THE PREVALENCE OF FURUNCULOSIS AND PLASMID-MEDIATED RESISTANCE OF ISOLATES OF *STAPHYLOCOCCUS AUREUS* FROM INFECTED INDIVIDUALS IN SOUTHWEST NIGERIA.

BY

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C E R T IF I C A T I O N

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DEDICATION

This thesis is wholly dedicated to the **Lion of the tribe of Judah**, the wisdom of God and the captain of the mighty throng.

ACKNOWLEDGEMENTS

God is highly exalted in His power, who is a teacher like Him? Job 36 verse 22.

Mine is life buoyed up by many hands. Like **Isaac Newton, "**if I have seen farther than men, it is because I have stood on the shoulders of giants". Fortuitous as this may be, it complicates such simple tasks as writing an acknowledgement. However, give it a try I shall.

I am clearly at a loss as to where and how to begin this acknowledgement in view of the fact that many people must have played unique roles (morally and otherwise) that culminated in the writing and finishing a thesis of this size in its series. Such roles included; a nod of approval, a word of encouragement, a smile of approval, a show of surprise, e.t.c. But whatever it was, it gave me the needed inspiration and determination to continue the works until the glorious ends.

My sincere gratitude goes to this amiable man of impeccable integrity , my ever listening and always caring supervisor, Dr. Olufemi Ezekiel Adeleke for reading and shaping this manual. 'igba odun, odun kan o'. Higher! higher! higher$^{3!}$ you shall go in Jesus name. I owe a debt of gratitude to Professor Itiola for his 'backbone' support till the end of this chronicles, higher! higher! you shall go sir, in Jesus name.

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An impetus of the determination that keeps me away from deterioration. Oh **God** of suddenly! I thank you for seeing me through. I am captured by your majesty. Holy, holy, holy and endless thanks unto your indescribable sacred name.

ABSTRACT

Furunculosis, a cosmopolitan infection of human skin caused by *Staphylococcus aureus*, is recurrent among most infected individuals. It is characterized by a honey crusted 'cropped' latent boil with potential to recur in a susceptible host. It is a common colonizer of the skin with a remarkable ability to hydrolyse β-lactam antibiotics, degrade skin lipid barrier and spread within the skin loci. In this study, the gender and age distributions of furunculosis, the antibiotic susceptibility pattern of its causative agent and the genetic basis of the recurrency were determined.

Exudates of 'cropped-boils' were obtained from 140 human volunteers (40 hospital reported and 100 non-reported cases of recurrent furunculosis) from different age range(1-100 years) for both genders within the six southwest states of Nigeria. The exudates were processed for isolation and identification of *S. aureus* by selective plating and biochemical tests. Antibiogram of the isolates was determined by disc-diffusion using multi-discs of eight standard antibiotics. Minimum Inhibitory Concentrations (MIC) of five selected antibiotics: amoxicillin-clavulanic acid, penicillin, ceftriaxone, cefuroxime and cloxacillin were determined by broth-dilution method. Detection of β-lactamase was carried out by cell-suspension iodometric method. Positive strains were processed for plasmid DNA isolation and molecular weight determination by lystostaphin cell lysis and agarose gel electrophoresis. Curing of R-plasmid DNA in selected bacterial strains was done by exposure to ethidium bromide prepared in 2-fold dilutions in nutrient broth from 6.25-100.00 µg/mL.

Transfer of resistance was done by conjugation between some resistant strains as donors and Carolina-typed sensitive *E. coli; E. coli 01 and E. coli 02* strains as recipients. The data were analysed using ANOVA at $p = 0.05$.

A total of 102 isolates comprising seventeen from each of the six southwest states were identified and selected. The gender distributions were 46.0% females and 54.0% males, comprising of 20.0% hospital reported and 80.0% non-reported for the highest prevalence females and 5.0% hospital reported and 95.0% non-reported for males. Recurrent furunculosis had the highest prevalence in males within the age groups 11-50 years and in females, age groups, 11-70 years. The isolates exhibited the lowest resistance of 8.0% to amoxicillin-clavulanic acid and 95.0% as the highest resistance to amoxicillin. Thirty of the isolates possessed β-lactamase in varying degrees out of which 29.0 were plasmid-borne. Of this number, 7.0 had multiple plasmid DNA of 2- 4 copies, ranging between 0.25 and 63.09 kb. The MIC of the antibiotics showed that the isolates were most susceptible to amoxicillin-clavulanic acid (3.90 – 250 µg/mL) and the highest resistance was recorded for penicillin G (7.8 – 500 µg/mL). Curing of R-plasmid DNA occurred at concentrations of 50.0µg/mL and 100.0µg/mL of ethidium bromide while conjugation was achieved in two out of seven competent cells, indicating a plasmid-borne resistance.

The prevalence of furunculosis varied across the different age groups. The high level multidrug resistance elicited by the strains of *Staphylococcus aureus* isolated was established to be due to the associated transferable R-plasmid encoded β-lactamase.

KEYWORDS Recurrent furunculosis, *Staphylococcus aureus* , β-lactam antibiotics, R- plasmid.

Word count: 485.

LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviaton	Full meaning
ATCC	- American Typed Culture Collections
BNF	- British National Formulary
BA	- Blood Agar
B. P.	- British Pharmacopoeia
EDTA	- Ethylene Diammonium Tetra-acetic Acid
F factor	- Fertility factor
5 ⟶ 3 - OH	- 5 prime to 3 prime hydroxyl group
HFT	- High Frequency Transfer
10/ml,	- International unit per milliliter
kb	- Kilobase
MCA	- MacConkey Agar
MDR	- Multiple Drug Resistance
MIC	- Minimum Inhibitory Concentration
MBC	-Minimum Bactericidal Concentration
MSA	- Mannitol Salt Agar
MW	- Molecular Weight
mM	- milliMole
NCTC	- National Collection of Typed Cultures
Penicillin G	- Benzylpencillin

Pencillin V	- Phenoxymethyl penicillin
R^+ (or F^+)	- Fertile (Male/donor) or drug-resistant cell
R^- (or F^-)	- Infertile (female/recipient) or drug sensitive cell.
r-determinants	- resistance determinants
R-Plasmid / R-factor)	– Resistance plasmid (or Resistance factor)
RTF	- Resistance Transfer Factor
6-APA	- 6-amino penicillanic acid
7-ACA	- 7-amino cephallosporanic acid
TE buffer	- Tris-EDTA buffer
TSA	- Tryptone Soya Agar
WHO	- World Health Organisation
µg/ml	- microgramme per milliltre
µI.	- microlitre
µm	- micrometer
β	Beta

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TE buffer - Tris-EDTA buffer

TSA - Tryptone Soya Agar

WHO - World Health Organisation

µg/ml - microgramme per milliltre

µl. - microlitre

µm - micrometer

β Beta

CHAPTER ONE

INTRODUCTION

Furunculosis is a very common skin disease which is usually not due to any disorder in blood, and is manifested as multiple boils(furuncles) on different parts of the body. Several boils joined together with tunnels under the skin are called carbuncle. Boil and carbuncle are painful, pus filled bumps that form under the skin when bacteria infect and inflame one or more of human hair follicles (Zimakoff *et.al.*, 1988).

Furuncles usually start as red, tender lump. The lumps are quickly filled with pus, growing larger, cropped and more painful until they rupture and drain. Although some furuncles disappear a few days after they occurred, most take about two weeks to heal. Furuncles can occur anywhere on the skin but appear mainly on the face, neck, armpits, buttocks or thighs, hair bearing areas where one mostly sweats or experiences friction. Anyone can develop cropped boil(furuncle) and carbuncles but people who have diabetes, suppressed immune system or acne are at great risk (Sosin *et.al.*, 1989).

As a boil gets larger, it develops a pus filled cavity, becoming an abscess. A pimple is a mini-boil and can also develops to an abscess and it occurs when the hair follicle or a tiny cut or scratch becomes infected with a bacterium usually *Staphylococcus aureus***.**

As a boil starts to develop, the body's immune system carries white cells in the blood to the site of the boil to attack and engulf the invading bacterial cells, a process known as phagocytosis.

The body also creates a fibrous wall as a barrier to contain the infectious agent. Once the furuncle reaches a certain size, this fibrous wall prevents antibiotics in the bloodstream from penetrating into the locus of infection.

The pus filled boils are made up of dead white cells and dead bacteria, and because this liquid forms under pressure, they becomes painful and will eventually burst, draining the pus, relieving pain and will then heal. The whole process can take two weeks, and often doctors will "lance" the furuncle early, making a deliberate hole in it to allow the pus to drain, to speed up the healing process (Ayliffe *et.al.*, 2006).

It is very common for boils to "crop", occurring as several boils that go through their life-cycle, heal and then occur weeks or months later (recurrent), a condition known as recurrent staphylococcal furunculosis (Gira *et. al.*, 2005). The condition can be very distressing and there may be need to exclude diabetes and other conditions through blood tests. It is not often due to the immune status of the host but rather to the continuous presence of the bacterium *Staphylococcus aureus* on the skin.

1.1. ***Staphylococcal* skin infections**

Skin infections due to *Staphylococcus aureus* are classified as primary or secondary. Primary skin infections are those occurring on apparently normal skin, and mainly comprise impetigo, folliculitis, furuncles (cropped boils), sycoe barbae, cellulitis, abscesses, paronychia and whitlows. The diagnosis of primary infection was based on clinical findings. Secondary skin infections are those arising in damaged skin (traumatized skin, or a pre-existing skin disease). The diagnosis of secondary skin infection was based on the presence of pus, local signs of inflammation (Khan, 1991).

An infected wound is an example of secondary skin infection. Infections ranging from superficial skin lesions to serious systemic infections have been associated with *Staphylococcus aureus* , while nosocomial infections have been attributed to epidemic strains with multi drug resistance (Price *et.al.*,1998). .

1.2. Epidemiology of furunculosis.

The incidence of cropped boils is uncertain. They are rare in children except in those with atopic eczema. Boils are more common in adolescents and in early adulthood, especially in boys, and the peak incidence for acne-vulgaris (Davies, 2007).

Predisposing factors to recurrent furunculosis infection include an infected wound, obesity, diabetes mellitus, skin disease, poor hygiene, debilitation, tight clothes, friction, and immunosuppressive therapy (Sungeg *et.al.*, 1998).

Staphylococcal colonization is more common on atopic eczema and may contribute to the pathogenesis. The evidence that links diabetes with furunculosis is conflicting but when boils affect people with diabetes, they tend to be more extensive.

Other conditions associated with furunculosis include obesity and immune compromise as with HIV, and treatment with immunosuppressive drugs. In adult, the use of topical steroids is associated with the development of folliculitis (Lyon *et al*., 1987)

1.3. Pathogenicity of furunculosis .

A boil as a seed of furunculosis starts as a hard, tender and red nodule surrounding a hair follicle. It enlarges and becomes ripe over several days as an abscess form.

Later it may discharge pus from its centre before healing and may leave a scar. Cropped boils arise in hair bearing areas, especially where there is friction, occlusion, and perspiration.

These include the neck, face, axillae, arms, wrists, fingers, buttocks and ano-genital region. Boils may be isolated or present as multiple lesions. The latter is particularly likely in the buttocks. There are sometimes uncommon symptoms such as fever and malaise (Sungeg *et.al.,* 1999).

1.4 Complications from furunculosis

There are a number of complications that can develop from furuncles and they Includes; cellulitis which is infection of outer skin, characterized by inflammation, tenderness, pain, redness, hot and swollen nodules. Other complications includes scarring, disfiguration, gangrene**,** when the blood supply to a tissue is cut off the tissue turns black and decays, necrotising soft tissue infections and impairment or congestion of dermal blood vessels (Gorwitz, 2007).

1.5 Antibiotic therapy

Patients suffering from recurrent boils need to guide against infestation of the skin with *Staphylococcus aureus*. Many types of treatment have been tried to prevent boils from cropping but without much success. In general, longer continuous courses of antibiotics, orally, do not seem very successful, presumably because they do not act on the *Staphylococcus aureus* living on the surface of the skin (Plorde and Sheris, 1974).

Staphylococcus aureus expresses its resistance to drugs including β–lactam antibiotics (e.g methicillim, oxacillin, penicillin and amoxicillin) through a variety of mechanisms: (1) Mutation in chromosomal genes (2) Acquisition of resistance genes as extra chromosomal plasmids, transducing particles, trasposons or other types of DNA. inserts.

Beginning with the use of penicillin in the 1940's, drug resistance has developed in staphylococci within a very short time after introduction of penicillin into clinical use. Some strains are now resistant to most conventional antibiotics while there is concern that new antibiotics have not been forthcoming(Clewell 2008).

New strategies in the pharmaceutical industry to find antimicrobial drugs involve identifying potential molecular targets in cells (such as the active site of enzymes involved in cell division), then developing inhibitors of the specific target molecule. Hopefully, this approach will turn up new antimicrobial agents for the battle against staphylococcal infections. In the past two decade, alternatives to vancomycin have been approved with the increase in Vancomycin Resistant *Staphylococcus aureus* (VRSA) isolates. (Stevens, *et al.*, 2005)

Hospital strains of *Staphylococcus aureus* are usually resistant to a variety of different antibiotics. A few strains are resistant to all clinically useful antibiotics except vancomycin, although, vancomycin-resistant strains are increasingly being reported. Methicillin resistance is widespread and most methicillin resistant strains of *Staphylococcus aureus* are also multiple drug resistant.

A plasmid associated with vancomycin resistance has been detected in *Enterococus faecalis* which can be transferred to *Staphylococcus aureus* in the laboratory, and it is speculated that this transfer may occur naturally (e.g. in the gastrointestinal tract) (Papanicolau *et.al.*, 1990).

In addition, *Staphylococcus aureus* exhibits resistance to antiseptics and disinfectants, such as quanternary ammonium compounds, which may aid its survival in the hospital environment.

Staphylococcal disease has been a perennial problem in the hospital environment since the beginning of the antibiotic era, such that during the 1950's and early 1960's, staphylococcal infection was synonymous with nosocomial infection (Lyon *et.al.*, 2008).

Though, *Staphylococcus aureus* was among the earliest bacterial species with recognizable extrachromosomal inheritance (Beveniste, 1973), yet, the genetic basis of bacterial drug-resistance, particularly in relation to severity in this organism remains a subject of interest and speculation (Olukoya *et al.*, 1995).

Underscoring the need for more studies on plasmid profiles of clinical strains of *Staphylococcus aureus.*

1.6. Phage therapy

Phage therapy is the therapeutic use of bacteriophages to treat pathogenic bacterial infection. In principle phage therapy results in less harm to the normal body flora and ecology than commonly used antibiotics, which often disrupt the normal gastrointestinal flora and result in opportunistic secondary infections by organisms such as *Clostridium difficile* (Thiel-Karl, 2004).

Phage therapy can be very effective in certain conditions and has some unique advantages over antibiotics. Bacteria also develop resistance to phages, but it is incomparably easier to develop new phage than new antibiotic. A potential benefit of phage therapy is freedom from the severe adverse effects of antibiotics.

As bacteria evolve resistance, the relevant phages naturally evolve alongside such that when multidrug resistance bacteria appear, there is phage to attack them . It only needs to be derived from the same environment (Pirisi,2000).

Phages have special advantage for localized use, because they penetrate deeper as long as the infection is present, rather than decrease rapidly in concentration that is common with antibiotics. The phages stop reproducing once the specific bacteria they target are destroyed (Shasha *et.al*, 2004).

Phage treated organisms do not develop secondary resistance, which is quite often in antibiotics treated organisms. With the increasing incidence of antibiotic resistant bacteria and a deficit in the development of new classes of antibiotics to counteract them, there is need to apply phages in a range of infections. Lytic phages are similar to antibiotics in that they have remarkable antibacterial activity.

However, therapeutic phages have some advantages over antibiotics, and have been reported to be more effective than antibiotics in treating certain infections in humans and experimentally infected animals (Thiel-Karl, 2004).

For example, in a study, *Staphylococcus aureus* phages were used to treat patients having purulent disease of the lungs and pleura, no side effects were observed in any of the patients, including those who received phages intravenously and it is of interest to note that phage therapy unlike antibiotic therapy is not constrained by series of factors like age, underlying disease, and allergies as in the appropriation of antibiotics (Pirisi, 2000).

1.7. Aims of this study

1. To collect pus samples from human volunteers identified with recurring incidents of furunculosis..

2. To determine antimicrobial susceptibility pattern of the isolates of *Staphylococcus aureus*

3 . To detect penicillin resistant and β-lactamase production among the isolates.

4. To carry out plasmid profiling and antibiotic resistance curing on selected drug resistant isolates

5. To carry out conjugational transfer of resistance with some resistant strains.

6. To make suggestion on antibiotic(s) that may be particularly suitable in the treatment of recurrent boil infection and make recommendations on preventive measures.

1.8. Justification for this study

Furunculosis, an age long common communal infection that cuts across tribes, race, sex and age groups is an infection with many predisposing factors like poor hygiene, infected wound, moisture, obesity, diabetes mellitus, skin disease, debilitation, tight clothes, friction and immunosuppressive therapy as well as industrial exposure to chemicals or oils. There is a scarcity of scientific information on the report and therapeutic management of recurrent cases of furuncles, thereby necessitating a study of this nature to provide baseline data for extended further works. In particular, boil of recurrent characteristic should attract clinical importance in view of the undesirable effects it has on the human host, namely; discomfort, disfiguration, repulsive attribute, complication potential and others. In spite of these negative effects, furunculosis of recurrent nature, from the available literature, is yet to attract the much needed attention by clinical microbiologists in terms of epidemiology, chemotherapy and genetic basis of the infection. This dearth of scientific study on recurrent boil infection and the need to curtail the infection have made it desirably imperative to embark on this study.

CHAPTER TWO

LITERATURE REVIEW

2.0. Origin of boils/furuncles

….. And the LORD said unto Moses and unto Aaron, take to you handfuls of ashes of the furnace, and let Moses sprinkle it toward the heaven in the sight of Pharaoh. And it shall become small dust in all the land of Egypt, and shall be boils(furuncles) breaking forth with sores upon man, and upon beast, throughout all the land of Egypt and it became boils(furuncles) breaking forth with sores upon man, and upon beast. And the magician could not stand before Moses because of the boils**;** for the boils(furuncles**)** were upon the magicians, and upon all the Egyptians (Exodus9 verses 6-11, (K.J.V).

And also with the physical suffering of Job "Satan went out from the presence of the LORD, and struck Job with painful boils (furuncles**)** from the sole of his foot to the crown of his head." This is as a result of an express permission for Satan from God to prove-test the stand of Job before God that testifies to his righteousness (Job2 verse 5-7) and lastly to the inflammation of king Hezekiah to whom God directed the prophet Isaiah to go and apply the poultice of fig tree for curing which he did (Isaiah38verse21).

Ever since those Biblical episodes, the incidence of boil/furuncle had come to stay. Remarkably, as a result of advancement in biological Sciences, the culprit monster in the power house of this painful – pus filled infection was traced to be *Staphylococcus aureus* (Jawetz *et.al.*, 2010) .

It is a Gram positive bacterium, that occurs in microscopic clusters resembling grapes. Bacteriological cultures of the nose and skin of normal humans invariably yield staphylococci. Robert Koch in 1878 was the first to describe staphylococci in human pus. In 1884, Rosenbach described the two pigmented colony types of staphylococci and proposed the appropriate nomenclatures; *Staphylococcus aureus* (Yellow) and *Staphylococcus albus* (white). The latter species is now named *Staphylococcus epidermidis* (Cohen and Kanock, 2004).

Although more than 20 species of *Staphylococcus* are described in Bergey's Manual (2001), *Staphylococcus aureus* and *Staphylococcus epidermidis* are significant in their interactions with humans because of their tendency to produce infections.

Staphylococcus aureus colonizes mainly the nasal passages, but it may be found regularly on most other anatomical loci of the body.

Staphylococcus aureus forms a fairly large yellow colony on rich medium and is often haemolytic on blood agar. *Staphylococcus aureus* is a facultative anaerobe that grows by aerobic respiration or by fermentation that yields principally lactic acid. It is a catalase positive, oxidase negative and coagulase positive organism that can grow at a temperature range of 15-45^{0}C and in NaCl concentrations as high as 15 percent^{w}/v. Nearly all the strains of *Staphylococcus aureus* produce the enzyme called coagulase and it is this enzyme that protects them from phagocytosis. *Staphylococcus aureus* should always be considered a potential pathogen (Yoshida and Eckstedt, 1968).

2.0.1. Pathogenesis of *staphylococcus aureus*

Staphylococcus aureus is a pathogen that is capable of causing disease utilizing a diverse armamentarium of virulence determinants. These determinants include both structural components and secreted cellular products. The polysaccharide capsule facilitates resistance to phagocytosis, the primary host defense mechanism against *Staph. aureus.* Surface proteins mediate staphylococcal attachment to selected host surfaces via tissue matrix molecules. Enterotoxins produce a sepsis syndrome by functioning as super antigens. Many of these molecules are under the control of a complex regulatory system that allows for the coordinated expression of different genes under different environmental conditions. Alteration of these regulatory genes affects the pathogen's capacity to cause disease. The nature of *Staphylococus aureus* infections varies from minor cutaneous soft tissue infections to life-threatening endovascular infections such as endocarditis. It has been increasingly recognized that different determinants have a unique role in the predilection of *Staphylococus aureus* for establishing infection at particular sites (Clewell, 2008).

Regardless of the primary site of infection there is a sequence of events necessary for infection to develop. In approximately a third of normal subjects *Staphylococus aureus* is a commensal colonizing the anterior nares. It causes disease only when there is a breach of a cutaneous or mucosal barrier. The staphylococci are then inoculated into a foreign site where colonization and proliferation occur. The ability to persist at these sites is a result of the organism's ability to persist in avascular tissues, avoid phagocytosis, elaborate biofilms and perhaps survive within epithelial cells (Cohen and Kurrock, 2004).

Depending on the virulence traits of the pathogen, the local host immune response as well as the primary site of infection, the organism may be contained or spread to additional sites establishing metastatic foci of infection(Kenneth, 2008).

2.0.2. The emergence of antibiotics

The first antibiotic, penicillin, was discovered in 1928 by Sir Alexander Fleming, who observed inhibition of staphylococci on an agar plate contaminated by a *Penicillium* mould. Fleming was searching for potential antibacterial compounds. He noticed that a patch of the mold *Penicillium notatum* had grown on a plate containing the bacterium *Staphylococcus* and that around the mould there was a zone where no *Staphylococcus* could grow.(Kenneth,2008). After more research, he was able to show that culture broth of the mold prevented growth of the *Staphylococcus* even when diluted up to 800 times. He named the active substance penicillin but was unable to isolate it. Several years later, in 1939, Ernst Chain and Howard Florey(1939) developed a way to isolate penicillin and used it to treat bacterial infection during the Second World War (Kenneth, 2008).

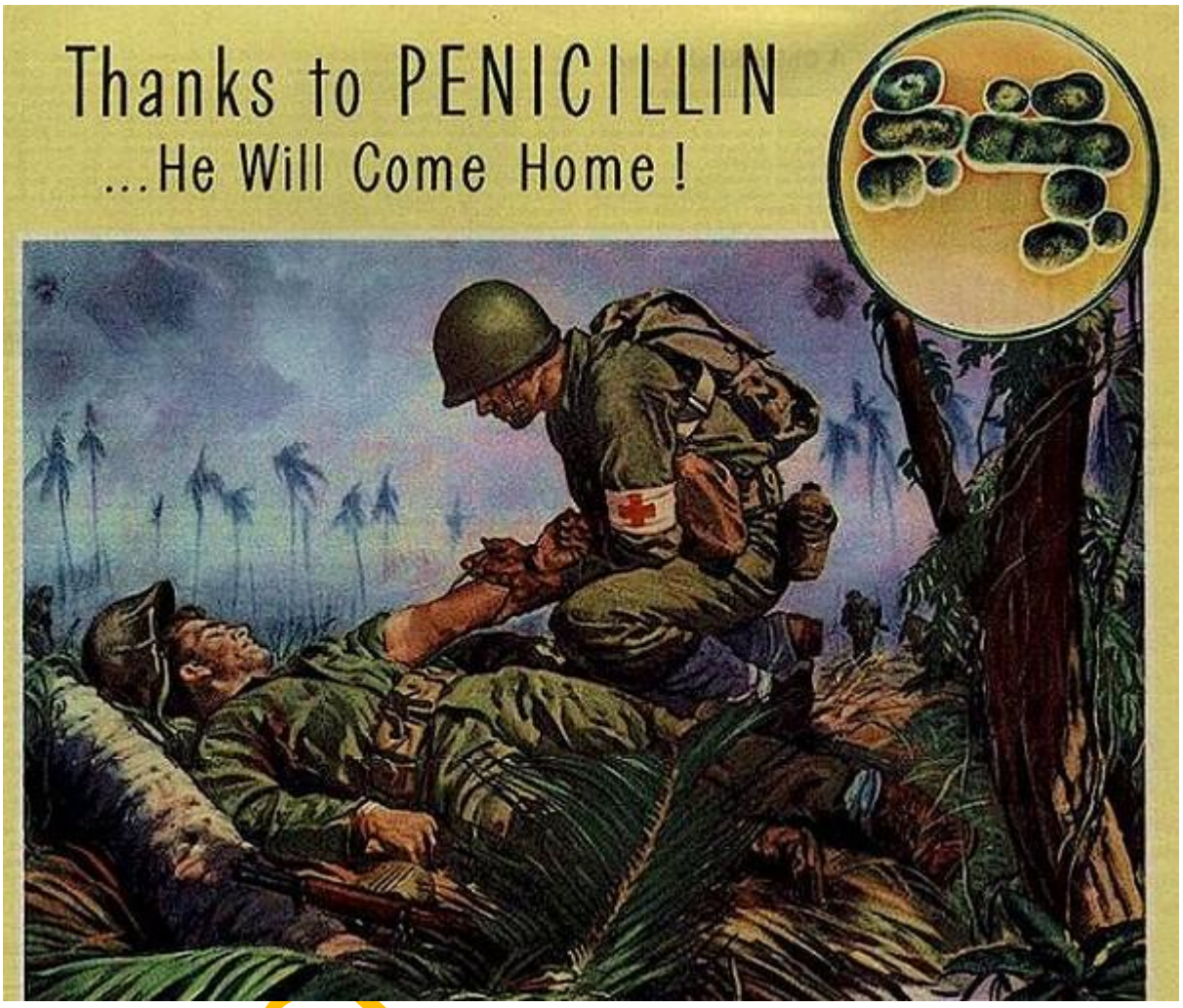

Figure 1.0. Kenneth Todar, 2008 The 2nd world war soldiers maiden therapeutic application of penicillin in curing infection

Penicillin came into clinical use in 1946 and made a huge impact on public health. For these discoveries Fleming, Chain and Florey were awarded the Nobel prize in 1945. Their discovery and development revolutionized modern medicine and paved the way for the development of many more natural antibiotics (Kenneth, 2008).

