BETA-LACTAMASE PRODUCTION AND PLASMID PROFILING OF ANTIBIOTIC RESISTANT WOUND ISOLATES FROM PATIENTS ATTENDING UNVERSITY COLLEGE HOSPITAL IBADAN.

MSC RESEARCH WORK

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Abstract: Background: Wound Infections Have Been A Problem In The Field Of Medicine For A Long Time Due To The Emergence And Re-Emergence Of Drug Resistance. The Assessment Of Beta-Lactamase Production, Plasmid Profiling And Corresponding Resistance Pattern In The Local Terrain Become Important For A Proper Understanding Of Wound Infection Burden And Its Epidemiology. The Study Aimed To Assess Beta-Lactamase Production Among Multidrug-Resistant Wound Isolates From Patients Attending University College Hospital (Uch), Ibadan. Methods: Sample Size Of 370 Was Determined Using The Cochran Formula. Exudates From Wounds Were Collected With Sterile Swab Sticks, Isolation And Identification Were Performed Using Standard Procedures. Antimicrobial Susceptibility Testing Was Carried Out Using The Kirby-Bauer Disc Diffusion Method, Beta Lactamase Production Was Carried Out Using The Starch Iodide Acidimetric Method. Double Disc Synergy Test And Combination Disk Were Used For Extended–Spectrum Beta-Lactamases (Esbl) And Metallo-Beta-Lactamase (Mbl) Detection Respectively. Plasmid Curing Of Two Beta-Lactamase Procedures Was Carried Out Using Sodium Dodecyl Sulphate (Sds). After Which, Post-Curing Antibiotics Susceptibility Testing Was Performed. Results: Result Shows 339 Isolates Of Which 56.9% Were Multidrug-Resistant. Beta-Lactamase Producers Were 60.7% From Which 5% And 14% Were Positive For Esbl And Mbl Test. Same Resistance Pattern To The Antibiotics Was Observed In Plasmid-Cured Isolates, Meaning That Their Resistance Was Not Plasmid-Mediated. Conclusion: The Establishment Of Beta-Lactamase Producing Capacity In The Wound Isolates In This Study Will Help Clinicians In The Appropriate Treatment Of Wound Infections.

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Chapter One
Introduction

1.1 Background to the Study

Emergence of multidrug-resistance bacteria has become a major problem[1]. The World Health Organization has categorized antibiotic resistance as one of the three most significant severe public health problems of the 21st century[2].Wound infection is a breach in the skin, and exposure of the subcutaneous tissues makes available good and suitable environment for microbial colonization and proliferation[3]. Wound infections can be caused by different bacteria groups,

which includes Gram-positive and Gram-negative. The Gram-positive bacteria includes *Staphylococcus aureus*, Coagulase-negative *Staphylococcus aureus*, *Enterococci* and Gram-negative bacteria which are *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter*, *Proteus mirabilis*, *Proteus vulgaris*, *Acinetobacter* specie, Candida and other *Streptococci*[4]. The increasing incidence of antibiotic resistant bacteria is a concern to both the clinicians and the patients due to obvious consequences such as treatment failures, prolonged patients' stay in hospital and nosocomial infections[3]. Meanwhile, the production of hydrolytic β-lactamase enzymes is the most prevalent resistance mechanism towards β-lactam antibiotics. Metallo-β-lactamases constitute a troublesome group of enzymes, since they present a broad-spectrum profile, they hydrolyse penicillin, cephalosporin and carbapenems, but not monobactams an example is aztreonam. Extended-spectrum beta-lactamases (ESBLs) constitute a growing class of plasmid-mediated ß-lactamases which confer resistance to broad spectrum beta-lactam antibiotics[5].The infections caused by extended-spectrum beta-lactamase (ESBL) producing bacteria, constitute severe problems[6].

Carbapenems is a broad-spectrum beta-lactam (β - lactam) antibiotic, developed as one of the last resort antibiotics for treatment of serious and life threatening infections caused by multitude of multidrug resistant (MDR) Gram-negative bacterial infections, including extended spectrum- β-lactamase (ESBL)-producing Gram-negative bacteria[7,8].

However, since the introduction of these antibiotics in medicine, bacteria have developed mechanisms that have resulted in the emergence of the multi resistance. The current study therefore investigates production of beta lactamase and resistance pattern of wound isolates in patients assessing healthcare in UCH, Ibadan, Oyo state.

