospital-acquired (HA) *S. aureus* infections have increased, while antibiotic treatment is increasingly hampered by the spread of *S. aureus* strains, which are resistant to multiple antibiotics including methicillin (Harbarth *et al*., 2005; Witte *et al*., 2007). The prevalence of community associated MRSA in Nigeria is on the increase. Taiwo *et al*. (2005) reported 29% in Ilorin while Ghebremedhin *et al*. (2009) documented 47% in University College Hospital, Ibadan. The later authors maintained that the prevalence of MRSA in general and CA-MRSA might be underestimated due to limited number of patients use for the study. Fadeyi *et al*. (2010) screened 198 healthcare workers screened of which 104 had MRSA either in the nose, hand or both giving a carriage rate of 52.5%. They maintained that nasal carriage (38.9%) was higher than hand (25.3%) and that Doctors (22.7%) and Nurses (16.7%) were the predominant carriers.

In a retrospective study, conducted during 2009 in the northwest part of Nigeria, including several clinical specimens, the prevalence of MRSA was 11% (Nwankwo and Nasiru, 2011). Another study in the south-west part of Nigeria, which did not mention the exact study period, reported a prevalence of MRSA of 41% for among several types of samples (Terry *et al*., 2012) Most of these MRSA isolates (93%) were hospital-acquired. The PVL toxin was detected in 24% of the MRSA isolates. Finally, a study conducted in the north-eastern part of the country, which did not mention the study period, reported a prevalence of MRSA of 28% for various clinical specimens, with 43% of MRSA originating from outpatients and 57% from inpatients (Ghamba *et al*., 2012). Because, most of these studies were based on phenotypic method of methicillin detection, more researches based on molecular techniques will reveal the true epidemiology of MRSA in this country, although, there are few molecular works on MRSA in Nigeria (Shittu *et al*., 2009, Ghebremedhin *et al*., 2009).

2.27 *mec* DNA

An extra chromosomal DNA of size 30-50kb called mec which is absent in susceptible strains of staphylococci is present in methicillin-resistant strains (Hiramatsu *et al*.; 1996).It is usually located close to pur-nov-his gene cluster on the *S. aureus* chromosome (Kuhl *et al*.; 1978). *Mec* composition includes *mecA*, its regulatory elements (*mecI* and *mecR1*) and about 20-45kb mec associated DNA.

2.28 Methicillin resistance determinant (*mecA*)

mecA is an inducible 76-kDa PBP responsible for Methicillin resistance in *staphylococcus* species. There are no *mec*A elements in susceptible strains of *staphylococcus* species. On other hand, susceptible and resistant strains of *S. aureus* produce four major PBPs, PBPs 1,2, 3, and 4, with equivalent molecular masses of 85, 81, 75,and 45 kDa (Georgopapadakou and Liu, 1980; Reynolds, 1988). PBPs are membrane bound DD-peptidases that have originated from serine proteases, with similar biochemical activity as that of the serine proteases (Waxman and Strominger, 1983; Ghuysen, 1994). These enzymes are involved in the reaction that cross-links the peptidoglycan of the bacterial cell wall. ß-Lactam antibiotics are substrates which covalently bind to the PBP active-site serine, thus hindering the action of the enzyme at concentrations that are equivalent to the MICs. PBPs 1, 2, and 3, have high affinity for most ß-lactam antibiotics and are required for cell growth and for the survival of susceptible strains. Attachment of ß-lactams to these PBPs is lethal (Georgopapadakou *et al.*, 1986; Chambers and Sachdeva, 1990).

In methicillin-resistant strains, PBP 2a, with low affinity for ß-lactam antibiotics (Brown and Reynolds, 1980, Hartman and Tomasz, 1984, Hayes *et al*., 1981, Utsui and Yokota, 1985), substitutes for the essential functions of high-affinity PBPs at concentration of antibiotic that is otherwise lethal. *mecA* is highly conserved among staphylococcal species (Archer *et al*., 1994, Beck *et al*., 1986, Reynolds and Fuller, 1986; Chambers, 1987). The same penicillin binding motifs as those found in the penicillin-binding domains of high-affinity PBPs also are present in PBP 2a (Wu *et al*.; 1994), but the reason for its low affinity is unknown. The *mecA* promoter region and its regulatory genes are similar in sequence to the similar regions of the staphylococcal ß-lactamase (Matsuhashi *et al*., 1986, Song *et al*., 1987). The origins of *mec* are not well understood. A *mecA* homolog with

88% amino acid similarity to *mecA* of methicillin-resistant staphylococci has been identified in *Staphylococcus sciuri* (Wu *et al*., 1996).

2.29 Regulatory elements of methicillin resistance (*mecI* and *mecR1*)

mecI and *mecR1* are regulatory genes controlling *mecA* expression and are located immediately upstream from the *mecA* promoter (Tesch *et al*., 1990; Hiramatsu *et al*., 1992). The molecular organization, structure, function, and mechanism of regulation of these elements are similar to staphylococcal ß-lactamase regulatory elements, *blaI* and *blaR1*. *blaI* is a DNA-binding protein that represses ß-lactamase gene transcription while blaR1 is a signal-transducing PBP that in the presence of ß-lactam antibiotic leads to ß-lactamase gene transcription, although the definite sequence of event by which this occurs is yet uncovered. *mecI* and *mecR1* perform similar regulatory roles for *mecA.* Quite a number of clinical isolates before 1970 (the COL strain is an example) have deletions of the penicillin-binding domain of *mecR1* and the complete downstream *mecI* (Hűrlimann-Dalel *et al*., 1992; Suzuki *et al*., 1993; Archer *et al*., 1994). PBP 2a production in these isolates is constitutive, provided that the inducible ß-lactamase is absent in the strain. If an inducible ß-lactamase is present in strains with *mecR1* deletions, PBP 2a also is inducible because of the presence of plasmid-encoded ß-lactamase regulatory genes which co regulates PBP 2a production (Ubukata *et al*., 1989, Hackbarth and Chambers, 1993).

Strains isolated since 1980 do have regulatory gene deletions but exhibit *mecI* polymorphisms and *mecA* promoter mutations (Hiramatsu *et al*., 1996). Complete and fully functional *mec* regulatory genes seem to strongly repress the production of PBP 2a (Kuwahara-Arai *et al*., 1996, Ryffel *et al*., 1992). PBP 2a in such strains greatly delays production and relatively little amounts of gene transcript and thus of protein are produced, and they may assume methicillin susceptible. Insertional inactivation of *mec* regulatory elements in one such strongly repressed strain was shown to increase the production of PBP 2a and result in homogeneous resistance ((Kuwahara-Arai *et al*., 1996, Niemeyer *et al*., 1996). However, regulatory elements other than *mecI* and *mecR1* also are required for strong repression of PBP 2a, because the same *mecI* and *mecR1* elements may have different effects on *mecA* transcription and resistance phenotype depending upon the genetic background (Niemeyer *et al*., 1996). Moreover, provided that some PBP 2a is

produced, there is no clear relationship between amounts of PBP 2a, whether it is constitutive or inducible, and whether resistance is homogeneous or heterogeneous. Yet, it seems likely that *mecI* polymorphisms and *mecA* promoter mutations found in most clinical isolates of methicillin-resistant *S. aureus* reflect the selective pressure of ß-lactam antibiotics for mutants lacking strong repressor activity, so that the amount of PBP 2a produced is sufficient to offer a survival advantage. PBP 2a production in strains with *mecI* or *mecA* promoter mutations is under the control of both *mec* and ß-lactamase regulatory genes (Hackbarth and Chambers, 1993).

2.30 *mec*-Associated DNA

mecA, and its regulatory genes are encoded by approximately 5 kb of DNA that is itself found within 25 to 50 kb of additional DNA that may contain up to 100 open reading frames (Beck *et al*.; 1986, Hiramatsu *et al*.; 1996). Transposons and insertion sequences are also present, including Tn*554* (which contains *ermA*, the gene encoding inducible erythromycin resistance (Archer and Niemeyer, 1994; Hiramatsu *et al*.; 1996; Berger-Bachi, 1997). The distance between *mecA* and IS*431mec* is not constant due to mutation that may occur in this region. IS*431* is an extremely common insertion sequence in the staphylococcal chromosome and plasmids and can be associated with a lots of resistance determinants, including mercury, cadmium, and tetracycline resistance (Gillespie *et al*.; 1987; Matthews and Stewart, 1988; Skinner *et al*.; 1988). For example, the *aadD* gene, which encodes an enzyme for Tobramycin resistance, is located on plasmid pUB110 (Dubin *et al*.; 1991). The ability of IS*431* elements through homologous recombination to trap and cluster resistance determinants with similar IS elements explains the multiple drug resistance phenotype that is characteristic of methicillin-resistant staphylococci.

2.31 Staphylococcal cassette chromosome (*SCCmec*)

SCC*mec* elements are unique genomic islands that are found in staphylococcal species, have two essential components, the *ccr* gene complex (*ccr*) and the mec gene complex (*mec*) (Hiramatsu *et al*., 2001, Ito *et al*., 2003). The ccr gene complex is composed of ccr genes and surrounding open reading frames (ORFs). For now, three phylogenetically distinct *ccr* genes, *ccrA*, *ccrB*, and *ccrC*, have been identified in *S. aureus* with DNA sequence similarities less than 50%. The *ccrA* and *ccrB* genes that have been identified to

date have been classified into four allotypes. In general, *ccr* genes with nucleotide identities more than 95% are assigned to the same allotype, whereas *ccr* genes that belong to different allotypes show nucleotide identities between 60% and 82%. All *ccrC* variants identified to date have 87% similarity; thus, there is only one *ccrC* allotype. Several *mec* and *ccr* allotypes have been found among SCC*mec* elements, which has led to the following classification (Ito *et al*.; 2003): Type I SCC*mec*, carrying class B *mec* and Type 1 *ccr*; Type II SCC*mec*, with class A *mec* and Type 2 *ccr*; Type III SCC*mec*, with class A *mec* and Type 3 *ccr*; Type IV SCC*mec*, with class B *mec* and Type 2 *ccr*; and Type V SCC*mec*, class C2 *mec* and Type 5 *ccr*. MRSA has become increasingly prevalent in community- acquired infections (CDC, 1999; Suggs *et al*., 1999). SCCmec typing and genotyping have provided strong evidence for the independent origins of health care-associated MRSA and community-acquired MRSA (C-MRSA).

2.32 Methods in typing methicillin-resistant staphyloccoci

Diagnostic microbiology laboratories and reference laboratories are key for identifying outbreaks of MRSA. New rapid techniques for the identification and characterization of MRSA have been developed. However a number of alternative methods including broth based methods, chromogenic media, rapid screening kits, molecular assays and automated systems are increasingly being used. Isolation from screening swabs can be a lengthy procedure, due to the number of 'contaminating' organisms that are present in swabs from non-sterile sites. This notwithstanding, the bacterium generally must be cultured via blood, urine, sputum, or other body fluid cultures, and cultured in the lab in sufficient quantities to perform these confirmatory tests first. Consequently, there is no quick and easy method to diagnose a MRSA infection. Phenotypic detection of MRSA historically, has been a challenge right from its discovery in the 1960s. This problem has been attributed to heterogenous expression of methicillin resistance by MRSA strains which complicate its detection in clinical microbiology laboratories. Resistance can easily be missed if inappropriate or improperly stored β-lactam antibiotic is tested, if the incubation is too brief (e.g. 18 hours instead of 24 hours), if temperature of 37^{o}C is used or if the inoculum is too low (Thornsberry *et al*., 1973). On the other hand, methods to enhance sensitivity in detection of methicillin resistance, such as increase salt concentration of media and prolonged (48hours) incubation, can lead to erroneous categorization of

susceptible strains as resistant. Several methods for detection of methicillin resistance in the clinical laboratories and for which sufficient scientific data are available to judge their sensitivity and reliability are the disk diffusion, broth dilution, agar screen, Etest and several automated systems.

2.32.1 Disk diffusion susceptibility testing

This method, can be performed using 1µg oxacillin disk, 1 µg nafcillin disk, 5µg methicillin disk and very recently 10µg or 30µg cefoxitin disk.16 Incubation temperatures of 30–35^{o}C give similar result for oxacillin, methicillin and nafcillin though oxacillin gives the best standardized and reliable result because of its stability (Taiwo, 2009). The Clinical and Laboratory Standards Institute (formerly called National Committee for Clinical and Laboratory Standards) specifies optimum temperature of 35^{o}C for testing of staphylococci to oxacillin15 and full 24 hours incubation. For cefoxitin, high accuracy has been found using incubation temperature not more than 36^{o}C (Skov *et al*., 2006) and 18 hours incubation time. Recommended culture medium for disk diffusion susceptibility testing is Mueller-Hinton (MH) agar although Iso-Sensitest and Mannitol salt agar (MSA) have been reliably used. The sensitivity of detection of methicillin resistance in MRSA with 1µg oxacillin or 5µg methicillin disk in not supplemented with MH agar at 35^{o}C is 95-100% (Taiwo, 2009). One drawback of the disk diffusion test is that the use of standard inoculums (107/CFU) may not detect some hetero-resistant MRSA strains. In this case, high-inoculum (109/CFU) disk diffusion has been found useful in detecting these hetero-resistant strains.

2.32.2 Broth microdilution MIC susceptibility testing

The standard broth microdilution test uses bacterial inoculum of approximately 5 x 105 CFU/ml and cation supplemented MH broth containing 2% sodium chloride (Taiwo, 2009) to determine the minimum inhibitory concentration (MIC) of oxacillin for the isolate. Incubation is at 35^{o}C for 24 hours and oxacillin, methicillin or nafcillin can be used but cephalosporins and cloxacillin should not be used because they give inaccurate results. Although addition of 5% NaCl may slightly improve detection of resistance; at this concentration, higher rates of borderline or falsely resistant results occur for susceptible strains. Incubation for 18 hours renders the test too insensitive and because of

β-lactamase effect, incubation for 48 hours is not recommended (McDougal and Thornsberry, 1986). In most studies, sensitivity of detecting methicillin resistance by this method exceeds 95% and specificity approaches 100%.

2.32.3 E-test (Epsilom-test) MIC method

In this method, an E-test strip (AB Biodisk, Solna, Sweden) containing graded concentration of β-lactam antibiotic is aseptically placed on an agar plate, which entire surface has been inoculated with a standardized inoculum (0.5 Mc Farland standard) of the bacterium. The agar plate is incubated for 18–24 hours at 35– 37^{o}C and the MIC is read as the point of intersection between edge of zone of inhibition and the E-test strip. Oxacillin E-test for detection of methicillin resistance in *S. aureus* has been standardized for use with MH agar supplemented with 2% NaCl and incubation at 35^{o}C for full 24 hours but recently cefoxitin E-test strip, which has comparable sensitivity and specificity with oxacillin E-test, is performed with unsupplemented MH agar and incubation temperature of 35^{o}C or 36^{o}C for 18 hours (Skov *et al*., 2006). E-test method has been shown to have excellent correlation with broth microdilution and the agar screen method for the detection of MRSA.

2.32.4 Agar screen method

This is one of the earliest reference methods used to define methicillin resistance in staphylococci. In this method, a bacterial suspension (104CFU/ml) is inoculated or spotted onto agar containing a β-lactam antibiotic. Growth of any colony on this drug containing agar after 24 hours incubation is indicative of resistance. The most frequently used media is MH agar supplemented with 2-4% NaCl containing 6µg/ml oxacillin or 10µg/ml methicillin. The sensitivity of agar screen method for detection of MRSA approaches 100%. Incubation for 48 hours is not recommended because β-lactamase producing susceptible *S. aureus* may grow after 24 hours (McDougal and Thornsberry, 1986). Even with several methodologies under various culture conditions, the sensitivity and specificity of this technique are consistently high and correlate well with other reference methods.

2.32.5 Population analysis profile

In this method, the proportion of heterogeneous cells in an overnight broth culture of a particular isolate is calculated on the basis of the ratio of colony-forming unit (CFU) growing on agar plates containing a range of concentrations of β-lactam antibiotic such as methicillin or oxacillin, to the number of CFU growing on drug-free agar plates after 24 or 48 hours incubation. By plotting the number of CFU at each concentration tested, it is possible to classify isolates as belonging to four expression classes, depending upon the degree of heterogeneity or homogeneity of methicillin-resistant cells in the population. This method correlates well with the *mec* gene analysis and is a reference phenotypic method of detecting methicillin resistance (Taiwo, 2009).

2.32.6 Automated systems

Several automated systems for detection of methicillin resistance, mostly based on broth microdilution methods, are in use. These include Autobac, Sceptor, AutoMicrobic and Vitek GPS-SA Card systems (Putland and Guinness, 1985). Variable results have been reported regarding the accurate detection of methicillin resistance with these commercial kits due to changes in software over time, variations in reference methodology used and poor definition as to the relative distribution of isolates expressing homogenous and various degrees of heterogeneous resistance. Laboratories using automated systems should therefore use either a confirmatory reference method (e.g. disk diffusion, broth dilution, agar screen) or conduct a trial comparing automated system with a reference method to document accuracy for the strains present within a particular hospital or community.

2.32.7 Serotyping

Serotyping is based on the fact that the strains of same species can differ in the antigenic determinants expressed on the cell surface such as lipopolysaccharides, membrane proteins and capsular polysaccharides. Serotyping is performed using several serological tests such as bacterial agglutination, latex agglutination, co-agglutination, and enzyme labeling assays. The technique is reproducible but has poor discriminatory power. Reports show that 11 *S. aureus* capsular types have been identified but almost every strain of MRSA is capsular type 5 and 8 (Schlichting *et al.*, 1993). This method has a limited role in epidemiological investigation of MRSA.

2.32.8 Zymotyping

Zymotyping is based on the electrophoretic properties of esterase enzymes. MRSA strains have been shown to possess three esterase enzymes namely A, B and C. Each of them varies in its electrophoretic mobility. The enzymes are extracted from the strais and electrophoressed on polyacrylamide gel. Results are analysed according to the differences observed in the mobility of esterases in different strains. The technique is labour intensive, and shows poor discriminatory power for MRSA (Gaston *et al*., 1988; Schlichting *et al*., 1993).

2.32.9 Whole cell protein typing

In this method the proteins are extracted from the culture of a strain, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained to compare with those of other strains. A large number of bands are produced in whole cell protein electrophoresis of MRSA strains but the differences, even between unrelated strains are small leading to poor discrimination of MRSA strains (Gaston *et al*., 1988).

2.32.10 Phage typing

Phage typing technique was standardised by the International Subcommittee on Phage Typing of staphylococci (Blair and Williams, 1961). Strains are classified according to their susceptibility to a set of phages selected. An internationally accepted set of 23 phages is used for typing human strains of *Staphylococcus aureus*. It is important to follow internationally recommended protocols for propagation and maintenance of phages, and also for interpretation of results to achieve internationally comparable results (Marples and Rosdahl, 1997). The technique requires maintenance of biologically active phages and is available only at reference centres. This technique has a fair amount of reproducibility, discriminatory power, ease of interpretation and is cost effective, though it is time consuming and technically demanding. The main disadvantage of phage typing is that a high proportion of MRSA strains are not typeable (Coia *et al*., 1990; Mathur and Mehndiratta, 2000). Additional locally isolated phages have been tried by various centres to overcome the problem of non-typeability. Specific phages for MRSA were found to be useful in increasing the typeability and discrimination of MRSA strains (Mathur and Mehndiratta, 2000). Phage typing has been reported to be valuable in the identification of

known epidemic strains among endemic strains and is preferred as first line approach in epidemiological investigation of MRSA strains (Amorim *et al*., 2007; Murchan *et al*., 2004).