In 1946, penicillin became generally available for treatment of bacterial infections, especially those caused by staphylococci and streptococci. Initially, the antibiotic was effective against all sorts of infections caused by these two Gram-positive bacteria. Penicillin had unbelievable ability to kill these bacterial pathogens without harming the host that harbored them. Incidentally, a significant fraction of all human infections are caused by these two bacteria (e.g. sore throat, pneumonia, scarlet fever, septicemia, skin infections, wound infections, etc.).In the late 1940s and early 1950s, new antibiotics were introduced, including streptomycin, chloramphenicol and tetracycline, and the age of antibiotic chemotherapy became full blown (Garrod and Lambert, 1981).

These antibiotics were effective against the full array of bacterial pathogens including Gram-positive and Gram-negative bacteria, intracellular parasites, and the tuberculosis bacillus. Synthetic antimicrobial agents such as the "sulfa drugs"(sulfonamides) and anti-tuberculosis drugs, such as para aminosalicylic acid (PAS) and isoniazid (INH), were also brought into wide-use. (Kenneth, 2008)

2.0.3. The β-lactam antibiotics

β-lactam antibiotics are among the most commonly prescribed drugs, grouped together based upon a shared feature of beta-lactam ring with a 3-carbon and 1-nitrogen constitution. β-lactam antibiotics include; Penicillin, Cephalosporins, Cephamycins, Carbapenems, monobactams and β-lactamase inhibitors (Pichichero,2005).

An intact β-lactam ring is required for these antibiotics to exert thier bactericidal activity. The penicillins and related cephalosporins destroy bacteria through inhibition of bacterial cell wall synthesis. Although several mechanisms may be responsible for this inhibition, the most important is probably the inhibition of the terminal peptidoglycan cross-linking. This terminal event in bacterial cell wall formation is essential for many bacteria, especially the Gram-positive organisms. Two areas of activity have been emphasized during the development of new β-lactam antibiotics. The first area of emphasis is in developing compounds with extended Gram-negative spectrum.

The second area of interest has been in the development of β-lactamase resistant antibiotics. Due to emergence of β-lactamase enzymes, the newer classes of beta-lactam antibiotics are either resistant to or at least partially resistant to this form of enzyme degradation. β-lactam antibiotics are commonly classified into groups according to antimicrobial properties (Holten and Onusko, 2000).

(1)

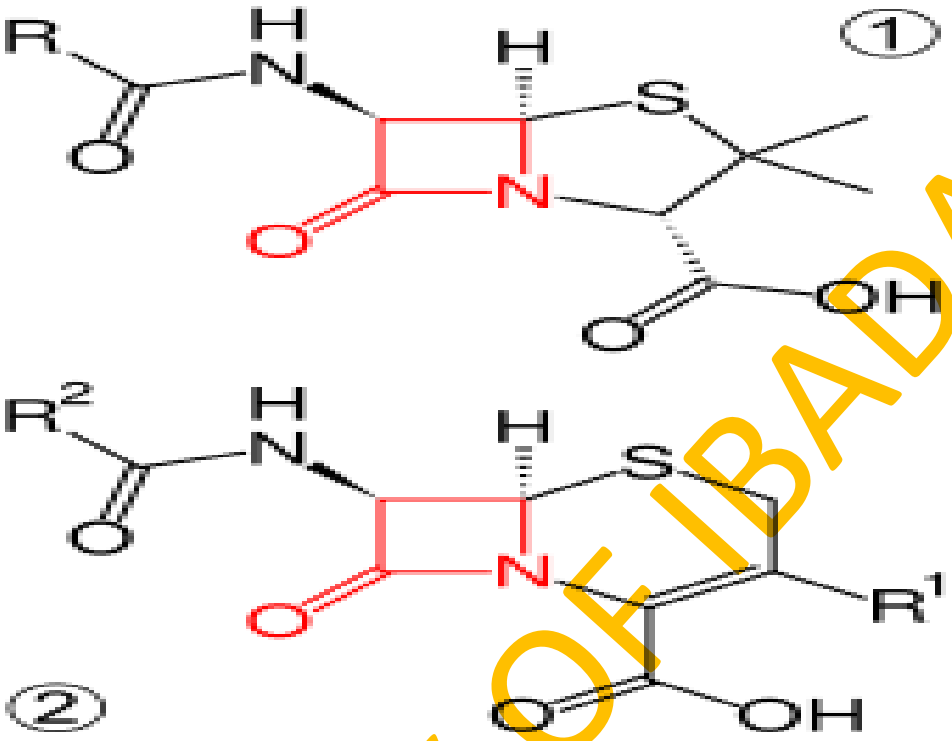

Figure 2.0. Tbe β-Lactam ring.

The above chemical structures 1 and 2 are the general structure of penicillins and cephalosporins respectively showing the **β-lactam ring in red**. The β-lactam antibiotics are so broad, and can be grouped according to their spectrum of activity, pharmacokinetics, and their cost, Up until 2003, when measured by sales, more than half of all commercially available antibiotics in use were β-lactam compounds.

2.0.4 Mechanism of action

β-lactam antibiotics inhibit the growth of sensitive bacteria by inactivating enzymes located in the bacterial cell membrane, which are involved in the third stage of cell wall synthesis. It is during this stage that linear strands of peptioglycan are cross – linked into a fishnet like polymer that surrounds the bacterial cell wall and confers osmotic stability in the hypertonic milieu of the infected patient .β-lactams inhibit the enzyme involved in different aspect of cell wall synthesis (Raghava, 2002).

Specifically, the cross linking of peptide on the mucossaccharide chain is prevented. If the cell walls are improperly made such cell walls allow water to flow into the cell causing it to burst. Penicillin binds at the active site of the transpeptidase enzyme that cross links the peptidoglycan strands. It does this by mimicking the D-alanyl D-alanine residues that would normally bind to this site. Penicillin irreversibly inhibits the enzyme transpeptidase by reacting with a serine residue in transpeptidase such that the growth of the bacterial cell wall is inhibited. Since mammalian cells do not have the same type of cell walls, penicillin specifically inhibits only bacterial cell wall synthesis (Bonfiglio and Livermore, 1991).

2.0.5. Penicillin G

Penicillin G (and its sodium, potassium, procaine, N-benzyl-beta-phenyethylamine and N-N^1- dibenzyl-ethyl diamine salts) is the most potent of all penicillin derivatives, it has several shortcomings and is effective only against Gram-positive bacteria.

It may be broken down in the stomach gastric acids and is poorly and irregularly absorbed into the blood stream. In addition, many disease producing staphylococci are able to produce an penicillinase capable of inactivating penicillin G. Various semi synthetic derivatives have been produced which overcome these shortcomings (Bobrowsky, 1974).

Powerful electron-attracting groups attached to the amino acid side chain provide steric hindrance which interferes with the enzyme attachment which would inactivate penicillin as shown above. If the polar character increases as it occurs in ampicillin or carbenicillin, there is greater activity against Gram-negative bacteria.

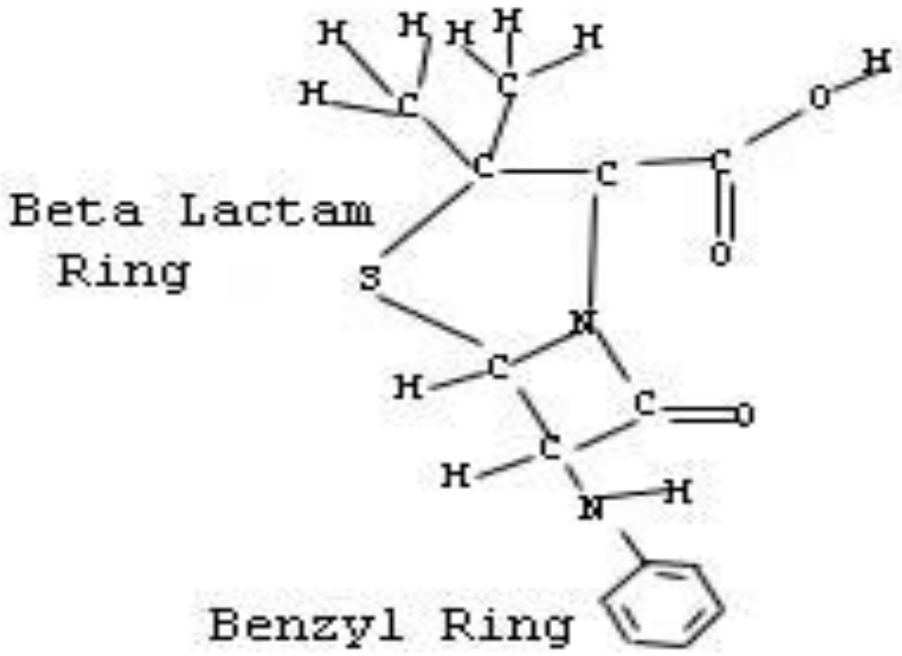

Figure 3.0.: Penicillin G.

2.0.6. Carboxyl penicillins

The carboxyl penicillins are a group of β-lactam antibiotics that comprise carbenicillin and ticarcillin The carboxyl penicillins feature the β-lactam backbone of penicillins but also a carboxylic acid or carboxylic acid ester group in the variable side chain The carboxyl penicillins exhibit activity against Gram-negative bacteria including *Pseudomonas aeruginosa* and *Proteus spp*. but are inactive against certain Gram positive pathogens such as *Staphylococcus aureus, Enterococcus faecalis,* and *Lysteria monocytogens.* The carboxyl penicillins are β-lactamase sensitive. When it is appropriated in synergism with suitable antibiotics namely gentamicin an aminoglycoside, they elicit an excellent antibacterial profile (Gerald, *et.al.*, 1986).

2.0.7. Isoxazolyl penicillins

The Isoxazolyl penicillin group consist of cloxacillin, flucloxacillin and di-cloxacillin which are resistant to penicillinase and gastric acid fluid in the gut, and are considerably more active against sensitive staphylococci than methicillin. Three members of this series; *BRL*.1400 5-methyl-3-phenyl-4-isoxazolylpenicillin *BRL*. 1577 5-methyl-3-*p*-chlorphenyl-4-isoxazolylpenicillin and *BRL*.1621 5-methyl-3-*o*-chlorphenyl-4-isoxazolylpenicillin have been investigated for antibacterial activity and for stability to, induction of, affinity for, and inhibitory action on, the penicillinases of *Staphylococcus aureus* and *Bacillus. Cereus* (Sutherland, 1970)

Isoxazolyl penicillin, are active against penicillinase-producing strains of *Staphylococcus aureus* and is well absorbed in man after oral and intramuscular administration. In current clinical use, they have proved to be active against Gram-positive cocci, including penicillin-resistant staphylococci. The extent of binding of flucloxacillin to the protein of human serum was similar to that of oxacillin and cloxacillin and less than that of dicloxacillin (Sutherland, 1970).

2.0.8. Amidino penicillins

These are semi-synthetic penicillins and mecillinam is a typical representative. They are potent on Gram –negative bacteria such as *Salmonella, Shigella, E coli, Klebsiella spp* and *Proteus* but have a lesser activity on *Pseudomona aeruginosa* (Cole, 1969).

The amidinopenicillins are more active against Gram-negative bacilli than against Gram-positive organisms. Amidinocillin is much less active against staphylococci and streptococci than is ampicillin, although the relatively high concentrations reached in the urine may be effective in eradicating Gram-positive urinary tract organisms. The Gram-negative and Gram-positive anaerobic bacteria are resistant to amidinocillin and pivamidinocillin, (Matshuashi *et.al.*,,1974).

2.0.9. Acylamino penicillins

The acylamino penicillins are also effective against many strains of *Pseudomonas aeruginosa* . They are destroyed by fewer strains, than the carboxyl penicillins, mezlocillin, piperacillin, azlocillin, ureidopenicillin and other members of this group are also more effective against *Klebsiella pneumoniae* and other Enterobacteriaeaiae.These drugs approach ampicillin in efficacy against gram-positive cocci, including enterococci. Examples of acylamino pencillin are piperacillin/tazobactam and 2-4,Dioxo-1-pyrimidinyl acylamino penicillin (Saltmarsh, 2004).

2.1.0. Acylureido penicillins

These are brands of penicillin family that posses improved broad spectrum activity against Gram-positive and Gram-negative bacteria and also are active on some facultative anaerobes notably *Pseudomona aeruginosa.* They include; piperacillin, azlocillin and mexlocillin. It has been reported that piperacillin has a considerable activity against β-lactamase sensitive *Actinobacter spp.* (Novaspratt, 1988).

2.1.1. Aminocillins

Aminocillins, of which amoxicillin is the most commonly prescribed, are semisynthetic penicillins. The amoxicillin retains the primary β-lactam structure of penicillin but differ from the parent compound by virtue of extended spectrum (more Gram-negative coverage) and ease of administration.

Many semi-synthetic penicillins now exist which provide extended range of treatment. However, only amoxicillin is used routinely to treat acute otitis media (Tan and File, 1995).

2.1.2. Monobactams

The monobactam family contains a cyclic β-lactam structure which is unique in that it is completely synthesized . Aztreonam is perhaps the most widely representative of the monobactam family. The drug is active against many of the Gram-negative aerobic organisms and is essentially resistant to beta-lactamase/cephalosporinase – degradation. Currently, no monobactams are available for treatment of children (Holten, 2000).

2.1.3. The clavams

These are regarded as novel β-lactam antibiotics because of substitute sulphur atom for oxygen atom. Clavams possess very weak antibacterial activity but can inhibit β-lactamases produced by Gram-negative and Gram-positive bacteria and have been reported to protect amoxicillin from destruction by β-lactamase producing bacteria when tested in vitro and in vivo. Clavulanic acid is an example of Clavams . When amoxicillin and clavulanic acid are combined in 2:1 ratio, (250mg:125mg) to produce augmentine^{R}, the pharmacokinetic and antibacterial actions are improved such that the β-lactamases of Gram-negative bacilli are inhibited while amoxicillin is shielded from degradation from bacterial cells (Roy *et al*., 1999).

2.1.4. Cephalosporins

Cephalosporin compounds were first isolated from cultures of *Cephalosporium acremonium* from a sewer in Sardinia in 1948 by Italian scientist Giuseppe Brotzu. The first agent, cephalothin, was launched by Eli Lilly in 1964. Cephalosporins are derived from cephalosporin C which is an acid-stable molecule. The Cephalosporin ring structure is derived from 7 aminocephalosporanic acid (7-ACA) while the penicillins are derived from 6 – amino penicillanic acid (6-APA). Both structures contain the basic β-lactam ring, but the cephalosporin structure allows for more activity on Gram-negative bacteria than the penicillins and aminocillins. Substitution at the "R" sites allows for variation in the spectrum of activity and duration of action (Novaspratt, 1988).

Cephalosporins are grouped into "generations" based on the time of their manufacture and also, on their pharmacological properties as well as stability to β-lactamases from Gram-negative bacteria. The first cephalosporins were designated first generation while later, more extended spectrum cephalosporins were classified as second generation cephalosporins. Currently, four generations of cephalosporins are recognized. Significantly, each newer generation of cephalosporins has greater Gram-negative antimicrobial properties than the preceding generation (Puroit *et.al.*, 2008).

Conversely, the "older" generation of cephalosporins have greater Gram- positive coverage including *Staphylococcus and Streptococcus*.

Most second and third generation cephalosporins haven't been approved for use in patients less than six months of age.

The first generation cephalosporins include; Cephalexin, Cephapirin, Cephalotin, Cephazolin and Cephradine. The second generation ones are Cefuroxime and Cephnoranide (Zinacef^{R}) while the third generation Cephalosporins include Ceftazadime(Fortum$^{(R)}$) , Ceptizoxime (Cefizox^{R}), latamoxef (monolactam^{R}), and Cefotaxime (Claforan^{R}).

Based on the rate of administration and stability to β-lactamase, cephalosporins are grouped as follows:

- Parenterally administered, high β–lactamase resistant cephalosporins, comprising cefuroxime, cefotaxime, cefamandole and lefoxitin.
- Parenterally administered, β-lactamase sensitive cephalosporins including cephapirin, cefazolin, cephaloridine, cephacetrile and cefazedone.
- Orally administered, β-lactamase sensitive cephalosporins including cefradine, cephalexin, and cefactor (Jawetz *et. al*., 2010).

2.1.5. Oxalocephalosporins.

These are broad spectrum antibiotics with oxygen atom replacing sulphur atom.Moxalactam is a typical representative. It is stable to βeta-lactamases and very active against most of the members of *Enterobacteriaceae and Pseudomonas aeruginosa* (Purohit, 2008).

2.1.6. Thienamycins

Thienamycin, one of the most potent naturally produced antibiotics known thus far, was discovered in *Streptomyces cattleya* in 1976. Thienamycin has excellent activity against both Gram-positive and Gram-negative bacteria and is resistant to bacterial β–lactamase enzymes. Thienamycin was the first among the naturally occurring class of carbapenem antibiotics to be discovered and isolated. In vitro, thienamycin employs a similar mode of action as other penicillins through disrupting the cell wall synthesis (peptidoglycan biosynthesis) of various Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aerugnosa* and others. Spratt *et al.*(1993) found that, although thienamycin binds to all of the penicillin-binding proteins (PBP) in *Escherichia coli*, it preferentially binds to PBP-1, and PBP-2, which are both associated with the elongation of the cell wall (Zimmerman *et.al.*, 1977).

Unlike penicillin which are rendered ineffective through rapid hydrolysis by β–lactamase enzymes present in some strains of bacteria, thienamycin remains antimicrobially active. Neu *et al*.,1980, found that thienamycin displayed high activity against bacteria that were resistant to other β–lactamase stable compounds particularly the cephalosporins, highlighting the superiority of thienamycin as an antibiotic among β-lactamase inhibitors(Zimmerman *et.al.*, 1977)..

Thienamycin itself is extremely unstable and decomposes in aqueous solution. Consequently, thienamycin is not applicable for clinical treatment of bacterial infections, rather, stable derivatives of thienamycin were produced, for medicinal use. One of such, imipenem, was formulated in 1985. Imipenem, a N-formimidoyl derivative of thienamycin, is rapidly metabolized by the renal dihydropeptidase enzyme found in the human body. To prevent its rapid degradation, impenem is normally co-administered with cilastatin. Cilastatin is a chemical compound which inhibits the human enzyme dehydropeptidase found in the kidney and is responsible for degrading the antibiotic imipenem. Cilastatin is therefore combined intravenously with imipenem in order to protect it from dihydropeptidase and prolong its antibacterial effect. However, cilastatin itself does not have antibiotic activity (Keynan *et. al.*, 1995).

2.1.7. Multidrug resistant *Staphylococcus aureus (MRSA).*

Over the years, and continuing into the present almost every known bacterial pathogen has developed resistance to one or more antibiotics in clinical use.

There has probably been a gene pool in nature for resistance to antibiotic as long as there has been for antibiotic production, for most microbes that are antibiotic producers are resistant to their own antibiotic (Indalo,1997)..

Evidence also began to accumulate that bacteria could pass genes for drug resistance between strains and even between species. For example, antibiotic-resistance genes of staphylococci are carried on plasmids that can be exchanged with *Bacillus, Streptococcus* and *Enterococcus* providing the means for acquiring additional genes and gene combinations. Some are carried on transposons, segments of DNA that can exist either in the chromosome or in plasmids. In any case, it is clear that genes for antibiotic resistance can be exchanged between strains and species of bacteria by means of the processes of horizontal gene transmission (HGT) (Clewell, 2008).

It is not surprising that resistance to penicillin in some strains of staphylococci was recognized almost immediately after introduction of the drug in 1946. Likewise, very soon after their introduction in the late 1940s, resistance to streptomycin, chloramphenicol and tetracycline was noted. By 1953, during a *Shigella* outbreak in Japan, a strain of the dysentery bacillus (*Shigella dysenteriae*) was isolated which was multiple drug resistant, exhibiting resistance to chloramphenicol, tetracycline, streptomycin and the sulfonamides (Raghava, 2002).

2.1.8. Multiple drug resistant organisms

Multiple drug resistant organisms are resistant to treatment with several, often unrelated, antimicrobial agents Some of the most important types of multiple drug resistant organisms that have been encountered-include *Shigella*, ***S****taphylococcus aureus*, *Vancomycin-ResistantEnterococci*,(VRE) and *Extended-Spectrum beta*-lactamase(ESBLs)Pseudomonas. *Shigella* has been reported with resistance to methicillin and oxacillin. *Penicillin-resistant-Streptococcus pneumonia* (PRSP), (MRSA) methicillin resistant *Staphylococcus aureus* and VRE are the most commonly encountered multiple drug resistant organisms in patients residing in non-hospital healthcare facilities such as nursing homes and other long-term care facilities. *Penicillin-resistant-Streptococcus pneumoniae*.**(**PRSP**)** are more common in patients seeking care in outpatient settings such as physicians' offices and clinics, than in pediatric settings. Extended-Spectrum β-lactamase organisms are most often encountered in the hospital (intensive care) setting, but MRSA and VRE also have a significant nosocomial ecology (Sweetman *et.al.*, 2005).

2.1.9 Mechanisms of antibiotic resistance in bacteria

Antibiotics are very commonly used substances to eradicate bacterial infections by bacteriostatic or even bactericidal effect. They act at a very specific stage (target), although other less important or secondary interactions can occur. In the past 60 years, an alarming increase in the resistance of bacteria that cause community acquired infections has also been documented, especially among staphylococci and pneumococci (*Streptococcus pneumoniae),* which are prevalent causes of disease and mortality(Clewell, 1981).

The mechanisms that have been responsible for the resistance include alteration of cell membranes, anti-metabolite activity, inhibition of nucleic acid synthesis, inhibition of protein synthesis and other several mechanisms have evolved in bacteria which confer them with antibiotic resistance. These mechanisms can either chemically modify antibiotics, render them inactive through physical removal from the cell, or modify target site so that it is no longer recognized by the antibiotic. The most common mode is enzymatic inactivation of the antibiotic. An existing cellular enzyme is modified to react with the antibiotic in such a way that it no longer affects the microorganism. An alternative strategy utilized by many bacteria is the alteration of the antibiotic target site. In the past 60 years, antibiotics have been critical in the fight against infectious diseases caused by bacteria and other microbes.

Antimicrobial chemotherapy has been a leading cause for the dramatic rise of average life expectancy in the 20th Century (Clewell *et.al.,* 2008).

However, disease-causing microbes that have become resistant to antibiotic drug therapy are increasing public health problem. Wound infections, gonorrhea, tuberculosis, pneumonia, septicaemia and childhood ear infections are just a few of the diseases that have become hard to treat with commonly prescribed antibiotics. A part of the problem is that bacteria and other microbes that cause infections are remarkably resilient and have developed several ways to resist antibiotics and other antimicrobial drugs (Kenneth, 2008).

Another part of the problem is due to increasing use, and of often abuse or misuse, of existing antibiotics in human and veterinary medicine and in agriculture. Agricultural practices account for over 60% of antibiotic usage in the U.S.,contributing an additional 18,000 tons per year to the antibiotic burden in the environment. Nowadays, about 70 percent of the bacteria that cause infections in hospitals are resistant to at least one of the drugs most commonly used for treatment. Some organisms are resistant to all approved antibiotics and can only be treated with experimental and potentially toxic drugs (Lyon and Skurray, 1987).

In a recent study, 25% of bacterial pneumonia cases were shown to be resistant to penicillin, and an additional 25% of cases were resistant to more than one antibiotic. Microbial development of resistance as well as economic incentives have resulted in research and development in the search for new antibiotics in order to maintain a pool of effective drugs at all times. While the development of resistant strains is inevitable, the slack ways in which antibiotics are administered and used have greatly exacerbated the process. These and other mechanisms are illustrated as shown in figures 4 and 5 below (Kenneth, 2008).

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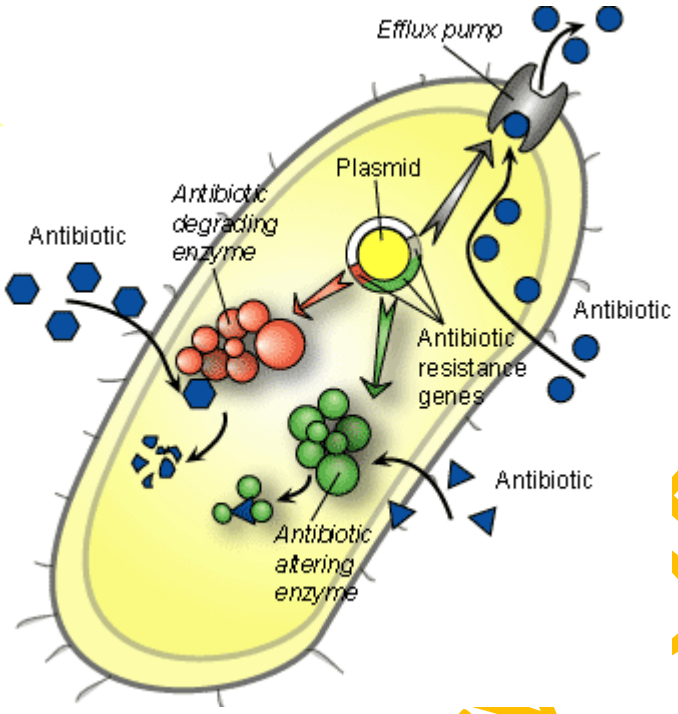


Figure 4.0 The organogram of the mechanisms of antibiotic resistance

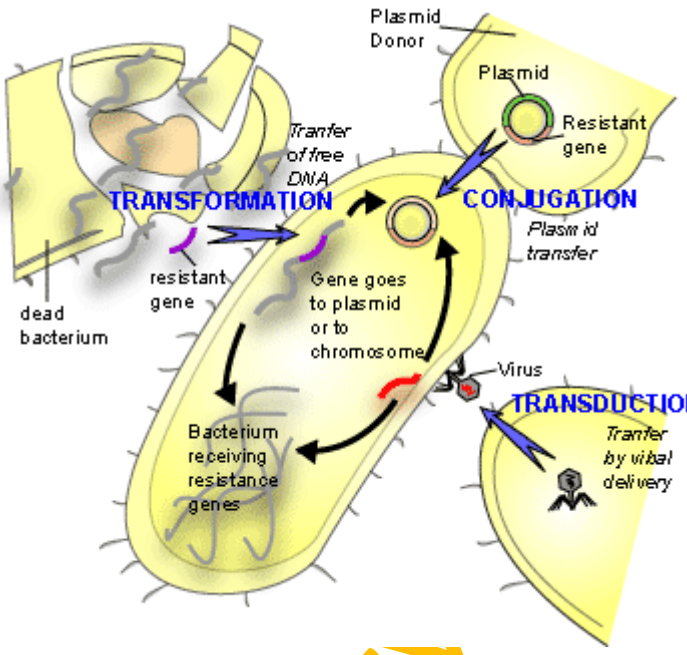


Figure 5.0. The organogram of the mechanisms of antibiotic resistance

2.10..0 Combating the development of antibiotic resistance

Bacterial resistance appears to be an ever-increasing problem and is threatening to spiral out of control. The scare caused by the rapid spread of β-lactam resistant *Staphylococcus aureus* among hospitals has been a cause for concern.. Since infections are generally polymicrobial, pharmaceutical companies must constantly research, develop and test new antimicrobials in order to maintain a pool of effective drugs in the market (Clewell, 2008). Towards combating the development of antibiotic resistance in microorganisms, recommendations have been made as follows:

- Caution in the veterinary use of antibiotics as feed additives given to farm animals to promote animal growth and to prevent infections rather than cure infections. The use of such antibiotics contributes to the emergence of antibiotic-resistant bacteria that threaten human health and decrease the effectiveness of the same antibiotics used . There is need for the use of right antibiotic in an infectious situation as determined by antibiotic sensitivity testing, when possible
- Rational antibiotic prescription. Unnecessary antibiotic prescriptions have been identified as causes of an enhanced rate of resistance development. Unnecessary prescriptions of antibiotics are made when antibiotics are prescribed for viral infections, even though antibiotics have no effect on most viruses naturally.