1.2 Statement of the Problem

Most of the wound samples brought to the medical laboratory for analyses of possible aetiologies come down with alarming resistance pattern to antibiotics during their antimicrobial susceptibility testing[9]. Wound samples when analysed in the clinical microbiological laboratories most often than not produce results indicative of antibiotic resistances to majority of antibiotics used during the susceptibility testing[10]. This has become a great concern to the laboratorians and clinicians.

The indiscriminate use of antibiotics has also led to the increase in multi-drug resistant organisms (MDRO)[11]. Other factors reported as fuelling the spread of ESBLs in developing economies include extensive self-medication, self-prescribing and non-prescription use of antimicrobials, poor hygienic conditions even in the hospital environment and very low infection control practice[12]. Knowledge on local antimicrobial resistance trends among wound isolates is important not only in guiding clinicians to prescribe appropriate antibiotics but also for evidence-based recommendations in empirical antibiotic treatment of wound and other infections[13,14]. The lack of a comprehensive surveillance and good antibiotic stewardship programs in Nigeria has largely contributed to increase in the disease burden of ESBL and MBL infections. This evidence based prescribing is highly needed everywhere antibiotics are prescribed. Thus this would be provided in this research work.

Moreover, most of the routine diagnostic laboratories in Nigeria seldom test for Beta lactamase production. Hence, the knowledge and local awareness of the burden of ESBL production with its attendant antimicrobial resistant pattern is low. It therefore goes unnoticed and continues to spread unabated with the associated high expenses and mortalities.

1.3 Research Questions

The following were the research questions that came to mind:

1. What are the likely causative agents of bacterial wound infection?
2. Are these bacterial susceptible to commonly used antibiotics?
3. Are these bacteria capable of carrying out ESBL and MBL activities?
4. Are there differences in the ESBL and MBL activities of these wound isolate from different wards?
5. Is the presence of plasmids a major pointer to the resistance capabilities of these isolates?

1.4 Aim and Objectives of the Study

The main aim of the study was to investigate beta-lactamase production and plasmid profiling of antibiotic-resistant wound isolates from patients attending University College Hospital, Ibadan. The specific objectives of the study were to:

1 Isolate and identify the causative bacteria responsible for wound infections in patient assessing healthcare in University college Hospital, UCH.

2 Investigate the antibiotic susceptibility pattern of the isolates.

3 Determine the Beta lactamase activity of the isolates (ESBL and MBL).

4 Compare ESBL and MBL production of wound isolates between different wards in the Hospital.

5 Determine the presence of plasmids in the resistant isolates and post curing susceptibility testing on the isolates.

1.5 Hypotheses of the Study

Hypothesis one:

Null hypothesis (H_0)

There was no significant relationship between the antibiotic-resistance pattern and Beta lactamase production activities of the wound bacterial isolates.

Alternate hypothesis (H_i)

There was a marked significant relationship between the antibiotic-resistance pattern and Beta lactamase production activities of the wound bacterial isolates.

Hypothesis Two:

Null hypothesis (H_0)

There was no relationship between antibiotic-resistance pattern and possession of plasmids in the wound bacterial isolates.

Alternate hypothesis (H_i)

There was a relationship between antibiotic-resistance pattern and possession of plasmids in the wound bacterial isolates.

1.6 Justification of the Study

Wound infections have been a problem in the field of medicine for a long time. Advances in control of infections have not completely eradicated this problem because of the emergence of drug resistance[15].Antibiotic resistance pattern in organisms causing wound infections is increasing. Many factors may have contributed to such a level of resistance, including the misuse of antibiotics by health professionals and unskilled practitioners[11]. In Nigeria and other developing countries, it is a common practice that antibiotics can be purchased without a prescription, which leads to the misuse of antibiotics, thus contributing to the emergence and spread of antimicrobial resistivity.

The assessment of Beta lactamase production, plasmid profiling and its corresponding resistance pattern in the local terrain becomes important for proper understanding of the disease burden and epidemiology. It is also critical to planning and efficient implementation of hospital infection prevention and control measures to curb further occurrence and transmission of the bacteria. However, this study will provide evidence - based information on the antibiotic resistance profile of wound isolates from UCH, Ibadan and also investigate plasmid presence in isolates and isolates' abilities to produce beta lactamase enzyme which confers resistance on the isolates. Thus, making it imperative to educate individuals on control measures on the spread of resistance bacteria in the environment. Hence, this study aims to investigate the plasmid harbouring and prevalence of ESBL and MBL producing organisms from wound infections and

1.7 Scope of the Study

This cross-sectional study analysed only wound samples, irrespective of the source, wards or units, sexes or ages of the patient provided it is within the hospital complex.