2.33 Genotypic method of detecting methicillin resistance

The 'gold standard' for MRSA detection/confirmation is one of the several genotypic methods for detecting the *mecA* gene that encodes the abnormal penicillin binding protein 2' (PBP 2') responsible for methicillin resistance. In the clinical laboratory, *mecA* gene can be detected using the three hours DNA hybridization probe (Skov *et al*., 1999). This is highly sensitive and specific and correlates with phenotypic susceptibility testing and other genotyping methods. However, the polymerase chain reaction (PCR) is now the most frequently used genotypic method to identify and confirm MRSA in clinical sample or culture media. This is done by amplifying internal fragments of *mecA* gene using a pair of closely spaced oligonucleotides (primers). Several PCR protocols are available for amplification and this procedure remains the confirmatory reference methodology for MRSA.

2.33.1 Plasmid profiling and Restriction endonuclease analysis of plasmids (REAP)

Plasmids are variable components of many staphylococcal genomes, classified into four and fifteen incompatibility groups and often responsible for antibiotic resistance ((Novick, 1990). For typing Methicillin resistant staphylococci, the isolated plasmid DNA is separated by agarose gel electrophoresis and the number of and size of plasmids are determined. This method complements the restriction analysis of plasmids in epidemiological studies allowing detection of possible loss or gain during the study. Since extra- chromosomal DNA shows high degree of variation, plasmid analysis is convenient for the study of relatively recent epidemiological relationships as against the chromosomal DNA which reflects more reliable relationships over a long period of time. In REAP analysis, plasmid DNA is (separately/or combine) digested with restriction suitable enzymes and the digests are electrophoresed through agarose gel and pattern of restriction fragments produced for each enzyme are used to determine the composite stain types. In Nigeria, Taiwo *et al*. (2005) used restriction enzyme analysis of plasmid (REAP) DNA to characterize an MRSA oubreak in University of Ilorin Teaching Hospital in 2001. The

investigators were able to characterize an epidemic MRSA strain with a unique REAP profile that was responsible for a short outbreak of infection in the hospital that year. The shortfall of this method is that some MRSA strains lack plasmids while in some, the plasmids are unstable and addition or lack of a plasmid may not necessarily designate a different strain. Plasmid fingerprinting has been largely replaced by more discriminatory genotyping methods. Due to instability of plasmids, the method shows only moderate reproducibility (Tenover *et al*., 1994) and hence an additional typing method is required for analysis (Hartstein *et al*., 1995).

2.33.2 Macrorestriction analysis

Total cellular DNA is isolated and digested with rare-cutting restriction enzyme e.g. SmaI or CspI in *S. aureus*. The restriction fragments usually in ranges of 1-700kb are generated which are separated by PFGE. Isolates with identical restriction profiles are assigned the same pulsotype while those that differ by one or two band shifts consistent with a single genetic event (e.g mutation resulting in loss or gain of restriction site, an insertion, a deletion or a chromosomal inversion) are assigned as subtypes. Isolates with three and more of such genetic differences are considered as different types. According to these criteria, genetic relationships among isolates are determined for epidemiological purposes (Tenover *et al*., 1995). In comparison with other typing methods, PFGE has shown an equal or greater discriminatory power (Maslow and Mulligan, 1996) and superior reproducibility (Tenover *et al*., 1997). PFGE has been used to determine interspecies and intraspecies genome variability in staphylococci (Tenover *et al*., 1995).

2.33.3 Selective restriction fragment hybridization

This method is used for detection of RFLP by Southern hybridization. Total DNA is isolated and digested with frequent- cutting enzyme e.g ClaI (Kreiswirth *et al*., 1993). The resulting fragments are separated with agarose gel electrophoresis, transferred to a nylon membrane and hybridized with radioactively or non radioactively labeled probes that target various genes or transpozons e.g *mecA*, Tn554, arg or aacA-aphD of *S. aureus* (Kreiswirth *et al*., 1993).

2.33.4 Gene-specific PCR

Amplification of conserved gene sequences by PCR reaction is used for identification of staphylococcal species and their genotypes. In *S. aureus*, the genes encoding 16SrRNA, factors essential for Methicillin resistance (*femA*, *femB* e.t.c) and staphylococcal thermonuclease (nuc) are frequently used for identification at species level (Chambers, 1997).

2.33.5 PCR-RFLP typing

A target DNA sequence corresponding to a specific gene or genomic region is amplified at high stringency using primers annealing to its terminal conserved regions. The amplicon is cut with restriction endonuclease and separated by agarose gel electrophoresis resulting in a specific RFLP pattern. This method is rapid, simple and reprocucible but has shown only moderate discrimination (Goh *et al*., 1992).

2.33.6 Multiplex PCR assay

This allows simultaneous amplification of several genes in one reaction mixture. It is use for detection of genes encoding various types of microbial surface components recognizing adhesive matrix molecule (MSCRAMMs), toxins or antibiotic resistance in staphylococci as well as SCCme element (Oliveria and de Lencastre, 2002).

2.33.7 Quantitative PCR

The real-time quantitative PCR system is based on the detection and quantitation of a fluorescent reporter whose signal increases in the direct proportion to the amount of PCR product in a reaction. General methods for quantitative detection of the amplicon, DNA-binding agents and fluorescent probes including TagMan ^{R} technology , molecular Beacons^{TM} and fluorescence resonance energy transfer (FRET) probes have been used in the study of staphylococci (Edwards *et al*., 2001; Yang *et al*.,2002).

2.33.8 Intergenic spacer length polymorphism (tDNA-ILP)

This method uses an internal transcribed spacer- PCR method based on the different distances separating conserved tRNA genes in staphylococcal species (Welsh and McClelland, 1992). Since tRNA genes contain sequence motifs that are highly conserved

among the eubacteria, consensus tRNA gene primers can be designed to explore intergenic length polymorphisms between species (Maes *et al*., 1997).

2.33.9 Arbitrarily primed PCR (AP-RAPD)

This is based on the use of short oligonucleotide primers with an arbitrary sequence under low stringency PCR conditions (Welsh and McClelland, 1990). Information on the target sequence is not required. A strain-specific DNA fingerprint comprised of amplified DNA fragments of various sizes is obtained. The discriminatory power varies according to the number and sequences of the arbitrary primers and amplication conditions, but it is inferior to PFGE in typing of MRSA strains (Saulnier *et al*., 1993). The disadvantages include lack of consensus rules for interpretation of results and reproducibility (Struelens *et al*., 1993) as well as evaluation based on virtual rather than by using gel analysis software (Burr and pepper, 1997). This method is adequate for rapid comparative typing but less suitable for library typing in surveillance programs.

2.33.10 DNA sequencing

Nucleic acid sequencing is a direct method for the detection of DNA sequence polymorphism. It is the most sensitve method with high discriminatory power. Its disadvantage is the time required and the cost of the procedure currently limiting its use. In staphylococci, like other bacterial species, most often ribosomal genes are sequenced for the identification purposes (Sasakki *et al*., 1997). Nucleotitde sequences are highly conserved and therefore, make it possible to determine phylogenetic relationships (Ludwig and Schleifer, 1992).

2.33.11 Multilocus sequence typing (MLST)

This is an investigatory tool in many studies of *S. aureus* evolution and epidemiology, and has had a large impact on the field of bacterial strain typing. This provide a new approach to molecular epidemiology that can identify and track the global spread of virulent or antibiotic-resistant isolates of bacterial pathogens using internet (Enright and Spratt, 1999). This technique is a highly discriminatory method of characterizing bacterial isolates on the basis of the sequences of 450bp internal fragments of seven housekeeping genes (Maiden *et al*., 1998) : carbamate kinase (EC 2.7.2.2; gmk), shikimate 5-dehydrogenase (EC1.1.1.25; aroE), glycerol kinase (EC 2.7.1.30; glpF), guanylate kinase

(EC 2.7.4.8; gmk), phosphate acetyltransferase (EC 2..3.1.8; pta), triose-phosphate isomerase (EC 5.3.1.1; tpi) and acetyl-coA C-acetyltransferase (EC 2.3.1.9; yqiL). MLST is useful for identification of MRSA and MSSA clones among isolates from patients with serious community and hospital acquired infections (Enright *et al*., 2000). It is also suitable for studying both evolution of MRSA pandemic clones (Oliveira *et al*., 2001 and 2002) and local epidemiology (Huygens *et al*., 2002). It also, has high discriminating power and reproducibility. However, its procedure is technically demanding and expensive.

2.33.12 DNA microarrays

This provides considerable acceleration in molecular biology assays. Oligonuclotides anchored on the membrane or on the glass slide hybridize with labeled PCR products or with cDNA. The result is recorded by an apparatus- the scan is digitized and analyzed using appropriate software. The high density microarrays containing thousands of oligonucleotides constituting about 90% of the S aureus genome are used in the study of deletions in open reading frames (Somerville *et al*., 2002) and regulation of the virulence factor production (Dunman *et al*., 2001). The microarray containing 16SrDNA probes can be used for species identification of bacteria (Wilson *et al*., 2002).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site and sample collection

The study area is Ibadan, south-west Nigeria. Ibadan is the capital of Oyo state and is located 3°5´ East of the Greenwich meridian and latitude 7°23´ north of the equator (Lawal *et al*., 2009). Clinical specimens which consist of wound swabs, ear swabs, eye swabs, high vagina swabs, throat swab and semen collected from patients admitted to University College Hospital (U.C.H) Ibadan and sent to Microbiology department for processing were used for the study. All clinical specimens were collected by Medical Officers on duty. However, community samples were collected from skin and nares of apparently healthy individuals in University of Ibadan, Air, bedrail, tables and walls of private suits within U.C.H, and waste water drainages around student union building and Microbiology Department University of Ibadan. Commercial sterile swab sticks were used to collect samples from nares and skin of humans and private suit surfaces. The water samples were collected in sterile sampling bottles. All samples were transported to the laboratory for processing.

3.2 Media preparation

Media used were Mueller-Hinton Agar (for sensitivity)**,** blood agar (BA), Mannitol Salt Agar (MSA), Nutrient Agar (NA), Tryptone Soya Agar (TSA) and nutrient broth. All the media were prepared according to the manufacturer's instructions by weighing the specified amount of each medium into 100ml of distilled water. The media were then homogenized autoclaved at 121^{o}C for 15munites. The sterilized media were allowed to cool to 45^{o}C before dispensing into petridishes to solidify. The petriplates were kept in refrigerator at 4^{0}C until when needed.

3.3 Isolation procedure

All specimens and samples on swab sticks were streaked on prepared plates of Mannitol Salt Agar and incubated at 37^{o}C for 18-24hours. Colonies showing yellow colouration or whitish color were picked and sub-cultured on TSA, BA or NA and incubated at 37^{o}C between 18-24 hours. For waste water samples, 1ml of the sample were serially diluted (4-fold) and 1ml of final dilution was plated out on Mannitol Salt Agar. Morphologically distinct colonies were sub-cultured on fresh plates. Pure colonies of bacteria stains obtained were stored on nutrient agar slants at 4^{o}C for subsequent studies.

3.4 Gram staining and Microscopy

About 20µl of sterile water was dropped on clean grease free slide and smeared with a fresh bacterial growth colony. This was heat-fixed by passing over a flame. The dried smears were covered with drops of crystal violet stain for 30-40 seconds. These were rapidly washed off with clean water. The slides were then covered with solution of Lugol's iodine for 30-40 seconds. Again, they were washed with clean water. Thereafter, the slides were decolorized with 95% ethanol for few seconds to remove the primary stain and washed immediately with water. The slides were subsequently counter stained with two drops of safranin solution for 30 seconds followed by rinsing with water. The back of the slides were cleaned and placed in a draining rack for the smear to air-dry. Each Gam stained cells was viewed first with X40 objective to check the distribution of cells, and then with oil immersion objective to look for the irregular clusters (Monica, 2002).

3.5 Tube coagulase test

Three test tubes were taken and labeled "test", "negative control" and "positive control". A serial dilution (1 in 10) of human plasma with physiological saline was made. In each tube 0.5 ml of the diluted human plasma was dispensed. Subsequently, the tube labeled test was added with 0.1 ml of overnight broth culture of test bacteria. The tube labeled positive control was also treated with 0.1 ml of overnight broth culture of known *S. aureus*. However, the tube labeled negative control was added with only 0.1 ml of sterile broth. All the tubes were incubated at 37^{o}C and observed up to four hours. Positive result was indicated by gelling of the plasma, which remains in place even after inverting the

tube. Test that remained negative after four hours at 37°C, was kept at room temperature for overnight incubation.

3.6 Catalase test

Two drops of hydrogen peroxide (3% H_2O_2) were dropped on a slide and a sterile wire loop made of platinum was used to pick a colony from overnight growth culture of the test organism and then mixed. An immediate bubbling indicates positive result.

3.7 Haemolysis Test

Mueller-Hinton agar plates were prepared according to the manufacturer's instructions. Thereafter, 10ml of human blood was mixed with 90ml of Mueller-Hinton medium (10%) and the mixture was allowed to solidify. After drying, the Petriplates were inoculated with each organism using a sterile wire loop. The plates were incubated at 37°C for between 18-24hrs. Haemolysis (clearing of zone around the growth area of bacterial colonies) and non-haemolytic (non-clearing of zones around the colonies) was checked.

3.8 Aerobic acid production from sugar utilization

The sugar solution was prepared by weighing 5.0g of each sugar into 1000ml of broth containing 0.08g of methyl red as an indicator (Appendix IV). The sugar solution was homogenized on hot plate and 5ml of the homogenized solutions were distributed into test tubes containing Durham tubes (inverted position). The sugar solutions were sterilized using autoclave at 121°C for 10minutes. Thereafter, the solutions were allowed to cool and then inoculated with the test organisms while one without inoculation served as control. The inoculated sugar solutions and the control were incubated for between 24-72hours at 37°C. The results were recorded after incubation. The interpretative criterion for sugar utilization was change in colour of the indicator from red to yellow colouration.

3.9 Standardization of inoculum size

Bacterial slant were sub-cultured and incubated on nutrient agar plates overnight. Thereafter, Mueller-Hinton broths were prepared according to the manufacturer's instructions and inoculated with single colony of the test organisms from the overnight plate culture and again incubated overnight at 37°C. Overnight broth cultures of the test

organisms were diluted with sterilized distill water to match the 0.5McFarland standard. This suspension had approximately 1 to 2 × 10^{8} CFU/ml.

3.10 Application of disc and interpretation of result

A sterile swab stick was dipped into the adjusted suspension and pressed firmly on the lower wall of the tube. This was streaked over the surface of an already prepared Mueller-Hinton Agar plates (as described above) and the antibiotic discs: Vancomycin (30µg), Methicillin (10µg), Cefoxitin (10µg), Oxacillin (1µg) (Oxoid®) and Cotrimoxazole (25µg), Chloramphenicol (10µg), Cloxacillin (5µg), Erythromycin (5µg), Gentamicin (10µg), Streptomycin (10µg), Augmentin (30µg) and Tetracycline (10µg) (Abtek®) were applied onto the inoculated plates maintaining a distance of 30mm edge to edge. For methicillin resistance detection, the Mueller-Hinton Agar medium was supplemented with 2% NaCl (2g of salt/100ml distilled water). The plates were incubation at 35°C (Cefoxitin, Oxacillin and Methicillin) and 37°C (other antibiotics) for 24hour. The diameter of the inhibition zones was measured to the nearest millimeter using ruler.

3.11 DNA Extraction by lysis method

The method described by Pitcher *et al*. (1989) was modified by using 10mM Tris-HCL, pH 8 as lysis buffer in place of 5M guanidine thiocyanate. Nutrient broths were prepared according to the manufacturer's instructions and a colony of each bacterial strain was then inoculated. The inoculated broths were incubated overnight at 37°C. Overnight culture of each isolate was transferred into a 1.5ml Eppendorff tube and centrifuged for 5 minutes at 13,000rpm.The sediment was washed twice in normal saline and re-centrifuged at 13,000rpm at 10°C for 15minutes. This was followed by addition of 200µl of lysis buffer (10mM Tris-HCL, pH 8) and vortexed. This was incubated for 37°C for 2 hours. After incubation, cell lysis was carried out by addition of lysis buffer (400µl) containing 50mM Tris-HCL, 100mM EDTA, 1% SDS, pH 8 and 1mg/ml of proteinase-K. The content was mixed by inverting the tubes and then incubated at 50°C in water bath for 1hour. After digestion with proteinase-K, 500µl of a mixture of phenol and chloroforms (1:1 v/v) was added and emulsified by vigorous shaking for 30 second. The phases were separated by centrifugation at 13000rpm at 4°C for 15 minutes. The aqueous (top) phase was removed and placed in another tube. The high-molecular-weight DNA in the aqueous phase was

precipitated by addition chloroform: chloroform: isoamyl alcohol in a ratio of 24:1 v/v and mixed by invasion. The precipitated DNA was washed twice in 80% ethanol. The DNA was air-dried and suspended in 100ml of distilled water. This was placed in 56^{o}C water-bath for at least 30 minutes.

3.12 DNA Extraction by boiling method

This method of DNA extraction was described by Perez-roth *at al*. (2001) was also employed. Colonies from an overnight culture of the organisms on Tryptone Soya Agar (TSA) were emulsified in sterile double distilled water (100µl) contained in 1.5ml Eppendorff tubes until turbidity equivalent to 0.5 McFarland standards was obtained. These were transferred into a heating block set at 100^{o}C for 15minutes. After boiling, the mixtures were then centrifuged for 5minute at 2400rpm. The supernatant were transferred into fresh sterilized Eppendorff tubes and stored at -20^{o}C in a refrigerator until when required.

3.13 Purity determination

The purity of the DNA was checked using an automated Nanodrop spectrophotometer (3300) connected to a computer. This procedure involved blanking the machine first with sterile distilled water which has optical density of zero. Subsequently, 20µl of the extracted DNA samples was transferred into the optical sensitive aperture of the machine (loading chamber). The machine arithmetically gave the required purity as well as the quantities of each DNA per micro liter. Samples with optical density in the range of 1.40-2.0 were used for PCR reactions. However, DNA samples of all isolates below this range of optical density were repeated.

3.14 Preparation of oligonucleotide primer solutions

All primers used were synthesized by Zigma-Aldrich, Germany (Table 1). In the laminar flow hood, solutions of the oligonucleotide primers (µM) were prepared by multiplying the concentration of the dried oligonucleotidede primers in nanomole of each forward and reverse primer (given by manufacturer in the primer data sheet) by ten. The products gave an equivalent of micro-litre of sterilized double distilled (molecular biology grade water) required to 100µM solution. After mixing the equivalent quantity of water, the solution was mixed on a vortex and centrifuged at 12000rpm for 5minutes at 4^{o}C. This was the

stock solution which was incubated 30minutes on ice. To avoid repeated thawing, 50µl (working solution) were taken from the stocked for PCR reactions while the stocked were kept in freezer at -20^{o}C

3.15 Preparation of PCR reaction solution

The 1X one Taq standard reaction buffer (BioLabs, New England) contains 20mM Tris-HCl, 1.8mM $MgCl_2$, 22mM NH_4Cl, 22mM KCl, 0.06% IGEPL CA-630, 0.05% Tween 20, pH 8.9. To prepare PCR mixture, 5µl standard reaction buffer was added to 0.5µl (10mM) concentration of each of the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP). Subsequently, the quantity of water, and DNA samples (1-2µl) was added. The forward and reverse primers (0.1-.0.25µl) Taq polymerase (0.125 U) was added last. The mixture was vortexed and then centrifuged in a mini-centrifuge 1200rpm for 10s.

3.16 Preparation of Agarose Gel Electrophoresis

Exaclty 1.20g weight of agarose powder was added to 150ml TBE buffer (1X:89mM Tris base,89mM boric acid, 2mM EDTA) in a conical flask and melted in a microwave oven for 2 minutes. The agarose solution was allowed to cool to 50 – 60^{o}C (5µl of a 10mg/ml solution of ethidum bromide was added at this stage), mixed by swirling and poured into a UV-transparent micro titre tray which contain forming comb. The gel was allowed stand for 10 minutes to solidify. This was transferred into the gel tank and covered with TBE buffer. Samples were loaded with loading buffer using a pipette into the wells. A standard molecular-weight DNA maker was then added to adjacent lane. Following electrophoresis at 100V (PCR) and 80V (plasmid) for 1hour gels was examined for bands by photo documentation system (TranUV, illuminator Biorad).