  This gives the opportunity for indigenous bacteria to acquire resistance that can be passed on to pathogens. The development of guidelines for antimicrobial use by primary and secondary care is also of paramount importance (Raghava,*et.al.*,2002). Compliance with antibiotic prescriptions. Lack of compliance with antibiotic

prescriptions may leave some bacteria alive or may expose them to sub-inhibitory concentrations of antibiotics for a prolonged period of time. *Mycobacterium tuberculosis* is a slow growing bacterium which infects the lung and causes tuberculosis, a disease that kills more adults than any other infectious disease. Due to the slow growing nature of the infection, treatment program lasts for months or even years. As a result of this, 5% of strains now observed are completely resistant to all known treatments. In the pharmaceutical industry, past and current strategies to combat resistance have not been effective. Pharmaceutical companies are seeking new, less costly strategies to develop antibiotics(Kenneth, 2008).

- Use of combination antibiotic therapy. Some clinical studies of bacterial infection, including endocarditis, Gram-negative bacteraemia, and neutropenic sepsis, and animal models of severe infection have supported the possibility of clinically relevant antimicrobial synergism with appropriate combinations of antibiotics (Woodford, 2007)

  However, current guidelines do not recommend combination therapy except for the express purposes of mixed resistance and broadening coverage when resistant pathogens are a concern. Effective treatment thus requires the use of a broad-spectrum antibiotic or combination therapy. The use of one β-lactam/β-lactamase inhibitor combination such as ampicillin/sulbactam, has been demonstrated to be more cost-effective than treatment with β-lactamase-stable antibiotics, such as cefoxitin and imipenem/cilastatin, for this indication.

- There is need for pharmaceutical companies to constantly research into, develop and test new antimicrobials in order to maintain a pool of effective drugs in circulation (Lode, 2008).

2.10.1. Plasmid

The term plasmid was first introduced by an American molecular biologist Joshua Lederberg in 1952. Joshua Lederberg discovered plasmids, arguably in the late 1940s, when attempting to determine the mediators of bacterial conjugation (mating) in *E. coli*. Before the recognition of biological significance of plasmid, clinicians assumed that conventional mutations caused clinical antibiotic resistance, a remarkable observation by Watanabe(1969) that dysentery bacillus could become resistant to three or more antibiotics simultaneously during treatment with only one antibiotic. This was attributed to acquisition of a plasmid, leading to the realization that plasmids were a major source of antibiotic resistance in bacteria. Plasmids serve as important tools in genetics and biotechnology laboratory where they are commonly used to multiply or *express* particular genes (Lipps, 2008).

Another major use of plasmids is to make large amounts of proteins. In this case, researchers grow bacteria containing a plasmid harboring the gene of interest. Just as the bacteria produces proteins to confer their antibiotic resistance, they can also be induced to produce large amounts of proteins from the inserted gene. This is a cheap and easy way of mass-producing a gene or the protein which then codes for, for example, insulin or even antibiotics. However, a plasmid can only contain inserts of about 1–10 kb (Novick, 1969).

Plasmid is an extra-chromosomal DNA molecule which is capable of replicating independently of the chromosomal DNA or in synchrony with host chromosomal DNA.. In many cases, it is circular and double-stranded. Plasmids usually occur naturally in bacteria, but are sometimes found in eukaryotic organisms (e.g., the *2µm* ring in *Saccharomyces cerevisiae* (Kandavelou, 2008) .

Plasmid molecular size varies from 1 to over 200 kilobase pairs (kbp). The number of identical plasmids within a single cell can be zero, one, or even thousands under some circumstances.

Plasmids can be considered to be independent life-forms similar to viruses, since both are capable of autonomous replication in suitable (host) environments.

However the plasmid-host relationship tends to be more symbiotic than parasitic (although this can also occur for viruses, for example with endoviruses) since plasmids can endow their hosts with useful packages of DNA to assist mutual survival in times of severe stress (Clewell *et. al.*, 1981).

For example, plasmids can confer antibiotic resistance on host bacteria, that may then survive along with their life-saving guests which can be carried along into future host generations.

Plasmids used in genetic engineering are called vectors, serving as important tools in genetics and biotechnology laboratories, where they are commonly used to replicate or *express* particular genes. Many plasmids are commercially available for such uses. For example, SeaKem® LE and NuSieve® gel to gel plasmid kit (Kandavelou, 2008).

2.10.2 Classification of plasmid

Plasmids are classified according to the functions associated with them as follows:

Resistance or (R plasmid) . These contain genes that can build resistance against antibiotics or poisons and are historically known as R-factors, before the nature of plasmids was understood. An example is *Escherichia coli* K12J53 that transfers Ampicillin^{r}, Kanamycin^{r} and Tetracyclin^{r} resistance. Fertility(-F-)plasmids, which contain tra-genes that are capable of conjugation.

Col-plasmids, which contain genes that code for the production of bacteriocin, proteins that kill other bacteria . Bacteriocingenic factor is a factor/gene that directs the host bacterium to synthesize bacteriocin that could kill bacteria belonging to the same or closely related species. Example is col-E1 (*Escherichia coli* JC411) that kill other *Escherichia coli* and closely related enterobacteria with bacteriocin (Davis *et al*., 1973).

Degradative plasmids, which afford the digestion of unusual substances, e.g., toluene or salicylic acid and an example is *Lysinibacillus sphaericus*wh22 strain that degrade Dichloro-methane, an organic compound.

Virulence plasmids, which mediate virulence in pathogenic bacteria and an example is virulence plasmid-encoded YopK in *Yersinia pseudotuberculosis* that causes systemic infection in mice and also the Ti plasmid of *Agrobacterium tumefaciens* that induces crown gal disease on dicotyledons of some plants **(**Purohit, 2008).

Plasmids can belong to more than one of these functional groups. Plasmids that exist only as one or a few copies in each bacterium are, upon cell division, in danger of being lost in one of the segregating bacteria. Such single-copy plasmids have systems

which attempt to actively distribute a copy to both daughter cells. Some plasmids include an addiction system or "postsegregational killing system (PSK)", such as the hok/sok (host killing/suppressor of killing) system of plasmid R1 in *Escherichia coli*. They produce both a long-lived poison and a short-lived antidote. Daughter cells that retain a copy of the plasmid survive, while a daughter cell that fails to inherit the plasmid dies or suffers a reduced growth-rate because of the lingering poison from the parent cell (Gerdes, 1986).

2.10.3. Mechanisms of plasmid DNA transfer.

Bacterial conjugation.

Bacterial conjugation is the process of bacterial mating, resulting in the sexual exchange of DNA. To do this, one of the bacteria has to carry an F-plasmid absent in the other. The F-plasmid (also called F-factor) is an episome .It carries its own origin of replication called oriV. There can only be one copy of the F-plasmid in a bacterium termed (F+,) either free or integrated. Among other genetic information, the F-plasmid carries a tra and a trb loci, which together are about 33 kb long and consist of about 40 genes. The tra locus includes the pil**i** gene that can attach themselves to the surface of F-negative bacteria and initiate the mating(Noble and Rahaman, 1986). The pili themselves do not seem to be the structures through which the actual exchange of DNA takes place; rather, some proteins coded in the tra or trb loci seem to open a channel called conjugation tube between the two bacteria. The transfer of DNA always runs from the (F+**)** towards the (F-**)** bacterium. If the F-plasmid is free, a nick is produced in one of the circular DNA strands of the plasmid. Then, the single-stranded DNA of the nicked strand is inserted into the recipient bacterium (5'-end first). The now single-stranded plasmid is filled up with a complementary strand by a rolling circle mechanism. If the F-plasmid is integrated, the transfered DNA consists of the F-plasmid sequence plus an amount of chomosomal DNA from the donor bacterium. The amount of chromosomal DNA that is transferred depends on the time of contact among bacteria, normally within 100 minutes on genetic map (Clewell, 2008).

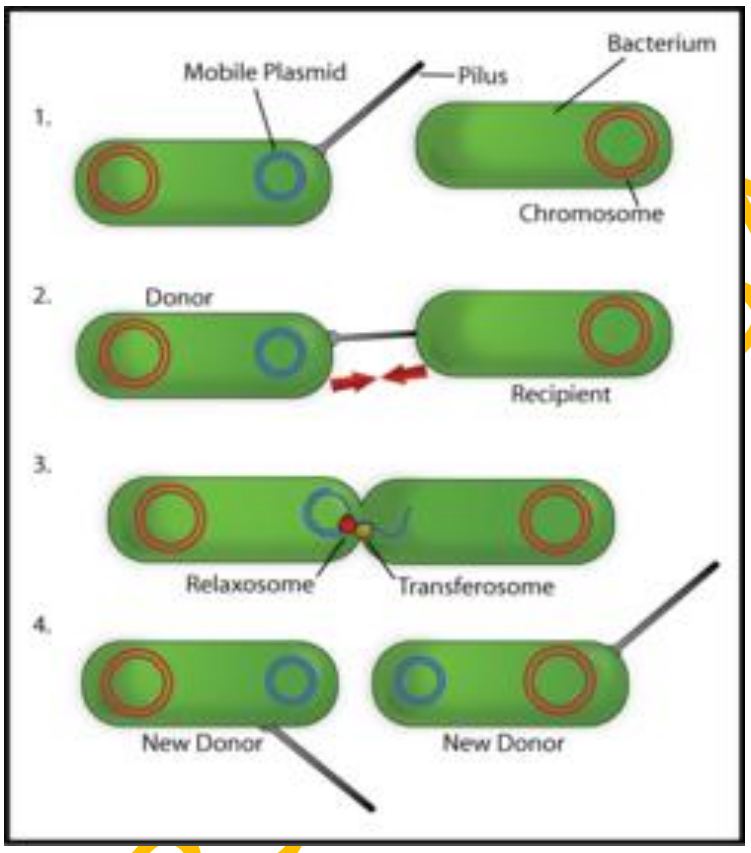


Figure 6.0 Noble and Rahaman,1986 (Conjugation in plasmid).

The transferred DNA can be integrated into the recipient DNA by recombination and this imported gene can confer drug resistance capacity to the recipient bacteria.

2.10.4. Hfr (carvalli) and Hfr (hayes).

Bacteria with an integrated F-plasmid do recombine unusually often, which is why they are said to exhibit Hfr-(high frequency recombination. Carvilli (1953) found that after a cross(conjugation) between Af met x Bf thre Leu, the recombinations are produced at a frequency of about 1000 times more in comparison with F strains. Carvalli named them as Hfr as they possess the quality of high frequency of recombination. In 1953, Hayes also isolated similar kind of strains, whose characters are restricted to a limited part of the donor genome and F can be converted permanently into Hfr with the loss of the capability of its own transfer (Purohit *et.al*., 2008).

2.10.5. Transformation

Transformation was first demonstrated in 1928 by Frederick Griffith, an English bacteriologist searching for a vaccine against bacterial pneumonia. Griffith discovered that a non-virulent strain of *Streptococcus pneumoniae* could be transformed into a virulent one by exposure to strains of virulent *Streptococcus pneumoniae* that had been killed with heat. In 1944 it was demonstrated that the transforming factor was genetic, when Oswald Avery, Colin MacLeod, and McCarty showed gene transfer in *Streptococcus pneumoniae*. Avery, Macleod and McCarty called the uptake and incorporation of DNA by bacteria "transformation."

In molecular biology, transformation is the genetic alteration of a cell resulting from the uptake, genomic incorporation and expression of foreign genetic material (DNA). Transformation of animal cells is usually called transfection. RNA may also be transferred into cells using similar methods, but this does not normally produce heritable change and so is not true transformation. Bacterial transformation may be referred to as a stable genetic change brought about by taking up naked DNA (DNA without associated cells or proteins), and competence refers to the state of being able to take up exogenous DNA from the environment. Two different forms of competence should be distinguished: natural and artificial . Some bacteria (around 1% of all species) are naturally capable of taking up DNA under laboratory conditions; many more may be able to take it up in their natural environments. Such species carry sets of genes specifying machinery for bringing DNA across the cell's membrane or membranes (Purohit, 2008).

Artificial competence is not encoded in the cell's genes. Instead it is induced by laboratory procedures in which cells are passively made permeable to DNA, using conditions that do not normally occur in nature¨ Chilling cells in the presence of divalent cations such as Ca^{2+} (in $CaCl_2$) prepares the cell walls to become permeable to plasmid DNA. Cells are incubated on ice with the DNA and then briefly heat shocked (e.g. 42 °C for 30–120 seconds), which causes the DNA to enter the cell. This method works very well for circular plasmid DNAs. An excellent preparation of competent cells will give 10^8 colonies per microgram of plasmid. However, cells that are naturally competent are usually transformed more efficiently with linear DNA than with plasmids (Kandavelou, 2008) .

2.10.6. Transduction

Transduction is the process by which DNA is transferred from one bacterium to another by a virus called bacteriophage. This is a common tool used by molecular biologists to stably introduce a foreign gene into a host cell's genome. When bacteriophages infect a bacterial cell, their normal mode of reproduction is to harness the replicational, transcriptional, and translation machinery of the host bacterial cell to make numerous virions, or complete viral particles, including the viral DNA or RNA and the protein coat (Purohit, 2008). If the lysogenic cycle is adopted, the phage chromosome is integrated into the bacterial chromosome, where it can remain dormant for thousands of generations. If the lysogen (e.g. UV light) is induced, the phage genome is excised from the bacterial chromosome and initiates the lytic cycle, which culminates in lysis of the cell and the release of phage particles. The lytic cycle leads to the production of new phage particles which are released by lysis of the host. However, the packaging of bacteriophage DNA has low fidelity and small pieces of bacterial DNA, together with the bacteriophage genome, may become packaged into the bacteriophage genome. At the same time, some phage genes are left behind in the bacterial chromosome. There are generally two types of recombination events that can lead to this incorporation of viral DNA into bacteria DNA (lysonenic or lytic) leading to two modes of recombination which can be generalized or specialized transduction. Generalized transduction is the process by which any bacterial gene may be transferred to another bacterium via a bacteriophage, and typically carries only bacterial DNA and no viral DNA. In essence, this is the packaging of bacterial DNA into a viral envelope while Specialized transduction is the process by which genes that are near the bacteriophage genome may be transferred to another bacterium via a bacteriophage.

The genes that get transferred (donor genes) always depend on where the phage genome is located on the chromosome . Both can be useful in galactosemia, correcting genetic diseases by direct modification of genetic errors and can aid the transfer of antibiotic resistance (Jones *et.al.*,1998) .

2.10.7. Recombinant(rDNA).

rDNA is a form of DNA that does not exist naturally, which is created by combining DNA sequences that would not normally occur together. In terms of genetic modification recombinant DNA (rDNA) is introduced through the addition of relevant DNA into an existing organismal DNA, such as the plasmids of bacteria, to code for or alter different traits for a specific purpose, such as antibiotic resistance. It differs from genetic recombination in that it does not occur through processes within the cell, but is engineered. In order to be useful, the rDNA molecules have to be made to replicate and function genetically within a cell. Small DNA fragments can be inserted into the plasmids, which are then introduced into bacterial cells. As the bacteria reproduce, so do the recombinant plasmids. The result is a bacterial colony in which the foreign gene has been cloned (Purohit, 2008). The major tools of recombinant DNA technology are bacterial enzymes called restriction enzymes. Enzymes called recombinases catalyze natural recombination reactions. RecA, the recombinase found in *E. coli*, is responsible for the repair of DNA double strand breaks (DSBs). Each enzyme recognizes a short, specific nucleotide sequence in DNA molecules, and cuts the backbones of the molecules at that sequence. The result is a set of double-stranded DNA fragments with single-stranded ends, called "sticky ends." Sticky ends are not really sticky; however, the bases on the sticky ends form base pairs with the complementary bases on other DNA molecules.

Thus, the sticky ends of DNA fragments can be used to join DNA pieces originating from different sources. A recombinant protein is protein that is derived from recombinant DNA (John, 1986).

2.10.8. Origin and make-up of R-plasmid.

The origin of R-plamids can not be traced to a specific source. since there are channels of speculations which can result in resistance factors such as indiscriminate use of antibiotics without prescription in agriculture, industries and hospitals coupled with the lack of some environmental policy that can mount a selective pressure on the microbial world of R-plasmids in an environment devoid of antibiotics.

Since microbes themselves are capable of producing antibiotics to selectively put out other competitors, R-plasmids/factors, can be selected from this action. Some bacterial strains are known to produce enzymes which are R-plasmids mediated enzymes.

One of such can be found in *Bacillus circulans* that produces neomycin – phoshotrasferase enzyme and butrocin antibiotic chemical configuration which is similar to neomycin (Clewell, 1981).

The presence of *Pseudomonas* spp., as one of the opportunistic microflora in the human intestinal tract and its low resistance to Sulfonamide, arose a suspicion to the possibility of R-plasmids evolution. Tsuomo Watanabe, a Japaneese biologist in 1963 raised a speculation, pointing to the likelihood of the R-factors as originating from *Pseudomonas* or some other genus outside the Kauffmann's *Enterobacteriaceae.*

The curiosity raised by Watanabe as to the close linkage on the host chromosome in the location of only the resistant factors but no other detectable marker with such close linkage, could assist in establishing the pick-up origin hypothesis.

The Peculiar resistance of *Pseudomona aeruginosa* to most antibiotics should not be suprising if all other R-plasmids are just variants of R-plasmids from *Pseudomonas spp.*

Pseudomonas aeruginosa can therefore be recognized as an index strain in the evaluation of new chemotherapeutic agents for antibacterial activities. The family of *Enterobacteriaceae* also elicits various resistance to various antibiotics namely; tetracycline chloramphenicol, sulphonamide and streptomycin. Hypothetically, episome(plasmids integrated into the bacterial chromosome) mediated transfer – were said to be borne on R-factor (Watanabe, 1969).

It can be said that drug resistance factor exists as two distinct components: The R-factors bearing resistance – (r) determinants and an agent for transferability, RTF, unmindful of their conjugation initiated transmissibility. So, the term R-factors, Resistance transfer agents and R-determinants can be interchangeably used (Detta *et al.*, 1971).

2.10.9. The β-lactamases

β-lactamases are enzymes produced by some bacteria and are responsible for the bacterial resistance to β-lactam antibiotics which have a common element in their molecular structure: a four-atom ring known as a β-lactam. The lactamase enzyme breaks that ring open, deactivating the molecule's antibacterial properties. Numerous chromosomal and plasmid-mediated β-lactamases are known and may be classified by their sequences or phenotypic properties. The ability of a βeta-lactamase to cause resistance varies with its activity, quantity, and cellular location and, for Gram-negative organisms, the permeability of the producer strain. β-lactamases sometimes cause obvious resistance

to substrate drugs in routine tests; often, however, these enzymes reduce susceptibility of the host bacteria thereby developing resistance to the antibiotic (Bush, 2000).

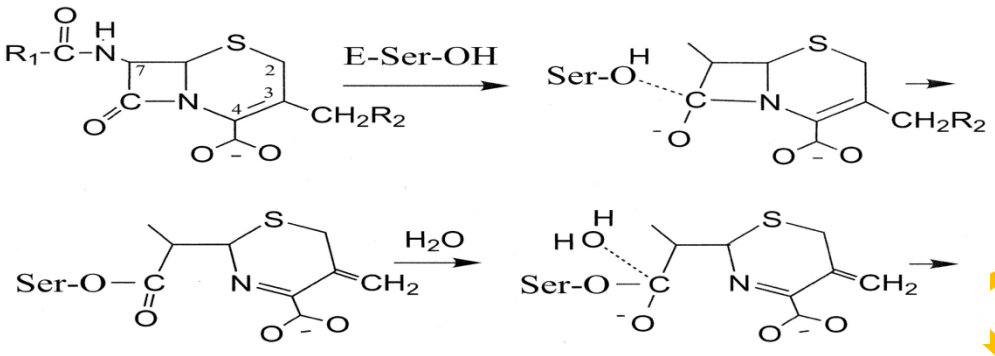
2.10.10. Penicillinase

Penicillinase is a specific type of β-lactamase, showing specificity for penicillins for hydrolysis of their beta-lactam ring. Molecular weights of the various penicillinase tend to cluster near 50,000kda. Penicillinase was the first β–lactamase to be identified: it was first isolated by Abraham and Chain in 1940 from Gram-negative *Escherichia coli* even before penicillin entered clinical use, but penicillinase production quickly spread to bacteria that previously did not produce it or only produced it rarely (Asheshov, 1969).

2.10.11 Cephalosporinase

This is an enzyme that hydrolyzes the -CO-NH-bond in the β-lactam ring of cephalosporins, converting it to an inactive product. Only a cephalosporinase producing bacterium is judged cephalosporinase-positive.

Nakamura and Nimoru (1990) cited instances when mediated class C enzyme cephalosporinase caused resistance to cephalosporins and when the enzyme induced peritoneal infection in mice. The generalized hydrolytic reaction in the conversion of β-lactam to inactive products .



Active cephalosporins

inactive cephalosporins
(amide bond in β-lactam ring hydrolysed)

Figure 7.0. Hydrolysis of β-lactam antibiotic

2.10.12 .The origin of β-lactamases

There are various embodiments of knowledge with suggestions on the origin of β-lactamase enzymes. Akiba and Yokota (1961) suggested the widespread and uncontrollable use of drugs with selective pressure in favour of organisms having genes that code for resistance. Some organisms were natural sources of antibiotics e.g. penicillins and cephalosporins, and as a result of selective pressure of the antibiotics on them, synthesis of β-lactamases ensues and their spreads to other microorganisms sharing the same environmental milieu.

The genuity of this suggestion depends on actual showcasing of the secretion of antibiotics under natural conditions to establish the survival value of antibiotics to microorganisms under such conditions and the selective advantage of enzymes such as β-lactamases.

The possibilities of a physiological role and the existence of a natural, physiological substrate besides the antibiotics are offered in support of the suggestion of selective pressure. Against the background knowledge that organisms bearing β–lactamases usually produce β-lactamase enzymes in response to antibiotics imposed selective pressure, the possibilities become farther from genuity (Bobrowsky, 1974).

2.10.13. The β-lactam resistant *staphylococcus aureus*

Two main types of β-lactmase Resistant *Staphylococcus aureus* occur: (i) strains arising de novo in patients with no established risk factors: these are true community acquired βeta-lactamase resistant *Staphylococcus aureus:* (ii) hospital strains having spread to the patients with risk factors such as recent hospitalization or surgery, an underlying

chronic disease, immune-suppression or injecting narcotic use (Papanicolau,1999).

On the basis of the criteria reviewed by Plorde *et. al. (*1974) β-lactamase resistant *Staphylococcus aureus* was classified as hospital acquired if any of the following features were present: hospitalization in the previous 2 years; presence of percutaneous lines, involving-devices or catheters, a household member hospitalized during the previous 2 years: a household member employed in a health care facility: chronic disease such as diabetes mellitus, human immunodeficiency virus infection, cancer, renal haemodialysis, pulmonary or cardiac failure, immunosuppression or organ transplantation; infecting narcotic use; and frequent (at least once a week) home-based care (nursery, physiotherapy, etc). Patients with none of the above listed features were considered to have true Community Acquired β-lactamase Resistant Staphylococcus infection. The first serious emergence of antibiotic resistant *Staph***.** occurred with a specific strain referred to as Methicillin-Resistant *Staphylococcus aureus*, abbreviated as MRSA. This strain expressed a modified penicillin-binding protein encoded by *mecA* gene and is present in 4 forms of *Staph*. cassette.

Consequently, vancomycin (a powerful antibiotic) became the primary antibiotic used to combat *Staphylococcus* infection. In 1997, a strain of *Staphylococcus. aureus* resistant to vancomycin was isolated, and people are once again exposed to the threat of untreatable staphylococcus infection. MRSA strains are currently a very significant healthcare problem. The sequencing of the *Staphylococcus aureus* genome will hopefully provide insight into how the organism generates such a variety of toxins, and aid researchers in developing ways of combating the versatile bacterium (Kenneth, 2008).

2.10.14. Extended-spectrum β-lactamase (ESBL) - producing Gram-negative bacteria

Extended-spectrum β-lactamases (ESβLs) are plasmid-associated β lactamases that have recently been found in the *Enterobacteriaceae*. ESβLs are capable of hydrolyzing penicillins, many narrow spectrum cephalosporins, many extended-spectrum cephalosporins, oxyimino-cephalosporins (cefotaxime, ceftazidime), and monobactams(aztreonam). β-lactamase inhibitors (e.g. clavulanic acid) generally inhibit ESβL producing strains. ESβL producing isolates are most commonly *Klebsiella ssp*, predominantly *Klebsiella pneumoniae*, and *E. coli*, but they have been found throughout the *Enterobacteriaeae.* Because ESβL enzymes are plasmid mediated, the genes encoding these enzymes are easily transferable among different bacteria. Most of these plasmids not only contain DNA encoding ESβL enzymes but also carry genes conferring resistance to several non-ß-lactam antibiotics. Consequently, most ESβL isolates are resistant to many classes of antibiotics. The most frequent resistance found in ESβL-producing organisms is the resistance to aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and sulfamethoxazole-trimethoprim combination. Treatment of these multiple drug-resistant organisms is a therapeutic challenge. ESβL producing strains have been isolated from abscesses, blood, catheter tips, lung, peritoneal fluid, sputum, and throat cultures. They apparently have a world-wide distribution (Kenneth, 2008).

2.10.15. Chemotherapy against β-lactamase resistance

Chemotherapy is the practice of bombarding the host microbial infected loci with an array of prescribed antibiotics which can be natural or synthetic with a therapeutic threshold taking into cognizance the dosage adjustment relative to the adverse reaction and probable toxicity to be elicited (Tom, 1997). Resistance to several penicillins is caused by β-lactamase enzymes disintegrating the antibiotic thereby preventing them from exerting any action on the bacteria. The enzyme forms stable non-active complex, thereby preventing the degradation of amoxicillin. Amoxicillin has activity against penicillin sensitive Gram-positive bacteria as well as Gram-negative bacteria. Amoxicillin is sensitive to destruction by β-lactamases and therefore when administered alone, it is not effective against β-lactamase producing bacteria such as *Klebsiella* and P*roteus spp*. Clavulanate is a naturally occurring non-competitive inhibitor of beta-lactamases that will only assist in the destruction of bacteria that produce beta-lactamase enzyme, but other form of resistance such as alteration of penicillin-binding protein, are not affected (Garrod *et al.*, 1981).