1.8 Limitations of the Study

Supply of Antibiotics disc and agar for the assay was delayed unnecessarily due to Covid -19 pandemic and the policies made on importations in the country. Moreover, paucity of funds could not allow for carrying out plasmid isolation for all the identified organisms, only few selected organisms were used for plasmid profiling.

Endnotes

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Chapter Two
Review of Related Literature
2.1 Wound Infections

The wound infection is a breach in the skin, and exposure of the subcutaneous tissues provides conducive environment for microbial colonization and proliferation[1].In wound infections, bacteria deposit and multiply in tissue this is an associated host reaction[2]. The main reason of wound infection is the breach in the skin that let different cell types enters the wound that initiates an inflammatory response. Signs of redness, pain, swelling, and fever are characteristics of the inflammatory response in the wound[3].

The wound is an injury to living tissues caused by a cut, puncture, bite or other impact on the skin surface. An infection occurs when germs enter wounds[4]. Wound infections are responsible for significant human mortality and morbidity worldwide. Wound infection could result in sepsis, limb loss, long hospital stays and higher cost[5]. These wounds are expected to heal within an expected period, depending on the category of the wound and degree of surgery. The prime closure of a clean, surgical wound would be expected to require superficial intervention to enable healing to progress naturally and quickly without difficulties in the surgical wound[6].

Wound infections can result in prolonged hospital stay, increased trauma care and high treatment costs if not well attended to[1]. Surgical wound infection is clinically defined as purulent discharge from the surgical wound or the insertion wound of the drain, or spreading cellulitis from the wound[6]. Patients may be affected with resistant bacteria or acquire resistance during or prior to antimicrobial exposure during treatment or in food residues[7].Surgical site infections (SSIs) is one of the most known postoperative complications and cause significant postoperative morbidity, mortality, prolong hospital stay and increase in hospital cost[8]. Burn wounds are highly susceptible to colonisation and infection and this is a major problem in the management of burn victims. Infected burn wounds are not only accompanied with a delay in epidermal maturation and deep scar formation, infected patients also tend to stay longer in the hospital and have a higher mortality rate as a result of sepsis[9] .

Wounds can however be classified into two main types, open and closed wound. Open wounds could be described as incisions, lacerations, puncture wounds, gunshot wounds and abrasions. Closed wounds include contusions more commonly known as bruises, hematomas crush injury. Most times contaminating microbes are eliminated by the host immune system and do not persist, but species that grow and divide may become established, causing wound colonization and infection. Infection in a wound delays healing and may cause wound breakdown, herniation, or complete wound dehiscence[10].

Wound infection varies with the type of surgery and surgical operations and they have been classified into, clean, clean-contaminated, contaminated and dirty. A clean wound is an incision through un-inflamed tissue

in which the wound is primarily closed. In this wound type only closed drainage systems are used and there is no breach in aseptic technique and the viscus is not opened. A clean-contaminated wound is one (that is otherwise clean) created at emergency surgery and in which the un-inflamed upper gastrointestinal tract, normal gall bladder and urinary bladder are opened but there is no spillage of contents and there is minor break in aseptic technique. Contaminated wounds however are traumatic wounds that are less than 6 hours old and one in which the inflamed upper gastrointestinal tract and obstructed urinary bladder are opened or spillage of contents occurs. In these wounds there are major breaks in sterile technique. Dirty wounds are associated with presence of pus and may include intra-peritoneal abscess formation or visceral perforation and traumatic wounds more than six (6) hours old[11,12].
Wound infections are found to be a major problem in our health care facilities. They are mostly common hospital acquired infections resulting into extended length of stay in the hospital with high cost and frequently observed in surgical patients[13].