3.17 PCR amplification of 16S-rRNA gene of *staphylococcus* species

An aliquot of 2.0µl of DNA suspension was added to make up 25µl of PCR mixture consisting of 5µl standard reaction buffer (20mM Tris-HCl, 1.8mM $MgCl_2$, 22mM NH_4Cl, 22mM KCl, 0.06% IGEPL CA-630, 0.05% Tween 20, pH 8.9), a 0.5µl (10mM) concentration of each of the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 0.25µl of TsaG422 as forward primer and Tsag765 as the reverse primer (genus specific),16.875 of water and 0.125 U of *Taq* DNA polymerase. DNA

amplification was carried out on PCR system 9700 thermocycler with the following thermal cycling profile: Initial denaturation at 94^{o}C for 1minutes, denaturation 94^{o}C for 30 seconds, annealing 58^{0}C for 30 seconds, extention 68^{o}C for 1 minute and final extension 68^{o}C for 5minutes with a programmable period of 30 cycles. After PCR amplification, 10µl of PCR product was removed and subjected to agarose gel electrophoresis (see 3.16).

3.18 PCR amplification of universal 16S-rRNA gene of bacteria

A PCR mixture with the same cycling parameters as described in (3.17**)** was employed; however, S4D2E forward and reverse primers were used. After PCR amplification, 10µl of PCR product was resolved by agarose gel electrophoresis. The remaining amplicons were used for RFLP.

3.19 Restriction fragment length polymorphism (RFLP)

Ten (10) µl of each amplified product was mixed with 2.5 µl of 10X buffer, 11.2µl of H_2O and digested with 1.0 U of Hind III restriction enzymes. The mixture was incubated at 37°C for 1-2 h. The sizes of the restriction fragments were determined by agarose gel electrophoresis and photographed using photo documentation system. The interpretation criterion for identifying different strains is a single band difference.

3.20 PCR-RFLP by double digestion with restriction endonucleases

Ten (10) µl of each amplified product was mixed 2.5 µl of 10X buffer, 10.5µl of H_2O and digested with 1.0 U of Hind III and EcoRI restriction enzymes simultaneously. The mixture was incubated at 37°C for 1-2 h. The sizes of the restriction fragments were determined by electrophoresis and photographed using photo documentation system. The interpretation criterion for identifying different strains is a single or more band difference.

3.21 Identification of *S. aureus* using *femB* as gene target

A PCR mixture with the same cycling parameters as described in (3.17**)** but with *femB* as forward and reverse primers and annealing temperature, 45^{o}C were used. After PCR amplification, 10µl of PCR product was resolved by agarose gel electrophoresis.

3.22 Identification of coagulase negative staphylococci

S. haemolyticus, *S. capitis*, *S. warneri*, *S. epidermidis* and *S. saprophyticus* forward and reverse primers were used for their target gene sequences. An aliquot of 2.0µl of DNA suspension of each organism was added up to make 25µl of PCR mixture containing forward and reverse primers of each orgamism (Table 1), 5µl standard reaction buffer [20mM Tris-HCl, 1.8mM $MgCl_2$, 22mM NH_4Cl, 22mM KCl, 0.06% IGEPL CA-630, 0.05% Tween 20, pH 8.9)] and 0.5µl (10mM) concentration of each of the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP). Following the same thermal cycling profile as in (3.5.7**)** but with annealing temperatures corresponding to each primer pair was used. After PCR amplification, 10µl of PCR product was removed and subjected to agarose gel electrophoresis. Following electrophoresis at 80V for 1hour, gels was examined for bands by a photo documentation system.

3.23 Detection of resistance genes (*mecA*, *blaZ* and *vanA* genes) by multiplex PCR

A 100mM concentration of each primer was made according to the manufacturer's instruction. From this concentration, 50µl of 100mM of both forward and reverse primers were transferred into 1.5ml eppendorff tubes which served as working solution. An aliquot of 2.0µl of DNA suspension was added to 25µl of PCR mixture consisting of 5.0µl reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8)], a 0.5µl (10mM concentration) of each of the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 6.0µl (25 mM) $MgCl_2$ 0.25µl of forward and reverse (*blaZ*, *vanA* and *mecA*) primers, and 0.125 U of *Taq* DNA polymerase. Multiplex Polymerase Chain Reaction assays were carried out with a negative control containing all of the reagents without DNA template. DNA amplification was carried out using 9700 thermocycler with the following thermal cycling profile as shown below: Initial denaturation at 95^{o}C for 3minutes, denaturation 94^{o}C for 30 seconds, annealing 45^{o}C for 30 seconds, extension 72^{o}C for 30 seconds and final extension 72^{o}C for 7 minutes with a programmable period of 30 cycles. After PCR amplification, 10µl of PCR product was resolved by agarose gel electrophoresis as described before (3.16).

3.24 Detection of vancomycin resistance (*vanA* gene)

To confirm that *vanA* gene was absent in the isolates studied, a uniplex PCR was performed as below: An aliquot of 2.0µl of DNA suspension was added made up 25µl of PCR mixture (as in multiplex above), 0.2µl of each *vanA* forward and reverse primers, with the following thermal cycling (as in multiplex above) and was resolved by agarose gel electrophoresis.

3.25 Detection of *mecA* regulatory genes by multiplex PCR

An aliquot of 2.0µl of DNA suspension was made up to 25µl of PCR mixture containing 0.25µl of each forward and reverse primers with the same cycling parameters as in (amplification of 16S-rRNA gene of *staphylococcus* species) but with annealing temperature of 50^{o}C. PCR product was resolved by agarose gel electrophoresis.

3.26 Identification of chromosomal cassette (*SCCmec*)

The *SCCmec* was determined by using PCR amplification of a sequence covering the right junction of the *SCCmec* elements and the adjacent chromosomal region encoding the species-specific open reading frame (ORFX) according to the following the same protocols above but with annealing temperature of 43^{o}C. PCR product was resolved by agarose gel electrophoresis.

3.27 Detection of Panton-Valentine leukocidin (PVL)

An aliquot of 2.0µl of DNA suspension was made up to 25µl of PCR mixture as in multiplex PCR above with annealing temperature of 64^{o}C. PCR product was resolved by agarose gel electrophoresis.

3.28 Plasmid extraction using modified method (Mini Prep)

Mini Prep method of Lech and Brent (1987) was used as described below: Overnight broth culture of the organisms (1.5ml) was transferred into Eppendorff tubes and spinned for 1 minute at 13, 000rpm. The supernatant was decanted and then vortexed at high speed to re-suspend the cells. About 300µl of TENS solution (Tris 25mM, EDTA 10Mm, NaOH 0.1N and SDS 0.5%) was added and mixed by inversion for 3-5 minutes until the solution became sticky. A volume of 150µl of 3.0M sodium acetate (pH 5.2) was added and vortexed. This was followed by spinning for 5minutes in a micro-centrifuge to pellet

cell debris and chromosomal DNA. The supernatants were transferred to fresh eppendorff tubes and 900µl of ice-cold absolute ethanol was added. This was spinned for another 10 minutes to pellet plasmid DNA. The supernatants was discarded while the pellet was washed twice with 1ml of 70% ethanol and dried. The pellet was re-suspended in 40µl of distilled water.

3.29 Running plasmid on gel electrophoresis

Ten (10µl) microlitre of the extracted plasmid was mixed with a loading buffer. This was then transferred into already prepared agarose gel (0.8%) stained with ethidium bromide. The set up was run at 80V for one hour and photographed on a TranUV illuminator (Biorad). Plasmid size was determined by comparing with H111 lambda DNA marker.

3.10 Preparation of curing agent (acridine orange solutions)

Exactly 2.5mg of acridine orange was transferred into 10ml universal plastic bottle containing 5ml of distilled water. The solution was homogenized and served as working solvent (water) for preparation of nutrient broth. An equivalent weight of nutrient broth required to prepare 5ml of nutrient broth was weighed and transferred into screw capped glass bottle. The acridine orange solution was then poured into the bottle capped. The procedure was the same for preparing 0.25mg/ml and 0.125mg/ml except that 1.25mg and 0.625mg gram of acridine orange powder will be weighed. The medium was then autoclaved at 121^{o}C for 15munites.

3.31 Plasmid curing

Three different concentrations of acridine orange solution (0.5mg/ml, 0.25mg/ml and 0.125mg/ml) were prepared as described above. Each of the concentrations was used to prepare nutrient broth in 1.5ml eppendorff tubes. The broth were sterilized at 121^{o}C for 15minutes and allowed to cool. Each organism was inoculated into the different concentrations and then incubated for 72 hours. After incubation, the organisms were plated out onto nutrient agar and then sub-cultured into freshly prepared nutrient broth. This was incubated for 18-24 hours. Plasmid extraction was repeated to check for plasmid. Absence of plasmid after gel electrophoresis is an indication of plasmid lost. This method was described by Sheikh *et al*. (2003)

3.32 Statistical analysis

Susceptibility of the isolates to antibiotics was analysed by descriptive statistics and Chi-Square at $P \leq 0.5$.

Table 3.1: Primers used in this study including its sequences, target genes, expected size of genes and sources

Primers	Sequences 5'-3'	Target genes	Size (bp)	Sources
FemB F	TTA CAG AGT TAA CTG TAA CC	*femB*	651	Perez-orth *et al*., 2001
R	ATA CAA ATC CAG CAC GCT CT			
mecA F	GTA GAA ATG ACT GAA CGT CCG ATA A	*mecA*	310	Perez-orth *et al*., 2001
R	CCA ATT CCA CAT TGT TTC GGT CTA A			
mecR1 F	ACC AAA CCC GAC AAC TAC	*mecR1*	784	Sanjay *et al*., 2004
R	TTC ACA TGT GAT AGT TCA TGT AG			
mecI F	GGT TAT GTT GAA ACG AAA G	*mecI*	637	Sanjay *et al*., 2004
R	GGT GTT ATT ACA AGC ATT ATT G			
vanA F	CAT GAA TAG AAT AAA AGT TGC AAT A	*vanA*	3010	Clark *et al*., 1993
R	CCC CTT TAA CGC TAA TAC GAT CAA			
blaZ F	ACT TCA ACA CCT GCT GCT TTC	*blaZ*	173	Ho chan lee *et al*., 2001
R	TGA CCA CTT TTA TCA GCA ACC			
Shae F	GTT GAG GGA ACA GAT	*S. haemolyticus*	85	Tadayuki *et al*., 2007
R	CAG CTG TTT GAA TAT CTT			
TstaG422	GGC CGT GTT GAA CGT GGT CAA ATC A	Genus 16SrRNA of *Staphylococcus*	370	Morot-Bizot *et al*., 2004
Tstag765	TIA CCA TTT CAG TAC CTT CTG GTA A			
Scap F	GCT AAT TTA GAT AGC GTA CCT TCA	*S. capitis*	208	Tadayuki *et al*., 2007
R	CAG ATC CAA AGC GTG CA			
Swar F	TGT AGC TAA CTT AGA TAG TGT TCC TTC T	*S. warneri*	63	Tadayuki *et al*., 2007
R	CCG CCA CCG TTA TTT CTT			
S4D2 F	ACG GCT ACC TTG TTA CGA CTT	16SrRNA gene	1500	Ashraf *et al*., 2002
S4D2 E	AGA GTT TGA TCC TGG CTC AG			
rjmec F	TAT GAT ATG CTT CTC C	*SCCmec* genes	400	Cuny and Witte, 2005
ORFX1R	AAC GTT TAG GCC CAT ACA CCA			
S ep F	ATC AAA AAG TTG GCG AAC CTT TTC A	*S. epidermidis*	124	Martineau *et al*., 1996

Table 1 Continues: Primers used in this study including its sequences, target genes, expected size of genes and sources

	R	CAA AAG AGC GTG GAG AAA AGT ATC A			
S sap	F	TCA AAA AGT TTT CTA AAA AAT TTA C	*S. saprophyticus*	221	Tadayuki *et al*., 2007
	R	ACG GGC GTC CAC AAA ATC AAT AGG A			
Xyl	F	AAC G CG CAA CGT GAT AAA ATT AAT G	*S. xylosus*	539	Tadayuki *et al*., 2007
	R	AAC GCG CAA CAG CAA TTA CG			
Pvl	F	ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A	PVL gene	433	Kader *et al*., 2011
	R	GCA TCA AGT GTA TTG GAT AGC AAA AGC			

CHAPTER FOUR

RESULTS

4.1 Sources of isolates

In table 4.1, total of 55 clinical *staphylococcus* species were isolated of which *Staphylococcus epidermidis* from wound infections accounted for 36.4% (20/55), followed by eye infections 20.0% (11/55), then semen 14.5% (8/55), and ear 10.9% (6/55). Sputum, throat, soft tissue and high vagina swab (HVS) each had 1.8% (1/55) *S. epidermidis*. Only urethra specimen had *S. xylosus* 1.8% (1/55). In wound infection, 5.5% (3/55) *S. aureus* were isolated while 1.8% (1/55) *S. aureus* was recovered from eye and ear specimens. In community isolates (Table 4.2), *S. epidermidis* constituted the largest percentage, 96.2% (51/53), with 71.70% (38/53) recovered from human nostril, 17.0% (9/53) in waste water, 1.9% (1/53) on air, 1.9% (1/53) on skin and 3.8% (2/53) in private suite surfaces. *Staphylococcus aureus* 1.9% (1/53) was isolated in both nostril and private suite surfaces.

4.2 Morphological and biochemical characteristics staphylococci

On tryptone Soya Agar, colonies were opaque and some white, or creamy and occasionally yellow or orange. On blood agar, however, the colonies were white. The isolates under light microscope were gram positive, non-sporing and spherical in shape occurring singly, in pairs and in irregular clusters. The biochemical characteristics revealed that *staphylococcus* species vary in their metabolism to various sugars **(**Table 4.3a and 4b). All species tested were catalase-positive with few isolates being coagulase positive while others were coagulase negative. Some of the species were strongly haemolytic, some partially haemolytic while others non-hemolytic. Some species encountered in this study fermented glucose, fructose, galactose, mannose, sucrose and maltose very well but some failed to utilize mannitol. All failed to ferment xylose. There was variation in the ability to produce gas.

The isolates were tentatively classified into three group; (a) those that were coagulase positive, utilized all the sugars except xylose, and produced no gas (b) those that were coagulase negative and failed to utilize mannitol with variation in their haemolytic activities, and produced gas from lactose (c) those that were coagulase negative, but produce acid and gas from Galactose, Mannose, Maltose and Lactose (Table 4.3b). *Staphylococcus aureus* met the criteria to belong to the first group while *S. epidermidis* belongs to the second group. Because, gas production was never used to separate Staphylococci (literature search), *S. capitis* and *S. xylosus* were close in their sugar utilization pattern and were assigned to belong to either of the last group. However, by using scheme of Kloo and Schleifer (1975) which mainly used sugar utilization pattern for species discrimination, the isolates were classified into three groups. As shown in table 4a and 4b, 100% *S. epidermidis* produced acid from Glucose, Fructose, Galactose, Mannose, Maltose and Lactose and had no effect on Mannitol and Xylose, (group 1). *S. aureus* differed from *S. epidermidis* by its ability to utilize all the sugars except Xylose and coagulase production (Group 2) while *S. xylosus* was differentiated from *S. aureus* by its inability to produce coagulase (Group 3).

Table 4.1: Distribution of clinical isolates according to their sources

Sources	*S. epidermidis*	*S. xylosus*	*S. aureus*
HVS	1	0	0
Semen	8	0	0
Ear	6	0	1
Eye	11	0	1
Soft tissue	1	0	0
Sputum	1	0	0
Throat	1	0	0
Urethra	0	1	0
Wound	20	0	3
Total	49	1	5

Table 4.2: **Distributions of community isolates according to their sources**

Sources	*S. epidermidis*	*S. xylosus*	*S. aureus*
Air	1	0	0
Water	9	0	0
Nostril	38	0	1
Skin	1	0	0
Private suite surface	2	0	1
Total	51	0	2

Table 4.3a: Morphological and biochemical characteristics of isolates from community samples

Serial No	Isolate code	Source	Gram	Cat	Coa	Hem	GLU	FRU	GAL	MNO	MAL	LAC	MAN	SUC	XYL	Probable organism
1	Com1	nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
2	Com6	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
3	Com7	Nostril	+	+	-	+	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
4	Com8	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
5	Com10	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
6	Com11	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
7	Com12	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
8	Com16	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
9	Com18	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
10	Com19	Skin	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
11	Com26	nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
12	Com29	nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
13	Com38	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
14	Com39	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
15	Com40	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
16	Com41	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
17	Com43	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
18	Com45	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
19	Com46	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
20	Com49	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
21	Com52	nostril	+	+	+	+	+	+	+	+	+	+	+	+	-	*S. aureus*
22	Com55	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
23	Com56	Nostril	+	+	-	+	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
24	Com58	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
25	Com59	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
26	Com60	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
27	Com63	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
28	Com68	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
29	Com69	Nostril	+	+	-	+	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
30	Com80	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*

Table 4.3a Continues: Morphological and biochemical characteristics of isolates from community samples

31	Com81	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
32	Com82	Air	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
33	Com88	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
34	Com89	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
35	Com91	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
36	Com92	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
37	Com93	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
38	Com95	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
39	Com96	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
40	Com97	Nostril	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
41	Wat5	Water	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
42	Wat3	Water	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
43	Wat8	Water	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
44	Wat7	Water	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
45	Wat10	Water	+	+	-	+	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
46	Wat19	Water	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
47	Wat4	Water	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
48	Wat30A	Water	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
49	Wat9	Water	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
50	Wat4	Water	+	+	+	+	+	+	+	+	+	+g	+	+	-	*S. epidermidis*
51	C36	P Suit	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
52	C41	P Suit	+	+	+	+	+	+	+	+	+	+	+	+	-	*S. aureus*
53	C56	P Suit	+	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*

Keys

+= produce acid only on sugars, - = produce no acid only on sugar, Coa=Coagulase, Cat=catalase, Hem=Haemolysis, Gram= Gram reaction, GLU=Glucose, FRU=Fructose, GAL=Galactose, MNO=Mannose, MAL=Maltose, LAC=Lactose, MAN=Mannitol, SUC=Sucrose and XYL=Xylose, +g=gas production

.