Clavulanic acid, the first β-lactamase inhibitor detected in the United State is still being combined with amoxicillin(augmentin$^{(R)}$) and ticarcillin(Timentin$^{(R)}$). It was isolated from cultures of *Streptomyces clavigerus***.** It is an irreversible inhibitor of β-lactamases that had been shown to protect penicillins from destruction by β-lactamase when tested in vitro and in vivo. Clinical and pharmacokinetic studies of the antibacterial action of this drug combination have indicated it to inhibit β-lactamases in a number of Gram-negative bacilli, and amoxicillin was readily protected from degradation by whole bacterial cells.

Clavulanic acid has been shown to inhibit β-lactamases from numerous pathogenic organisms, when combined with amoxicillin (augmentin$^{(R)}$) but it does not inhibit chromosomal-mediated enzyme (Richard Skyes Type1) produced by some enterobacteriaceae and *Pseudomonas spp*, The mechanism of action of clavulanic acid depends primarily on the specific β-lactamase being inhibited. Although competitive inhibition is seen, clavulanic acid most often acts as a suicide inhibitor .

Sulbactam is another β-lactamase inhibitor, it has a weak intrinsic antibacterial activity but acts synergistically with β–lactamase labile antibiotics against most β-lactamase producing organisms such as Methicillin Resistant **S**taphylococcus aureus, when combined with ampicillin (Unasyn). In the case of MRSA, the resistance mechanism is more complex and less well understood (Lode, 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.0. Materials

3.1. Glassware: The glassware used in this research work included; test-tubes, Pasteur pipettes, universal bottles, bijou bottles, Petri-plates, McCartney borttles, conical flasks, flat bottom flasks, Durham tubes and various items of sterilizable resins. All the items of glassware used were of Pyrex made.

3.2. Equipment:

Oven sterilizer (Gallenkamp, United Kingdom) . It was used for the sterilization of glassware and Petri-plates at a temperature of 160^0C for 1hr.

Microscope (Olympus). It was used for viewing bacterial stained smears.

Incubator(Gallenkamp, U. K). This equipment served the purpose of obtaining optimal growth of bacterial cultures at a specific temperature, generally 37^0C.

Inoculating hood. It was used for aseptic inoculation of clinical sample

Autoclave(Gallenkamp, U. K.). This equipment was used for the sterilization of media and glass consumables.

Shaker Bath (Gallenkamp, U. K). It was used to accelerate and maintain bacterial culture in optimal growth.

Refrigerated High Speed Centrifuge(MSE).: It was used for separation of cultures and preparation of biochemical liquid suspensions.

Weighing balances; H80 and PC 400, (Mettler, Switzerland). They were used for obtaining the weights of culture media, antibiotic powder and some other materials.

Refrigerator. It was used for preservation of bacterial cultures at a temperature of 4^0C

Eppendorf tubes(U.S.A.). It was used for centrifugation of overnight culture in the process of plasmid extraction.

Vortex Mixer(Griffin and George Limited (Ltd.), London). The equipment was used for homogenization of culture preparations.

P^H Meter 7020(EIL, England) This apparatus was used for measurement of acidity and alkalinity of medium.

Ultraviolet (UV) light transilluminator. It was used for viewing plasmid bands.

Electrothermal Water Bath(Electrothermal, London). The equipment was used to dissolve culture media, relevant reagents and to achieve required slight temperature changes.

Hospital Sterile Swab(EVEPON, Nigeria) It was used for collection of clinical specimens.

3.2.1 Chemicals:

The chemicals used included; barium chloride, benzylpenicillin (penicillin G), bromophenol blue, carbol fuchsin, chloroform, ethanol, Gram's (Lugol's) iodine, hydrogen peroxide, hydrochloric acid,(conc.), isopropyl alcohol, phenol red, potassium iodide, phosphate buffer, potassium acetate, acetic acid, agarose powder, Tris base, acrylamide gel and distilled water.

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3.2.2. Culture media: The culture media used were Nutrient agar, Luria Bertani agar, DNAse agar, Sensitivity Test Agar, Blood agar and Mannitol Salt Agar. Their compositions and methods of preparation are stated in appendix 2 .

3.2.3. Reagents:

Crystal violet, iodine, potassium iodide, safranin, basic fuchsin, phenol crystal, ethanol(95%) , sodium chloride, Tris-EDTATE buffer, glacial acetic acid. Their compositions and methods of preparation are stated in appendix 3 .

3.2.4. Antibiotics:

The antimicrobial discs containing the following antimicrobial agents were applied (Oxoid) cloxacilln 5µg; amoxicillin-clavulanate 30µg; amoxycillin 10µg; tetracycline 30µg; gentamicin 10µg; erythromycin 15µg and cotrimozaxole 25µg .

The antibiotic used in the antibiogram and MIC determination were obtained locally both as multi-discs and powders for injection; amoxicillin, B.P. (Beecham Laboratories, London), Cloxacillin(Bombay India); cefuroxime,B.P. (Glaxo operation, U.K.) cefotaxime B.P.(Hoechst,Nigeria) ampicillin,B.P. (Britlodge Ltd.,U.K.) and penicillin G,(B.P).(Kamfarma Laboratories, Switzerland) (Difco) and powdered samples.

3.3 Methods

3.3.1. Collection of samples

Pus samples of exudates from cropped boils were collected from a total of one hundred and forty volunteers within the community and hospitals located in the South-Western region of Nigeria namely: Ibadan, Ondo, Ikeja, Osogbo, Isagamu, Ekiti. The volunteers were of varied age, sex and loci of recurrent furunculosis as noted in the interview with the infected volunteers.

The samples were collected with cotton-wool swab moistened with sterile 0.1% bacteriological peptone water, and either processed immediately or preserved in a refrigerator at 4^0C till the second day.

3.3.2 Isolation of **microorganism**(s).

Each swab specimen was plated on Blood Agar, by streaking , followed by incubation at 37^0C for 24-48hours under aerobic condition.

The colonies observed were Gram-stained .Those that stained Gram –positive cocci occurring in groups of four or more were sub-cultured mannitol salt agar(MSA). The discrete colonies obtained were then processed for the isolation and identification of *Staphylococcus aureus*.

3.3.3. Preparation of 0.1m phosphate buffer .

Solution A: 1.36g of potassium dihydrogen phosphate (KH_2P0_4) was dissolved in 100ml, of distilled water, pH 5.5,

Solution B: 1.42g of disodium hydrogen phosphate, anhydrous (Na_2HP0_4), was dissolved in 100ml of distilled water, pH 8.8.

34ml of solution A was then mixed with 66ml, of solution B to give phosphate buffer of pH 7.0. The buffer was then dispensed in 10ml, amount into clean universal bottles and sterilized by autoclaving. Thereafter, buffer was stored away in the refrigerator until when needed.

3.3.4. Preparation of iodine reagent

Iodine crystals – 0.506g

Potassium iodide _ 13.30g

The two substances were dissolved in 25ml of distilled water, starting with potassium iodide. The mixture was shaken well and stored in a brown bottle away from light until when the reagent was needed.

3.3.5. Preparation of starch solution

The starch solution was prepared fresh as 1% (w/v) aqueous concentration, by dissolving 0.25g of soluble starch in 25ml of sterile distilled water. The mixture was boiled in an electro-thermal water bath, with intermittent stirring, to give whitish gelatinous solution. It was allowed to cool before use.

3.4. Biochemical characterization of isolates

3.4.1. Gram staining

Bacterial smear was prepared for every colony and fixed with Bunsen burner flame, for Gram-staining. The smear was flooded with crystal violet which was allowed to react for 1 minute .The excess stain was rinsed off and replaced with Gram"s iodine solution serving as mordant.

After 30 seconds the mordant was replaced with acetone which was allowed in drops on the smear to decolorize the smear . At the point when the blue stain stopped issuing out from the smear, the alcohol was rinsed off and replaced with carbol fuchsin basic, allowed to react for 20seconds. The stain was rinsed off. The preparation was blotted dry and examined under oil immersion ×100 objective lens of the Olympus light microscope.

Bacterial cells observed as spheres that retained blue/purple color of the primary stain, mostly in clusters of four or more were noted for further identification (Harold,2007).

3.4.2. Catalase test

A sterile capillary tube was aseptically dipped into 3%Hydrogen peroxide and a drop of Hydrogen peroxide from the capillary tube was put on a clean microscope slide. A bit of bacterial colony was then smeared on the hydrogen peroxide.

Effervescence of bubbles indicated a positive test for *Staph. aureus* due to the breakdown of Hydrogen peroxide and liberation of oxygen gas:

$2H_20_2 \rightarrow 2H_20 + 0_2$ (Harold,2007)

3.4.3. Coagulase test

Overnight broth culture of isolated colony, 0.1ml., and 0.9ml of a 10^{-1} saline dilution of human plasma were pippeted into sterile test- tubes. The mixtures were incubated at 37^0C and examined hourly for 6 hours, for clumping as a sign of positive test, and control tubes without any organism were also examined. When necessary ,incubation was prolonged beyond 6 hours since *Staphylococcus aureus* is not known to produce spontaneous clotting due to utilization of citrate that may have been used as an anticoagulant.(Gillespie,1943)

3.4.4. Oxidase test

A bacterial smear from mannitol salt agar (MSA) was swabbed on a piece of sterile filter paper moistened with fresh normal saline preparation of oxidase reagent (NNNN-tetramethyl-paraphenylenediamine hydrochloride.) a chromogenic reducing agent, acting as an artificial electron acceptor, which will turn purple if oxidized cytochrome oxidase is present. Purple color within 10 seconds was recorded as negative (John, 2008) .

3.4.5. Starch hydrolysis

The respective isolates of *Staph aureus* were streaked on plate count agar fortified with soluble starch, and incubated at 37^{o}C for 48hrs.

Hydrolysis of starch was tested by flooding the slide preparation with Gram's iodine. Blue black color around the isolates depict the inability of the organism to hydrolyse starch(John, 2008).

3.4.6. Haemolysis test:

Defibrinated/oxalated human blood measuring 4ml was aseptically added to 25ml of liquefied nutrient agar in a 25ml volume universal bottle, the mixture was rotated gently, then poured into sterile culture plate, and allowed to set, followed by drying at 37^{o}C inside a freshly disinfected incubator. A loop-full of overnight broth culture of isolated colony was then streaked on the medium,incubated at 37^{o}C for 24 hours and then examined for haemolysis.

Observation

Clear zones around the colonies were observed to indicate the haemolytic property of the *Staphylococcus aureus* under investigation. (Harold,2007).

3.4.7. Gelatin hydrolysis/liquefaction

Gelatin powder was dissolved as 15% in nutrient broth and autoclaved at 110^{o}C for 25mins .cooled Nutrient gelatin was inoculated with wire-loop from 24hr-old broth culture and incubated for maximum period of 7days,along with a control (uninoculated nutrient gelatin).

After incubation, tubes were examined for hydrolysis by immersing the tubes in the ice bath for 1hour. The control tube in which no hydrolysis has taken place became solidified, a negative test. Those that were hydrolyzed remained fluid and therefore, gelatin hydrolysis positive(Victoria,1996).

3.4.8. Deoxyribonuclease test (DNAse)

Pour–plates of DNase agar were prepared, allowed to set and dry. Every isolate of *Staphylococcus aureus* was then inoculated on DNase agar plate and incubated for 24hours at 37^0C .A positive result was indicated by a complete colour change from green into a milky turbid, hydrolytic cleared zone. Lack of this observation showed DNase negative test(Smith,1969).

3.4.9. Sugar fermentation test

A 10 ml volume of the fermentable sterile sugar broth was dispensed into each tube containing an inverted Durham tube and autoclaved at 121^OC for 5 minutes. Following autoclaving , the cooled sugar broth was inoculated with a sterile wire loop from a bacterial colony suspension and then incubated along with a control (uninoculated sugar broth) for 24 -48 hours. The tests and control were then examined for acid and gas production.

A colour change from pink to yellow and a displacement of broth in the Durham tube gave positive result while those that showed no change were negative(Harold,2007).

3.5.0. Purity testing

Purity check was carried out at periodic and regular interval of two months on the strains of *Staphylococcus aureus* preserved on Nutrient agar slant by means of a few biochemical characterizations, mainly: mannitol salt agar culture, coagulase test and haemolysis test.

3.5. The antibiogram

The antibiotic susceptibility profile of every isolate of *Staphylococcus aureus* was determined as follows;

3.5.1. Antibiotic susceptibility test:

The antibiogram was determined by modified disk-diffusion technique . All the isolates of *Staphylococcus. aureus* were tested for the sensitivity to the following antibiotics; amoxicillin(10µg),cotrimoxazole(25µg),gentamicin(10µg),chroramphenicol(30µg),amoxicillin-clavulanate (30µg), erythromycin (15µg), tetracycline(30µg) (Oxoid product). A volume of 0.1ml of the overnight broth culture of every isolate was pipetted into 9.9ml of sterile distilled water in the test tubes to make 10^{-2} dilution of the organism. From this dilution, 0.1ml of the diluted culture was pipetted into the 20ml of sterile melted and cooled (45^{o}C) sensitivity test agar(STA) (Oxoid) was aseptically poured into the sterile culture plate, and were allowed to set. The antibiotic multi-discs were aseptically placed in each plate which were then left on the laboratory bench for 45-60mins to allow pre-incubation diffusion. They were them incubated at 37^{0}C for 24 hours.

The zones of growth inhibition were recorded and analyzed according to CLSI breakpoint zones of growth inhibition diameter as either sensitive or resistance using *Staphylococcus .aureus ATCC29213* as a control strain.

3..6. .0 .Determination of the minimum inhibitory concentration(mic).

MIC was determined by tube-broth dilution for Penicillin G, Amoxycillin-Clavulanate, Cefotaxime, Cloxacillin and Ceftriaxone against the 30 β-lactamase positive strains. Each antibiotic was prepared as 1000 µg /ml. stock by dissolving 10mg in 10ml. of sterile distilled water. 5ml of double strength nutrient broth was added to 5mls of 1000 µg /ml antibiotic stock to give 500 µg /ml which was then diluted successively in double-fold decreasing concentration, to 0.47 µg /ml in the 11^{th} tube. Every concentration was prepared in duplicate. 0.1ml of 1:100 dilution of the organism was taken into each of the tubes, with the exception of the 11^{th} tube which serves as negative control that contained sterile nutrient broth and the drug (no organism) while the 12^{th} tube which serves as positive control contained nutrient broth and the organism only. The tests and controls were incubated for 24hours at 37^0C, before been checked for growth. The lowest concentration that showed no growth was taken as the MIC of the antibiotic(Harold, 2007).

3..7. Minimum bactericidal concentration(mbc)

The dilutions that fail to show growth during the MIC dilution were each streaked on nutrient agar plate and incubated at 37^0C for 24 hours . They were then examined for growth. The least dilution that failed to form colonies on plate culture was taken as the minimum bactericidal concentration(Harold,2007).

3.8. Detection of β-lactamase

Iodometric (cell-suspension) method

Overnight nutrient broth culture of each strain was sub-cultured by streaking on nutrient agar plate followed by incubation at 37^0C for 18-24 hrs. A cell suspension was prepared in triplicates by emulsifying bacterial colonies with a sterile wire-loop in 0.5ml of freshly prepared phosphate buffered solution containing penicillin G (10,000 units or 0.06mg per ml.). The cell density was determined to be about 1 x 10^9 cells/ml, with McFarland standards (Kolmer *et al.*, 1951). The suspension, contained in small sterile test-tubes, are homogenized on a vortex mixer briefly. The standard strain's suspension and ordinary penicillin G phosphate buffered solution served as control. The test and control tubes were incubated at room temperature of 28^0C for a minimum period of 1hr. Thereafter, two drops of freshly prepared 1% aqueous starch solution were added to each suspension. The mixture was shaken gently and briefly, after which one drop of iodine solution was added without shaking the mixture. The mixtures were allowed to stand at room temperature (28^oC) for 10 minutes, for a color change from blue-black to colorless for a positive test. The results were interpreted as: negative, where there was no color change within 10 minutes.

3.9.. Extraction and estimation of plasmid DNA

Plasmid DNA was isolated as described by Bamboo and Dolly(1979), and Kudos and Liu(1981) by the use of lystostaphin for lyzing the cell wall. Each *S. aureus* strain was inoculated into 3ml trypt soy broth and incubated overnight on a roller drum at 37^0C. About 1.5ml of each overnight broth culture was transferred into Eppendorf tubes and centrifuged for one minute at 15000 rev/min at room temperature(28^0C). The supernatant was discarded and 2µl of lystostaphin solution (1.0µg/ml in distilled water) added to the pellet. Tubes were capped, vortexed and placed in ice for 30 minutes followed by adding 200µl of alkaline detergent solution (0.2N NaOH; 1% Sodium dodecyl sulphate). The tubes were inverted several times and then kept in the waterbath for 5 minutes. 150µl of 3M sodium acetate (pH 4.8) was added and tubes were inverted several times to mix and then kept on ice for at least 10 mins. The tubes were centrifuged at room temperature(28^0C) at 1500 r.p.m. for five minutes and the supernatant was transferred into fresh Eppendorf tubes. One milliliter of 95% ice cold ethanol was added to the tubes, which were then kept at -20^0C for five minutes. After five minutes, they were centrifuged at 15000 r.p.m for 3 minutes; the supernatant was discarded and the sediment was resuspended in 50µl of Tris EDTA (10mM Tris HCL and 1mM EDTA, pH 8.0). five microlitre of the contents were then loaded into wells of 0.8.% agarose gels containing ethidium bromide. A 23.13 kb DNA ladder was run alongside with test isolates as a molecular size marker.

Electrophoresis was carried in Tris Acetate EDTA buffer containing ethidium bromide (20ml of 50 x EDTA and 6.0µl of 10µg/µl ethidium bromide per litre) at 30mA (90V) for 4 hours. Plasmids were viewed on an ultra violet (UV) Transilluminator and photographs were taken using a Polaroid camera. Films were exposed for 90 seconds in the studio and later developed. Plasmid sizes were estimated from a standard curve drawn with the molecular sizes of the 1.0kb DNA ladder against their migration distance.(Olukoya,1995).

3.10. Calculation of molecular weight of fragments

The fragment bands observed in the isolates of *Staphylococcus aureus* were directly compared with those of the molecular weight marker (Hind III digest of lambda phage from Roche Diagnostics, Germany), which has molecular weights of 23.130, 9.416, 6.557, 4.361, 2.322, 2.027, 0.564 kilobase .

3.11. Curing of antibiotic resistance with ethidium bromide.

The modified method of Julian and Willam (1984) as reported by Adeleke *et.al.,*(2002) was used with some further modifications.. Ethidium bromide was prepared in nutrient broth in serial double-fold dilutions of 100.00, 50.00, 25.00, 12.50 and 6.25 µg/ml. A sub-culture of each of thirty β-lactamase positive strains of *Staphylococcus aurues* inoculated into the mixture, followed by vortexing.

The mixtures were incubated overnight at 37^0C and following the incubation, each mutagen-exposed culture was plated on nutrient agar and incubated similarly. Colonies were then randomly selected from each of the four plates per strain, for sensitivity testing. Every colony selected for sensitivity testing was grown overnight in 5ml nutrient broth which was diluted in 10^{-2} in sterile distilled water. After shaking, pour-plates were prepared by seeding using 0.1ml of the diluted culture in molten nutrient agar. Wells were later dug on a set medium with size 3 cork-borer. The wells were then filled each with MIC of each of the test antibiotics .

3.12. R-plasmid transfer by conjugation

3.12.1 Preparation of competent *Staphylococcus aureus* cell.

Overnight pure culture of *Staphylococcus aureus* was inoculated in 10ml of Luria Betani broth and incubated on a shaker at 37^0C until the OD_{650} = was obtained.

The preparation was chilled on ice for 5minutes and kept cold for the rest of the preparation, the cells were harvested by centrifuging at 500rpm for 5minutes and the supernatant was discarded . The subnatant(pellets) was re-suspended in 5ml ice-cold 0.1M $CaCl_2$ by agitating while in ice bath, recentrifuged at 5000rpm for 5 minutes .

The preparation was resuspended in 1ml cold 0.1M $CaCl_2$ and stored on ice for 2hrs as competent cell bearing no plasmid.

Lyophilized culture of Escherichia coli strains (donor and receipient) were reconstituted with 0.1ml of sterile Luria Betani(LB) broth. The donor strain was streaked on a plate of LB agar medium containing streptomycin(concentration 10µg/ml) and the recipient strain on LB agar medium containing ampicillin (concentration 10 µg/ml) both were incubated at 37^0C for 24 hours to confirm the resistance status of the organisms to the antibiotics. With the plates showing no growth, the donour and recipient strains were plated separately on LB agar+str +amp and incubated at 37^0C for 24 Hrs.(Robert,1989).

The strains of *Staphylococcus aureus* obtained from boil specimen were plated on streptomycin fortified and ampicillin fortified medium and streptomycin-ampicillin supplemented manitol salt agar (MSA) medium to examine their resistant status.

Streptomycin sensitive(but ampicillin resistant) *Esch. coli* recipient strain was plated on LB agar medium supplemented with ampicillin while ampicillin sensitive(but streptomycin resistant) *Esch. coli* donor strain was plated on Luria broth supplemented with streptomycin. Growth was noticed in both.

The recipient *Esch. coli strain* (ampicillin resistant, streptomycin sensitive) was mated with the donor strain of *Staphylococcus aureus*(streptomycin sensitive, ampicillin sensitive) on plain Luria medium containing no antibiotic, and were mated and incubated at 37^0C for 24hrs.

Five colonies of each pure bacterial culture were picked separately from the mated plate and plated on LB agar medium supplemented separately on the antibiotic they were sensitive to, presence of growth on each separate plates was interpreted as an indication of plasmid transfer while absence of growth signified no plasmid transfer.

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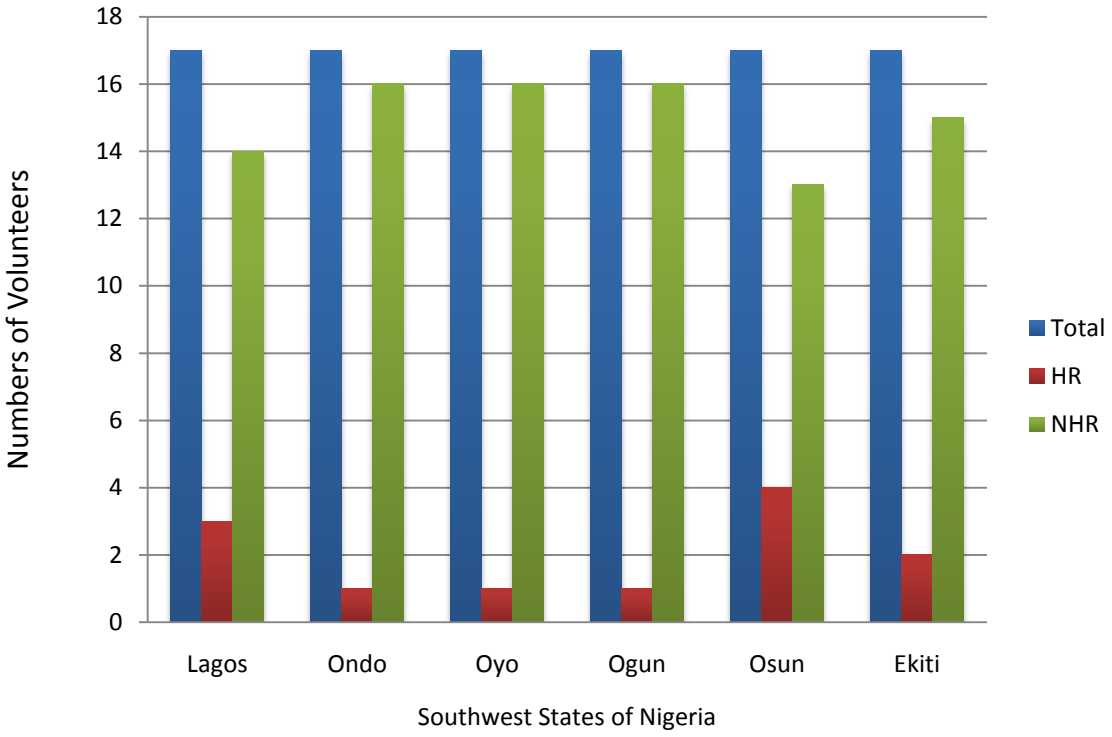
CHAPTER FOUR

RESULTS

4.0. Isolation and identification of *Staphylococcus aureus* from the clinical samples.

Six catchment zones were identified in southwest Nigeria for the collection of exudates of cropped boils from infected volunteers (Fig.8.0). Out of 140 clinical samples screened from the infected donor, 102 isolates of *Staphylococcus aureus* were identified and selected, with each zone producing 17 isolates of *Staphylococcus aureus* each from the specimens collected. The ratio of gender distribution (Table 1.0.). was found to be forty-six females to fifty six males (46:56) while the ratio of non-hospital reported to the hospital reported samples was found to be 90:12(Table 2.0) in this study. They were of varied age and loci of collection as shown in the pathological distribution (Appendix 10.) The age of the smallest patient, a female infant, was three years while the oldest was eighty three years, an hospitalized reported case.



Key: HR: Hospital Reported NHR: Non-Hospital Reported

Fig 8.0. Percentage distribution of furunculosis among the southwest Nigeria.

Table 1.0. Gender and anatomical distribution numbers of isolates of *Staph. aureus*

Anatomical loci	Sex				Total	
	Male		Female			
	Isolate numbers	Percentage %	Isolate numbers	Percentage %	Isolate numbers	Percentage %
Armpit	7	6.9	6	5.9	13	12.7
Breast	0	.0	3	2.9	3	2.9
Buttock	2	1.9	3	2.9	5	4.9
Cheek	4	3.9	0	.0	4	3.9
Chin	3	2.9	3	2.9	6	5.9
Ear	13	12.9	5	4.9	18	17.6
Elbow	2	1.9	3	2.9	5	4.9
Eyelid	2	1.9	2	2.0	4	3.9
Forearm	1	0.9	3	2.9	4	3.9
Head	6	5.9	1	1.0	7	6.9
Knee	1	0.9	2	2.0	3	2.9
Leg	1	0.9	0	.0	1	1.0
Lip	2	1.9	1	1.0	2	2.0
Nose	7	6.9	9	8.8	16	15.7
Thigh	3	2.9	2	2.0	5	4.9
Upper arm	2	1.9.0	3	2.9	5	4.9
Total	**56**	**54.9**	**46**	**45.1**	**102**	**100.0**

Table 2.0

Age distribution of the volunteers in relation to non-hospital reported and reported furunculosis.