2.2 Causative Pathogens of Wound Infection

Most wounds were caused by shrapnel and shells that were contaminated with soil and this caused horrific wounds that resulted in high number of emergencies. The fact that it took quite a long time for soldiers to be retrieved from front line, coupled with poor sanitation with crowded living conditions made treatment of wound infections very difficult[14.]
Wounds are contaminated by pathogens and body commensals ranging from bacteria to other infectious entities such as fungi and parasites, *Escherichia coli*, *Klebsiella species* and *Proteus* species[13]. Burn wounds are highly susceptible to colonisation by microorganisms and infection and this is a major problem in the management of burn victims. Infected burn wounds are not only associated with a delay in epidermal maturation and deep scar formation, infected patients also tend to stay much longer in the hospital and have a higher mortality rate due to sepsis when compared with non-infected patients[15]. Wound infections can be caused by different bacteria groups, which includes Gram-positive and Gram-negative. The Gram-positive bacteria includes Staphylococcus *aureus*, coagulase-negative *Staphylococcus aureus*, *Enterococci* and Gram-negative bacteria which are *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter*, *P. mirabilis*, *P. vulgaris*, *Acinetobacter* specie, Candida and other *Streptococci*[16]. The commonest organism causing wound infection was staphylococcus aureus followed by other gram negative bacilli[17]. But in India Gram-negative bacilli were predominantly isolated compared to Gram-positive pathogens[18,19]. In wound infections, bacteria deposit and multiply in tissue this is an associated host reaction[20].
Enterobacteriaceae are Gram-negative, facultative anaerobes, and non-sporing bacilli. This bacterial group have become one of the most important causes of Wound, nosocomial (community-acquired infections), urinary tract, respiratory tract, and blood stream infections. Increasing rates of antimicrobial resistance have become a worldwide problem majorly caused by enterobateriacea[21, 22].
Pseudomonas aeruginosa is a gram negative aerobic rod; this organism has been of great threat in surgical site wound infection due to its records of staggering resistance to extended spectrum antibiotics[23.]
Staphylococcus aureus is also an important pathogen causing skin, wound and burn infections, septicaemia and endocarditis and all other infections involving antibiotic resistant strains, this has great impact on human health[24]. Coagulase negative *Staphylococcus* (CoNS), a component of the normal skin flora has become an important opportunistic pathogen in foreign body sites causing both nosocomial and community acquire infections. Most developed countries have reported an increase in colonisation and infection in hospitalized patients by coagulase negative *Staphylococcus (CoNS*) while there are scanty data in developing countries[25].
All wounds can be contaminated from endogenous sources of the patient (e.g. nasopharynx and gastrointestinal tract), the surrounding skin or the immediate environment. The local environment is of particular importance for patients admitted in healthcare settings. Microbes in such wounds create a continuum from initial contamination all the way through colonization to fully blown infection. For this infection to occur, factors including the virulence characteristics of microbes, selection pressures, the host immune system, age and comorbid conditions of a patient play a critical role[26].
Skin serves as a first line defence (innate immunity) in battle against microbes. Despite this, wound breaks the integrity of the skin, and creates an open field for a microbe thereby circumventing the innate immunity and infection is established. It is very clear that Wound infections have resulted in considerable morbidity, mortality, prolonged hospitalization and escalation of direct and indirect healthcare costs[27, 28].
Wound provides a warm, moist and conducive nutritive environment to microbial colonization, proliferation, and infection. It can occur during trauma, accident, burn, surgical procedures or as a result of chronic disease conditions such as diabetes Mellitus and leprosy[29, 30]. Many different bacterial species live on human skin, in the nasopharynx, gastrointestinal

tract, and other parts of the body with little potential for causing disease, because of the first line of defence within the body. Despite the defence, any breach in the skin surface whether trauma, accident, surgical operation, or burn provides an open door for bacterial infections[31, 32].The most common isolates in all types of wounds are *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus vulgaris, Escherichia coli, Klebsiella* spp, *Enterococcus* spp[33].

2.3. Wound Treatment in the Pre-Antibiotic Era

2.3.1 Brief History

Prior to the development of antibiotics, infection was a major cause of morbidity and mortality and treatment was a great challenge. During the First World War, new infection problems arose, this led to the development of the most rapid changes in the understanding of infections and their treatments all of which happened before the development of antibiotics[34].

In 1918 a group of surgeons took cultures of wound specimens from their patients and found that 90.3% of the wounds were infected[35]. Captain Pettit looked particularly at gas gangrene and found 53% infected of wounds infected with anaerobic bacteria, however he does not mention numbers infected with aerobic bacteria [36]. Wounds were likely to be contaminated with multiple organisms. Alexander Flemming grew 10 different bacteria from one patient's single wound over a period of 67 days[37]. It was promptly noted that more effective treatments were required.

2.3.2 Wound Treatment

Iodine tincture was applied to the wounds of the soldiers, but it is a weak antiseptic. In the hospitals and casualty stations, potassium permanganate; another mild antiseptic, was used on suppurating wounds. Stronger antiseptics; carbolic acid, mercury per chloride and hydrogen peroxide, were also developed but their use was limited due to their corrosive properties. And saline, invented during the first part of the war, was also found very effective against infection when soaked in dressings.

By 1915, infection had been identified as a major cause of mortality for which something needed to be done. Large numbers of medical trails were set up in Britain and France. H.D. Dakin noted that sodium hypo chloride was an effective germicide but irritating to skin. He found out that addition of boric acid neutralizes the solution, making it effective but also non-irritating[38].