Table 4.3b: Morphological and biochemical characteristics of isolates from clinical samples

	Isolate code	Sources	Gram	Coa	Hem	GLU	FRU	GAL	MNO	MAL	LACT	MAN	SUC	XYL	Probable organism
1	100	Wound	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
2	S10	Semen	+	-	+	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
3	131	Wound	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
4	149	Wound	+	-	+	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
5	S21	Semen	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
6	157	Eye	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
7	167	Ear	+	+	+	+	+	+	+	+	+	+	+	-	*S. aureus*
8	181	Eye	+	-	+	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
9	201	Wound	+	+	+	+	+	+	+	+	+	-	+	-	*S. aureus*
10	204	Wound	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
11	260	Wound	+	-	+	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
12	283	Eye	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
13	292	Wound	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
14	380	Sputum	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
15	413	Wound	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
16	S5	Semen	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
17	493	Eye	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
18	611	Wound	+	+	-	+	+	+	+	+	+	-	+	-	*S. aureus*
19	615	HVS	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
20	634	Eye	+	-	+	+	+	+	+	+	+	+	+	-	*S. aureus*
21	641	Ear	+	-	+	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
22	S20	Semen	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
23	S3	Semen	+	-	+	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
24	698	Ear	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
25	6N	Throat	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
26	S24	Semen	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
27	S12	Semen	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
28	781	Ear	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
29	798	Wound	+	-	+	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
30	821	Eye	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*

Table 4.3b Continues: Morphological and biochemical characteristics of isolates from clinical samples

31	S11	Semen	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
32	A	Eye	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
33	AA	Wound	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
34	ON	Wound	+	-	+	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
35	OO	Wound	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
36	650	Wound	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
37	652	Wound	+	+	-	+	+	+	+	+	+	-	+	-	*S. aureus*
38	146	Wound	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
39	667	Ear	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
40	995	Ear	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
41	779	Wound	+	+	-	+	+	+	+	+	+	+	+	-	*S. aureus*
42	48	Eye	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
43	681	Urethra	+	-	+	+	+	+g	+g	+g	+g	+g	+	-	*S. xylosus*
44	441	Wound	+	-	+	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
45	153	Wound	+	-	+	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
46	76	Wound	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
47	1079	Eye	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
48	4020	Eye	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
49	998	Wound	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
50	463	Wound	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
51	467	Ear	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
52	922	Wound	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
53	668	Eye	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
54	263	Soft tissue	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*
55	699	Eye	+	-	-	+	+	+	+	+	+g	-	+	-	*S. epidermidis*

Keys

+= produce acid only on sugars, -=produce no acid only on sugars, +g=produce acid and gas, Coa=Coagulase, Cat=catalase, Hem=Haemolysis, Gram= Gram reaction, GLU=Glucose, FRU=Fructose, GAL=Galactose, MNO=Mannose, MAL=Maltose, LAC=Lactose, MAN=Mannitol, SUC=Sucrose and XYL=Xylose.

4.3 Susceptibility to antibiotics

The phenotypic resistance test of the clinical isolate of staphylococci to methicillin indicated that 40 (72.7 %) of these species were resistant to antibiotics, comprising of 35 (63.6 %) *S. epidermidis*, 4 *S. aureus* (7.3 %) and 1(1.8 %) *S. xylosus*. While in community isolates, 33 (62.3 %) were resistant to antibiotics made of 32 (60.4 %) *S. epidermidis* and 1(1.9 %) *S. aureus*. In the clinical isolates of *S. epidermidis*, 30.9, 32.7, 34.5, 40.0, 41.8, 60.0, 76.4, and 89.1 % were resistant to Chloramphenicol (CHL), Vamcomycin (VAN), Streptomycin (STR), Erythromycin (ERY), Gentamycin (GEN), Tetracycline (TET) Cotrimoxazole (COT), and Cloxacillin (CXC) respectively (Table 5). Correspondingly, in community isolates of *S. epidermidis*, 28.3, 3.8, 32.1, 50.9, 26.4, 58.5, 90.6 and 92.5 % were resistant to these antibiotics. In the clinical isolates of *S. aureus*, 3.6, 5.5, 5.5, 7.3, 7.3, 7.3, 9.1 and 9.1 % were resistant to VAN, ERY, CHL, STR, GEN, TET, COT and CXC respectively. In community isolates, 1.9 % *S. aureus* were resistant to COT, CHL, ERY, GEN and STR respectively while 3.8 % were resistant to CXC. Among clinical isolates of *S. xylosus*, 1.8 % were resistant to all the antibiotics except CHL and STR. In Table 4.4, among the antibiotics used, there was no significant difference in the resistance patterns between the clinical and community isolates of *Staphylococcus* species except streptomycin and erythromycin ($p \leq 0.05$) (Table 4.4). However, the clinical isolates, *S. epidermidis* 10.9, 16.4, 18.2, 21.8 and 25.5 % were classified as intermediate for TET, GEN, CHL, STR and ERY while in community, 1.9, 22.6, 22.6, 30.2 and 35.8 % were found as intermidiate for TET, GEN, CHL, ERY and STR respectively (Table 4.5 and Table 4.6). Multiple resistance was also observed amongst the clinical and community isolates. In clinical isolates, 3 organisms (1 *S. aureus*, 2 *S. epidermidis*) were resistant to eight and seven different antibiotic classes respectively (Table 4.5) while in community isolates, only two isolates (2 *S. epidermidis*) were resistant to seven. Also, 13 (10 *S. epidermidis*, 2 *S. aureus*, 1 *S. xylosus*) and 8 (8 *S. epidermidis*) clinical isolates were resistant to six and five antibiotic classes respectively (Table 4.6). Correspondingly, 2 (2 *S. epidermidis*) and 14 (14 *S. epidermidis*) of community isolates were resistant the same number of antibiotics.

Table 4.4: Comparison of percentage resistance of clinical and community isolates to various antibiotics.

Antibiotics	Clinical						Community				
	S. epidermidis	%	*S. aureus*	%	*S. xylosus*	%	*S. epidermidis*	%	*S. aureus*	%	*P*
COT	42/55	76.4	5/55	9.1	1/55	1.8/53	48/53	90.6	1/53	1.9	0.457
CHL	17/55	30.9	3/55	5.5	0	0	15/53	28.3	1/53	1.9	0.411
CXC	49/55	89.1	5/55	9.1	1/55	1.8/53	49/53	92.5	2/53	3.8	-
ERY	22/55	40.0	3/55	5.5	1/55	1.8/53	27/53	50.9	1/53	1.9	0.025[a]
GEN	23/55	41.8	4/55	7.3	1/55	1.8/53	14/53	26.4	1/53	1.9	0.487
STR	19/55	34.5	4/55	7.3	0	0	17/53	32.1	1/53	1.9	0.019[a]
AUG	47/55	85.5	5/55	9.1	1/55	1.8/53	50/53	94.3	2/53	3.8	0.842
TET	33/55	60.0	4/55	7.3	1/55	1.8/53	31/53	58.5	0	0	0.296
VAN	18/55	32.7	2/55	3.6	1/55	1.8/53	2/53	3.8	0	0	0.281
MET	35/55	63.6	4/55	7.3	1/55	1.8/53	32/53	60.4	1/53	1.9	0.209

Keys:

P= level of significance a= significant at P≤ 0.05, COT= Cotrimoxazole, CHL= Chloramphenicol CXC =Cloxacillin, ERY =Erythromycin, GEN =Gentamicin, STR =Streptomycin TET =Tetracycline, VAN =Vancomycin, MET=Methicillin

Table 4.5: Antibiotic resistance pattern of clinical isolate of *staphylococcus* species

Antibiotics	Sources & frequency of organism type
COT,CHL,CXC,ERY,GEN,STR,TET,VAN	Wound(1) S.ep ,eye(1) S.au, semen(1) S.ep
COT,CHL,CXC,ERY,GEN,STR,TET	Wound(1) S.ep, ear(1) S.ep
COT,CHL,CXC,ERY,STR,TET,VAN	Wound(1) S.au
COT,CHL,CXC,ERY,GEN,TET	Ear(2) S.ep Wound(2) S.ep
COT,CXC,ERY,GEN,STR,TET	Semen(1)wound(2) S.au & S.ep, urethra(1) S.xy eye(1) S.ep
COT,CHL,ERY,GEN,STR,TET	Ear(1) S.ep
COT,CHL,CXC,GEN,STR,TET	Ear(1) S.ep semen(1) S.ep, Wound(1) S.ep
COT,CXC,STR,TET,VAN	semen(1) S.ep
COT,CHL,CXC,ERY,TET	Eye(1) S.ep
COT,CHL,CXC,GEN,TET	Wound(1) S.ep
COT,CXC,ERY,GEN,TET	Semen(1) S.ep
COT,CHL,CXC,ERY,GEN	Wound(1) S.ep
COT,CXC,ERY,GEN,STR	Wound(1) S.ep
COT,CXC,GEN,STR,TET	Eye(1) S.ep
COT,CXC,ERY,TET,VAN	Soft tissue(1) S.ep
COT,CXC,GEN,STR	Wound(1) S.au
COT,CXC,STR,TET	Wound(2) S.ep ,eye(1) S.ep
COT,CXC,TET	Wound(6) S.ep
COT,CXC,ERY	HVS(1) S.ep, throat(1) S.ep
CXC,ERY,AUG	Semen(1) S.ep
COT,CHL,CXC	Ear(1) S.ep, Eye(1) S.ep
COT,CXC,GEN	Eye(1) S.ep

Keys:

COT= Cotrimoxazole

CHL= Chloramphenicol

CXC =Cloxacillin

ERY =Erythromycin

GEN =Gentamicin

STR =Streptomycin

TET =Tetracycline

VAN =Vancomycin

S.au=*S. aureus*

S. ep=*S. epidermidis*

S.xy=*S. xylosus*

Table 4.6: Antibiotic resistance pattern of community isolates of *staphylococcus* species

Antibiotics	Sources & frequency of organism type
COT,CHL,CXC,ERY,GEN,STR,TET	Nostril(2) S.ep
COT,CHL,CXC,ERY,GEN,TET	Nostril (1) S.ep
COT,CHL,CXC,ERY,STR,TET	Waste water (1) S.ep
COT,CXC,ERY,STR,VAN	Nostril (1) S.ep
COT,CHL,CXC,ERY,TET	Waste water (1) S.ep, Nostril (1) S.ep
COT,CHL,CXC,ERY,GEN	Nostril (1) S.ep
COT,CHL,CXC,ERY,VAN	Nostril (1) S.ep
COT,CXC,ERY,GEN,TET	Outdoor air(1) S.ep, Waste water (1) S.ep, Nostril (3) S.ep
COT,CXC,ERY,STR,TET	Waste water (1) S.ep
COT,CXC,GEN,STR,TET	Nostril (1) S.ep
COT,CXC,GEN,STR,TET	Nostril (3) S.ep
COT,CHL,CXC,TET	Waste water (1) S.ep, Nostril (1) S.ep
COT,CXC,STR,ERY	Nostril (2) S.ep
COT,CXC,ERY,TET	Nostril (1) S.ep
COT,ERY,STR,TET	Nostril (1) S.ep
COT,CXC,ERY,STR	Nostril (1) S.ep
COT,CXC,STR,TET	Nostril (2) S.au &S.ep
COT,CXC,GEN,TET	Nostril (1) S.ep
COT,CXC,GEN	Waste water (2) S.ep
COT,CHL,ERY	Waste water (1) S.ep
CXC,ERY,STR	Waste water (1) S.ep
CXC,ERY,TET	Waste water (1) S.ep
CXC,GEN,STR	Nostril (1) S.ep
COT,CXC,TET	private suite surface(1) S.ep, Nostril (3) S.ep
CHL,CXC,ERY	private suite surface(1) S.au,
COT,CXC,ERY	Nostril (2) S.ep
COT,CXC,STR	Nostril (3) S.ep
COT,ERY,TET	Nostril (1) S.ep
COT,CHL,TET	Nostril (1) S.ep

Keys:

COT= Cotrimoxazole

TET =Tetracycline

VAN =Vancomycin

CHL= Chloramphenicol

CXC =Cloxacillin

ERY =Erythromycin

GEN =Gentamicin

STR =Streptomycin

S.au=*S. aureus*

S. ep=*S. epidermidis*

4.4 Amplicon of 16S-rRNA of *staphylococcus* species

All the staphylococcal isolates gave positive amplification with the genus specific primers targeting its 16S-rRNA gene. Plate 4.1 shows the gel electrophoresis of amplification products. This procedure eliminated non-staphylococcus bacteria that may interfere with the identification processes. The primers amplified the expected product size (370bp).

4.5 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

The 16SrRNA genes of all isolates showing positive amplification with genus specific primers were re-amplified with universal bacterial 16SrRNA primers (Plate 4.2) and then digested with restriction enzyme Hind 111. The Hind 111 classified these isolates into three distinct RFLP patterns; 1250-1050-450bp, 1050-450 and 1125bp (plate 4.3).

4.6 Confirmation of the chromosomal distinct RFLP

The primers targeting *S. aureus* and other coagulase negative staphylococci (*S. saprophyticus*, *S. warneri*, *S. capitis*, *S. haemolyticus*, *S. epidermidis* and *S. xylosus*) were used individually to amplify their corresponding genes. The primers targeting *S. epidermidis* (Plate 4.4), *S. aureus*, (plate 4.5) and *S. xylosus* (plate 4.6) showed positive amplification. The RFLP patterns representing *S. epidermidis*, (lane 4, 5 and 6), *S. aureus*, (lane 1 and 9) and *S. xylosus* (lane 8) as shown in Plate 4.3 below. In an attempt to ensure species discrimination, amplicons previously produced (370bp) were digested with Hind 111 restriction enzyme and this could not yield any differential result with respect to speciation (plate 4.7). However, when both Hind 111 and EcoR1 were simultaneously used to digest the amplicon, the products were classified into two distinct RFLP patterns with molecular weight 220-50bp (pattern 1) and 370bp (pattern 2). By comparing the pattern of digestion on gel with known species, background pattern 1 represents *S. aureus* (lane 1, 8, 13, 18, 20 and 33) and pattern 2 Coagulase negative staphylococci (*S. xylosus*, and *S. epdermidis*) as shown in Plate 4.8.

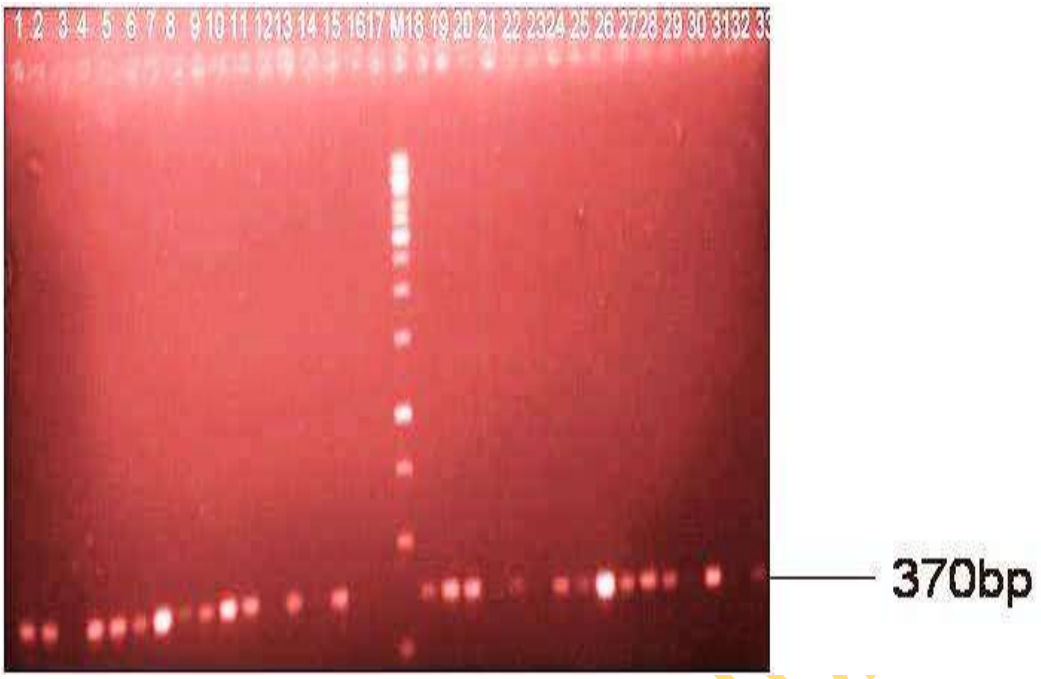

Plate 4.1: Gel electrophotogram showing amplicon of *staphylococcus* species when its 16SrRNA genus specific primer was used in PCR

Lane	Isolate	lane	Isolate	Lane	Isolate
M	Molecular marker (1kbp)	14	Non-staphylococcal isolate	28	S10
1	*S. aureus* (ATCC29535)	15	wat8	29	260
2	131	16	Non-staphylococcal isolate	30	S12
3	Com93	17	Non-staphylococcal isolate	31	Com60
4	Com56	18	167	32	Com93
5	Com7	19	S20	33	611
6	667	20	634		
7	C36	21	Non-staphylococcal isolates		
8	C41	22	Com88		
9	S24	23	Non-staphylococcal isolates		
10	100	24	4020		
11	S17	25	181		
12	Non-staphylococcal isolate	26	641		
13	779	27	Com1		

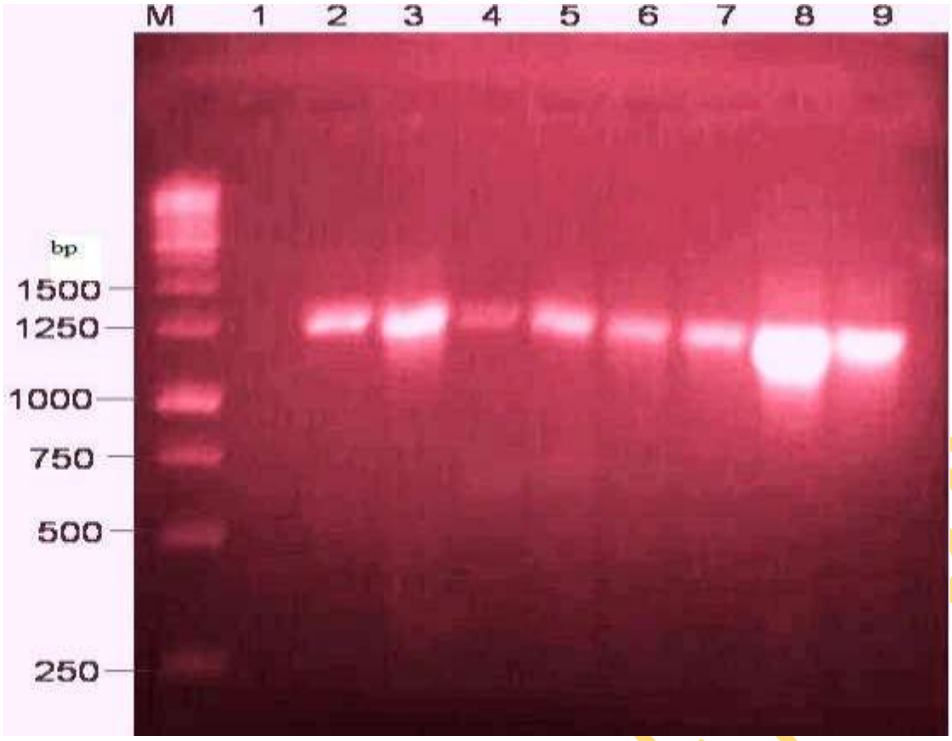

Plate 4.2: Gel Gel electrophotogram showing amplicon of *staphylococcus* species when 16SrRNA universal primer was used in PCR

Lane	Isolates
M	Molecular marker (1kb)
1	Buffer (Control)
2	*S. aureus* (ATCC29535)
3	260
4	AA
5	A
6	131
7	634
8	38
9	652

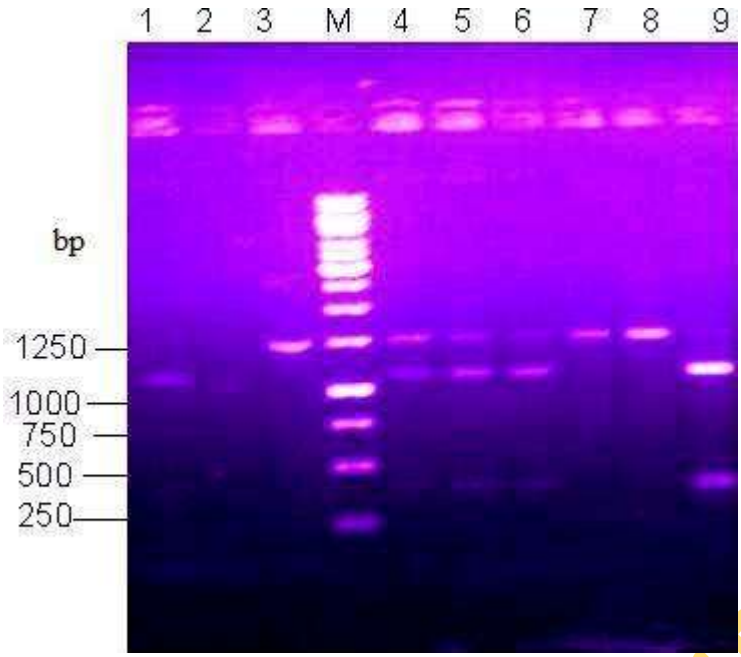

Plate 4.3: Gel Electrophotogram showing RFLP patterns of *staphylococcus* species after digestion with Hind 111 enzyme