Age distribution (years)	Total number of Samples	Non-hospital reported	Hospital reported
1-10	9	4	5
11-20	27	25	2
21-30	21	21	0
31-40	15	15	0
41-50	16	14	2
51-60	6	5	1
61-70	4	4	0
71-80	1	1	0
81-90	3	1	2
91-100	0	0	0
Total	102	90	12

4.0.1 Biochemical characterization

In the biochemical characterization tests performed on the isolates, expected results were obtained for *Staphylococcus aureus* though, there were some deviations by some isolates. They showed pronounced growth on mannitol salt agar. Seventy-five percent of the isolates changed the color of the medium from pink to yellow within 18 to 24 hours of incubation, while the remaining 25% did not. Catalase test performed on the isolate gave 100% positive results. The coagulase test showed 90% positive, while 95% of the isolates exhibited haemolysis on blood agar as shown in figure 9.0. In the sugar fermentation test performed on the isolates, 87% of the isolates fermented lactose while 75% fermented glucose and mannitol respectively. The proteolytic property of *Staphylococcus aureus* recorded 92% gelatin hydrolysis . In examining the ability of the isolates to hydrolyze starch, 12% of the isolates hydrolyzed nutrient starch medium while 88% did not.

The DNase test performed on all the isolates to detect the DNase enzyme, gave 87% DNase positive and 13% negative. Figure 9.0 shows the biochemical characterization profiles of the isolates.

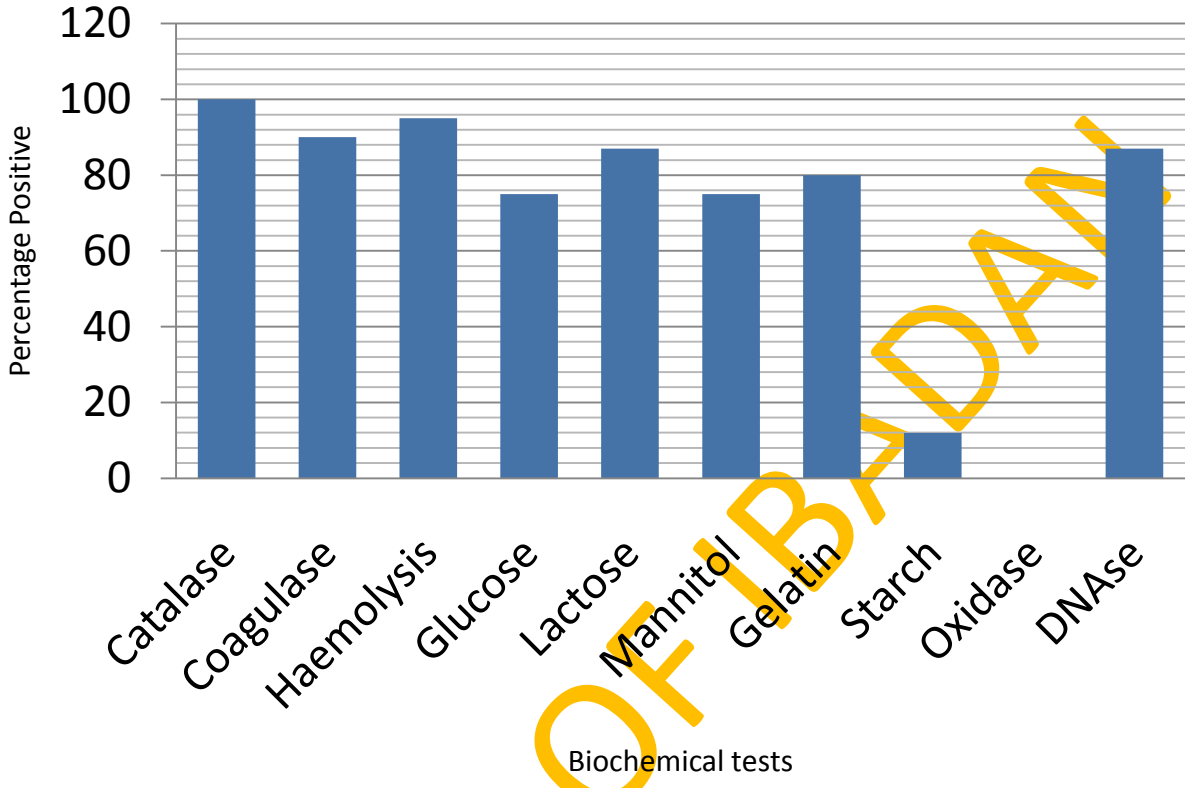


Figure 9.0. Biochemical characterization profiles of *Staphylococcus aureus.*

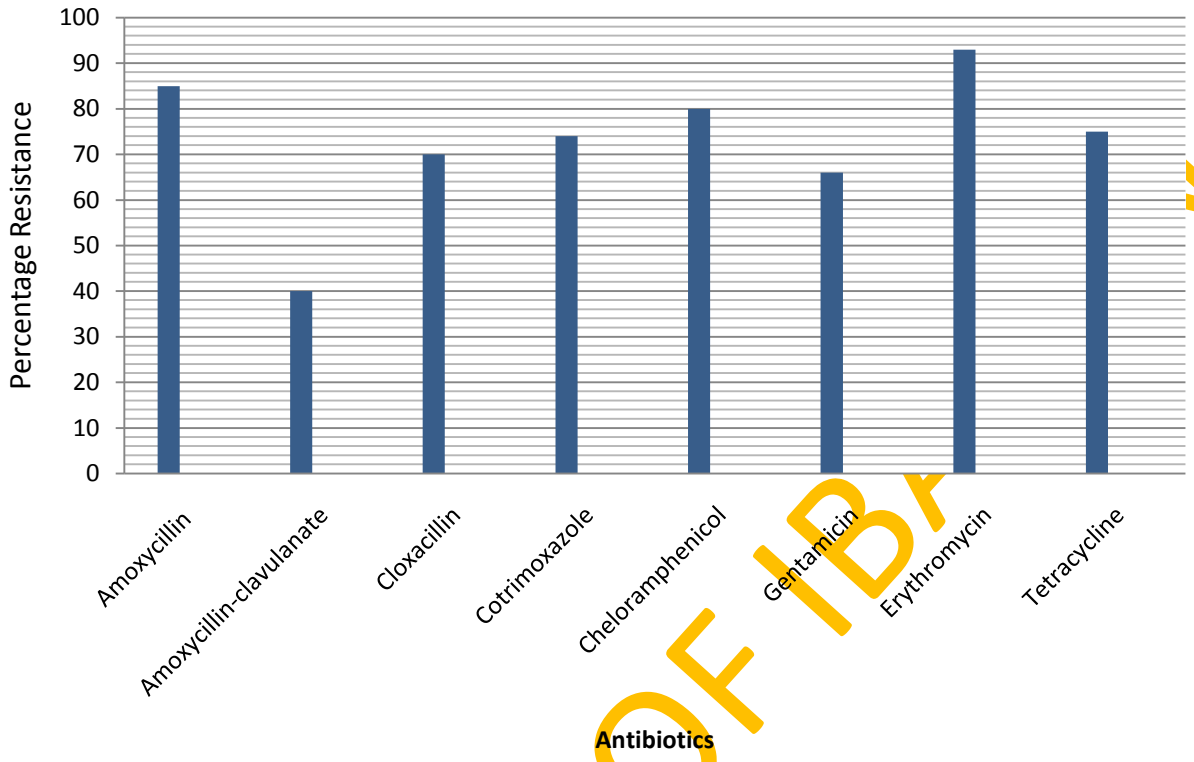


Figure 10.0. Resistance patterns of isolates of *Staphylococcus aureus*.

4.0.2. Antibiogram of the *Staphylococcus aureus* isolates

The antibiogram produced the antibiotic susceptibility profile of the *Staph. aureus* isolates for the Gram-positive antibiotic discs tested. The zones of growth inhibition were recorded and analyzed according to (CLSI 2011) zones of growth inhibition diameter (breakpoint) using *Staphylococcus aureus ATCC29213* as a control strain.

In the antibiogram study, the percentage resistance of the isolates to the antibiotics used was found to be lowest for amoxicillin-clavulanic acid 39% while 64% was recorded for gentamicin. Tetracycline resistance was 81% while erythromycin resistance was 58%. Resistance to cloxacillin was 68% resistance and the highest resistance of 83% was to amoxicillin.. Cotrimoxazole, a double blocker antibiotic showed 72% while chloramphenicol elicited 78%. The antibiotic resistance pattern shared by the isolates showed that no particular resistance pattern cut across all the sources as shown in (Table 19) in appendix 14.

One hundred and two isolates were screened for β-lactamase enzyme production, out of which 30 were β-lactamase positive while the remaining 72 strains were negative. *Staphylococcus aureus* ATCC 29213 was used as control

4.0.3. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration(MBC) of 5-selected antibiotics against selected strains of *Staphylococcus aureus*.

In the determination of MIC and MBC using five selected antibiotics against the 30 β-lactamase positive isolates, varied resistance were recorded. Five of the isolates exhibited high resistance (each with MIC 250 µg/ml) while four strains showed low resistance (each with MIC 3.90 µg/ml) to amoxicillin-clavulanate . 7% of the selected strains recorded MIC of 31.25 µg/ml while 43% of the strains had MIC of 250µg/ml using cloxacillin.. The MBC recorded for cloxacillin in this study ranged between 31.25 - 500 µg/ml.

Six isolates exhibited high resistance to cefotaxime at 250 µg/ml, a third generation cephalosporin while four isolates recorded MIC of 15.63 µg/ml. The MBC recorded for cefotaxime in this study, ranged between 31.25 - 500 µg/ml

Ten of the isolates were highly resistant to ceftriaxone with MIC 125 µg/ml while 6 isolates elicited 31.25 µg/ml to the same antibiotic. Ceftriaxone exhibited MBC range of 62.5-500 µg/ml. Penicillin G recorded the MIC of 500 µg/ml in this study, while *Staphylococcus aureus* (Sa06) exhibited low resistance (7.8 µg/ml MIC) to penicillin G. The MBC range of 31.25 - 650 µg/ml were recorded for penicillin G.

The reference strain *Staphylococcus aureus ATCC29213* recorded varied MIC, ranging from 5.2-12.0 µg/ml as shown in Table 3.0. The analysis of variance showed the degree of significance of the antibiotics used(Table 4.0).

Table 3.0

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of selected antibiotics against the clinical strains of *Staphylococcus aureus.*

STRAIN	AMX-CLV		CLX		CFT		CFZ		PEN.G	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
SA01	125	250	62.5	125	62.5	125	125	250	125	250
SA02	7.8	31.25	250	500	250	500	250	500	31.25	62.5
SA03	250	500	250	500	31.25	62.5	125	250	62.5	125
SA04	250	500	125	250	250	500	250	500	31.25	62.5
SA05	3.90	31.25	31.25	62.5	62.5	125	62.5	125	31.25	62.5
SA06	3.90	7.8	250	500	125	250	62.5	125	7.8	31.25
SA07	250	500	125	250	250	500	125	250	62.5	125
SA08	15.63	62.5	62.5	125	62.5	125	62.5	125	250	500
SA10	15.63	62.5	125	250	62.5	125	62.5	125	31.25	62.5
SA11	250	500	250	500	125	250	62.5	125	62.5	250
SA13	7.8	31.25	125	250	15.63	62.5	31.25	62.5	31.25	62.5
SA16	15.63	31.25	62.5	125	62.5	125	31.25	62.5	62.5	125
SA18	1.93	7.8	250	500	62.5	250	125	500	31.25	62.5
SA23	7.8	15.63	250	500	31.25	125	31.25	62.5	62.5	125
SA24	15.63	31.25	250	500	15.63	62.5	62.5	125	62,5	125
SA25	3.90	7.8	125	250	125	250	62.5	125	31.25	62.5
SA33	62.5	125	250	500	15.63	62.5	250	500	125	250
SA40	15.63	31.25	62.5	125	62.5	125	62.5	125	125	250
SA45	31.25	62.5	250	500	125	250	125	250	31.25	62.5
SA46	3.90	15.63	250	500	31.25	62.5	125	250	15.63	31.25
SA50	15.63	31.25	125	250	15.63	31.25	125	250	62.5	125
SA51	15.63	31.25	125	250	31.25	62.5	62.5	125	31.25	62.5
SA52	31.25	62.5	250	500	125	250	31.25	62.5	62.5	125
SA53	250	500	125	250	31.25	62.5	62.5	125	62.5	125
SA62	31.25	62.5	125	250	125	250	31.25	62.5	500	650
SA63	31.25	250	125	250	125	250	125	250	31.25	62.5
SA64	125	250	15.63	31.25	250	500	250	500	500	650
SA91	125	250	62.5	125	250	500	125	250	31.25	62.5
SA94	125	250	31.25	62.5	250	500	125	250	125	250
SA97	31.25	62.5	250	500	31.25	62.5	31.25	62.5	31.25	125

Staph aureus ATCC 29213 had MIC ranges from 5.2-12 as a control organism

Key: *SA: Staphylococcus aureus*

AMX-CLV: Amoxycillin-clavulanate CLX: Cloxacillin CFT: Cefotaxime

CFZ: Ceftriaxone PEN.G : Penicillin.

Table 4.0

Comparism of MIC and MBC significance of the selected antibiotics using Analysis Of Variance (ANOVA)

Minimum Inhibitory Concentration				**Minimum Bactericidal Concentration**				
Antibiotics	N	Subset for alpha = .05		**Antibiotics**	N	Subset for alpha = .05		
		1	2			1	2	3
Penicillin G	30	63, 1523		PenicillinG	30	144.5310		
Amx-clv	30	78, 9890		Amx-clv	30	152.3430		
Cefotaxime	30	95, 8340		Cefotaxime	30		248.3333	
Ceftriaxone	30	102, 0800		Ceftriaxone	30		308.3333	
Cloxacillin	30		162.500	Cloxacillin	30			391.6667
Sig.		105	1.000	Sig.		.835	.183	1.000

	Sum of squares	**df**	**Mean square**	**F**	**Sign**
MIC	171775.2 1027859	4 145	42943 7088.683	6,058	0.05
MBC	1325905	4	331476.323	15.829	0.00

P-value = 0.05

4.0.4.Plasmid profilling.

Plasmid mediated resistance was determined on both the β-lactamase positive isolates and selected β- lactamase negatives. DNA molecular weight marker (HindIII) was used as a dendrogram to determine the unknown molecular weight(Table 20.) in appendix 15.

Of the thirty isolates that possessed β-lactamase, twenty-nine were plasmid -borne among which seven isolates had multiple plasmid DNA copies . The molecular weight ranged between 0.25kb and 63.09kb among the β-lactamase positive isolates, while the number of plasmid copies ranged between 1 and 4.(Table 5.0). The molecular weight of plasmid DNA for β- lactamase negatives isolates ranged between 13.18kb and 50.12kb as shown in Tables 6.0.

The plasmid DNA bands pattern for the local isolates elicited varied band weights after electrophoresis as shown in figure 11, 12, 13, 14 and 15 respectively. The distance travelled by the plasmid DNA of the electrophoresed isolates correlates with the molecular weights of plasmid DNA isolated.

Table 5.0

Plasmid DNA extracted from β-lactamase positive strains of *Staphylococcus aureus*.

Strains numbers	Plasmid Copies	Molecular weight graphical values	Molecular weights (base pair)	Molecularweight (kilobase)
SA01	3	2.90	794.3282	0.79
		3.05	1122.0.85	1.12
		4.36	23120.6479	23.13
SA02	1	4.36	23120.6479	23.13
SA03	2	2.40	251.18864	0.25
		3.10	1258.5254	1.26
SA04	4	2.90	795.3282	0.79
		3.05	1122.01845	1.12
		4.36	23120.6479	23.13
SA05	3	2.75	562.3413252	0.56
		2.78	602.559586	0.60
		3.00	1000.00	1.00
SA06	3	2.95	891.2502	0.89
		3.05	1122.018	1.12
		4.48	30199.577	30.20
SA07	1	4.36	22908.67653	2.29
SA08	1	4.42	26302.680	26.30
SA10	1	4.16	14454.40	14.45
SA11	1	4.20	15848.9319	15.85
SA13	2	4.20	15848.9319	15.85
		1.14	13803.8426	13.80
SA16	2	4.20	15848.93192	15.85
		4.59	38904.5145	38.90
SA18	1	4.16	14454.40	14.45
SA23	1	4.61	40738.03	40.74
SA25	1	4.55	35481.34	35.48
SA33	1	4.70	50118.72	50.12
SA40	1	4.80	63095.73	63.09
SA41	1	4.61	40738.03	40.74
SA45	1	4.42	26302.68	26.30
SA46	1	4.45	28183.83	28.18
SA50	1	4.45	28183.83	28.18

Table 5.0 (cotnd). Plasmid DNA extracted from β-lactamase positive strains of *Staphylococcus aureus*

SA52	1	4.49	30902.95	30.90
SA53	1	4.45	28183.83	28.18
SA62	1	4.50	31622.78	31.62
SA64	1	4.50	31622.78	31.62
SA91	1	4.68	47683.01	47.86
SA94	1	4.61	40738.03	40.74
SA97	1	4.52	33113.11	33.11
SA98	1	4.45	28183.83	28.18

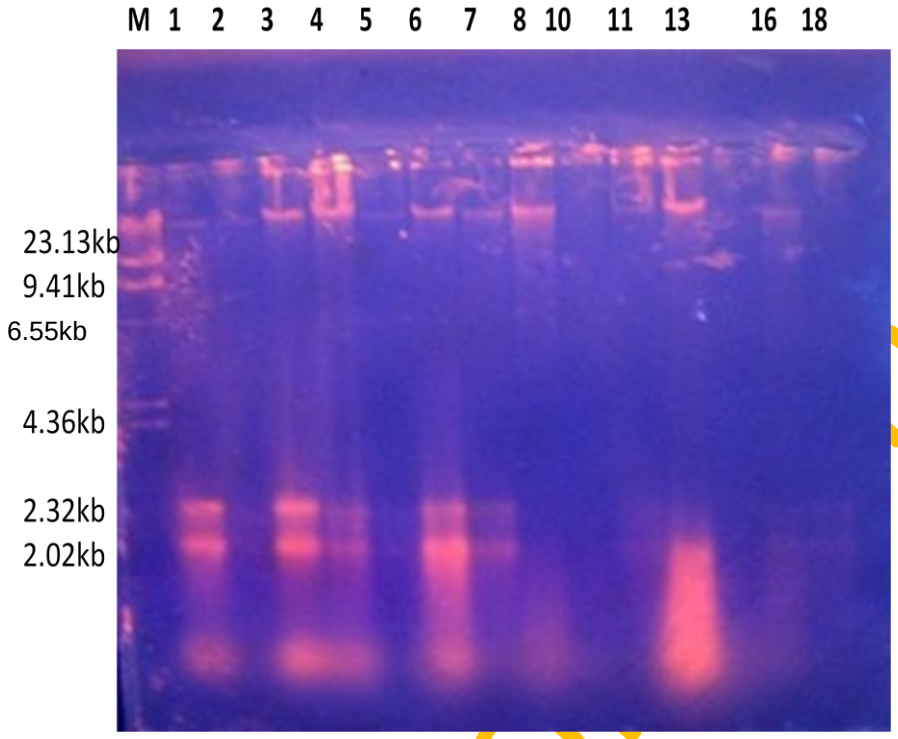


Fig 11.0 Agarose gel electrophoretic U.V. transilluminated photograph of the 13 β-lactamase positive *Staphylococcus aureus*. Numbers on the right denote the molecular mass of reference plasmids in kilobases.
Lane 1 to 18 are the plasmid profiles of the selected isolates.

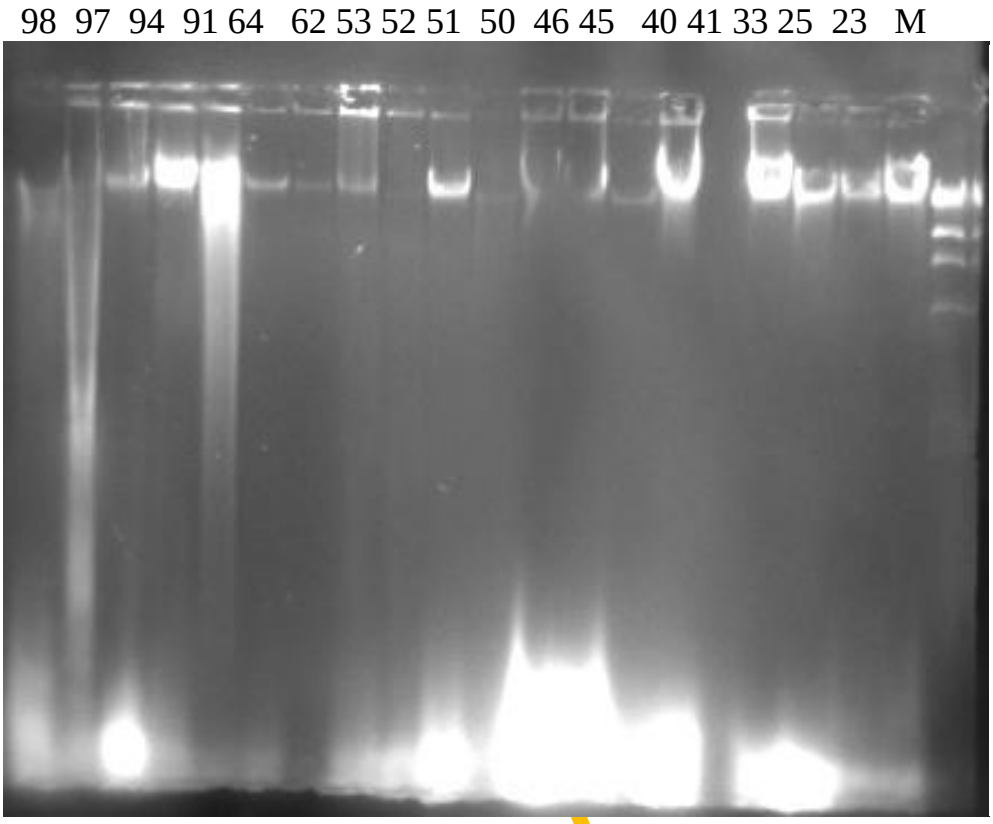


Fig 12.0 Agarose gel electrophoretic U.V. transilluminated photograph of the 17 β-lactamase positive *Staphylococcus aureus.*
Lane 23 to 98 are plasmid profiles of the selected isolates.

Table 6.0.

. Plasmid DNA extracted from selected β-lactamase negative strains of *Staphylococcus aureus*.

Strains numbers	Plasmid copies	Molec. weight graphical values	Molecularweights(base pair)	(Kilobase)
29	1	4.49	303902.95	30.39
30	1	4.40	25118.86	25.12
32	1	4.52	3313.11	33.11
34	1	4.50	31622.78	31.62
35	1	4.50	31622.78	31.62
36	1	4.35	22387.21	22.39
37	1	4.35	22387.21	22.39
38	0	0.00	0.00	0.00
39	1	4.58	38018.93	38.02
41	1	4.32	20892.93	38.02
44	0	0	0	0
47	1	4.40	25118.86	25.12
49	1	4.18	38018.93	20.89
57	1	4.40	25118.86	25.12
60	1	4.70	50118.72	50.12
65	1	4.49	30902.95	30.90
70	1	4.42	26302.68	26.30
77	1	4.25	17782.79	17.78
81	1	4.49	30902.95	30.90
82	1	4.45	28183.83	28.18
84	1	4.40	25118.83	28.18
85	1	4.19	15488.12	15.49
86	1	4.12	13182.56	13.18
89	1	4.12	13182.56	13.18
90	1	4.16	4454.40	14.45

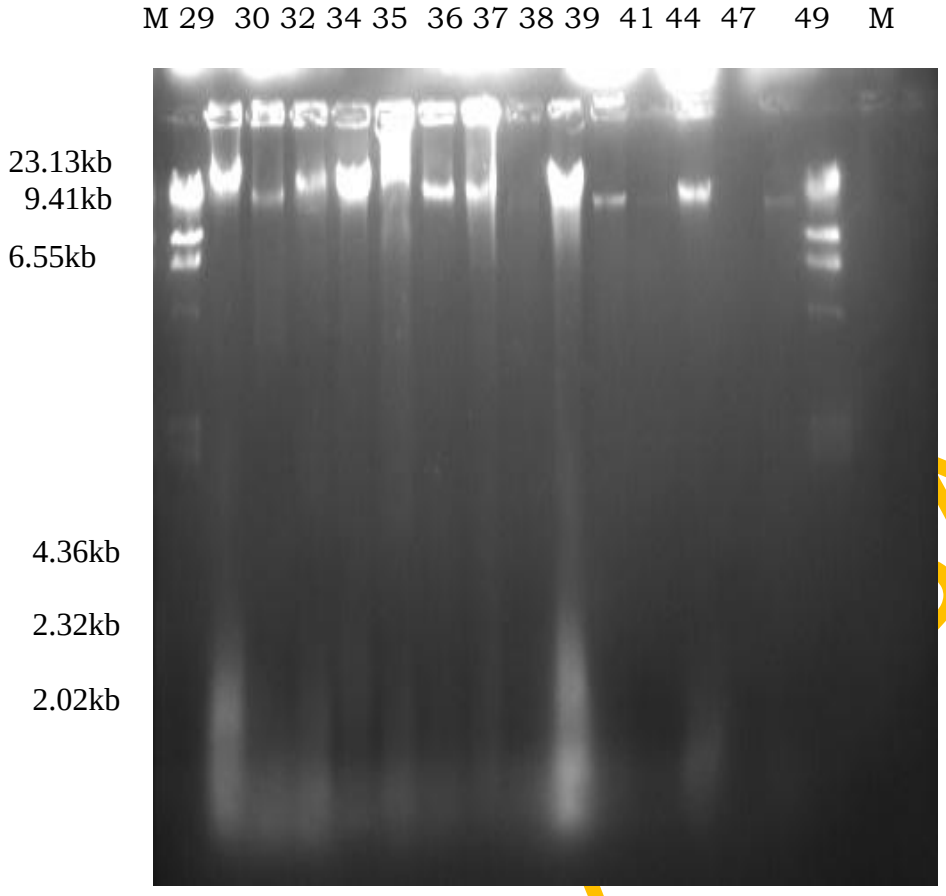


Fig 13.0 Agarose gel electrophoretic U.V. transilluminated photograph of the 13 β-lactamase negative *Staphylococcus aureus.* Lane 29 to 49 are plasmid profiles of selected isolates.