In a separate trial, A. Carrel discovered that Dakin's hypochlorite solution's antiseptic activity was short-lived, so it had to be renewed periodically[39]. An Indian rubber tube was invented by Carrel, with many holes along its circumference. This could be put along the wound, covered with towel and the antiseptic solution was continually poured down into it. The fluid was absorbed by the towel and maintained contact with the wound surface[39]. This became known as the Carrel-Dakin treatment. Unfortunately, it took a long time for the preparation to reach out to the front hospitals and so it was not used until the end of the war. Until then, the most widely used treatment remained carbolic acid solutions.

2.3.3 Hygiene and Sanitation

During the First World War, the importance of avoiding the spread of infection was also clear. Flies became such a problem that a committee was set up to eradicate them, producing pamphlets advising on the burning of rubbish and on positioning and depths of toilet trenches [39]. In principle, hygiene and sanitation became a high priority, but owing to crowded conditions and inadequate washing facilities, it was difficult in practice.

In hospitals, disinfectants were used and mattresses and beddings were sprayed with formalin solution and then steamed. Blankets and woolen articles were soaked in cresol solution and cotton wool and linen were boiled[40].

2.3.4 Tetanus and Gas Gangrene

Tetanus and Gas Gangrene are two of the major wound infections affecting soldiers during the war. Tetanus is usually caused by Clostridium tetani, which is found naturally in cultivated soils, so the wounds of soldiers were highly infected. There were 15.9 cases of tetanus per 1,000 injured in September 1914. It had risen to 31.8 cases per 1000 cases by the beginning of October 1914[41].

Even prior to World War I, tetanus was a well-understood disease. In 1885, by inoculating tetani with garden soil, Nicolaier developed the disease in mice, isolating the bacillus from the wound[41]. Behring and Kitasato later showed in 1890 that animals could be immunized by repeated injection of nonlethal doses of the bacillus toxin against the disease[41]. However, it was during the First World War that this was actually put into effect on a wide scale in humans. At the end of October 1914, the 'antitetanic serum' was introduced. The number of tetanus cases fell to 1.7 per 1000 wounded in November 1914 and 0.9 per 1000 wounded in December 1914, respectively[41].

Gas gangrene is known to be caused by *Clostridium bacillus*, mainly *C. perfringens, C. novyi* and *C. septicum*. The treatment procedure is surgical washing and debridement, and today antibiotics are used these days. Escritt remembers attempting on the ship to cure the disease. We used to get a lot of gas gangrene, you know, and it was found that the safest cure was to cut wounds to a depth of around a quarter of an inch around the edges and this had to be done within four hours onset of the injury. In that way, the organism in

those tissues is been cut out before they penetrated fully[42].

Gas gangrene was also a concern in the field clinics; the only goal of the surgeon was to open and clean the wound, or cut out the mortifying limb before the dreaded gangrene found its way into the vital parts of the body[43].' Research has gone into seeking a particular cure. A specific anti-gas gangrene serum was developed by Professor Weinberg of the Pasteur Institute in Paris and was given to most of the patients suffering from the disease in 1918. It was administered subcutaneously together with saline and camphorated oil[44].

Recipients of serum had a higher survival rate than those who did not receive it, but it was not successful in all cases and, sadly, until the end of the war, gas gangrene remained a major concern. It was not until antibiotics were developed that gas gangrene could be treated effectively, although today it is still a cause of critical illness.

2.3.5 Bacteriophage Typing

William Twort Initially described this nearly a century ago, and independently discovered shortly afterwards by Felix d'Herelle (considered by many to be the inventor of bacteriophages and their therapeutic implication: phage therapy), phages are tiny viruses that demonstrate the ability to kill target bacteria while cell lines from other organisms were preserved. Since its inception, the application of phages had since been proposed as a therapy to treat acute and chronic infections with initial successes due to the specificity of cellular target hosts, firstly mentioned in the disciplines of dermatology, ophthalmology, urology, stomatology, pediatrics, Otolaryngology and surgery[45]. The only available therapy in the 1920s and most of the 1930s was serum therapy for selected pathogens such as pneumococci and diphtheria and because of this, there was an enormous preference over phage therapy as a treatment for bacterial diseases at the initial stage and this was very understandable. This concept of the therapeutic use of phages to combat bacterial infection was met with resistance from the public and medical community alike[46]. The Pharmacy and Chemistry Council concluded that the proof of the therapeutic benefit of lytic filtrates was, for the most part, inconsistent, unconvincing and proposed further studies to stick to its value. This occurred after finding that, in multiple studies published at the time, there was a lack of adequate controls coupled with contradictory discoveries[47].