Lane	Isolates
M	Molecular marker (100bp)
1	*S. aureus* (ATCC29535)
2	181
3	undigested amplicon
4	380
5	260
6	AA
7	undigested amplicon
8	681
9	634

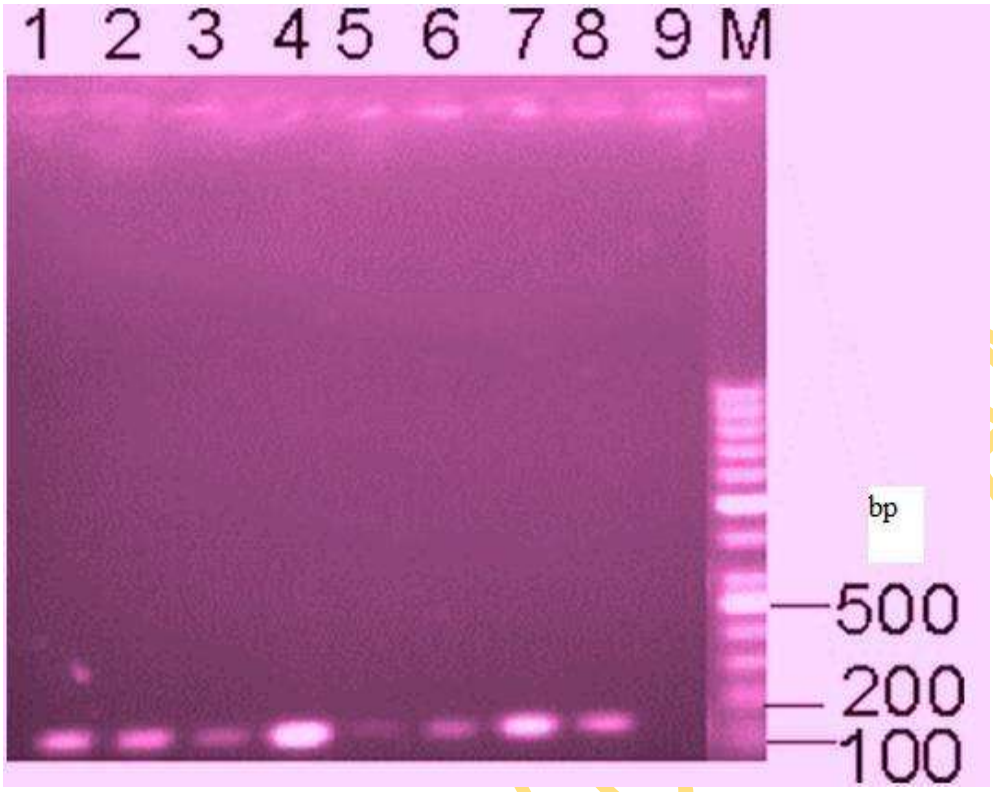

Plate 4.4: Gel Electrophotogram showing amplicon of *S. epidermidis* using its species specific primers in PCR

Lane	Isolates
M	Molecular marker (100bp)
1	131
2	Com29
3	Com11
4	Com74
5	Com7
6	AA
7	S12
8	Com49
9	*S. aureus* (ATCC29535)

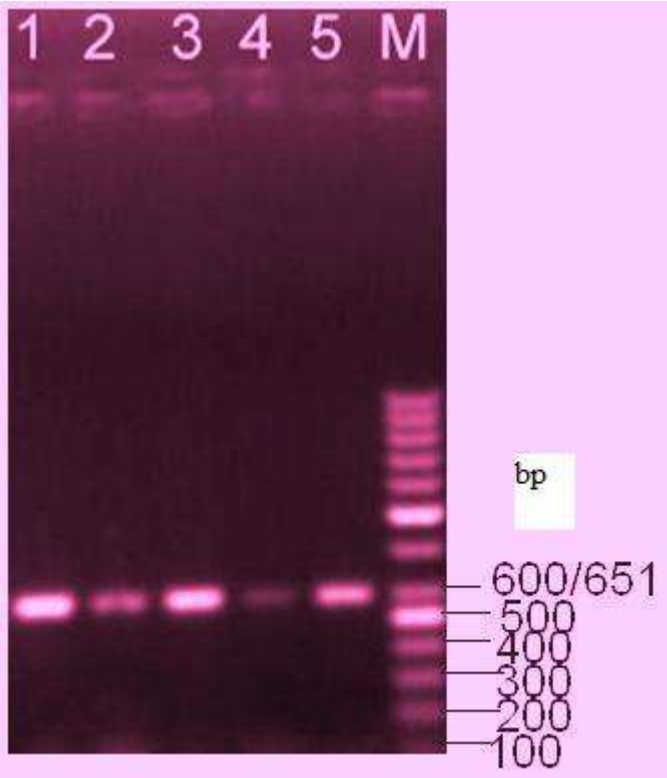

Plate 4.5: Gel Electrophotogram showing amplicon of *S. aureus* using species specific primers in PCR

Lane	Isolates
M	Molecular marker (100bp)
1	*S. aureus* (ATCC29535)
2	634
3	167
4	Com52
5	779

Plate 4.6: Gel Electrophotogram showing amplicon of *S. xylosus* using its species specific primer in PCR

M =100bp molecular ladder, 1= *S. xylosus* (isolate 681)

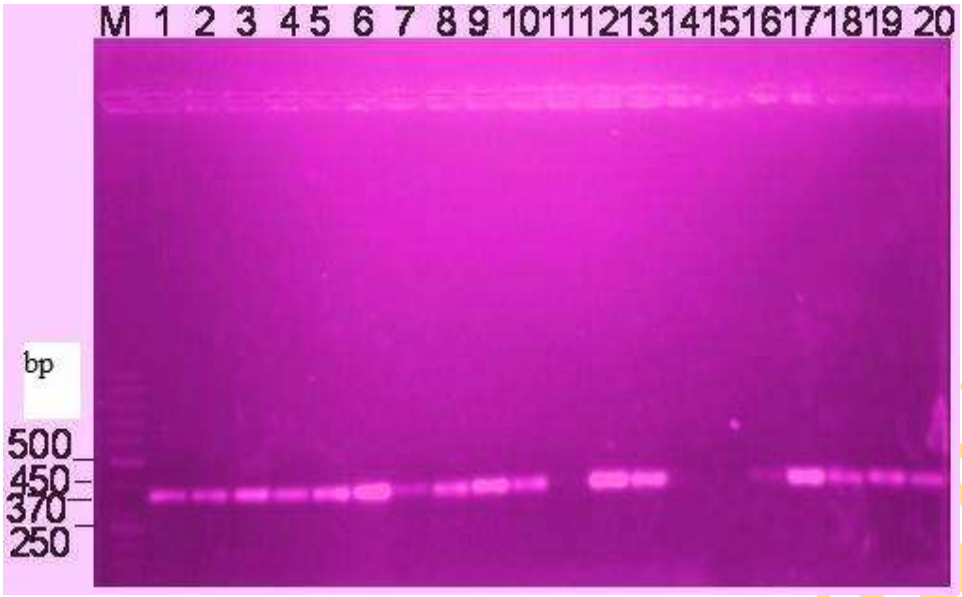

Plate 4.7: Gel Electrophotogram showing absence of restriction when Hind 111 was used to digest amplicon produced using genus specific primers

Lane	Isolates	Lane	Isolates
M	Molecular marker (1kb)	11	S17
1	634 (*S. aureus*)	12	Com88
2	131	13	779
3	Com93	14	641
4	Com56	15	wat8
5	Com7	16	4020
6	OO	17	181
7	C36	18	167
8	C41	19	S20
9	S24	20	*S. aureus* (ATCC29535)
10	100		

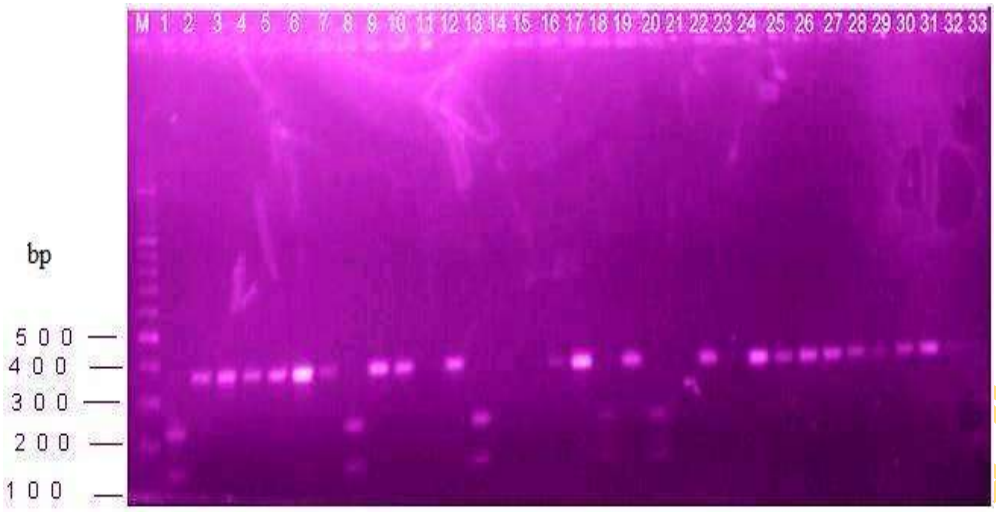

Plate 4.8: Gel Electrophotogram showing RFLP patterns of *Staphylococcus* species after double digestion with Hind111 and EcoR1

Lane	Isolate	lane	Isolate	Lane	Isolate
M	Molecular marker (100bp)	14		28	S10
1	*S. aureus* (ATCC29535)	15		29	260
2	131	16	4020	30	S12
3	Com93	17	181	31	Com60
4	Com56	18	167 (*S. aureus*)	32	Com93
5	Com7	19	S20	33	611(*S. aureus*)
6	667	20	634 (*S. aureus* mec+)		
7	C36	21	681		
8	C41 (*S. aureus*)	22	S21		
9	S24	23	283		
10	100	24	com111		
11		25	S3		
12	Com88	26	A		
13	779 (*S. aureus*)	27	Com1		

4.7 Methicillin resistance

A total of 55 clinical and 53 community isolates of *Staphylococcus* species were investigated for methicillin resistance using genotypic methods. Two clinical isolates had visible bands on gel electrophoresis when *mecA* specific primers were used to amplify the target gene. The result is shown in Plate 4.9a (lane 15 and 18). To avoid false negative, specific primers (mecI and mecR1) targeting the *mecA* regulatory elements (*mecI* and *mecR1*) and primers (rjmecF and ORFX1R) targeting all chromosomal cassettes (*SCCmec*) were subsequently used to amplify the respective gene targets. All the isolates showing no visible bands were screened for the regulatory element in a multiplex format .Unexpectedly was found a strain of *S. aureus* showing one of the regulatory elements (isolate 167). To confirm this result, one of the mec positive isolates together with four *S. aureus* including *mecA* negative control (*S. aureus*, ATCC92535) were repeated in a multiplex format. Again, bands from both *mecA* positive and the isolate were seen while control and other isolates showed no bands (Plate 4.10). These figures represent approximately 5.5 % prevalence (3/55) in the clinical setting. The amplification of chromosomal cassette failed due large difference in annealing temperature of forward and reverse primers used.

4.8 Vancomycin Resistance

Of all the clinical and community isolates studied, no *vanA* associated resistance was detected when the specific primers targeting the *vanA* gene were used. The expected size of this gene is 3010bp which failed amplification in both multiplex and uniplex PCR format for several trials.

4.9 β-lactamase gene

Among the clinical isolates investigated, 9(16.4 %) had detectable β-lactamase genes (plate 4.9a and 4.9b, Table 4.7) which could be responsible for resistance phenotype expressed by some of the isolates to some β-lactam antibiotics. In community isolates however, only 1 (1.8 %) had this gene.

4.10 Staphylococcal Cassette Chromosome mec typing

The detection of chromosomal cassette in s*taphylococcus* species is an evidence of methicillin associated resistance. In this study, however, the SCCmec failed to amplify the sequence of 400bp. This problem was caused by wide difference in the annealing temperature (Tm ^{0}C) of forward primer (44.6 ^{0}C) and reverse primer (65.1^{0}C).

4.11 Panton Valentine Leukocidin detection

The origins of three *mecA* positive isolates were determined by accessing the presence of Panton Valentine Leukocidin using the specific primers. However, no amplification was found after repeated trials. Hence, in community isolates however, no single methicillin resistance was found amongst all the strains studied.

Table 4.7: Percentage yield of the target genes in multiplex and uniplex PCR analysis

Gene target	Clinical isolates	Community isolates
mecA	2(3.6%)	0.0%
mecR1	3(5.5%)	0.0%
mecI	0.0%	0.0%
blaZ	9(16.4%)	1.8%
PVL	0.0%	0.0%
SCCmec	Failed	Failed

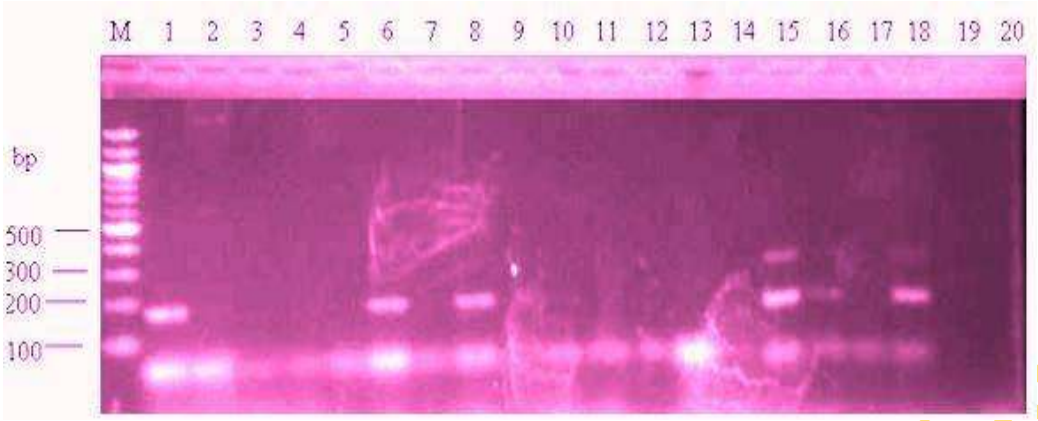

Plate 4.9a: Gel Electrophotogram showing multiplex PCR products for the identification of *mecA*, *vanA* and *blaZ* genes

Lane	Isolates	Status
M	Molecular marker	
1	146	β-lactamase +ve and mecA -ve
2	493	β-lactamase -ve and mecA -ve
3	667	β-lactamase -ve and mecA -ve
4	OO	β-lactamase -ve and mecA -ve
5	C36	β-lactamase -ve and mecA -ve
6	615	β-lactamase +ve and mecA -ve
7	S20	β-lactamase -ve and mecA -ve
8	Com29	β-lactamase +ve and mecA -ve
9	153	β-lactamase +ve and mecA -ve
10	Com7	β-lactamase -ve and mecA -ve
11	Com89	β-lactamase -ve and mecA -ve
12	Com60	β-lactamase -ve and mecA -ve
13	Com96	β-lactamase -ve and mecA -ve
14	292	β-lactamase -ve and mecA -ve
15	634	β-lactamase +ve and mecA+ve
16	A	β-lactamase +ve and mecA -ve
17	641	β-lactamase -ve and mecA -ve
18	AA	β-lactamase +ve and mecA+ve
19	Com31	β-lactamase -ve and mecA -ve
20	water	Negative control

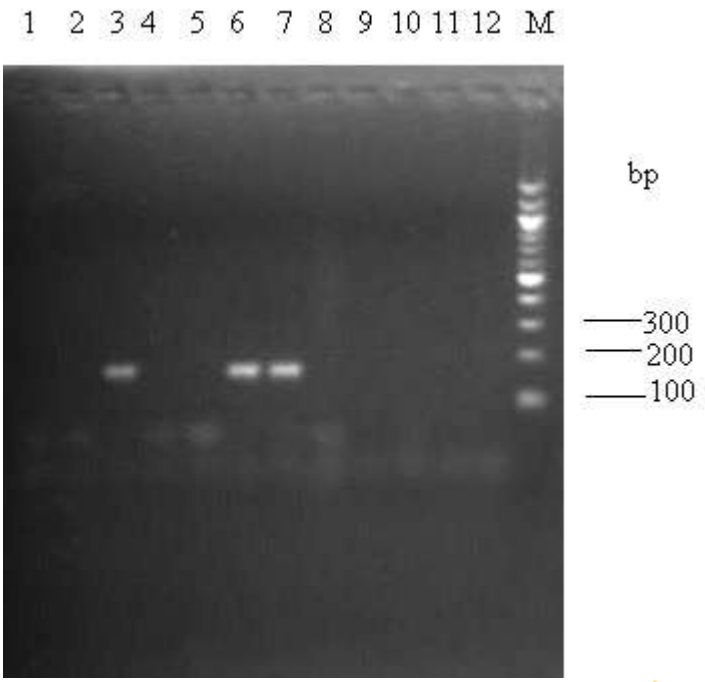

Plate 4.9b: Gel Electrophotogram showing multiplex PCR for the identification of *mecA*, *vanA* and *blaZ* genes.

Lane	Isolates	Status
M	Molecular marker	
1	441	β-lactamase -ve and mecA -ve
2	167	β-lactamase -ve and mecA -ve
3	153	β-lactamase +ve and mecA -ve
4	48	β-lactamase -ve and mecA -ve
5	6N	β-lactamase -ve and mecA -ve
6	380	β-lactamase +ve and mecA -ve
7	260	β-lactamase +ve and mecA -ve
8	681	β-lactamase -ve and mecA -ve
9	995	β-lactamase -ve and mecA -ve
10	S21	β-lactamase -ve and mecA -ve
11	779	β-lactamase -ve and mecA-ve
12	76	β-lactamase -ve and mecA -ve

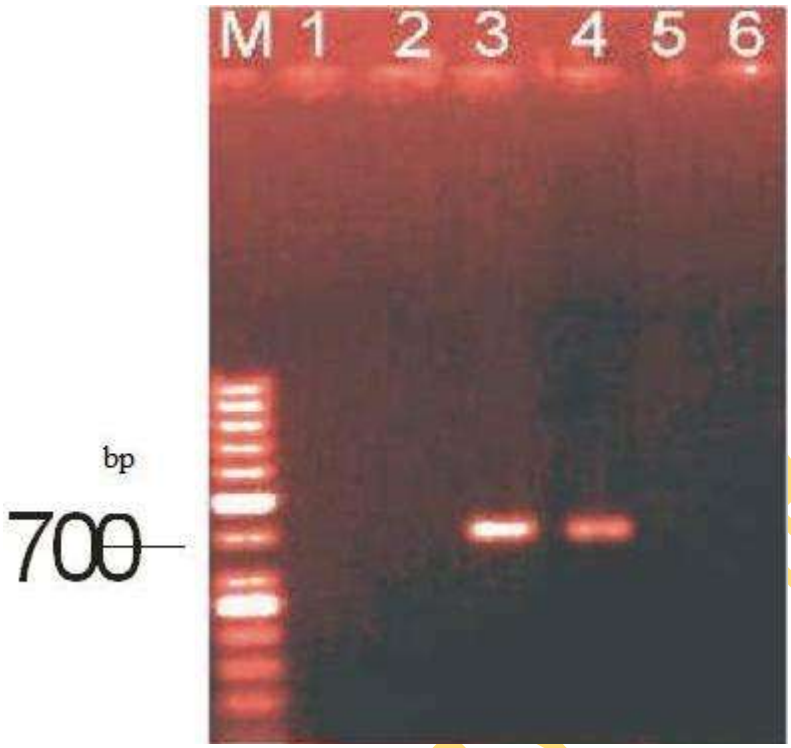

Plate 4.10: Gel Electrophotogram showing multiplex PCR product for detection of *mecA* regulatory elements

M= Molecular marker (100bp), lane 1= *S. aureus* (ATCC92535), 2=611(*S. aureus*) 3=634(*mecA* positive), 4=167(isolate under investigation), 5=201(*S. aureus*), 6=C41 (*S. aureus*).