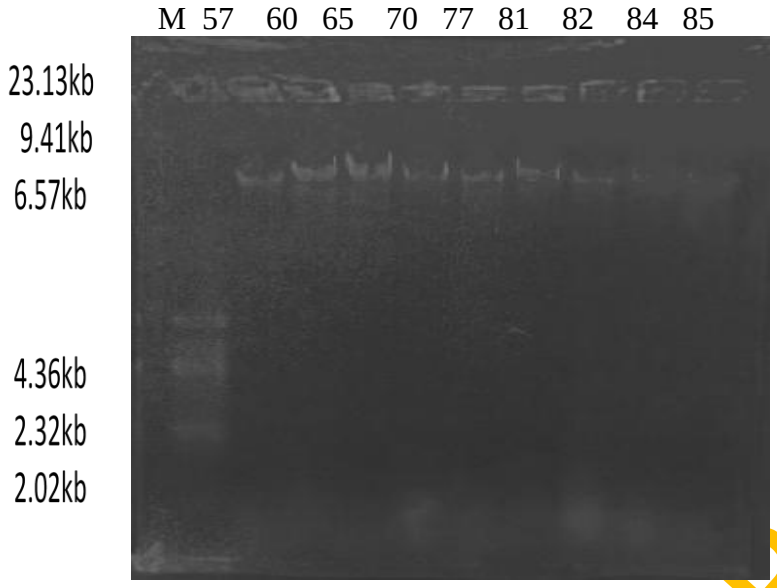


Fig 14.0. Agarose gel electrophoretic U.V. transilluminated photograph of the 9 β-lactamase negative *Staphylococcus aureus*
Lane 57 to 85 are plasmid profiles of the selected isolates.

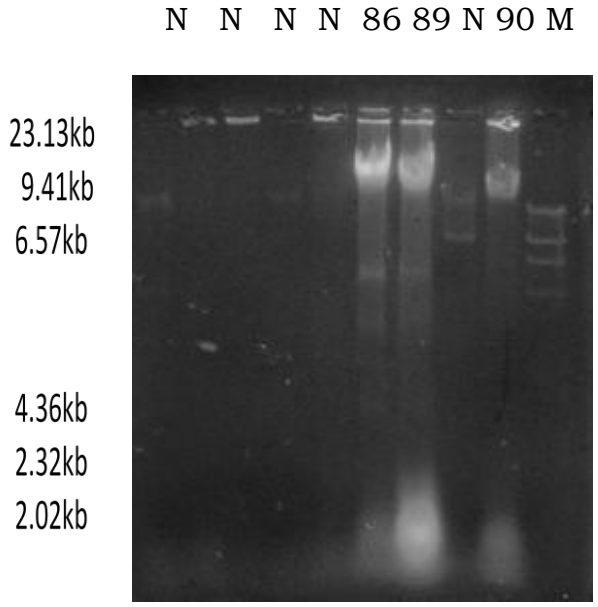


Fig 15.0 Agarose gel electrophoretic U.V. transilluminated photograph of the 3 β-lactamase negative *Staphylococcus aureus.*
Lane 86 to 90 are plasmid profiles of the selected isolates.

Staphylococcus aureus Sa 04 and Sa 06 had the highest inhibition zone 28mm to amoxicillin while the lowest zone of inhibition of 8mm were recorded for amoxicillin, cotimoxazole and chloramphenicol respectively(Table 7.0). Seven β-lactamase positive strains with multiple copies of R-plasmid DNA varied from 2- 4 were selected for plasmid curing. The varied concentrations of ethidium bromide in Nutrient-broth-ethidium bromide mixture produced different effects on bacterial growth (Table 8.0). Two of the seven strains, *(SA03 & SA06)* could not grow in all the concentrations tested while one strain *(SA04)* grew in all the concentrations. Four strains grew either in 12.5µg/ml or 6.25µg/ml or both of these concentrations tested. Sub-cultures of bacterial growth from ethidium bromide broth to Nutrient broth mixture produced colonies on mutagen free nutrient agar. Table 9.0 elicited the antibiogram of selected isolates after curing, it was observed that amoxicillin had the highest sensitivity (32mm) against *Sa 04* , closely followed by *Sa 02, 03, 16* with 30mm zone of inhibition. However, cloxacillin had the lowest (14mm) zone of inhibition against *Sa 04*.

Table 7.0

Antibiogram of the selected isolates of *Staphylococcus aureus* before curing

Strain nos	Amx 10µg	Amx-clv 30µg	Clx 5µg	Cmx 25µg	Chl 10µg	Gen 10µg	Ery 15µg	Tet 30µg
SA 01	8	12	14	8	10	12	12	14
SA 03	14	12	7	10	12	12	13	14
SA 04	28	18	9	10	8	11	12	14
SA 05	26	18	8	16	16	12	12	12
SA 06	28	19	9	10	12	12	13	14
SA 13	25	14	8	9	11	10	11	12
SA 16	14	12	8	10	12	10	10	14

Key: AMX: Amoxycillin **AMX-CLV:** Amoxycillin-clavulanic acid

CMX: Cotrimoxazole **CHL:** Chloramphenicol **CLX:** Cloxacillin

GEN: Gentamicin **ERY:** Erythromycin **TET:** Tetracycline

Zone of growth inhibition measured in millimeter in diameter and Interpreted with CLSI(2011) manuals**.**

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Table 8.0 **The curing effect of ethidium bromide on the resistant isolates of *Staphylococcus aureus*.**

	Growth in Ethidium bromide Broth					Subculture on Nutrient agar				
Strains of *S. aureus*	100	50	25	12.5	6.25	100	50	25	12.5	6.25
SA01	-	-	-	+	+	-	-	-	+	+
SA03	-	-	-	-	-	-	-	-	-	-
SA04	+	+	+	+	+	+	+	+	+	+
SA05	-	-	-	-	+	-	-	-	-	+
SA06	-	-	-	-	-	-	-	-	-	-
SA13	-	-	-	-	±	-	-	-	-	+
SA16	-	-	-	±	±	-	-	-	-	+

Key: Ethidium bromide concentration 100, 50, 25, 12.5 and 6.25µg/ml
N.A: Nutrient agar. *SA: Staphylococcus aureus*.
+ Growth - No growth
± Weak growth *S.A. Staphylococcus aureus*

Table 9.0

Antibiogram of the selected isolates of *Staphylococcus aureus* after curing

Strain nos	Amx 10µg	Amx-clv 30µg	Clx 5µg	Cmx 25µg	Chl 10µg	Gen 10µg	Ery 15µg	Tet 30µg
SA 01	30	20	18	18	20	16	24	20
SA 04	32	24	14	18	20	16	24	20
SA 05	30	22	18	18	22	15	23	21
SA 13	30	25	18	16	20	16	23	19
SA 16	30	22	20	20	19	15	24	22

Key: AMX: Amoxycillin **AMX-CLV:** Amoxycillin-clavulanic acid**.**

CMX: Cotrimoxazole **CHL:** Chloramphenicol **CLX:** Cloxacillin

GEN: Gentamicin **ERY:** Erythromycin **TET:** Tetracycline

SA*: Staphylococcus aureus*

CLSI (2011) : Zone of growth inhibition interpretation.

4.0.5 Transfer of resistance by conjugation

Seven strains with multiple copies of plasmid DNA were selected for conjugative studies, four of the selected strains were sensitive to ampicillin but resistant to streptomycin while the remaining three were resistant to ampicillin but sensitive to streptomycin (Table 10).

Table 10.0.

Selected strains for conjugative study

Strain number	Pathological source	β-lactamase	Plasmid numbers	Amp	Str
SA01	Neck	+	3	S	R
SA03	Armpit	+	3	S	R
SA04	Buttock	+	4	R	S
SA05	Breast	+	3	R	S
SA06	Thigh	+	2	R	S
SA13	Armpit	+	2	S	R
SA16	Eyelid	+	2	S	R

The Carolina typed *Esch. coli* amp^{R} and str^{R} with resistant and donor status grew on antibiotic free Luria Betani media. The medium was then supplemented with the respective antibiotic and none of the strains shows growth Table 11.0.

Table 11. 0.

Resistance transfer by conjugation

	Strain 01		***Strain 01***		***Strain 02***		***Strain 02***	
Contentof Medium	**Expect.**	**Observ.**	**Expect.**	**Observ.**	**Expect.**	**Observ.**	**Expect.**	**Obser**
LB agar	+	+	+	+	+	+	+	+
LBagar+Str	+	+	+	+	-	-	-	-
LBagar+Amp	-	-	-	-	+	+	+	+
LBagar+Str+Amp	-	-	-	-	-	-	-	-

Key**:** Donor strain *: Esch coli (Str^r)*
Recipient strain: *Esch coli (Amp^r)*

The streptomycin resistant but ampicillin sensitive strains were selected(after tested on ampicillin and streptomycin) from the *Staphylococcus aureus* with donor plasmid status (strain 01,03, 13, 16) and were mated with ampicillin resistant but streptomycin sensitive *strain 02 Esch. coli* as shown in (Table 12) .

Table 12.0

Growth on mating plate

	Strain1+*E.coli02* amp^r	Strain3 +*E.coli02* amp^r	Strain 13+*E.coli* 02 amp^r	Strain 16+*E.coli* 02 amp^r
Content of plate				
LB agar	+	+	+	+
LB agar+Str +Amp	-	-	-	-

The results of the transconjugant cell growth after mating showed no significant transconjugation .

Table 13. 0.

Transconjugants cell growth

	STRAIN 01		***STRAIN 03***		***STRAIN 13***		***STRAIN 16***	
Content of plate	**Expect.**	**Observ.**	**Expect.**	**Observ.**	**Expect.**	**Observ.**	**Expect.**	**Observ.**
LB agar	+	+	+	+	+	+	+	+
LB agar+Str	+	-	+	-	+	-	+	-
LB agar+Amp	+	-	+	-	+	-	+	-
LB agar+Str+Amp	+	-	+	-	+	-	+	-

Table 14.0.

Competency of *Staphylococcus aureus* after treatment with Calcium chloride

	STRAIN 01		***STRAIN 03***		***STRAIN 13***		***STRAIN 16***	
content of plate	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**
LB agar+Str	+	+	+	-	+	-	+	+
LB agar+Amp	+	+	+	-	+	-	+	+
agar+Str+Amp	+	+	+	-	+	-	+	+

The transconjugant cell growth showed no recombination(Table 13) When the selected *Staphylococcus aureus* were made competent and mated with recipient *Esch.coli* with donor status. Transconjugation were noticed in the strain 1 and 16 *E.coli*,(Table 14), which served as an evidenced of conjugation in the aided plasmid-borne *Staphylococcus aureus*

CHAPTER FIVE

DISCUSSION

The prevalence of recurrent furunculosis in this study from the data obtained showed that cases of recurrent furuncolosis were resident more in the community than in the hospitals considering that 88..23% of the samples were obtained from the community against the 11.57% from the hospital cases. The sharp contrast in the samples collected from the two settings could be attributed to the preference of the populace to indulge in self management of furunculosis. The percentage gender distributions were 46% females and 54% males , comprising of 20% hospital reported and 80% non hospital reported for females and 5% hospital reported and 95% non hospital reported for males. This ratio of the gender distribution of the infection, may reflect the greater premium feminine folks placed on their skin relative to males, in contrast to the cases of urinary tract infection, where females usually maintain the lead due to the closeness of their urethra to the bladder.

In a survey conducted in this study, the general prevalence of furunculosis could be a reflection of lack of social infrastructures in the environment associated with the individual cultural and hygiene practice which are considered to be amongst the epidemiological factors aiding the spread and control of an infection. It has been recognized that people with hyperacious sebaceous secretion due to their genetic make-up harbor more lipid materials on their body than those without.

Hence, the predisposition of the former to attract microbes-laden particles into their body that would give rise to development of boil. It could also reflect on puberty symptoms as well as some profession that are associated with frequent contact with oil.

In the summary of pathological distribution of recurrent furunculosis in this study, the highest occurrence was found in the ear, and every other anatomical locus of the body has varied percentage, an indication of the ability of *Staphylococcus aureus* to cause infection in almost every part of the body, thus agreeing with the widely acclaimed status of *Staphylococcus aureus* as a common aetiologic of medical importance (Kenneth,2008).

The cultural and biochemical characterizations employed, afforded the identification of *Staphylococcus aureus* as reported by Harold *et.al. (*2007). In particular the detection of the enzyme catalase in all the *Staphylococcus aureus* isolates coupled with their lack of oxidase confirmed that the organism that was studied belonged to the genus, *Staphylococcus*.

The heat stability, resistant and pathogenic properties of *Staphylococcus aureus* have been attributed by some workers to the possession of coagulase and deoxyribonuclease (DNAse)

(Smith,1969). The occurrence of these two enzymes in the isolates studied (87% DNAse and 90% coagulase) corroborates the report of many workers on *Staphylococcus aureus*. In this study, 87% were DNAse and 90% coagulase positive. In the test for haemolysis, 95% of the isolates

produced alpha-toxin indicated by a wide zone of clear (β-type) haemolysis on blood agar thus confirming the human origin of the *Staph aureus* isolates in agreement with the report of Mathew (2009) that the Staphylococci detected with alpha and gamma haemolysins were of human origin.

In this study, 90% of the isolates exhibited production of coagulase including the control strain, ATCC29213, an indication that some exceptions could be expected among the strains of the organism., which means some of them are coagulase negative while other are not..

Gelatin hydrolysis is a characteristic property of *Staphylococcus aureus* due to proteolytic enzyme, which breaks down host protein and establishes infections (John,2008). Though, little emphasis is often placed on ability of staphylococci to liquefy gelatin, 92% of the test strains liquefied gelatin. The *Staphylococcus aureus* produced proteolytic enzyme as observed from their effect on the enzyme gelatinase, an indicator of pathogenicity.

In the antibiotic susceptibility testing, the antibiogram produced the antibiotic susceptibility profile of the *Staph aureus* isolates against the Gram-positive antibiotics tested. Guided by CLSI standard interpretative chart (2011), the zones of growth inhibition diameter, observed were categorized as either sensitive or resistant.

In this study, 83.33% of the isolates were resistant to amoxicillin thereby supporting the reduced activity of this antibiotic against Gram-positive bacteria relative to Gram-negative bacteria..

The reduced resistance of the isolates to amoxicillin clavulanate (39%) recorded in the antibiogram showed the efficacy of these β-lactams antibiotics. The potency of amoxycillinclavulanate can be attributed to the synergistic effect of the two components of clavulanic acid with amoxicillin whereby clavulanic acid acts as an irreversible inhibitor of β-lactamase enzyme (Lode,2008).

The overall resistance of the isolates of *Staphylococcus aureus* showed that amoxicillin 85%, tetracycline 75% , erythromycin 83% ,cotrimoxazole 74% and cloxacillin (70%) and were ineffective against the isolates. The reduced susceptibility of *Staphylococcus aureus* to cloxacillin recorded 18.62% could be due to genetic variation of the isolates while 80% was recorded for chloramphenicol on *Staphylococcus aureus*. Akiba and Yokota (1969) in their studies on resistant strains of *Esch. coli* reported enzyme inhibition of chloramphenicol in the lysates of the resistant strains. These workers affirmed the inhibition of β-galactosidase that reduced permeability influx of the chloramphenicol in the resistant strains of *Esch. coli.* to the cell as being plasmid-borne.

In this study, β-lactamase, an heterocyclic enzyme capable of cleaving the β-lactam ring of some antibiotics by hydrolysis was verified in the isolates of *Staph aureus*, 30 of the 102 isolates were β-lactamase positive. The resistance recorded among the β-lactamase negative strains of *Staphylococcus aureus* could be due to the establishment of a permeability block in the cells preventing the penetration of the required concentration of the antibiotic to the cell (Bonfiglio,1991).

The proportion of the resistant strains in this study corroborates the view of some workers of threat to therapeutic failure associated with β-lactam antibiotics (Kenneth, 2008).

The magnitude of β-lactamase producing strains agreed also with the view of Abdul and Dipak (2005) on the increasing trend of β-lactamase production mediated by R-plasmid in drug resistance especially in *Staphylococcus aureus* .

Cloxacillin, elicited the MIC's range of between 15.63 µg/ml. - 250 µg/ml. Cefotaxime and ceftriaxone which are third generation cephalosporins, produced the MIC that ranged between 15.63 and 250 µg/ml against the 30 β-lactamase positive strains *of Staphylococci aureus.* The potency of these cepahlosporins reported by some workers, was contradicted in this result. Cefotaxime and cefuroxime have been recognized for their improved potency being less susceptible to inactivation by β-lactamase than the first and second generation cephalosporins.

The higher MIC's recorded for the two cephalosporins in respect of the β-lactamase producing strains of *Staph aureus* agreed with the findings of Asbel (2000), on the epidemic of multiple cephalosporin resistant strains of bacteria in the hospital environment due to frequent use. The high MIC of 31.25 -500 µg/ml obtained for penicillin G with the exception of *SA06* that elicited MIC 7.8 µg/ml in this study underscores the high level resistance to this antibiotic by *Staphylococcus aureus*.

In this study, the least MIC and MBC recorded for the 5 selected antibiotics against the 30 β-lactamase producing strain were 3.90 and 7.8µg/ml as elicited by the strains tested against amoxicillin-clavulanate as obtained with the tested strains, while the highest MIC and MBC o 500 and 650µg/ml respectively were recorded for penicillin.. The MIC values showed the high level resistance of *Staph aureus* isolates tested as reported earlier by different workers.

In the plasmid profiling, the isolation of plasmid DNA in the study support the contribution of high resistance observed with the plasmid bearing strains. Plasmid DNA were obtained from 29 of the 30 β-lactamase positive strains, each with one plasmid copy or more, thereby supporting the recognition of R-plasmid as agent that bears the information to synthesize antibiotic degrading enzyme (Clewell,2008).

The high level resistance of *Staph aureus* to the five selected antibiotics and the associated transferable R-plasmid encoding β-lactamase could account for the treatment failure associated with recurrent furunculosis.. It is also possible that the inappropriate usage of the antibiotics could also be a contributory factor to the recurrent furunculosis in this study.

The R-plasmid DNA in this study has a varied molecular weights with different copy numbers and sizes. The observed variation agreed with the findings of Olukoya *et.al*. (1995) and Adeleke *et.al*. (2002) who isolated plasmid from strains of *Staphylococcus aureus* of varied clinical origin..

In this study, the molecular weights determined for most of the plasmid DNA isolates were reasonably closer and similar, in size ; 23.13, 15.85, and 28.18 kb for the plasmids from the strains of *Staphylococcus aureus* detected with β-lactamase enzymes. This observation is in line with the reported suggestion that plasmid mediated antibiotic resistance is of epidemiological relevance. This is useful in characterization of many infectious diseases especially with particular reference to *Staphylococcus aureus*.(Lyon, 1987; Adeleke *et al.*, 1997) .

The significant rate (71%) of curing of antibiotic resistance by ethidium bromide in this study recorded for the multiple resistant strains selected, agrees with the findings of Bouchard *et al.* (1961) on resistant *Staphylococcus aureus.* Also Darini (1996) reported a high frequency of ethidium bromide cure on staphylococcal plasmid thereby serving as an evidence of a plasmid-mediated resistance . It is remarkable that most of the resistant strains tested retained

their resistance to cloxacillin, a β- lactamase stable antibiotic, after exposure to the ethidium bromide showing that the cloxacillin resistance reported in this study could be due to chromosomal mediation or other factors other than plasmid mediation. It is noteworthy also that strains of *Staph. aureus SA03* and *SA06* isolated from armpit and breast pathological sites were the only strains that lost their resistance to all the concentrations of the mutagen tested.

Ethidium bromide, a trypanocidal chemical substance and an intercalating agent impairing nucleic acid synthesis through a mediation of frame-shift mutation, had been reported to have an antibacterial effect and exhibit phenotypic changes such as loss of enzyme activity and antibiotic resistance or ability to sporulate which formed the basis of its use as a curing agent for antibiotic resistance in this study as reported for staphylococcal extra-chromosomal replicon marker(Bouanchand *et al.*, 1968).

A further step taken in this study to ascertain the plasmid mediation of the antibiotic resistance observed through conjugational experiment for the transfer of plasmid DNA. Of the 4 multi-resistan strains of *Staphylococcus. aureus* with a donor and recipient status selected for this study, There was no plasmid transfer among the clinical isolates. However, after subjecting clinical isolates to competency treatment in $Cacl_2$, plasmid transfer was observed in strain 1 and 16. The conjugation observed in 2 of the 4 multi-drug resistance strains of *Staph aureus* when made competent with $CaCl_2$ could be attributed to genetic variation of the isolates or the inability of $CaCl_2$ to effect competency in those 2 other isolates of *Staph aureus* that fail to exhibited conjugation. As such, conjugation may have a selective property in mediating transfer of resistance in bacteria of different Genera due to some limiting factors.

The thought of Kenneth (2008) on the enlightenment, legislation and enforcement of the law regulating the indiscriminate use of antibiotics in the veterinary and impregnation of antibiotic in the toddler toys as a measure to reduce the incidence of multiple resistant bacteria in the human community is quite relevant towards the control of antibiotic resistance.

The high MIC's of the selected antibiotics tested against the isolates of *Staphylococcus aureus* and the multiple copies of plasmid DNA extracted with varied molecular weight as well as one form of antibiotic misuse or the other are suggested to account for the recurrent nature of furunculosis.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

The results obtained on the pathological distribution of the 102 clinical isolates of *Staphylococcus aureus* studied, followed the trend of expected epidemiological distribution data of the non-hospital reported and hospital reported cases of furunculosis.

The observed gender distributions data of the 102 isolates of *Staphylococcus aureus* studied, in which males and females ratio showed 56 to 46 respectively, correlates with the gender with the suggestion that females do pay more attention to skin care than males. The higher frequency of the non-hospital reported (90 isolates) relative to the hospital reported (12 isolates) infection also buttressed the self-management approach to the treatment of furunculosis. It was observed that age range has no influence on recurrent furunculosis in this study in which the youngest age was 3years while 83 years was recorded for the oldest patient.The different biochemical characterizations that detected out *Staphylococcus aureus* in the specimen collected identified this organism as a major causative agent of boil infections, thereby underscoring the pathogenic potential of the organism. The magnitudes of minimum inhibitory concentrations of the antibiotics tested in this study, coupled with the pronounced antibiotic resistance of these isolates even to the third generation antibiotic having extended β-lactamase spectrum and the variation in the resistance pattern elicited by the isolates served as an indication of varied loci of collection.

Staphylococcus aureus in this study, showed a lower resistance to cotrimoxazole unlike amoxicillin-clavulanate , which could be due to antibiotic site of action . The susceptibility of the resistant strains of the *Staphylococcus aureus* to the curing agent serves as a strong evidence of plasmid-mediated resistance on the strains of the *Staphylococcus aureus* studied. The high rate (71%) of curing of antibiotic resistance by ethidium bromide in this study agrees with the findings of many workers on resistant staphylococci and it is also an indication of a strong mutagenic effect of the curing agent . The observation of the two strains that could not revert their antibiotic resistance completely after exposure to ethidium bromide emphasize the worrisome trend in the resistance of *Staphylococcus aureus* to β-lactam antibiotics.

Bacterial conjugation for the transfer of plasmid DNA coding for antibiotic resistance was not observed with the clinical strain of *Staphylococcus aureus and Escherichia coli*, which may be traceable to the staphylococcal cell wall barrier. However, there was an horizontal transfer of plasmid DNA, occurring between different clinical strains of *Staph. aureus*. in agreement with the finding of Jevenand (1997) on the same organism. The isolation of β-lactamase encoded plasmid DNA from resistant clinical strains of *Staphylococcus aureus* studied coupled with the elimination of antibiotic resistance and possibility of resistant plasmid transfer among the same species of *Staphylococcus aureus* has contributed to removing the speculation surrounding the genetic basis of antibiotic resistance in *Staphylococcus aureus*.

Futhermore, this study give an account on the spread of recurrent furuncles with respect to patients and gender, along with the associated isolation of plasmid DNA as a major reasons for the recurrence of furuncles in infected subjects.

In southwest Nigeria, the observed high rate in self-medication with antibiotics, coupled with lack of information on the subject matter accounted for the spread of the antibiotic resistance gene complicates antibiotic therapy. , For these reason, there is need for public enlightenment on the appropriate therapeutic management of furunculosis.

6.0.1. **Contribution to knowledge**.

- The enzyme responsible for the inactivation of β-lactams antibiotics was discovered.
- The enzyme discovered was found to be plasmid borne.
- Plasmid DNA of varied molecular sizes and copies was discovered
- Possibilities of conjugation was achieved
- Amoxycilllin-clavulanate (augmentin^{R}) from the panel of antibiotic appropriated was found to be effective against the aetiologic agent of recurrent furunculosis in this study.
- Poor hygiene and cultural practice was found from the survey conducted to be one of the contributory factor responsible for the spread of recurrent furunculosis.
- Therapeutic management of recurrent furunculosis with amoxicillin-clavulanate and improved hygiene practice is therefore recommended ,

REFERENCE

Abdul, H.K.and Dipak, K.P. (2005). Antibiotic susceptibility and R-plasmid mediated drug resistance in *Staphylococcus aureus*. *Medical Journal of Islamic World Academy of Science* ***15****:31-34.*

Adeleke, O. E. (1997). A study of plasmid-mediated resistance in clinical strains of *Staphylococcus aureus* to βeta-lactam antibiotics, *Ph.D. thesis Pp 21 -90.*

Adeleke, O.E. and Odelola, H.I.(2002). Plasmid profiles of multidrug resistant

Asheshov, E.H.(1969). The genetics of penicillinase production in *Staphylococcus aureus* strain PS80. *J Gen . Microbiol.* ***59****(3):289–301.*

Ayliffe, G.A.J., Livingstone, R. and Lowbury, E.J..L.(2006). Antibiotic-resistant *Staphylococcus aureus* in dermatology and burns wards. *J.Clin.Pathol.* ***(30)*** *:40-44.*

Barret, F., Casy, J.I, Wicox, C.(1970). Bacteriophage types and antibiotic susceptibility

Behulah's Bilingual and Parallel Bible **(KJV):** *Exodus9:6-7,Job2:5-7,Isaiah38:21*

36 *: 277-283.*

Beveniste, R .and Davies, J .(1973).Mechanisms of antibiotic resistance in bacteria. *Ann. Rev Biochem .* ***42*** *: 471*

Bobrowski, M.M. (1974). Bacterial β-1actamases and their significance in resistance to penicillins and cephalosporins. *Postepy Med. Doshiadczalnej.* ***28****(4): 587.*

Bonfiglio, G, Livermore D.M.(1991). β-lactamase types amongst *Staphylococcus aureus* isolates in relation to susceptibility to β-Lactam inhibitor combinations *Antimicrob Chemother.****33:****465-81.*

Bouanchaud, D.H, Scavizzi, M.R. and Chabbert, Y.A.(1968). Elimination by ethidium bromide of antibiotic resistance in enterobacteria and staphylococci. *J. Gen. Microbiol .* ***54*** *: 417-425.*

British Society of Antimicrobial Chemotherapy (2002). Disc diffusion method for antimicrobial susceptibility testing. *Br. Soc. Antimicrobiol. Chemother.* ***2****:1-46.*

Bush L.M, and Johnson, C.C.(2000) Ureidopenicilllins and βeta-lactamase inhibitor combinations. Infect. *Dis. Clin. North. Am.****14****:409.*

Clewell, B.D.(1981) . Plasmid, drug resistance and gene transfer in the genius *Streptococcus. Microbiol. Rev.****45:****409-411.*

Clewell, B. D. (2008). Antibiotics resistance in bacteria. Origins and Emergence. *http:/www/scitopics.com/antibiotic resistance in bacteria origins and emergence.html.*

Clinical and Laboratory Standards Institute(2011): antimicrobial susceptibility testing standards, M02-A10 and M07-A8.