The emergence of the antibiotic chemotherapy age with the introduction of sulfa drugs in the 1930s and later penicillin in the 1940s further dampened enthusiasm on phage research and therapy was largely relegated to medical history in the western countries[46]. However, phage therapy remained an active area of research and development in the former USSR, Poland, and to a lesser extent India. Remarkably, over the last decade, the emergence of multidrug resistant bacteria has led researchers to re-consider this century-old approach and take a fresh look at phage therapy as a "novelle" and potentially viable treatment option for difficult to treat bacterial pathogens[46].

2.4 Discovery of Antibiotics

Antibiotics are the 'wonder drugs' used to fight microbes[48]. The beginning of modern "antibiotic era" was synonymously associated with two names Alexander Fleming and Paul Ehrlich[49]. Antibiotics were considered a magic bullet that selectively targets microbes that were responsible for causing disease, but selectively would not affect the host. The period from the 1950s to 1970s was thus considered as the golden era for the discovery of novel antibiotics classes[50]. Millions of metric tons of newer classes of antibiotics have been produced in last 60 years since its inception. Antibiotics are either cytotoxic or cytostatic to the micro-organisms, allowing the body's natural defences, such as the immune system, to eliminate them. They act mostly by inhibiting the synthesis of a bacterial cell, synthesis of proteins, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), by a membrane disorganizing agent, or other specific actions[51]. Antibiotics may also enter the cell wall of the bacteria by binding to them, using the energy-dependent transport mechanisms in ribosomal sites, which subsequently lead to protein synthesis inhibition [52].

2.4.1 Mechanism of Antimicrobial Action

Antimicrobial agents can be divided majorly into groups based on the mechanism of antimicrobial activity. The main groups are: agents that inhibit cell wall synthesis, depolarize the cell membrane, inhibit protein synthesis, inhibit nuclei acid synthesis, and inhibit metabolic pathways in bacteria[53]. With such a wide range of mechanisms one would have believed there is better control over the organisms. To combat against infections or microbes, undoubtedly antibiotics had been a blessing to human civilization that has saved millions of people[54]. Multiple varieties of the antibiotics have been used for therapeutic purposes over a long period of time.

Table 1: Showing Antimicrobial groups based on mechanism of action and examples

Mechanism of action	Antimicrobial group
Cell wall synthesis Inhibitor	β-Lactams Carbapenems Cephalosporins Monobactams Penicillins Glycopeptides
Cell Membrane Depolarizer	Lipopeptides
Protein Synthesis Inhibitor	Bind to 30S Ribosomal Subunit Aminoglycosides Tetracyclines Bind to 50S Ribosomal Subunit Chloramphenicol Lincosamides Macrolides Oxazolidinones Streptogramins
Inhibitor of Nucleic Acid Synthesis	Quinolones Fluoroquinolones
Inhibitor of Metabolic Pathways	Sulfonamides Trimethoprim

2.4.2 Mechanism of Antibiotic Resistance

Resistance to antimicrobial agents has become a major source of morbidity and mortality around the globe. When antibiotics were first introduced in the 1900's, it was thought that we had won the war against microorganisms. It was soon discovered however, that the microorganisms were capable of developing resistance to any of the drugs that were used[54]. The main mechanisms of resistance are:

1. Limiting drug uptake
2. Modification of a drug target
3. Inactivation of a drug
4. Active efflux of a drug.

2.4.2.1 Limiting Drug Uptake.

There is a natural variation in bacteria's ability to limit antimicrobial agent uptake. A barrier to some kinds of molecules is provided by the structure and functions of the LPS layer in gram negative bacteria. This gives these bacteria innate resistance to large antimicrobial agents in some classes[55]. Gram positive bacteria on the other hand do not possess an outer membrane, and restricting drug access is not as prevalent. However, it has been found that Staphylococcus aureus (a gram-positive bacterium) recently has developed resistance to vancomycin. *Staphylococcus aureus* uses two mechanisms against vancomycin, one of which causes the bacteria to create a thickened cell wall that makes it difficult for the drug to penetrate the cell, and provides an intermediate resistance to vancomycin. Such strains are designated as VRSA strains[56]. Mycobacteria have a high-lipid outer membrane, and hydrophobic drugs such as Rifampicin and fluoroquinolones have better access to the cell, but hydrophilic drugs have less access to the cell[57]. Bacteria that lack a cell wall, such as Mycoplasma and related species, are intrinsically resistant to all drugs that target the cell wall including β-lactams and glycopeptides[58].