4.12 Plasmid profiling

A total of 35 clinical and 19 community isolates were investigated for plasmid profile. Of the 35 clinical isolates, 33 (94.3%) had plasmid of size 23130kb, while only 2 (5.7%) had no plasmid at all. In this manner, of the 19 community isolates, 17 (89.50%) had plasmid. Only 2 (10.5%) had no plasmid (Table 4.8 and Table 4.9). The plate 4.11 shows the plasmid profiles of the isolates on gel electrophoresis.

4.13 Plasmid curing

A total of 32 isolates were investigated plasmid lockout using acridine orange at three different concentrations and were run on agarose gel electrophoresis to see the outcome of the curing. The Table 4.10 shows the outcome of the plasmid curing with different concentrations of acridine orange. At concentration of 0.5mg/ml acridine orange, 50% (16/32) of the isolates were cured (Plate 4.12). When the concentration was reduced to 0.25mg/ml, however, 31.3% (10/32) were cured (Plate 4.13). Again, when the concentration was reduced to 0.125mg/ml, 18.8% (6/32) of the isolates were cured (Plate 4.14).

Table 4.8: Plasmid profile of clinical isolates

Serial No	Isolates	Plasmid status	No of Plasmids
1	413	+	1
2	652	+	1
3	6N	+	1
4	998	+	1
5	100	+	1
6	149	+	1
7	076	+	1
8	OO	+	1
9	463	+	1
10	283	+	1
11	650	+	1
12	131	+	1
13	AA	+	1
14	798	+	1
15	A	+	1
16	1079	+	1
17	167	-	0
18	955	+	1
19	821	-	0
20	668	+	1
21	641	+	1
22	699	+	1
23	S5	+	1
24	S24	+	1
25	S10	+	1
26	S11	+	1
27	S12	+	1
28	S21	+	1
29	467	+	1
30	922	+	1
31	ON	+	1
32	146	+	1
33	681	+	1
34	S20	+	1
35	611	+	1

Keys:
+ = Plasmid present

-= Plasmid absent

Table 4.9: Plasmid profile of community isolates

Serial No	Isolates	Plasmid status	No of plasmids
1	Com11	-	0
2	Com93	+	1
3	Com60	+	1
4	Com29	+	1
5	Com6	+	1
6	Com41	+	1
7	Com16	+	1
8	Wat9	+	1
9	Com7	+	1
10	Com38	+	1
11	Com89	+	1
12	Com96	+	1
13	Com92	-	0
14	Com56	+	1
15	Com45	+	1
16	Com10	+	1
17	Com74	+	1
18	Com88	+	1
19	Com8	+	1

Keys:
+ = Plasmid present

-= Plasmid absent

Table 4.10: Plasmid curing at three different concentrations of acridine orange

Serial No	Isolates	0.5mg/ml	0.25mg/ml	0.125mg/ml
1	998	+	+	+
2	100	+	+	+
3	076	+	+	+
4	OO	+	+	+
5	463	+	+	+
6	283	+	+	+
7	AA	+	+	+
8	1079	+	+	+
9	611	-	+	+
10	955	-	+	+
11	668	+	+	+
12	699	+	+	+
13	S10	+	+	+
14	467	+	+	+
15	ON	+	+	+
16	146	+	-	-
17	Com93	-	-	-
18	Com60	-	-	-
19	Com29	+	+	+
20	Com6	-	-	+
21	Com41	+	+	+
22	Com16	-	-	+
23	Wat9	-	-	+
24	Com7	-	-	-
25	Com89	-	+	+
26	Com96	-	-	+
27	Com56	-	+	+
28	Com45	-	+	+
29	Com10	-	+	+
30	Com74	-	-	+
31	Com88	-	-	-
32	Com8	-	+	-

Keys:
+ = Plasmid present

-= Plasmid absent

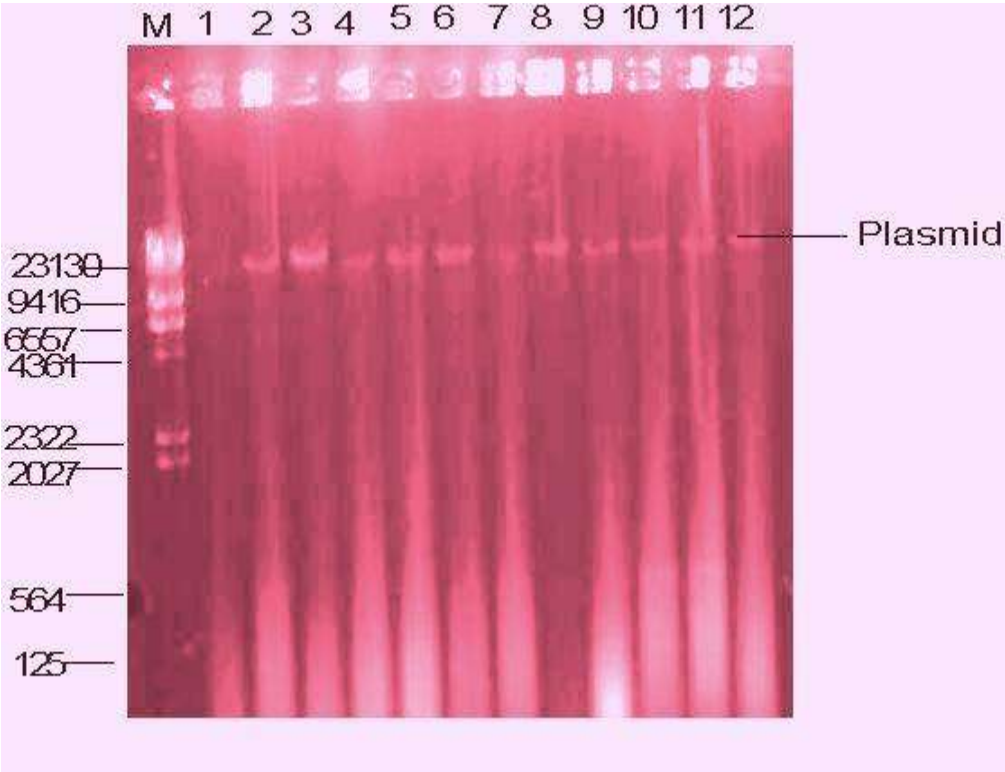

Plate 4.11: Gel Electrophotogram showing Plasmid profiles of the isolates

Lane	Isolates	Plasmid Status	Lane	Isolates	Plasmid Status
M	Hind111 lambda DNA	marker	7	652	+ve
1	Com11	-ve	8	Com6	+ve
2	413	+ve	9	6N	+ve
3	Com93	+ve	10	998	+ve
4	Com60	+ve	11	100	+ve
5	A	+ve	12	149	+ve
6	Com29	+ve			

Plate 4.12: Gel Electrophotogram showing Plasmid pfofile after curing at 0.5mg/ml of acridine orange

Lane	Isolates	Plasmid Status	Lane	Isolates	Plasmid Status
M	Hind111 lambda DNA	marker			
1	Com56	-ve	6	Com88	-ve
2	AA	+ve	7	Com96	-ve
3	OO	+ve	8	Com8	-ve
4	Com41	+ve	9	Com74	-ve
5	1079	+ve	10	611	+ve

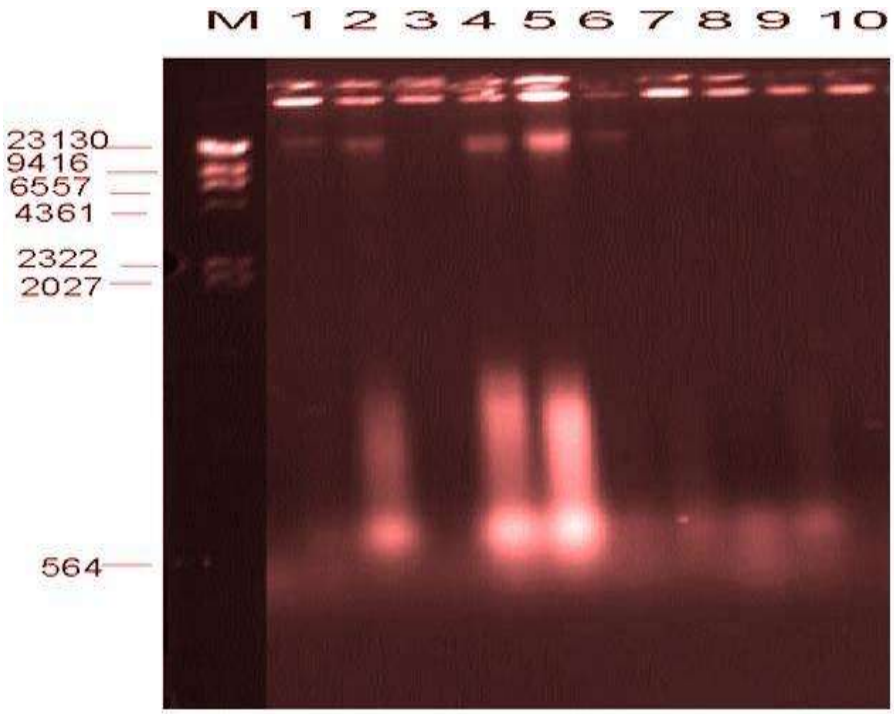

Plate 4.13: Gel Electrophotogram showing Plasmid pfofile after curing at 0.25mg/ml of acridine orange

Lane	Isolates	Plasmid Status	Lane	Isolates	Plasmid Status
M	Hind111 lambda DNA	marker			
1	Com56	+ve	6	611	+ve
2	AA	+ve	7	Com8	+ve
3	Com74	-ve	8	Com96	-ve
4	Com41	+ve	9	OO	+ve
5	1079	+ve	10	Com88	-ve

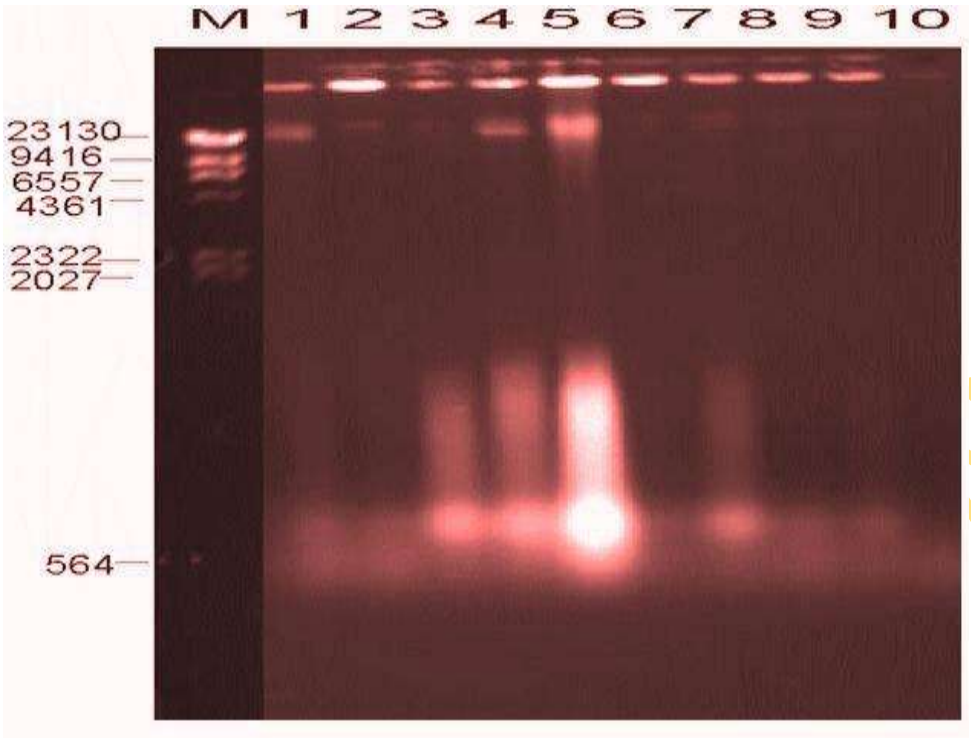

Plate 4.14: Gel Electrophotogram showing Plasmid pfofile after curing at 0.125mg/ml of acridine orange

Lane	Isolates	Plasmid Status	Lane	Isolates	Plasmid Status
M	Hind111 lambda DNA	marker			
1	Com56	+ve	6	611	+ve
2	AA	+ve	7	Com96	-ve
3	OO	+ve	8	Com8	+ve
4	Com41	+ve	9	Com74	-ve
5	1079	+ve	10	Com88	-ve

CHAPTER FIVE

DISCUSSION

5.1 Isolation and species distribution

The analysis of clinical and community samples obtained from air, selected waste water drainages and human swabs (eye, semen, ear, high vagina swab, throat, urethra, wound, nostril, skin) in the University of Ibadan and University College Hospital indicated that s*taphylococcus* species were widely distributed in both environments. The coagulase negative staphylococci (CNS) were the most frequently isolated organisms in this study particularly *S. epidermidis*. The 108 staphylococci identified were classified into three distinct species (*S. epidermidis*, *S. xylosus* and *S. aureus*). The distribution of *S. epidermidis* and *S. aureus* in this study was consistent with the study carried by Bannerman (2003) and Kwok and Chow (2003) which indicated that *S. epidermidis* was the most abundance in clinical specimen. The identification of *S. xylosus* in clinical specimen is consistent with reports which indicated that there was abundance of wider range of CNS in clinical samples (Schnitzler *et al*., 1997, Bannerman, 2003). In community specimen, Geetha *et al*. (2003) stated that CNS form larger part of cutaneous microflora hence, their prevalence. Coagulase-negative staphylococci before now were regarded as non-pathogenic skin flora and as culture contaminants. Contrary to this assumption, the occurrence of these species implicated as causative agents of nosocomial infections has been on the increase, with the increasing use of prosthetic devices and other invasive technologies (Shittu *et al*., 2004). Although *Staphylococcus epidermidis* accounted for the majority of CNS infections, other species have also been identified in association with human infections (Wallet *et al*., 2000).

5.2 Identification

The problems of identification of bacterial species lie in the power of discrimination between species and subspecies as well as reproducibility (Mehndiratta *et al*., 2009). In many clinical laboratories, CNS are not identified to species level and, in most cases, identification of clinically significant CNS is carried out through conventional methods and commercial identification kits. In this study, conventional identification method was used which was based on phenotypic and biochemical characteristics for the identification of staphylococci. However, *S. xylosus* displaying close biochemical characteristics with *S. capitis* was not distinguished by this method. This species was confirmed as *S. xylosus* by molecular method (PCR-FRLP) and this is consistent with Perl *et al*. (1994) who reported that the overall accuracy of conventional identification method is low and range from 50 to 70%. This is because of the limited number of stable features that can be used for species discrimination. Hence many taxa remain difficult to distinguish from one another and are misidentified by phenotypic tests (Couto *et al*., 2001); hence, the importance of using molecular method for bacterial identification and in disease surveillance cannot be over emphasized.

The use of nucleic acid targets, with their high sensitivity and specificity, provides an alternative technique for the accurate identification and classification of *staphylococcus* species. In this study, PCR-FLP of 16S-rRNA gene of bacteria was chosen which correctly discriminated the species of staphylococci encountered. This was consistent with the PCR-RFLP analysis of the internal transcribed spacer of the 16S rRNA gene, the intergenic spacer of the 16S-23S rRNA gene, the *tuf* gene, the *gap* gene, and the *groEL* gene developed for the identification of s*taphylococcus* species (Mendoza *et al*., 1998; Barros *et al*., 2007). In a related study, PCR-RFLP was successfully used to assess the relatedness of MRSA circulating in hospital and community environments which also identified the organisms based RFLP patterns (Awadalla *et al*., 2010). The PCR-RFLP analysis is useful for the taxonomy of staphylococci and proves to be easier, less expensive and less equipment dependent than sequencing. Although comparison of the 16S rRNA gene sequences has been useful in phylogenetic studies at the genus level, its use has been questioned in studies at the species level. This stems from the fact that closely related species may have identical 16S rRNA sequences or, alternatively, that

divergent 16S rRNA sequences may exist within a single organism (Stackebrandt and Goebel, 1994).

The RFLP patterns were confirmed using species specific primers. The PCR-RFLP has proved to be a useful tool in the identification of bacteria to species level as well as routine epidemiological and infection control measures. This technique correctly discriminated among species of the same genus. The significance of this work was more highlighted when double restriction using EcoR1 and Hind111 endonucleases were used to digest a PCR product of 370bp (staphylococci genus amplicon). This separated *S. aureus* from other CNS with high specificity and sensitivity. Product of 210-50bp indicates *S. aureus* while 370bp (no digestion) gives CNS. Therefore, double digestion can be used to separate coagulase positive staphylococci and coagulase negative staphylococci. Since Coagulase test could be sometimes misleading, this method could be substituted especially when isolating *S. aureus* amidst CNS from clinical samples.

5.3 Susceptibility to antibiotics

In this study, there was variability in the performance of disc diffusion using the three acceptable phenotypic test discs (methicillin, cefoxitin and oxacillin) when compared to molecular method. The variability in the performance of disc diffusion is attributable to the heterogeneous nature of phenotypic expression of resistance in MRSA isolates. The heterogeneous nature of methicillin resistance in staphylococci limits accuracy and reliability of phenotypic methods such as disc diffusion, broth and agar dilution tests (Prasad *et al*., 2000; Esma *et al*., 2009). In a number of studies, sensitivity and specificity of disc diffusion method have been reported between 61.3 - 100 % and 50 - 99.1 % respectively (Prasad *et al*., 2000, Udo *et al*., 2000). Early reports indicated that methicillin-resistant *S. aureus* (MRSA) were heterogeneous in their expression of resistance to β-lactam agents, in that large differences in the degree of resistance were seen among the individual cells in a population (Knox, 1961).

In addition to above factors, other attributable factors like growth conditions (Annear, 1968; Sinsimer *et al*., 2005), salt concentrations (Milne *et al*., 1993) inoculums size (Huang, 1993), medium compositions (Coombs *et al*., 1996) might have had considerable effects on the susceptibility test. In this study, incessant power supply during the course of

susceptibility testing might have also contributed significantly to the variability of the diffusion test. This is because steady incubation temperature was hardly maintained and this could have caused the organisms to grow below its optimum temperature; thus affecting its metabolism. Recent work has also shown that the susceptibility to antibiotics is highly dependent on the bacterial metabolism and that global metabolic regulators can modulate this phenotype (Fernando and Jose, 2013). This modulation includes situations in which bacteria can be more resistant or more susceptible to antibiotics. Understanding these processes will thus help in establishing novel therapeutic approaches based on the actual susceptibility shown by bacteria during infection, which might differ from that determined in the laboratory.

However, other situations in which bacteria become transiently resistant to antibiotics, in the absence of a genetic change, have been also described (Levin and Rozen, 2006). Among them, the most studied are drug indifference, the growth in biofilms, and the phenomenon of persistence. However, there are other situations wherein bacteria present changes in their susceptibility to antibiotics depending on their metabolic state. Whilst this phenotype of resistance to antibiotics has been in occasions attributed to a situation of growth arrest that precludes the activity of some bactericidal antibiotics as beta-lactams, growing number of evidences indicate that the link between the metabolic state of bacterial populations and their phenotype of susceptibility to antibiotics is far more complex (Fernando and Jose, 2013).