Cohen, P.R. and Kurrock R.(2004).Community-acquired methicillin-resistant *Staphylococcus aureus* skin infection: an emerging clinical problem. *J Am Acad Dermatol.* ***50****:277- 80.*

Cole,M. **(**1969). Penicillins and other acylamino compounds synthesized by the cell-bound penicillin acylase of *Escherichia coli J.Biochem 115(4):747-756 Ltd, London.* ***Pp*** *242-243.*

Cowan, S.T. (1938b). The classification of *Staphylococci* by prepitation and biological reactions. *J. Path. Bact.* ***46****: 31.*

Cowan, S.T. (1974). Cowan and Steel's manual for the identification of medical bacteria **2nd** edn. Cambridge University Press.

Cooke, F. and Wain, J. (2004). The emergence of antibiotic resistance in typhoid fever. Trav. Med. Infect. Dis. **2(2)**:67-74.

Davis, B.D, Dulbecco, R., Eisen, H.N. and Ginsberg, S.H(1973). *W.B Microbiology 2nd edition.* ***(20):*** *1105*

Davis, S.L, Perri, M.B. and Donabedian, S.M. (2007). Epidemiology and outcomes of community-associated methicillin-resistant *Staphylococcus aureus* infection *J. Clin. Microbiol.****120****: 1222-1224*

Detta, N., Hadges, R.W and Shaw, E.J. (1971). Properties of an R-factor from *Pseudomonas aeruginosa*. Am. *J. bacterial. **108**:1244-124*

Fons, M.T. Hege, M. Landire, P. Raibaud, R. Ducluzeau, and E. Maguin. (1997). Isolation and characterization of a plasmid from *Lactobacillus fermentum* conferring erythromycin resistance. *Plasmid **37**: 199-203* .

Garrod, L.P., Lambert, *H.P and O' Grady' F.(1981).* Antibiotic and Chemotherapy *5th edn. churchhill Livingstone **pp** 464-483.*

Gerdes, K., *Rasmussen. PH, Molin. S. (1986). "Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells", Proc Natl. Acad. Sci. U.S.A. **83 (10)**: 3116. doi:10.1073/pnas.83.10.3116* .

Gerald, P., Bodey and Beverly Deerhake(1971). In Vitro Studies of α-Carboxyl-3 Thienylmethyl Penicillin,a New Semisynthetic Penicillin**.** *Appl Microbiol.* ***21(1)****: 61–65.*

Gorwitz, R.J. (2007). The role of ancillary antimicrobial therapy for treatment of uncomplicated skin infections in the era of community-associated methicillin-*resistant Staphylococcus aureus. Clin Infect Dis .* ***44****:785-86.*

Gillespie, *M.T. and Skurray, R.A.(1986).* Plasmids in multi-resistant *Staphylococcus aureus. Microbiol.Sci****.(3):****53-58.*

Gira, ***A****.K., Reisenauer, A.H .and Hammock, L. et al.,(2005)* Furunculosis and familial deficiency of mannose-binding lectin. *Eur J Clin Invest.* ***35:****531-32.*

Harold, J.B.(2007). Microbiological applications: Laboratory Manual in General Microbiology.

Hayanga, A, Okello, A., Hussein, R., and Nyong'o A.(1997). Experience with methicillin resistant *Staphylococcus aureus in* Nairobi Hospital.

Holten. K.B, Onusko, E.M. (2000). Appropriate prescribing of oral β-lactam antibiotics". *American Family Physician* **62** (3): 611–20

Indalo, A.A. (1997) Antibiotic sale behaviour in Nairobi: a contributing factor to antimicrobial drug resistance. East Afr. Med. J.; **74**:171-173

Jevanand, H.R,, Ragavan, P.U.M and Gunapathi, R.S. (1997). Study of R-factors among multi- drug resistant *Salmonella typhi. Indian J. Med. Microbiol.* ***15:****37-39.*

Jawetz,M., Geo,F.,Karen.C. and Janet. S.(2010). Medical microbiology, *20th edn, Pp 339-367*

John, J.F. Jr, and Twitty, J.A.(1986).. Plasmids as epidemiological markers in noscomial gram-negative bacilli. Experience at a University and review of the literature. *Rev. Infects Dis.* ***8:*** *693-699.*

John, O.O.and Arundhati,A.K.,(2008). Medical Laboratory Science(Theory and Practice). *2nd Edition Pp525-725.*

Jones, Elizabeth, Hartl, and Daniel L. (1998). Genetics: principles and analysis. Boston: *Jones and Bartlett Publishers.* ***ISBN*** *0-7637-0489-X.*

Julian,M.T.,William, W.K.,(1984). A simple and rapid method for the elimination of R-Plasmid from enteric bacteria.*springerlink-current microniology,* ***vol.11 No3, 155-157***

Kado, C.I. and Liu, S.T. (1981). Rapid procedure for procedure and isolation of large and small plasmids. *J. Bacteriol.* ***145****:1365-1373*.

Kandavelou, K and Chandrasegaran, S. (2008). "Plasmids for Gene Therapy", Plasmids:Current Research and future Trends. *Casiter Academic Press.* ***ISBN 978****-1-904455-35-6.*

Kenneth Todar .(2008) Todars online Textbook of Bacteriology *Reviews.Pp1-5*

Keynan S, Hooper NM, Felici A, Amicosante G, Turner AJ (1995). "The renal membrane dipeptidas (dehydropeptidase I) inhibitor, cilastatin, inhibits the bacterial metallo-βeta- lactamase enzyme CphA". *Antimicrob. Agents Chemother.* **39** (7): 1629–31

Khan, M.A. (1991).The emergence of methicillin resistant *S. aureus* isolated from skin lesions. *Bangladesh J. Microbiol,* ***8:****21-25*.

Lipps, G. (editor). (2008). Plasmids: Current Research and Future Trends: *Caister Academic Press.* ***ISBN 978****-1-904455-35-6.*

Lode, H.M.,(2008). Rational antibiotics therapy and the position of ampicillin/sulbactam; *Int.J.animicr.agents.(32): 10-28*

Lyon, B.R. and Skurray, R.(1987). Antimicrobial resistance in *Staphylococcus aureus*: Genetic basis. *Microbiol. Rev****.51****:88-134*.

Lyon, B.R., Luorio, J.L., May, J.W., Skurray, R.A.(1984). Molecular epidemiology of multi-resistant *Staphylococcus aureus* in Australian hospitals. *J. Med Microb**17**:79-89.*

Macfie, J., Harvey., J.(1977). The treatment of acute superficial abscesses: a prospective clinical trial. *Br J Surg **64:**264.*

Matsuhashi, S., Kamiryo, T., Blumberg, P.M. (1974). Mechanism of action and development of resistance to a new amidino penicillin. *J Bacteriol. 117(2):578-87.*

Mathew,O.,Ojo (2009). Manual of pathogenic bacteria and fungi. *First edn.Pp 60-97*

Mayer, L.W.N.(1988). Use of plasmid profiles in epidemiological surveillance of disease outbreaks and in tracing antibiotic resistance. *Clin.Microbiol.Rev. **1**:288-243.*

Novick, R. P. (1969). Extra-chromosomal inheritance in bacteria. *Bacteriol. Rev.**33**(2): 210– 263.*

Noble, W.C and Rahman, M.(1986). Plasmid profiling of epidemic *Staphylococc*i from around 1960: a comparison of epidemiological techniques. *J. Hyg (Comb) .**97**:211-218.*

NovaSpratt, B., Cromie, K.D.(1988). Penicillin-binding proteins of gram-negative bacteria. *Rev Infect Dis. 10:699*

Olukoya, D.K, Asielue, J.O, and Olasupo. (1995) Plasmid profiles and antibiotic patterns of *Staphlococcus aureus* isolate from Nigeria. *Afr. J Med Sci. **24:** 135-139.*

Papanicolaou, G.A., Medeiros, A.A and Jacoby, G.A . (1990). Novel plasmid-mediated beta-lactamase (MIR-1) conferring resistance to oxyimino- and alpha-methoxy β-lactams in clinical isolates of *Klebsiella pneumonea*. *Antimicrob Agents Chemother. **34:**2200.*

Purohit,S.S.,Saluja,A.K.,Kakrani,H.N.(2008). Pharmaceutical microbiology. *Student edition:473-500*

Pitout, J.D. and Sanders.C.C.(1997).. Antimicrobial resistance with focus on β-lactam resistance in gram-negative bacilli. *Am J Med* ***103****:51*.

Pirisi, A.(2000) Phage therapy- advantages over antibiotics.*Lancet 356(9239): 14*

Plorde, J.J. and Sherris, J.C.(1974). Staphylococcal resistance to antibiotics: origin measurement and epidemiology. *Ann. N.Y. Acad. Sci.* ***236:****413-434.*

Price, M.F, McBride M.E, Wolf J.E Jr.(1998). Prevalence of methicilllin-resistant *Staphylococcus aureus* in a dermatology outpatient population. South Med J. **91**:369-71

Raghava, U. Jones, N.S.(,2002). Combating bacterial resistance in Otorhinolaryngology. *Clin Otolaryngol Allied Sci. ; 27(6):446-52*.

Robert, T. (1989). Introductory bacterial conjugation kit. *Teacher's manual.*

Roy, H. M, Ashish, S. Paradkar et al.(1999). Genes Specific for the Biosynthesis of Clavam Metabolites Antipodal to Clavulanic Acid Are Clustered with the Gene for Clavaminate Synthase 1 in *Streptomyces clavuligerus. Antimicrob Agents Chemother.* ***43(5):*** *1215–1224*

Shasha, S.M.,Sharon, n., Inbar,.M.(2004) Bacteriophages as antibacterial agents (in Hebrew) *Harefua 143(2):121-5.*

Smith, P.R.,Hancock,G.A.,Rhoden,D.L.(1969). Improved medium for detecting DNase producing bacteria.*Appl Microbiol.08: 991-993*

Skurray A.R., Rouch A.D.et.al,(1988). Multi resistant *Staphylococcus aureus*: genetic and evolution of epidemic Australian strains. *J. Antimicrob Chemother, 21:19-28.*

Sosin D.M, Gunn.RA., Ford W.L and Skaggs J.W.(1989). An outbreak of furunculosis among high school athletes. *Am J Sports Med.* ***17****:828.*

Stevens, D.L. and Bisno, A.L.(2005). Practice guidelines for the diagnosis and management of skin and soft-tissue infections. *Clin Infect Dis* . ***41****:1373.*

Sugeng, M.W., Ang. P., Tan, H.H. et. al., (1999). Characteristics of bacterial skin infections in children compared to adults at a tertiary dermatologic center. *Int J Dermatol.* ***381****:128-32.*

Sutherland, R. E., A. P. Croydon, and G. N. Rolinson(1970). Flucloxacillin, a New Isoxazolyl Penicillin, Compared with Oxacillin, Cloxacillin, and Dicloxacillin. *Br Med J. 1970 ; 4(5733): 455–460.*

Sweetman, S. and Martindale. (2005). The complete drug resistance. London: *Pharmaceutical Press. Electronic version.*

Tan, J.S and File, T.M Jr.(1995). Antipseudomonal penicillins. *Med Clin North Am.****79****:679.*

Thiel Karl (2004) Old dogma new tricks- 21st Century phage therapy.*Nature Biotechnology 22(1):31-36*

Tom Elliot, Mark Hastings, Urlrich Desselberger(1997). Lecture notes on medical microbiology. 3rd edn. *Pp177-194.*

Victoria Odepidan (1996). An outline of microbiological techniques. *1st Edition,Pp 1-30.*

Watanabe, T. and Fukasawa, T. (1969a). Episome-mediated transfer of drug-resistance in *Enterobacteriaceae*. I.Transfer of resistance factors by conjugation. *J. Bacteriol.* ***81****: 669-678.*

Willey, J.M., Sherwood, L.M., Woolverton, C.J., (2008). Presscott, Harley and Klein's Microbiology. *7th ed. McGraw-Hill Companies, Inc. pp. 357-383.*

Woodford, N., Ellington, M.J., (2007). The emergence of antibiotic resistance by mutation. *Clin Microbiol.Infect.**13**(1):5-18.*

Yoshida, K., and Eckstedt,R.D.(1968) Relation of mucoid growth of *Staphylococcus aureus* to clumping factor reaction, morphology in serum-soft agar and virulence.*J.Bacteriol.96:902*

Yokota, T. and Akiba, T. (1961c). Studies on the mechanism of transfer of drug-resistance in bacteria. VI. Biochemical aspects on a mechanism of resistance to chloramphenicol in the chloramphicol resistant and in the multiple-drug-resistant strains *of E. coli* . (In Japanese) *Med. Biol. (Tokyo)* ***58** : 172-175.*

Zimakoff, J, Rosdahl, V.T, Petersen, W. and Scheibel, J.(1988). Recurrent *Staphylococcal* furunculosis in families. *Scand J. Infect Dis. 20:403.*

Zimmerman *et al*,.(1977) Thienamycin: development of imipenem-cilastatin *Journal of Antimicrobial Chemotherapy Pp. 1-35*

APPENDIX 1

The list of the equipment and apparatus used in this research works are;

Apparatus and equipment

- Agarose gel casting trays and combs.
- Autoclave, Gallenkamp, U.K.
- Bijou bottles, Lagos, Nigeria.
- Binocular Light Microscope, Olympus, Switzerland.
- Chrome made improvised inoculating needle, Ibadan, Nigeria.
- Deep Freezer, Haier Thermocool, China.
- Desktop laboratory centrifuge, Gallenkamp, U.K.
- Disposable hand gloves and face masks, Indonesia.
- Dual U.V.UVT-2600 Transilluminator, U.S.A.
- Durham tubes, U.K.
- Electrophoresis tanks
- Electrophoretic machine.
- Eppendorf Microcentrifuge 5415 R, U. S A.
- Eppendorf Thermo mixer Compact, 1.5ml. U.S.A.
- Formica made Glass inoculating Hood, ESCO, Germany.
- Freeze dryer, Edward, England

APPENDIX 1 (CONTD)

- Gallenkamp Economy Incubator , U.K.
- High Performance U V Transilluminator, U.S.A.
- Hot Plate & Stirrer, Jenway 1000, Sweden.
- Ice Mixing Machine, Whipool, England.
- Incubator (Gallenkamp,U.K.)
- Microcentrifuge,Spectrafuge IM Labriet: U.S.A.
- Microwave.
- Mistral 4l, U.K.
- Non adsorbent sterile cotton wool, U.K.
- Oven sterilizer, Gallenkamp, U.K.
- P500B Powerpack Sigma, U.K.
- pH Meter 7020, EIL, England.
- Polaroid Camera and films, England.
- Pyrex made Glass flaks; test tubes, conical and volumetric flasks, U.K.
- Pyrex Petri dishes, England.
- Refrigerated High Speed Centrifuge,GL18B.U.S.A.
- Shake and Bake Hybridization, model 136400 Boeker, Germany.
- So-Low Ultralow freezer, U.S.A.
- Thermocycler, Techne, Germany.

APPENDIX 1 (CONTD)

- Thermometer, Techne, Germany.
- Uniscope Laboratory Incubator, Surgifield Medico, England.
- Universal bottle.
- Vortex Mixer, Stuart, Germany.
- Washing brush, Ibadan, Nigeria
- Water-bath with Shaker,GLS400, U.S.A.
- Weighing balance (Sartorious,Germany)
- Weighing Balances, Mettler H80, PC400 Sartorious Germany.

APPENDIX 2

Culture media

Luria Bertani (L.B) agar (Carolina Biologicals, U.S.A).

Approximate formular per litre

Tryptone	10.0g
Yeast extract	5.0g
Sodium Chloride	5.0g
Agar	15.0g

Suspend 35g of the powder in 1L of sterile distilled water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolved the powder. Autoclave at 121^0C for 5 minutes.

Luria Betani (LB) broth (Carolina Biologicals, U.S.A)

Approximate formular per litre

Pancreatic Digest Casein	10.0g
Yeast Extract	5.0g
Sodium Chloride	5.0g

Suspend 20g of the powder in 1L of sterile distilled water. Autoclave at 121^0C for 15 minutes.

APPENDIX 2 (CONTD)

Sensitivity Test Agar (Lab. M).

Peptone infusion solids	21.5g
Starch	0.6g
Sodium Chloride	5.0g
Disodium citrate	1.0g
Adenine Sulphate	0.01g
Guanine hydrochloride	0.01g
Uracil	0.01g
Xanthine	0.01g
Aneurine Hydrochloride	0.01g
Agar No. 2	12.0g
pH	7.4

Suspend 28.0g of the powder in 1L of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve powder. Autoclave at 121^0C for 15 minutes.

APPENDIX 2(CONTD)

DNase Agar (OXOID)

Approximate volume per litre

Tryptose	20.0
Sodium chloride	5.0
Deoxyribonucleic acid	2.0
Agar-agar	15
pH	7.4

Suspend 39.0g of the powder in 1L of purified water, add 4.0ml of methyl green. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve powder. Autoclave at 121^0C for 15 minutes.

APPENDIX 2 (CONTD)

Mannitol Salt Agar (Biotech)

Approximate volume per litre

Meat extract	10.0
Peptone	10.0
Mannitol	10.0
NaCl	75.0
Phenol red	0.025
Agar	15.0
p H	7.4

Suspend 108 g of the powder in 1L of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve powder. Autoclave at 121^0C for 15 minutes.

APPENDIX 2 (CONTD)

Nutrient agar (Biotech)

Approximate Formula per litre

Peptone	5.0g
Beef extract	3.0g
Sodium Chloride	8.0g
Agar No 2	12.0g
pH	7.4

Suspend 28.0g of the powder in 1L of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve powder. Autoclave at 121^0C for 15 minutes.

Bacteriological peptone water(Lab M)

Peptone powder	5.0g
Tryptone	5.0g
Sodium Chloride	5.0
pH	7.4

Suspend 15.0g of the powder in 1L of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve powder. Autoclave at 121^0C for 15minutes..

APPENDIX 2 (CONTD)

Nutrient broth (Oxoid)

Approximate formular per litre

Bacto beef extract	3g
Bacto peptone	5g
H_20	1litre.
pH	6.8 @25^oC

Suspend 8.0g of the powder in 1L of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve powder. Autoclave at 121^{0}C for 15 minutes.

Blood agar

Approximate formular per 20 milliliter.

Nutrient agar	20ml
5% Unclotted blood	1ml

The nutrient agar was prepared and sterilized and left to cool to 45^{0}C, onemillilitre of unclotted blood was aseptically added to the cooled nutrient agar and mixed gently but thoroughly. The mixture was then aseptically poured in a sterile petri dishes and allow to solidify.

APPENDIX 2 (CONTD)

Media pouring and antibiotic supplementation

Allow media to cool to about 47-52^{0}C before pouring the plates. For antibiotic supplemented plates, add the antibiotic solution when the medium is about to be poured, mix thoroughly to allow uniform distribution and pour the plates, flame to eliminate bubbles on the plate. All plain plates are stored at 4^{0}C for as long as 3 weeks while all antibiotic-containing plates were stored for not more than 1week.

APPENDIX 3

CHEMICALS REAGENTS

Hydrogen peroxide(H_2o_2)

This was prepared by weighing out 0.3g of the stock solution into a test tube and adding 10ml of distilled water to obtain a 3% H_2O_2 solution.

Gram-stains and reagents

Crystal	500m
Distilled water	100ml

Gram's iodine solution

Iodine	1.0g
Potassium iodide	2.0g
Distilled water	300ml

Safranin solution

Safranin	1.0g
Distilled water	100ml

Dilute carbol fuchsin

Basic fucsin	1.0g
Phenol crystal	5.0g
Ethanol(95%	10ml
Distilled water	100ml.

APPENDIX 3(CONTD)

Physiological saline

NaCl 0.85g

dH_20 100ml

HCl, 1.0M

Concentrated (12N) HCl 10ml

dH_2O 110ml

Filter and sterilize, store in refrigerator.

Phosphate buffered saline (PBS) 1l 10x stock

80g NaCl

2gKCl

11.5g $Na_2HPO_4.7H2O$

2g KH_2PO_4

Plasmid extraction buffer

Buffer#

50mM glucose

10mM EDTA

22mM Tris,pH8

APPENDIX 3(CONTD)

Buffer #2

0.2M NaoH

1% SDS

Buffer#3(Potassium acetate)200mls

120ml 5M Potassium acetate, autoclave, then add;

23ml glacial acetic acid

57ml sterile dH_20.

10%Stop mix

20% ficoll

0.4% Bromophenol

0.4% xylene cyanol

Phenol buffer

	1L	4L
Tris(Trisma Base)	24.2g	96.8g
0.5M EDTA	2ml	8ml

pH to 8.0, bring to volume with dH_20

APPENDIX 3(CONTD)

Equilibrated phenol

Warm frozen redistilled phenol in a water bath until completely melted. Add an equal volume of Phenol buffer(1M Tris, pH 8.0). Mix carefully, then let stand at 4^0C until two phases separate. Remove the aqueous layer from the top. Add an equal volume of 0.1M Tris, pH 8, shake, let the mixture stand at 4^0C until the phases separate.)

50mm Tris-Hcl (P^H 8.0)

1MNacl

1mM EDTA

0.1%SDS (Sodium Dodecyl Sulphate)

Saline: 0.85Nacl (Autoclaved and Stored)

TE buffer

1mM Na_2EDTA

10Mm Tris-Hcl (Ph8.0)(Autoclaved and Stored.

STE buffer

100mM Nacl

1mM NA_2EDTA

10Mm Tris-Hcl(pH8.0) (Autoclaved and Stored at 5^0C)

Lysozyme:2mg/ml in ET, prepared fresh.

APPENDIX 3 (CONTD)

Isopropanol- stock

10 M Ammonium acetat

3.0Molar Autoclaved and stored at 5^0C

APPENDIX 4.

Preparation of Mcfarland standards.

The McFarland Nephelometer turbidity standards were prepared as described by Kolmer et al; (1951).

McFarland standard numbers;

1 = 3 x 10^8 cells per ml

2 = 6 x 10^8

3 = 9 x 10^8

4 = 1.2 x 10^9

5 = 1.5 x 10^9

6 = 1.8 x 10^9

7 = 2.1 x 10^9

8 = 2.4 x 10^9

9 = 2.7 x 10^9

10 = 3.0 x 10^9

The cell-suspension generally approximated to 1.8 x 10^9 cells per ml for each strains of *Staphylococcus aureus* .

APPENDIX 5

MOLECULAR TERMINOLOGIES.

Molecular weight markers

Hind III-digested λ , of seven fragments: 23.130, 9.416, 6.557, 4.361, 2.322, 2.027, 0.564kb pairs..

The MW markers were products of Roche Diagnostics GmbH, Penzberg Germany.

Agarose Slab: The slab/well through which the agarose preparation were loaded on before electrical discharge.

Restriction Site: Sites cut by restriction endonucleases to provide the "major guide post" for locating gene of interest on recombinant plasmid.

Nitrocellulose: The nylon membrane through which the band of protein will be reflected for reading.

Ethidium Bromide: An intercalating dye for staining the agarose gel

10mg/ml stock, 10ml in foil covered tube at 5^0C

(One slab stock)

DNA Ladder: 123bp, 500bp, 1kb, Hind111 fragment

10M TE(pH8.1)	90µl
Stop Mix	90µl
DNA Ladder Stock	20 µl
	200 µl Total Volume

APPENDIX 5 (CONTD)

Chloroform: Isoanyl alcohol (24:1) 5ml/pair.It is a plasmid MINI-PREP solution (for extraction).

Stock Solutions: It is often convenient to prepare solutions in a concentrated form, such as 10×,20×,50× or 100×. Thus, if a 100× stock is M and it is used to prepare 1× solution, the final concentration will be 10mM.

Unit conversion: Expression of units in this text are often in millimolar (mM) or microgram(µg).This simply requires moving the decimal point to the appropriate number of places.

10mM = 0.010M, 10mg = 0.010g

0.1µg = 100µl, 10µg = 0.000010g.

U.V. illumination: A light box that enable the visualization of DNA in ethidium bromide .Gel Electrophoresis Tank: design for the samples to be run on the same gels.

APPENDIX 6

CURING AGENTS

Ethidium bromide formulation.

The ethidium bromide used was 95% pure, with formula weight (FW) of 394,32g.

To prepare a stock concentration of 500mg/ml of the curing agent.

95g of ethidium bromide is contained in 100g of the powder and 0.01g of 10mg will be contained in:

$$\frac{100}{95} = 100 \times \frac{1}{\frac{0.0105g}{95}} \times \frac{}{100} = 0.011g \text{ approx.}$$

Thus, 0.0105g (equivalent to 0.01g or 10mg) of the powder was weighed out and dissolved in 20ml, of sterile distilled water to produce 500mg/ml, stock concentration of ethidium bromide. From this stock the following concentrations were prepared, each in 10ml, over night broth culture.

100mg/ml: 2ml, of stock mixed with 8ml, of broth culture

50mg/ml: 1ml, of stock mixed with 9ml, of broth culture

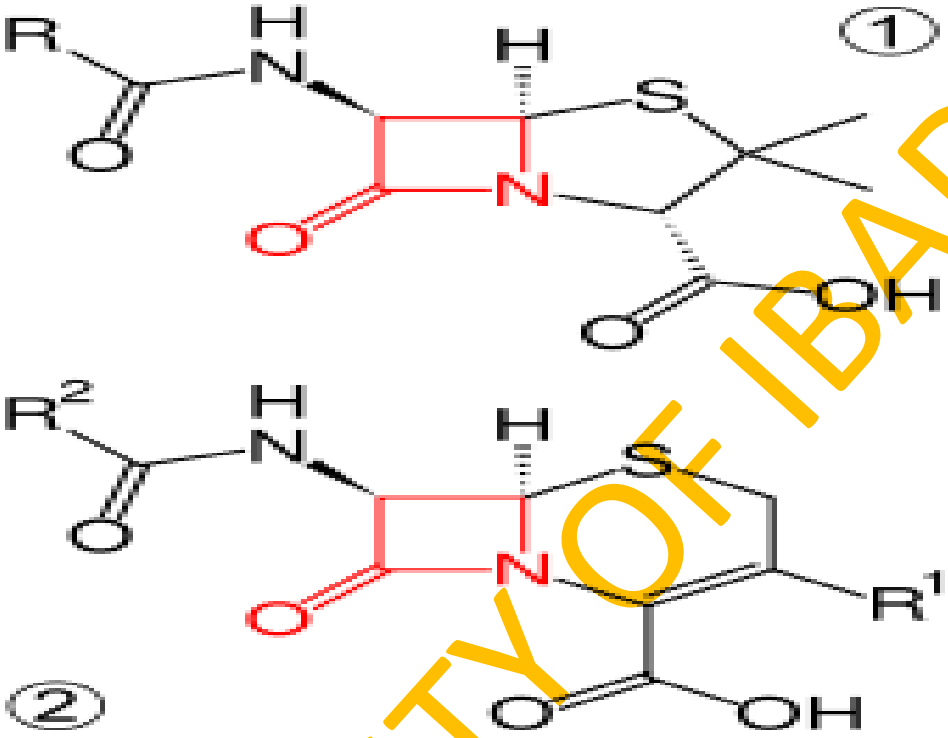
25mg/ml: 0.5ml of stock mixed with 9.5ml of broth culture

12mg.ml: 0.25ml of stock mixed with 9.75ml of broth culture

6.25mg/ml: 0.125ml of stock mixed with 9.875ml of broth culture.