Porin channels is another avenue by which some selected bacteria with large outer membranes control uptake of drugs into the cell. The porin channels in gram negative bacteria generally allow access to hydrophilic molecules[55]. There are two key ways in which changes in porin can restrict drug uptake: a decrease in the amount of porins present and mutations that alter the porin channel selectivity[57]. Formation of biofilm is another widely known phenomenon that some pathogenic bacteria can use to prevent the effect of the host immune system or the action of antimicrobials. These biofilms may contain a predominant organism (such as by Pseudomonas aeruginosa in the lung), or may consist of a wide variety of organisms, as seen in the biofilm community of normal flora in the gut. For antimicrobials to be effective against biofilms, they must be administered in much higher doses[59].

2.4.2.2. Modification of Drug Targets

There are multiple components in the bacterial cell that may be targets of antimicrobial agents; and there are just as many targets that may be modified by the bacteria to enable resistance to those drugs. An alteration in the structure and/or number of PBPs (penicillin-binding proteins) is one mechanism of resistance to β-lactam drugs used almost exclusively

by gram positive bacteria. PBPs are trans-peptidases involved in the construction of peptidoglycan in the cell wall. A change in the number (increase in PBPs that have a decrease in drug binding ability, or decrease in PBPs with normal drug binding) of PBPs impacts the amount of drug that can bind to that target. A change in structure (e.g. PBP2a in S. aureus by acquisition of the mecA gene) may decrease the ability of the drug to bind, or totally inhibit drug binding[60].

The glycopeptides (e.g. vancomycin) work also by inhibiting cell wall synthesis, and lipopeptides (e.g. daptomycin) work by depolarization of the cell membrane. Gram negative bacteria (thick LPS layer) have intrinsic resistance to these drugs[61]. Resistance to vancomycin has become a major issue in the enterococci (VRE—vancomycin-resistant enterococci) and in *Staphylococcus aureus* (MRSA). Resistance is mediated via acquisition of van genes which results in changes in the structure of peptidoglycan precursors that cause a reduction in the binding ability of vancomycin[60]. For drugs that target nucleic acid synthesis (fluoroquinolones), resistance is through modifications in DNA gyrase (gram negative bacteria—e.g. *gyrA*) or topoisomerase IV (gram positive bacteria—e.g. *grlA*). These mutations cause changes in the structure of gyrase and topoisomerase which reduce or eliminate the ability of the drug to bind to these components[62].

2.4.2.3. Drug Inactivation

There are two main ways in which bacteria inactivate drugs; by actual degradation of the drug, or by transfer of a chemical group to the drug structure. The β-lactamases are a very large group of drug-hydrolysing enzymes. An example of another drug that can be inactivated by hydrolyzation is tetracycline, via the *tetX* gene[63]. Drug inactivation by transfer of a chemical group to the drug most commonly uses transfer of acetyl, phosphoryl, and adenyl structure. There are a large number of transferases that have been identified. Acetylation is the most diversely used mechanism, and is known to be used against the aminoglycosides, chloramphenicol, the streptogramins, and the fluoroquinolones class. Phosphorylation and adenylating are known to be used originally against the aminoglycosides[63].