Variation in methicillin susceptibility test performance with Mueller-Hinton medium from different manufacturers (Hindle and Inderlied, 1985, Hindle and Warner, 1987) and of different batches from the same manufacturer, have been reported (Derek *et al*., 2005) while in disc diffusion tests with higher concentrations of NaCl, a heavy inoculum may also lead to increased false-resistant reports, particularly with oxacillin (Derek *et al*., 2005). Now, the consequent problems with methicillin susceptibility testing have been the subject of many subsequent publications and there are many recommendations, some conflicting, regarding the most reliable methods for routine use. This confusion is partly because there are marked differences between strains and different populations of strains were investigated in different studies (Tomasz *et al*., 1991). With these numerous factors

responsible for heterogeneity in resistance of *staphylococcus* species, the low sensitivity and specificity of phenotypic detection technique can be anticipated, hence, the use of molecular method for methicillin resistance study proved to be a powerful tool in epidemiological surveillance and phylogenetic studies.

The multiple antibiotics resistance of CNS observed in this study was similar to work of Geetha *et al*. (2003) in which multiple resistance among the CNS reported to be as high as 80.77%. According to these authors, the resistance was from chromosomal origin especially to antibiotics such as penicillin, methicillin, and ciprofloxacillin. The origin of resistance to Gentamycin, and Tetracycline was said to be confined only to plasmid, but because plasmid loci which can be transposable may have been transferred onto the chromosome in the isolates studied explaining the high level of chromosomal encoded resistance to these antibiotics (Geetha *et al*., 2003). The antibiogram pattern in this study showed that *S. epidermidis* tend to be resistance to a wider range of antibiotics and this is consistent with the report of Obi *et al.* (1993) who reported resistance of 77.0% of *S. epidermidis* in Lagos, Nigeria. In addition, cases of multi-drug-resistant CNS species in human infection have been reported (Stepanovic *et al*., 2002; Basaglia *et al*., 2003). The reason for the multiple antibiotic resistance including methicillin, cefoxitin and oxacillin (even though 99% were lacking *mecA* gene in their chromosomes) in CNS is unknown, but it could be that there were transfer of genetic elements occurring between CNS and *S. aureus*. The review by Pfaller and Henweldt (1988) indicates that *S. epidermidis* has become resistant to commonly used antibiotics which may serve as reservoir for antibiotic resistance strains in hospitals. These antibiotic resistant determinants can be transferred to new bacterial species as part of the large conjugative replicons which commonly code resistance to some aminoglycosides such as Gentamycin, Kanamycin (Neihart *et al*., 1988). Also, CNS carries a variety of multiple resistance genes on their plasmid which can be exchanged and spread amongst different species of staphylococci including *S. aureus* (Neihart *et al*., 1988).

It was observed that high proportion of the isolates were resistant to most of the antibiotics in study. For example, Tetracycline and cotrimoxazole historically had wide clinical application, inexpensive, orally administered and available from diverse sources where

they are sold with or without prescription in Nigeria. Moreover, they are listed in many developing countries as among the antibacterial agents that have been rendered ineffective, or for which there are serious concerns regarding bacterial resistance (Okeke, 2003). It appears that misuse and overuse of these antibiotics could have contributed to this trend in Nigeria. Therefore, to prevent treatment failures in the absence of data on antibiotic susceptibility testing, public enlightenment on the ineffectiveness of these antibiotics against staphylococcal infections and the enactment of effective drug policies in Nigeria are necessity. Limited treatment options and prolonged course of infection due to these CNS species could have severe consequences for patients. Full and accurate identification of CNS isolates in clinical samples is therefore of great importance for epidemiological purposes and infection control measures (Basaglia *et al*., 2003).

5.4 Molecular detection of resistance determinants

The detection of methicillin resistance in staphylococci depends on efficient production of the PBP2a and is modulated by chromosomal factors (Sadeghian *et al*., 2004). Depending on the genetic background of the strain that acquired *mecA*, resistance levels range from phenotypically susceptible to highly resistant. Maximal expression of resistance by PBP2a requires efficient and correct synthesis of the peptidoglycan precursor. Genes involved in cell wall precursor formation and turnover, regulation, transport and signal transduction may determine the level of resistance that is expressed (Sadeghian *et al*., 2004). The overall assessment of MRSA in this study by phenotypic methods involving use of methicillin, cefoxitin and oxacillin did not in any way correlate with genotypic method based on PCR. The detection of MRSA in this study by molecular method (5.5 %), however, was little higher than the finding of Shittu *et al*. (2009) who documented 2% MRSA (1/50) prevalence in Ife, Nigeria. The absence of methicillin resistance in community isolates may be due to low resistance recorded in the clinical settings and probably because horizontal gene transfer of *mecA* is a rare event as compare to genetic events occurring in plasmid associated resistance. The detection of methicillin resistance in both *S. aureus* and *S. epidermidis* in this study is a warning signal that in nearest future, high prevalence rates should be anticipated especially in the hospital settings. This low resistance in MRSA prevalence is comparable to some European countries, including Denmark, Finland, the Netherlands, Norway, and Sweden, which have a very low

prevalence of MRSA infections and fewer than 3% of clinical *S. aureus* isolates, are MRSA (Bocher *et al*., 2010). It should be noted that if this report was based on phenotypic method of assessing methicillin resistance, a range of 62.3-72.3% prevalence would have been reported as against 5.5% with molecular method. This could have been misleading, which may create unnecessary global fear in health institutions, thus highlighting the importance of molecular over phenotypic methods.

The appearance of vancomycin resistance determinant in MRSA with reduced susceptibility evoked the specter of a totally resistant *S. aureus* (Sadeghian *et al*., 2004). Vancomycin is the main antimicrobial agent available to treat serious infections with MRSA but unfortunately, decrease in vancomycin susceptibility of *S. aureus* and isolation of vancomycin intermediate and resistant *S. aureus* have recently been reported from many countries (Benjamin *et al*., 2010). Many reports from north India also recorded the emergence of low level and intermediate vancomycin resistance (Wootten *et al*., 2001, Assadullah *et al*., 2003, Bhateja *et al*., 2005, Menezes *et al*., 2008, Bijiyani and Purva, 2009). The experimental transfer of the *van*A gene cluster from *E. faecalis* to *S. aureus* (Noble *et al*., 1992) has raised fears about the occurrence of such genetic transfer in clinical isolates of methicillin resistant *S. aureus*. In this study, the vancomycin resistance determinant (*vanA*) was not found in any of the isolates studied. This could probably be as a result of rare or infrequent use of vancomycin for clinical purposes in this region. In addition, the presentation of this antibiotic makes its abuse very difficult by ordinary person (i.e. it requires professional). Hence, the antibiotic pressure for this drug is low within this region. Another possibility that may be accounting for the absence of this resistance determinant could be that the transfer of this resistance determinant from enterococci to *S. aureus* is a slow event because even in the developed countries were vancomycin is frequently use for treatment of patients, its incidence is relatively very low (Tenover *et al*., 2004).

β-lactamase confers resistance to penecillins, cephalosporins and related antibiotics by their ability to hydrolyze theses drugs before they reach their target sites. In Gram negative bacteria, these targets are called penicillin-binding proteins, are located on the outer surface of the inner membrane of the cell wall. β-lactamases are located at the

periplasm ready to intercept incoming β-lactam molecules (Sadeghian *et al*., 2004). The most successful β-lactamase genes are carried by the plasmids, which unlike the chromosomally-encoded counterparts, are usually produced constitutively so that changes in their ability to confer resistance stem from alteration in the structure of the protein rather than from deregulation of gene expression (Sadeghian *et al*., 2004). The assessment of beta-lactamase using molecular method was in accord with previous work done (Fagade *et al*., 2010) by conventional Iodometric method. The percentage of beta-lactamase detected in clinical isolates was seven times more compare to the community isolates. This is not surprising since antibiotic selective pressure is more pronounced in clinical environment compare to the community environment. This is also in accord with the previous work by Adriana *et al*. (2003) that reported high rates in clinical setting. What is surprising is the fact that majority of the *S. epidermidis* were resistant to majority of the antibiotics, even though they could not produce beta-lactamase. It may be assumed that resistance in these isolates is independent of their ability to produce this enzyme, and that resistance could involves constitutive rather than inducible.

It should be noted that two strains of *S. aureus* and one strain of *S. epidermidis* were methicillin resistant. To the best of my knowledge, this is the first time *S. epidermidis* that is *mecA* positive as confirmed by PCR is reported in Nigeria. There was similar documentation of methicillin resistance determinant found in *S. haemolyticus* and *S. scuiri* by Shittu *et al*. (2012) in Nigeria. In this context, it is noteworthy that 70 to 75% of all CNS worldwide are now resistant to methicillin (Diekema *et al*., 2001), thus representing a huge potential reservoir of resistance. This has an epidemiological significance as a pointer towards origin and mechanism of *mecA* transfer as well as rapid spread of these resistant determinants (*mecA* or chromosomal cassette SCCmec) to other coagulase negative staphylococci. At this point, genetic exchange must have occurred between *S. aureus* and *S. epidermidis* in this study, suggesting horizontal gene transfer. The question that is difficult to answer is: which of these species transferred its *mecA* gene to the other? It has been proposed that CNS serve as donors for the transfer of the *mecA* gene to *S. aureus* (Tesch *et al*., 1988, Archer *et al*., 1994, Wielders *et al*., 2001). The transfer of *mecA* from *S. epidermidis* to *S. aureus* was witnessed in vivo, suggesting that *mecA* may transfer more frequently to *Staphylococcus aureus*, (Wielders *et al*., 2001). Bloemendaal

et al. (2010) presented data that strongly support interspecies transfer of *SCCmec* in a patient undergoing antibiotic therapy, from *S. epidermidis* O7.1 to MSSA WKZ-1, to become MRSA WKZ-2. Therefore, it is possible to assume that *mecA* gene was transferred from *S. epidermidis* to *S. aureus* in this study.

5.5 Detection of *mecA* by its regulatory elements and *SCCmec*

It should be noted that the use of *mecA* gene only as target for assessing methicillin resistance may not be sufficient, because false negative could occur. False negative could result from use of high DNA concentration or when there is repression of PBP2a production due to fully functional regulatory elements leading to absence or little *mecA* production (Ryffel *et al*.; 1992; Kuwahara-Arai *et al*.; 1996). Other researchers have suggested that MSSA can arise from MRSA both in vivo and in vitro by a spontaneous loss of *mecA* (Deplano *et al*., 2000, Daskalaki *et al*., 2007). Loss of *mecA* has been reported to occur under stressful conditions in vitro, such as long-term storage in antibiotic-free medium, nutrient starvation, elevated temperatures, or UV irradiation (Inglis *et al*., 1990). In addition, Noto *et al*. (2008) have shown that exposure of MRSA isolates to vancomycin in vitro can result in a loss of *mecA* or portions of SCC*mec*. In this study, however, a potential MRSA was missed; not due to mution but because of absence of beta-lactamase regulatory elements which would have co-regulated with *mecR1* gene for *mecA* expression. This was similar to a report in Irish hospital, Dublin where 25 strains of multi resistant methicillin *Staphylococcus aureus* failed amplification with PCR *mecA*-specific primers, but 7 of the strains yielded *SCCmec* with PCR (Anna *et al*., 2008). This result could mean that these strains acquired the *SCCmec* from MRSA within the environment or that there was mutation leading to loss of *mecA*. The emergence of *mecA* variants (*mecC*) has compounded the molecular detection of methicillin resistance in staphylococci (CDC, 2012). In this report, *mecA* was investigated by two methods; an-in house PCR and the GenoType *staphylococcus* test, but results were negative indicating that any MRSA isolate with variant *mecA* cannot be identified using the conventional *mecA* type primers. This recent study highlighted the importance of *mecA* typing using various molecular techniques. In this study, the problem of false negative was overcome by assessing methicillin resistance at three different levels; *mecA*, *mec* regulatory elements (*mec1* and *mecR1*) and chromosomal cassette, SCCmec. The latter did not work because

of the wide different in annealing temperature ™ between the forward primer (rjmecF, Tm, 44.6 ^{0}C) and the reverse primer (ORFX1R, Tm, 65.1^{0}C). These primers were designed to detect the presence of all classes of chromosomal cassettes in staphylococci. However, primers designed for the regulatory elements were able to detect MRSA misidentified as methicillin negative *Staphylococcus aureus* by using *mecA* primers. Therefore, a true *mecA* negative organism is the one which fail to yield result after molecular assessment at these three levels (*mecA* and its associated elements).

5.6 Observed mutation of *mecA* regulatory elements

It has been reported that methicillin-resistant *S. epidermidis* strains appear relatively stable, with 57.9% of the isolates containing the whole regulatory region (Sadeghian *et al*., 2004). Alterations within the *mecA* were detected more often in other CNS, which also had a higher percentage with deletions of regulatory genes. On the other hand, Petinaki *et al*. (2001) identified MRSA among genetically heterogenous population, with several alterations and deletions of *mec* genes. In this study, deletion of the signal repressor gene (*mecI*) in the three *mecA* positive strains was observed. They failed to be amplified for several trials. This is in accordance with Sanjay *et al*. (2004) who reported a number of mutations in *mecI* and *mecA* from clinical isolates. The substantial reason for this is straight forward; its absence paved way for the beta-lactamase regulatory element (*blaI*) to co-regulate with the *mecR1* (signal transducer) for *mecA* gene expression, otherwise, its repressor activity would have resulted in lack of *mecA* gene. This is in accordance with several reports which indicate that for *mecA* expression, one of the beta-lactamase regulatory elements must co-regulate with the *mecA* regulatory elements (Hackbarth and Chambers, 1993; Adriana *et al*., 2003). In two isolates, however, resistance is inducible because of the presence of *mecR1* and beta-lactamase regulatory elements. It is only when there is deletion of *mecR1* that resistance becomes constitutive, provided that the inducible ß-lactamase is absent in the strain (Archer *et al*.; 1994, Hűrlimann-Dalel *et al*.; 1992, Suzuki *et al*.; 1993). It is possible to classify MRSA as beta-lactamse hyper producer because in most *mecA* positive strain, there is always beta-lactamase, although one of the isolates in this study lacked this gene.

5.7 Classification of *mecA* positive isolates

Panton-Valentine leukocidin (PVL) gene has attained significance with the worldwide emergence of community-acquired methicillin-resistant *S. aureus* (CA-MRSA) infections. In San Francisco, 70% of MRSA which were isolated from jail inmates or patients requiring surgical treatment at an outpatient clinic specializing in skin and soft-tissue infections carried PVL (Diep *et al*., 2004). François *et al*. (2003) reported that PVL locus represents a stable genetic marker for community associated methicillin *S. aureus*, CA-MRSA. For this reason, PVL status of the three methicillin positive isolates was assessed to determine whether they were clinically or community acquired. The *mecA* positive isolates were found to be of clinical origin as they failed to amplify any segment of Panton Valentine Leukocidin sequences using specific primers after several trials. This is contrary to the study by Ghebremedhin *et al*., 2009 in South-West where MRSA with PVL was identified, but is in agreement with a recent study by Shittu *et al*., 2011, where 40% *S. aureus* possessed the PVL genes but were not methicillin resistant (i.e. MSSA). According to the latter, PVL-producing MSSA affiliated to CC121 are known to be common in many countries including Nigeria, Togo and South Africa in sub-Saharan Africa. They maintained that the high proportion of PVL-positive MSSA observed in their study could increase the risk of inter-individual transmission (e.g skin-to-skin and skin-to-fomite contacts) which may represent important routes of spread in the various hospital settings. Contact with colonized and/or infected individuals as well as contaminated fomites in the spread of PVL positive *S. aureus* have been described as risk factors for community-associated MRSA (Cataldo, 2010). The absence of PVL in this study may probably as result of limited number of *S. aureus* encountered. The PVL locus is carried on a bacteriophage and is present in only a small percentage of *S. aureus* isolates from France, where this locus is associated with skin infections and occasionally, serves necrotizing pneumonia (Francois *et al*., 2003). In this study, the SCC*mec* IVa was not typed because of the small sample size of the *mecA* positive isolates.

5.8 Plasmid analysis

In study, plasmid analysis showed that the isolates had plasmid of size 23.13kb. This was similar to 23.13kb identified by Mostafizur *et al*. (2005) which harbor resistance determinant to β-lactam antibiotics. The homogeneity of the isolates (clinical and

community) with respect to antibiograms and plasmid profiles was an evidence of genetic transfer from a common source and this is likely to have arisen through horizontal gene transfer from a single strain or its derivates from hospital to hospital and hospital to community and verse versa. Evolutionary events through recombination or transposition might have resulted to emergence of these variant strains occurring now. The frequent use of antibiotics has led to selective pressure resulting to emergence of resistant determinants within many staphylococci as evidenced by outbreak of resistance mostly encountered following its introduction into clinical practice. Geetha *et al* (2003) reported the successful transfer of R-plasmid *in vivo* by mixed culture transfer on solid media. This depicts the epidemiological significance of normal staphylococcal habitat (body surfaces) in the emergence of antibiotic resistance as well as topical use of antibiotics which predispose the organisms to antibiotic selective pressure for plasmid gene expression. Reports on plasmid mediated resistance to chloramphenicol, gentamycin, tobramycin, and kannamycin in *Staphylococcus aureus* as well as *aureus* strains possessing plasmid copy of β- lactamase determinant has been documented (Punithavathi, and Krishnaveni, 2012). It is worth noting that as antimicrobial agents are developed, there is also corresponding increase in the number of beta-lactamases that are emerged (Davies and Davies, 2010).

CHAPTER SIX

CONCLUSIONS

6.1 Conclusions

The incidence of high level of multiple resistance among *staphylococcus* species (especially *S. epidermidis*) to commonly used antibiotics in this investigation is worrisome especially with the detection of methicillin resistant *S. epidermidis* (in this study), *S. haemolyticus* and *S. scuiri* (other studies). Originally, CNS are often reported without further speciation, assuming that they are contaminating clinical samples but are not involved in the primary infection. However, the detection *mecA* gene in these organisms is evidence that these bacteria may be responsible for primary infections in patients. These may carry a variety of multiple resistance genes on their plasmids that can be exchanged and spread amongst different species of staphylococci including *S. aureus* which could serve as reservoirs for antibiotic resistance strains in hospitals. In Nigeria, there are no systematic approaches of reporting infectious diseases. Most health institutions lack proper infection control programmes and there are no coordination of efforts among public health authorities. There is hence no national policy for screening, reporting and control of MRSA outbreaks. The tendency is that MRSA infections/ outbreaks in Nigeria are largely undetected and their contributions to mortality, morbidity and cost of care, associated with hospital-acquired infections are unknown. For these reasons, CNS should be as a matter of urgency considered as etiologic agents of infections following its detection in clinical samples.

As a result of the limited number of stable features that can be used for species discrimination due variability in the expression of metabolic activities and/or the morphological features of some staphylococcal species in conventional reference identification, it becomes difficult to precisely assign the strains to the species level. Moreover, conventional reference methods are too laborious and time consuming to be

used in clinical laboratories. Hence, in this study, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) supplemented with PCR species-specific primers has proven to be quite simple and fast with absolute typeability, reproducibility and good discriminatory power in identifying staphylococci to species level.

This technique is useful tool in the identification of bacteria to species level as well as routine epidemiological and infection control measures and therefore should be routely used in Microbilogy laboratory.