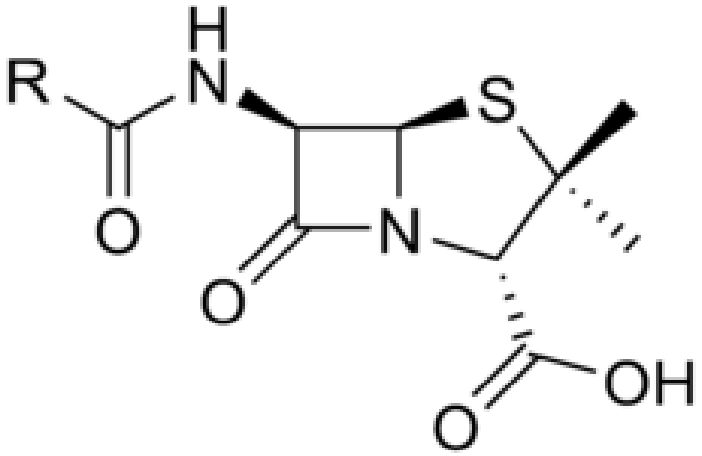
APPENDIX 7

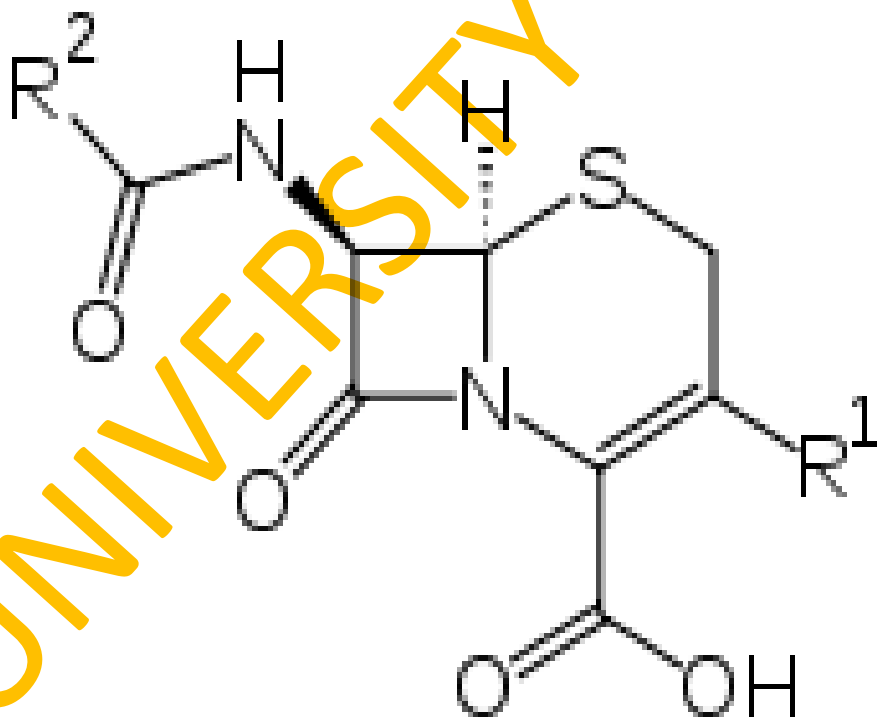
Molecular structures of β- lactams antibiotics showing the β-lactam ring in red



APPENDIX 8

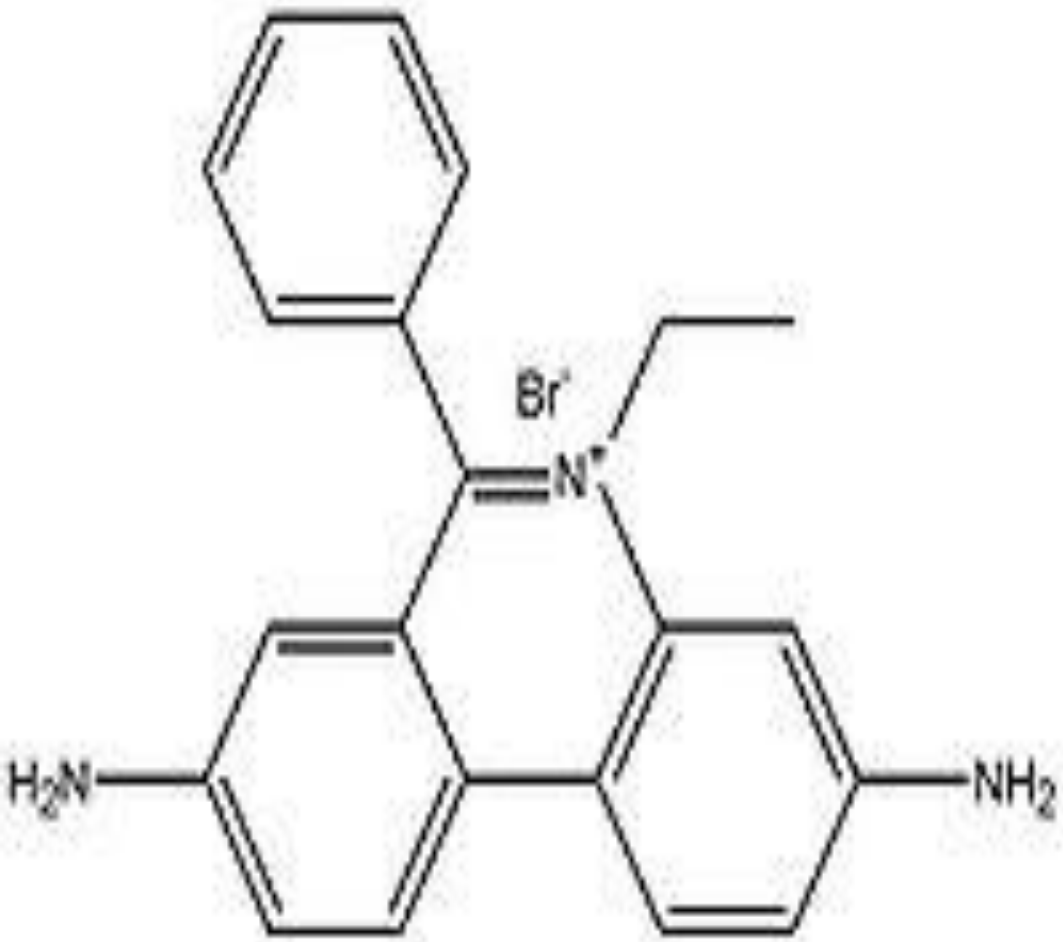
Molecular structure of penicillin nucleus and cephalosporins





APPENDIX 9

Structure of ethidium bromide



APPENDIX 10

Table 15.0

Pathological distribution of the isolates of *Staph aureus.*

NUMBER	SITE OF COLLECTION	AGE	SEX	NHR	HR	STATE OF COLLECTION	OF
1	Nose	15	F		√	Lagos	
2	Ear	32	M	√		Osun	
3	Armpit	18	F	√		Oyo	
4	Buttock	26	F	√		Ekiti	
5	Chin	6	F		√	Ekiti	
6	Breast	9	F	√		Ogun	
7	Thigh	37	M	√		Ogun	
8	Ear	55	M	√		Oyo	
9	Ear	33	M	√		Ogun	
10	Elbow	13	F	√		Ondo	
11	Ear	30	M	√		Osun	
12	Eyelid	6	F	√		Oyo	
13	Armpit	33	F	√		Ogun	
14	Ear	57	M	√		Lagos	
15	Armpit	10	F		√	Lagos	
16	Eyelid	24	M	√		Ekiti	
17	Thigh	18	F	√		Ekiti	
18	Cheek	41	M	√		Ondo	
19	Armpit	30	M	√		Ekiti	
20	Nose	15	F	√		Oyo	
21	Nose	39	F	√		Ondo	
22	Eyelid	6	F		√	Lagos	
23	Nose	45	F	√		Oyo	
24	Cheek	60	M	√		Lagos	
25	Head	74	M	√		Oyo	
26	Ear	48	F	√		Ekiti	
27	Buttock	70	F	√		Lagos	
28	Ear	20	M		√	Ekiti	
29	Head	40	M	√		Oyo	
30	Buttock	62	F	√		Ekiti	
31	Nose	13	F	√		Ekiti	
32	Ear	28	M	√		Ondo	
33	Armpit	52	M	√		Osun	
34	Knee	50	M	√		Ondo	
35	Thigh	20	F	√		Lagos	
36	Chin	43	F	√		Ogun	
37	Head	81	M	√		Oyo	
38	Armpit	14	M	√		Oyo	
39	Head	30	M	√		Ekiti	
40	Chin	42	M		√	Ogun	
41	Knee	21	F	√		Ondo	

Pathological distribution table(contd)

42	Fore arm	14	M	√		Ondo
43	Elbow	63	M	√		Ekiti
44	Elbow	41	F	√		Osun
45	Breast	8	F	√		Ondo
46	Ear	8	F		√	Ogun
47	Ear	68	M	√		Lagos
48	Nose	35	F	√		Lagos
49	Leg	43	M	√		Oyo
50	Ear	60	M	√		Ogun
51	Ear	33	M	√		Ogun
52	Thigh	33	M	√		Oyo
53	Ear	50	M	√		Ondo
54	Nose	40	M	√		Lagos
55	Nose	3	F		√	Ondo
56	Chin	24	F	√		Ekiti
57	Head	25	M	√		Ondo
58	Ear	42	M	√		Ondo
59	Nose	15	M	√		Ekiti
60	Nose	50	F	√		Lagos
61	Nose	44	M	√		Lagos
62	Nose	40	M	√		Lagos
63	Nose	14	M	√		Osun
64	Armpit	30	M	√		Ekiti
65	Nose	15	M	√		Ondo
66	Chin	30	M	√		Ogun
67	Knee	19	M	√		Lagos
68	Nose	16	M	√		Lagos
69	Elbow	30	M	√		Ekiti
70	Armpit	16	M	√		Oyo
71	Thigh	24	M	√		Ondo
72	Ear	12	F	√		Ogun
73	Cheek	23	M	√		Osun
74	Chin	14	M	√		Osun
75	Thigh	21	F	√		Oyo
76	Armpit	12	M	√		Ondo
77	Breast	8	F	√		Oyo
78	Lip	14	M	√		Lagos
79	Head	23	M	√		Osun
80	Knee	18	F	√		Osun
81	Nose	50	F		√	Lagos
82	Lip	25	M	√		Ogun
83	Ear	83	F		√	Ogun
84	Eyelid	25	M	√		Osun
85	Upper arm	23	F	√		Ondo
86	Upper arm	81	M		√	Ekiti
87	Buttock	16	M	√		Ogun
88	Head	33	F	√		Oyo

89	Cheek	50	M	√		Osun
90	Buttock	40	M	√		Osun
91	Forearm	60	F		√	Oyo
92	Forearm	20	F	√		Osun
93	Upper arm	47	F	√		Oyo
94	Upper arm	25	M	√		Osun
95	Fore arm	40	F	√		Osun
96	Elbow	20	F	√		Ondo
97	Lip	24	F	√		Ogun
98	Ear	44	F	√		Ogun
99	Armpit	18	F	√		Ogun
100	Armpit	31	F	√		Ekiti
101	Armpit	24	M	√		Osun
102	Armpit	18	F	√		Osun

APPENDIX 11

Table 16.0

Characterisation of the *Staphylococcus aureus* isolates obtained from furuncles

Strain No	Catalase	Coagulase	Haemolys.	Fermentation Glucose	Lactose	Mannitol	Gelatin hydrolysis	Gram stain	Starch Hydroly-sis	Oxidase	DNaseActivity	Comment
S1	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S2	+	+	+	+	+	+	+	+	+	–	+	S.aureus
S3	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S4	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S5	+	+	+	+	+	+	+	+	–	–	+	S aureus
S6	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S7	+	+	-	+	+	+	+	+	–	–	+	S. aureus
S8	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S9	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S10	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S11	+	+	+	+	+	+	+	+	+	–	+	S. aureus
S12	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S13	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S14	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S15	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S16	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S17	+	+		+	+	+	+	+	–	–	+	S. aureus
S18	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S19	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S20	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S21	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S22	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S23	+	+		+	+	+	+	+	–	–	+	S. aureus

Characterisation of the *Staphylococcus aureus* isolates obtained from boils/furuncles (contd)

S24											eus
S25	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S26	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S27	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S28	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S29	+	+		+	+	+	+	+	–	–	+	S aureus
S30	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S31	+	+	+	+	+	+	+	+	+	–	-	S. aureus
S32	+	+	+	+	+	+	+	+	+	–	+	S. aureus
S33	+	+	+	+	+	+	+	+	+	–	+	S. aureus
S34	+	+	+	+	+	+	+	+	+	–	+	S. aureus
S35	+	+	+	+	+	+	+	+	+	–	+	S. aureus
S36	+	+	+	+	+	+	+	+	+	–	-	S. aureus
S37	+	+	+	+	+	+	+	+	+	–	+	S. aureus
S38	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S39	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S40	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S41	+	+	+	+	-	+	+	+	–	–	+	S. aureus
S42	+	+	+	+	+	+	+	+	+	–	+	S. aureus
S43	+	+	+	+	+	+	+	+	+	–	+	S. aureus
S44	+	+	+	+	+	+	+	+	+	–	+	S. aureus
S45	+	+	+	+	+	+	+	+	–	–		S aureus
S46	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S47	+	+		+	-	+	+	+	–	–	+	S. aureus
S48	+	+	+	+	-	+	+	+	–	–	+	S. aureus
S49	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S50	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S51	+	+	+	+	-	+	+	+	–	–	+	S. aureus
S52	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S53	+	+	+	+	+	+	+	+	–	–	+	S aureus
S54	+	+	+	+	+	+	+	+	–	–	+	S .aureus
S55	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S56	+	+	+	+	+	+	+	+	–	–	-	S. aureus
S57	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S58	+	+	+	+	+	+	+	+	–	–	+	S. aureus
S59	+	+	+	+	+	+	+	+	–	–	+	S. aureus

Characterisation of the *Staphylococcus aureus* isolates obtained from furuncles (contd)

S60											s
S61											s
S62	+	+	+	+	-	+	+	+	–	-	S. aureus
S63	+	+	+	+	+	+	+	+	–	+	S. aureus
S64	+	+	+	+	+	+	+	+	–	-	S. aureus
S65	+	+	+	+	+	+	+	+	–	+	S. aureus
S66	+	+	+	+	+	+	+	+	–	+	S. aureus
S67	+	+	+	+	+	+	+	+	–	+	S. aureus
S68	+	+	+	+	+	+	+	+	–	-	S aureus
S69	+	+	+	+	+	+	+	+	–	+	S. aureus
S70	+	+	+	+	+	+	+	+	–	-	S. aureus
S71	+	+	+	+	+	+	+	+	–	+	S. aureus
S72	+	+	+	+	+	+	+	+	–	+	S. aureus
S73	+	+	+	+	+	+	+	+	–	-	S. aureus
S74	+	+	+	+	-	+	+	+	–	-	S. aureus
S75	+	+	+	+	-	+	+	+	–	+	S. aureus
S76	+	+	+	+	-	+	+	+	–	+	S. aureus
S77	+	+	+	+	+	+	+	+	–	-	S. aureus
S78	+	+	+	+	+	+	+	+	–	+	S. aureus
S79	+	+	+	+	+	+	+	+	–	+	S. aureus
S80	+	+	+	+	+	+	+	+	–	+	S. aureus
S81	+	+	+	+	+	+	+	+	–	+	S. aureus
S82	+	-	+	+	+	+	+	+	–	-	S. aureus
S83	+	+	+	+	+	+	+	+	–	+	S aureus
S84	+	+	+	+	+	+	+	+	–	+	S. aureus
S85	+	+	+	+	+	+	+	+	–	+	S. aureus
S86	+	+	+	+	+	+	+	+	–	-	S. aureus
S87	+	+	+	+	+	+	+	+	–	-	S. aureus
S88	+	+	+	+	+	+	+	+	–	+	S. aureus
S89	+	+	+	+	+	+	+	+	–	+	S. aureus
S90	+	+	+	+	+	+	+	+	–	+	S. aureus
S91	+	+	+	+	+	+	+	+	–	+	S. aureus
S92	+	+	+	+	+	+	+	+	–	-	S. aureus
S93	+	+	+	+	+	+	+	+	–	+	S. aureus
S94	+	+	+	+	-	+	+	+	–	-	S. aureus
S95	+	+	+	+	+	+	+	+	–	-	S. aureus

Characterisation of the *Staphylococcus aureus* isolates obtained from furuncles contd

S96	+	+	+	+	-	+	+	+	–	–	-	S. aureus
S97	+	+	+	+	-	+	+	+	–	–	-	S. aureus
S98	+	+	+	+	+	+	+	+	–	–	+	S aureus
S99	+	+	+	+	+	+	+	+	–	–	+	S .aureus
S100	+	+	+	+	-	+	+	+	–	–	+	S. aureus
S101	+	+	+	+	+	+	+	+	–	–	+	S .aureus
S102	+	+	+	+	-	+	+	+	–	–	+	S. aureus

-: Negative = -
Positive= +

$$\%+ve = \frac{\text{Total Nos of Positive} - \text{Total Nos of Negative}}{102} \times 100$$

APPENDIX 12

Table 17.0

Antibiotic susceptibility pattern of the *Staphylococcus aureus* isolates in relation to detection of β-lactamase

Strain Nos	β-lactamase production	Amx	Aug	Clx	Cmx	Chl	Gen	Ery	Tet 14
SA01	+	8	12	14	8	10	12	12	
SA02	+	14	20	22	18	18	16	28	19
SA03	+	14	12	7	10	12	12	13	14
SA04	+	28	18	9	10	8	11	12	14
SA05	+	26	18	8	16	12	12	12	12
SA06	+	28	19	9	10	12	12	13	14
SA07	+	17	24	22	26	10	10	23	20
SA08	+	29	24	7	9	12	12	23	26
SA09	-	25	26	22	16	12	12	24	19
SA10	+	20	28	23	10	12	12	23	12
SA11	+	30	32	14	20	10	8	27	20
SA12	-	28	21	8	10	10	16	23	22
SA13	+	25	14	8	9	12	10	11	12
SA14	-	13	24	9	18	10	10	24	10
SA15	-	R	23	20	9	12	12	12	8
SA16	+	14	12	8	10	12	10	10	14
SA17	-	R	23	14	8	10	11	23	14
SA18	+	32	27	9	10	11	12	8	10
SA19	-	14	19	20	9	10	11	10	10
SA20	-	8	20	22	20	12	12	10	10
SA21	-	12	20	9	10	10	20	29	12
SA22	-	15	10	28	8	12	8	23	20
SA23	+	15	10	8	26	20	8	29	8
SA24	+	26	10	14	10	12	11	10	19
SA25	+	28	10	19	8	10	20	12	14
SA26	-	29	12	24	10	25	9	25	10
SA27	-	12	13	9	10	12	16	25	10
SA28	-	11	24	9	10	12	9	25	12
SA29	-	13	28	22	22	10	10	30	19
SA30	-	14	23	9	10	12	16	26	10
SA31	-	30	12	23	10	10	16	27	10
SA32	-	30	21	22	26	12	17	12	20

SA33	+	29	23	8	20	20	10	24	8
SA34	-	24	20	24	8	8	12	31	28
SA35	-	24	12	8	10	12	10	28	12
SA36	-	29	34	9	8	10	15	23	19
SA37	-	24	31	20	16	10	10	26	29
SA38	-	28	12	20	10	12	12	29	8
SA39	-	21	22	20	10	24	22	23	19
SA40	+	24	23	8	8	24	10	29	9
SA41	+	30	13	22	10	12	10	24	12
SA42	-	21	15	9	20	12	12	26	20
SA43	-	23	10	9	8	25	10	22	28
SA44	-	12	22	8	9	12	22	25	28
SA45	+	16	28	8	10	28	12	28	14
SA46	-	20	19	23	10	12	12	29	10
SA47	-	15	23	22	22	12	10	10	11
SA48	-	18	23	9	10	10	12	12	12
SA49	-	18	12	9	16	12	10	27	10
SA50	+	17	21	9	8	20	22	24	12
SA51	+	16	23	8	9	10	10	11	10
SA52	+	15	26	8	10	10	25	31	12
SA53	+	23	12	22	20	10	10	12	12
SA54	-	12	32	29	20	21	10	28	12
SA55	-	23	20	22	10	10	20	12	10
SA56	-	12	13	22	9	10	10	24	12
SA57	-	23	24	9	10	12	17	12	10
SA58	-	30	14	8	10	25	10	24	12
SA59	-	12	21	9	22	12	12	11	14
SA60	-	12	26	9	20	12	12	24	10
SA61	-	14	12	8	10	20	24	28	12
SA62	-	15	23	8	20	12	12	10	14
SA63	+	34	23	22	10	10	10	10	12
SA64	+	28	13	23	9	10	10	12	12
SA65	-	28	24	8	10	12	21	25	12
SA66	-	12	28	8	9	26	12	12	14
SA67	-	8	10	9	10	12	12	10	11
SA68	-	18	12	9	10	10	10	12	12
SA69	-	13	29	8	9	12	10	25	10
SA70	-	17	22	8	10	18	10	24	14
SA71	-	18	18	9	10	12	12	25	12

SA72	-	23	23	9	9	10	10	12	11
SA73	-	19	26	8	16	8	10	10	10
SA74	-	29	18	8	10	8	20	12	12
SA75	-	20	23	9	8	24	23	12	12
SA76	-	20	24	9	10	12	12	10	12
SA77	-	32	15	8	21	10	10	12	12
SA78	-	8	14	9	10	12	18	10	14
SA79	-	24	20	9	10	18	24	8	8
SA80	-	23	23	8	16	12	20	9	8
SA81	-	10	18	9	8	10	16	12	12
SA82	-	19	27	8	10	24	28	12	12
SA83	-	29	29	22	20	12	20	10	14
SA84	-	28	18	8	10	10	10	12	12
SA85	-	12	20	8	28	12	24	10	12
SA86	-	19	12	8	10	10	10	12	8
SA87	-	32	28	9	10	19	10	12	12
SA88	-	19	28	9	10	10	12	10	12
SA89	-	28	29	8	16	10	10	11	14
SA90	-	16	12	8	10	10	22	12	11
SA91	+	23	20	8	10	12	12	10	10
SA92	-	12	23	9	8	12	10	12	8
SA93	-	23	27	8	10	10	12	10	8
SA94	+	14	10	9	10	18	18	12	14
SA95	-	34	20	9	20	10	16	12	12
SA96	-	16	20	8	10	12	12	11	12
SA97	+	23	12	9	25	12	10	12	14
8SA98	-	25	27	9	10	10	10	8	13
SA99	-	12	25	8	8	20	18	8	12
SA100	-	30	18	8	9	12	25	12	10
SA101	+	22	20	9	30	23	19	12	10
SA102	-	12	19	8	10	10	20	10	14

KEY: Zone of growth inhibition were interpreted according to CLSI performance standard for antimicrobial susceptibility testing.

Clx : Cloxacilln 5µg Aug : Augmentin 30µg

Amx: Amoxycillin 10µg Tet :Tetracycline 10µg

Gen: Gentamicin 10µg Ery: Erythromycin 5µg

Cmx: Cotrimozaxole25µg

APPENDIX 13

Table:18.

Clinical and **L**aboratory **S**tandards **I**nstitute antimicrobial Susceptibility testing Standard M100 – 521 Vol 31. No. 1. for – β-actamase *Staphylococcous aureus*

Antibiotic	**Dise size**	**Breakpoint**	
		Susceptibility	**Resistance**
Amoxycillin	10mg	≥ 29	< 28
Augimentin	30mg	≥ 20	≤ 19
Gentamicin	10mg	≥ 15	≤ 12
Erythromycin	15mg	≥ 23	≤ 13
Cotrimoxazole	23:75mg	≥ 16	≤ 10
Chloraphenicol	30mg	≥ 18	≤ 12
Cloxacillin	5mg	≥ 14	≤ 9
Tetracycline	30mg	≥ 19	≤ 14
Penicillin	10mg	≥ 29	≤ 28

APPENDIX 14

Table 19.0

Percentage Resistance of the Isolates of *Staphylococcus aureus* to the antibiotics.

Antibiotics	**% Resistance**
Amoxycillin	83
Amoxicillin-clavulanic acid	39
Cloxacillin	68
Cotrimoxazole	72
Chloraphenicol	78
Erythromycin	58
Gentamicin	64
Tetracycline	81

APPENDIX 15

Table 20.0

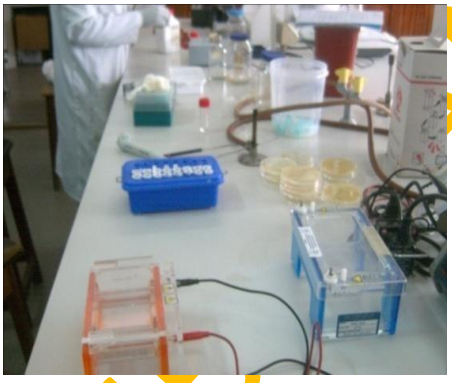
The DNA molecular weight marker (Hind iii)

LADDER λ	MOL. WEIGHT OF MARKER IN BASE PAIR	LOG MOLEC. WEIGHT. OF MARKER	MOBILITY (DISTANCE MOVED)	CONCENTRATION (NG/4µl)
01	23130	4.364	5.0	4477
02	9416	3.974	4.5	194
03	6557	3.817	4.2	135
04	4361	3.639	3.8	80
05	2322	3.366	2.8	48
06	2027	3.307	2.5	42
07	564	2.751	0.5	12.5

APPENDIX 16

Pictogram of some investigative tool in molecular biology laboratory.

Eppendorf centrifuge ice-bath



Electrophoresis Settings

Eppendorf micro-centrifuge

Ultra-violet incubator

Boiling an Agarose gel on electrothermal plate

Staph. aureus growth on M.S.A

APPENDIX 17

PUBLICATIONS FROM THE THESIS.

Okunye, O.L., Adeleke, O.E. and Adeyemi, O.H.(2011) Antibiotic Susceptibility Patterns Of The β-lactam Resistant Clinical isolates of *Staphlococcus aureus* from Recurrent Furuncles In South-West Nigeria. **S**ierra **L**eone **J**ournal of **B**iomedical **R**esearch ISSN 2076-6270 Vol.3(3) pp. 123-127,

Okunye, O. L, Adeleke, O.E. and Adeyemi,O.H.(2011) Curing of Antibiotic Resistance in Clinical strains of *Staphylococcus aureus* obtained from Recurrent Furunculosis in Southwest of Nigeria **I**nternational **J**ournal of **B**iological **S**cience **ASCN** 2011/328 (in press).