The assessment of MRSA in this study by phenotypic methods varied widely from genotypic method. Although, factors like growth conditions, salt concentrations, inoculums size, medium compositions might have contributed to such variation, however, heterogeneity in expression of resistance and marked differences between strains could be plausible factors. Hence, the choice of molecular methods for resistance detection and surveillance cannot be overemphasized. However, the use of *mecA* only as target for detecting methicillin resistance has drawback, due to false negative that could occur. False negative could result from use of high DNA concentration or when there is repression of PBP2a production or spontaneous loss of *mecA* as a result of mutation in mec complex. In this study, a potential MRSA was missed in a PCR analysis of *mecA* due to absence of beta-lactamase regulatory elements which would have co-regulated with *mecR1* for *mecA* expression. In three *mecA* positive isolates, *mecI* was found to have mutated, but contain functional signal transducer genes (*mecR1*). For these reasons, detection of methicllin resistance should include *mecA* regulatory elements (*mecR1* and *mecI*) in addition to *mecA* and *SCCmec* as target genes (marker).

MRSA infections have been associated with significant morbidity and mortality in health-care institutions, empirical antibiotic regimes are needed to manage patients suffering from MRSA/CN-MRS infections. Therefore, an accurate analysis of methicillin resistance and the determination of the origin of methicillin positive isolates may allow for provision of better antimicrobial therapy. An accurate and fast detection of resistant isolates should constitute a critical goal of clinical microbiology, and therefore, PCR assays have become an essential tool in laboratory programs. Hence, for analysis of resistance, epidemiological

studies and the detection of infectious and pathogenic organisms, molecular methods should be the choice.

6.2 Recommendations

For any case of epidemic, control measures have been the critical point of emphasi, therefore, there is need for proper clinical documentation of reported cases of MRSA, quarantine the subject(s) from the general population and treat the individual infected with MRSA. Also, organizing health education to both health care workers and the general public on the significance of MRSA should be a continuous exercise. This can be achieved through awareness campaigns and general population screening. It is important that MRSA reference laboratory be set up so that reported cases can be characterized for epidemiological purposes. For correct assessment of methicillin resistance, detection should be based on *mecA* detection supplemented with its regulatory elements or SCC*mec*. This will help to fast track any outbreak of epidemic MRSA clones. When all these are put in place, the incidence of methicillin resistant-staphylococci will be a mere history in Nigeria. Further studies should be carried out on MRSA strain typing to check which clone(s) are circulating within this country as well as explaining possession of PVL genes in community acquired MRSA isolates and its absence in clinical isolates.

6.3 Contributions to Knowledge

1. This study has shown that the resistance determinant (*blaZ*) predominantly found in clinical isolates are also emerging in the community isolates.
2. To my best knowledge, this is the first time *S. epidermidis* with methicillin resistant gene as confirmed by molecular method was identified in Nigeria.
3. This study established non-existance of vancomycin resistant gene circulating in both clinical and community isolates of s*taphylococcus* species.
4. This study has demostrated that methicllin resistance gene can be detected indirectly using *mecA* regulatory elements.
5. This study has introduced a system of separating *S. aureus* from other coagulase negative Staphylococci when s*taphylococcus* genus amplicon was double-digested with restriction enzymes (EcoR1 and H111).

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APPENDIX I

PREPARATION OF REAGENTS

Lysis buffer: pH 8.0

Solution A

Tris----------2.5g + 10ml d H_2O (1M)

$NaCl_2$----------0.585g + 10ml d H_2O (1M)

EDTA----------2.923g + 10ml d H_2O (1M)

Solution B

SDS----------0.2g + 5ml d H_2O (4%)

Na-acetate----------6.54g + 10ml d H_2O (5M)

Proteinase K----------10mg/ml

$C_1V_1=C_2V_2$

Where C_1= initial concentration and V_1 = initial volume

C_2= final concentration and V_2 = final volume

Tris

C_1= 1000mM, C_2= 50mM, V_2= 100ml and V_1=?

$V_1= C_2V_2/ C_1$

Hence, V_1= 50×100/1000 = 5ml

$NaCl_2$, V_1= 5ml

EDTA, V_1= 0.5ml

Overall solution A: 5ml Tris + 5ml $NaCl_2$ + 0.5ml EDTA into a measuring cylinder and add up to 100ml. Adjust to pH 8.0 using HCl and NaoH.

Overall solution B: Mix 4% SDS + 5M Na-acetate + 10mg/ml proteinase K.

Phenol-Chloroform-isoamylalchohol (24:1:1%): 24ml + 1ml + 1ml each respectively.

Chloroform-isoamylalchohol (24:1%): 24ml + 1ml each respectively.

Alcohol (70%): 70ml of alcohol + 30ml of d H_2O.

50X TAE (TRIS-ACETATE) STOCK

242.0 g Tris base

57.1 ml Glacial acetic acid

18.61 g Na_2EDTA .2H2O

To 1 liter with distilled water and sterilize.

5X TBE (TRIS-BORATE) STOCK

(1X=89 mM Tris base, 89 mM boric acid, 2 mM EDTA)

(0.5X=45 mM Tris-borate, 1 mM EDTA)

54.0 g Tris base

27.5 g Boric acid

3.72 g Na2EDTA .2H2O

To 1 liter with distilled water and sterilize.

Preparation of the 0.5 McFarland standards

Zero point five (0.5) mL of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2$. 2H2O) was added to 99.5 mL of 0.18 M H_2SO_4 (1% w/v) with constant stirring. The solution was thoroughly mixed to obtain an even suspension. By using cuvettes with water as a blank standard, the absorbance of the solution was measured in a spectrophotometer at a wavelength of 625 nm. The acceptable absorbance range for the standard was 0.08-0.13. The standard were distributed into screw-cap tubes and sealed tightly to prevent loss by evaporation.

APPENDIX II

Zone of inhibition (mm) produced by community isolates to various antibiotics

Isolates	COT 25(µg)	CHL 10(µg)	CXC 5(µg)	ERY5 (µg)	GEN 10(µg)	STR 10(µg)	AUG 30(µg)	TET 10(µg)	VAN 30(µg)	MET 10(µg)	FOX 10(µg)	OX 1(µg)
Com1	0.0	20.0	0.0	14.0	0.0	0.0	0.0	9.0	17.0	13.0	10.0	0.0
Com6	0.0	0.0	0.0	0.0	12.0	14.0	0.0	18.0	16.0	19.0	13.0	0.0
Com7	0.0	10.0	0.0	0.0	12.0	10.0	0.0	11.0	15.0	24.0	22.0	0.0
Com8	0.0	15.0	0.0	9.0	10.0	20.0	0.0	0.0	16.0	22.0	14.0	0.0
Com10	0.0	16.0	0.0	12.0	15.0	13.0	0.0	0.0	11.0	20.0	0.0	0.0
Com11	0.0	18.0	0.0	10.0	15.0	12.0	0.0	10.0	17.0	23.0	22.0	0.0
Com12	13.0	13.0	15.0	0.0	15.0	13.0	15.0	14.0	14.0	19.0	14.0	13.0
Com16	0.0	14.0	11.0	12.0	14.0	14.0	0.0	11.0	11.0	17.0	13.0	12.0
Com18	0.0	17.0	0.0	0.0	13.0	12.0	0.0	18.0	11.0	22.0	13.0	8.0
Com19	0.0	18.0	0.0	11.0	10.0	20.0	0.0	10.0	13.0	20.0	20.0	9.0
Com26	0.0	10.0	0.0	9.0	18.0	22.0	0.0	14.0	0.0	0.0	0.0	0.0
Com29	0.0	0.0	0.0	0.0	0.0	14.0	0.0	11.0	13.0	0.0	0.0	0.0
Com38	20.0	20.0	20.0	20.0	20.0	17.0	16.0	13.0	18.0	26.0	17.0	22.0
Com39	20.0	14.0	9.0	14.0	14.0	14.0	0.0	11.0	12.0	20.0	12.0	12.0
Com40	0.0	16.0	0.0	13.0	12.0	10.0	0.0	17.0	13.0	11.0	9.0	0.0
Com41	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	14.0	0.0	0.0	0.0
Com43	0.0	15.0	0.0	13.0	14.0	14.0	0.0	15.0	15.0	20.0	20.0	0.0
Com45	12.0	15.0	0.0	11.0	18.0	0.0	0.0	14.0	18.0	11.0	15.0	0.0
Com46	0.0	0.0	0.0	19.0	18.0	14.0	0.0	11.0	14.0	20.0	24.0	0.0
Com49	0.0	20.0	0.0	0.0	13.0	0.0	0.0	0.0	12.0	18.0	21.0	13.0
Com52	0.0	18.0	11.0	11.0	12.0	11.0	0.0	15.0	11.0	19.0	14.0	8.0
Com55	0.0	18.0	0.0	12.0	16.0	13.0	0.0	12.0	12.0	19.0	15.0	15.0
Com56	0.0	12.0	0.0	0.0	17.0	11.0	15.0	19.0	0.0	0.0	0.0	0.0
Com58	0.0	15.0	0.0	17.0	19.0	0.0	0.0	17.0	21.0	23.0	12.0	14.0
Com59	15.0	15.0	0.0	0.0	13.0	10.0	0.0	14.0	11.0	19.0	14.0	15.0
Com60	0.0	13.0	0.0	12.0	11.0	0.0	0.0	0.0	12.0	0.0	0.0	0.0
Com63	15.0	14.0	0.0	10.0	13.0	12.0	0.0	0.0	14.0	18.0	17.0	13.0
Com68	0.0	9.0	17.0	15.0	17.0	13.0	9.0	10.0	13.0	18.0	13.0	14.0
Com69	0.0	18.0	0.0	0.0	0.0	13.0	0.0	0.0	15.0	0.0	9.0	0.0
Com80	0.0	12.0	12.0	12.0	13.0	10.0	0.0	0.0	10.0	18.0	20.0	0.0
Com81	0.0	15.0	10.0	11.0	11.0	0.0	11.0	0.0	13.0	18.0	17.0	11.0
Com82	0.0	13.0	0.0	0.0	12.0	12.0	0.0	0.0	12.0	0.0	0.0	0.0
Com88	0.0	9.0	0.0	0.0	17.0	16.0	10.0	15.0	14.0	15.0	0.0	0.0
Com89	0.0	11.0	0.0	11.0	11.0	13.0	0.0	0.0	15.0	12.0	0.0	0.0
Com91	0.0	9.0	0.0	0.0	16.0	16.0	0.0	20.0	13.0	17.0	17.0	10.0
Com92	0.0	12.0	0.0	0.0	20.0	0.0	20.0	0.0	16.0	21.0	23.0	0.0
Com93	0.0	9.0	0.0	0.0	16.0	16.0	0.0	10.0	13.0	13.0	10.0	0.0
Wat5	0.0	9.0	0.0	0.0	16.0	16.0	0.0	10.0	13.0	13.0	10.0	0.0
Wat3	0.0	15.0	0.0	16.0	12.0	13.0	0.0	15.0	15.0	21.0	22.0	9.0
Wat8	0.0	10.0	0.0	18.0	13.0	18.0	0.0	9.0	12.0	19.0	20.0	0.0
Wat7	0.0	19.0	0.0	17.0	10.0	16.0	0.0	17.0	22.0	25.0	0.0	0.0

APPENDIX II Continues: Zone of inhibition (mm) produced by community isolates to various antibiotics

Wat10	0.0	0.0	0.0	0.0	22.0	20.0	0.0	25.0	28.0	0.0	30.0	0.0
Wat19	0.0	16.0	0.0	0.0	18.0	0.0	0.0	15.0	16.0	18.0	18.0	0.0
Wat4	0.0	0.0	0.0	0.0	18.0	0.0	0.0	0.0	18.0	18.0	24.0	0.0
Wat30A	0.0	18.0	0.0	0.0	15.0	0.0	0.0	0.0	13.0	15.0	14.0	0.0
Wat9	0.0	12.0	0.0	0.0	20.0	18.0	0.0	0.0	16.0	19.0	20.0	0.0
Com1	0.0	25.0	0.0	0.0	19.0	0.0	0.0	20.0	18.0	21.0	21.0	0.0
Com2	15.0	17.0	0.0	14.0	16.0	14.0	10.0	17.0	17.0	21.0	20.0	0.0
Com3	0.0	0.0	0.0	12.0	17.0	14.0	0.0	10.0	18.0	25.0	21.0	0.0
Com111	0.0	12.0	0.0	10.0	13.0	19.0	0.0	0.0	16.0	19.0	20.0	0.0
C36	0.0	17.0	0.0	13.0	14.0	16.0	10.0	0.0	20.0	25.0	22.0	14.0
C41	18.0	0.0	0.0	0.0	19.0	20.0	14.0	20.0	17.0	18.0	28.0	9.0
C56	18.0	23.0	0.0	21.0	17.0	18.0	0.0	19.0	19.0	17.0	30.0	0.0

APPENDIX III

Zone of inhibition (mm) produced by clinical isolates to various antibiotics

Isolates	COT 25(µg)	CHL 10(µg)	CXC 5(µg)	ERY5 (µg)	GEN 10(µg)	STR 10(µg)	AUG 30(µg)	TET 10(µg)	VAN 30(µg)	MET 10(µg)	FOX 10(µg)	OX 1(µg)
100	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
S10	0.0	15.0	0.0	14.0	14.0	11.0	0.0	8.0	16.0	15.0	14.0	5.0
131	0.0	14.0	0.0	17.0	19.0	18.0	5.0	9.0	17.0	20.0	19.0	3.0
149	0.0	12.0	0.0	17.0	19.0	15.0	0.0	12.0	17.0	22.0	22.0	10.0
S21	20.0	19.0	0.0	13.0	14.0	12.0	0.0	11.0	16.0	21.0	18.0	0.0
157	5.0	17.0	10.0	19.0	20.0	17.0	0.0	0.0	19.0	18.0	19.0	10.0
167	0.0	0.0	0.0	0.0	7.0	12.0	0.0	0.0	17.0	15.0	10.0	0.0
181	0.0	0.0	0.0	0.0	15.0	12.0	0.0	9.0	17.0	6.0	4.0	0.0
201	0.0	20.0	8.0	13.0	12.0	10.0	0.0	13.0	15.0	18.0	18.0	0.0
204	0.0	17.0	8.0	17.0	19.0	17.0	0.0	12.0	19.0	23.0	22.0	10.0
260	21.0	11.0	0.0	17.0	10.0	19.0	0.0	14.0	20.0	11.0	11.0	0.0
283	10.0	15.0	7.0	19.0	16.0	18.0	10.0	17.0	18.0	20.0	20.0	7.0
380	0.0	13.0	0.0	13.0	15.0	13.0	0.0	18.0	17.0	22.0	20.0	19.0
413	0.0	0.0	0.0	0.0	0.0	12.0	0.0	10.0	15.0	18.0	17.0	0.0
S5	0.0	13.0	0.0	0.0	0.0	0.0	0.0	0.0	13.0	0.0	0.0	0.0
493	0.0	10.0	0.0	12.0	10.0	14.0	0.0	9.0	16.0	12.0	0.0	0.0
611	0.0	15.0	0.0	0.0	10.0	11.0	0.0	9.0	11.0	15.0	15.0	10.0
615	0.0	12.0	0.0	0.0	17.0	17.0	0.0	18.0	14.0	23.0	24.0	0.0
634	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.0	10.0	15.0	17.0	0.0
641	9.0	0.0	0.0	0.0	0.0	0.0	0.0	10.0	13.0	0.0	0.0	0.0
S20	0.0	0.0	0.0	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
S3	0.0	16.0	0.0	10.0	11.0	17.0	0.0	0.0	13.0	14.0	16.0	0.0
698	0.0	0.0	0.0	12.0	0.0	0.0	0.0	0.0	11.0	10.0	10.0	0.0
6N	0.0	18.0	0.0	0.0	19.0	14.0	0.0	16.0	17.0	17.0	20.0	11.0
S24	0.0	8.0	0.0	14.0	10.0	0.0	0.0	10.0	15.0	12.0	0.0	0.0
S12	18.0	18.0	0.0	0.0	20.0	20.0	0.0	18.0	18.0	19.0	22.0	10.0
781	0.0	0.0	0.0	12.0	13.0	13.0	0.0	13.0	17.0	20.0	0.0	0.0
798	0.0	0.0	0.0	0.0	9.0	15.0	0.0	16.0	18.0	0.0	0.0	0.0
821	0.0	20.0	0.0	15.0	12.0	14.0	0.0	17.0	17.0	6.0	6.0	0.0
S11	18.0	15.0	0.0	11.0	14.0	12.0	0.0	15.0	13.0	15.0	15.0	12.0
A	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.0	15.0	15.0	0.0
ON	0.0	12.0	0.0	11.0	13.0	12.0	0.0	17.0	15.0	20.0	18.0	0.0
OO	0.0	15.0	0.0	13.0	13.0	10.0	0.0	9.0	13.0	19.0	16.0	0.0
650	12.0	0.0	0.0	17.0	10.0	19.0	0.0	10.0	18.0	18.0	19.0	10.0
652	0.0	14.0	0.0	17.0	19.0	15.0	15.0	0.0	19.0	20.0	19.0	10.0
146	0.0	17.0	5.0	15.0	17.0	18.0	4.0	5.0	13.0	19.0	19.0	15.0
667	0.0	16.0	9.0	0.0	12.0	10.0	0.0	7.0	13.0	19.0	15.0	13.0
995	0.0	0.0	0.0	0.0	0.0	0.0	15.0	0.0	17.0	0.0	0.0	0.0
779	0.0	15.0	0.0	12.0	14.0	11.0	0.0	12.0	15.0	23.0	21.0	0.0

APPENDIX III Continues: Zone of inhibition (mm) produced by clinical isolates to various antibiotics

48	0.0	12.0	0.0	12.0	14.0	11.0	0.0	0.0	12.0	21.0	20.0	13.0
681	0.0	16.0	0.0	10.0	11.0	17.0	0.0	0.0	13.0	14.0	16.0	0.0
441	0.0	13.0	0.0	0.0	0.0	0.0	0.0	0.0	13.0	0.0	0.0	0.0
153	11.0	0.0	0.0	0.0	14.0	0.0	0.0	5.0	0.0	0.0	0.0	0.0
76	0.0	0.0	0.0	0.0	0.0	14.0	0.0	0.0	18.0	0.0	0.0	0.0
4020	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.0	0.0	0.0	0.0
998	0.0	16.0	9.0	0.0	12.0	10.0	0.0	7.0	13.0	19.0	15.0	13.0
463	0.0	18.0	5.0	7.0	6.0	5.0	0.0	17.0	12.0	20.0	18.0	13.0
467	24.0	19.0	13.0	22.0	20.0	19.0	24.0	23.0	16.0	25.0	18.0	0.0
922	25.0	19.0	11.0	22.0	19.0	18.0	23.0	20.0	20.0	30.0	20.0	15.0
668	0.0	17.0	5.0	5.0	15.0	5.0	4.0	5.0	14.0	20.0	20.0	15.0
263	13.0	19.0	0.0	0.0	19.0	17.0	0.0	0.0	0.0	0.0	15.0	0.0
699	0.0	15.0	0.0	12.0	0.0	11.0	0.0	9.0	11.0	15.0	15.0	10.0
155	20.0	19.0	0.0	13.0	14.0	12.0	0.0	10.0	15.0	20.0	18.0	10.0

APPENDIX IV

Composition of media

Composition	Weight (g/l)
Mannitol Salt Agar (Oxoid)	
Lab-lemco powder	1.0
Peptone	10.0
Mannitol	75.0
NaCl	5.0
Phenol red	0.025
Agar	15.0
pH	7.5±0.2 (25^0C)
Tryptone Soya Agar (Oxoid)	
Casien	15.0
Soya bean	5.0
NaCl	5.0
Agar	15.0
pH	7.3±0.2 (25^0C)
Nutrient broth (Fischer)	
Lab-lemco powder	1.0

Yeast	2.0
NaCl	5.0
Phenol red	0.025
pH	7.5±0.2 (25^0C)

Mueller-Hinton Agar (Oxoid)	
Meat infusion	2.0
Casein hydrolysate	15.0
Starch	1.5
Agar-agar	13.0
pH	7.4±0.2 (25^0C)

Sugar solution	
Sugar	5.0
Peptone	10.0
NaCl	5.0
Methyl red	0.08