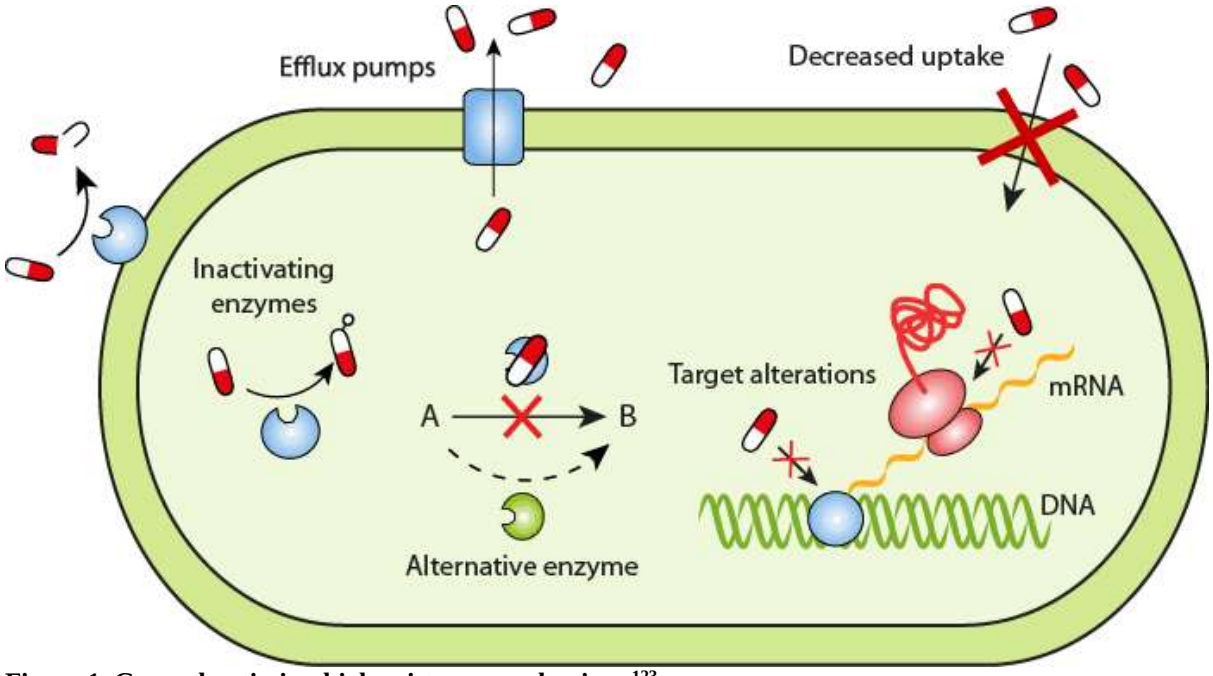

Figure 1. General antimicrobial resistance mechanisms[123].

2.4.2.4 Drug Efflux

Bacteria possess genes that are chromosomally encoded for efflux pumps. Some are expressed constitutively, and others are induced or over expressed (high-level resistance is usually through a mutation that modifies the transport channel) under certain environmental stimulus or when a suitable substrate is available. The efflux pumps function primarily to get rid of the bacterial cell of toxic substances, and many of these pumps will transport large specie of compounds (multi-drug [MDR] efflux pumps). The resistance capability of many of these pumps is influenced by what carbon source is available[55].

There are five major families of efflux pumps in bacteria classified based on structure and energy source:

1. The ATP-binding cassette (ABC) family
2. The multidrug and toxic compound extrusion (MATE) family
3. The small multidrug resistance (SMR) family
4. The major facilitator superfamily (MFS)
5. The resistance-nodulation-cell division (RND) family.

Efflux pumps found in gram positive bacteria may confer intrinsic resistance due to its being encoded on the chromosome. These pumps include members of the MATE and MFS families and efflux fluoroquinolones. There are also gram-positive efflux pumps known to be borne on plasmids. Currently, the characterized pumps in gram positive bacteria are from the MFS family[64]. Efflux pumps found mostly in gram negative bacteria are widely distributed and may come from all five of the families, with the most clinically significant pumps belonging to the RND family[64].

2.4.3 Origins of Resistance

Bacteria are known to have either intrinsic or acquired resistance to antimicrobials. Susceptibility and resistance are usually measured as a function of minimum inhibitory concentration (MIC), the minimal concentration of drug that will inhibit growth of the bacteria. The susceptibility is actually a range of the average MICs for any given drug across the same bacterial species. If that average MIC for a species is in the resistant part of the range, the species is considered to have intrinsic resistance to that drug. Bacteria may also acquire resistance genes from other similar organisms, and the level of resistance will vary depending on the species and the type of genes acquired[65].

2.4.3.1 Natural Resistance

Natural resistance could be intrinsic (always expressed in the species), or induced (the genes are naturally occurring in the bacteria, but are expressed only to resistance levels after exposure to an antibiotic). Intrinsic resistance may be defined as a trait that is shared universally within a bacterial species; it is usually independent of previous antibiotic exposure, and not related to horizontal gene transfer[66]. The most common bacterial mechanisms involved in intrinsic resistance are decreased permeability of the outer membrane (most specifically the lipopolysaccharide, LPS, in gram negative bacteria) and the natural activity of efflux pumps. Multidrug-efflux pumps are also a common mechanism of induced resistance[66].

2.4.3.2 Acquired Resistance

Acquisition of genetic material that confers resistance is possible through all of the major routes by which bacteria acquire any genetic material: transformation, transposition, and conjugation (all termed horizontal gene transfer—HGT); in addition, the bacteria may experience mutations to its own chromosomal DNA. The acquisition may be temporary or permanent. Plasmid-mediated transmission of gene resistance is the most common route for acquisition of outside genetic material; bacteriophage-borne transmission is fairly rare. Certain bacteria such as *Acinetobacter* spp. are naturally competent, and therefore capable of acquiring genetic material directly from the external environment. Internally, insertion sequences and integrin may move genetic material around, and stressors (starvation, UV radiation, chemicals, etc.) on the bacteria are common causes of genetic mutations (substitutions, deletions etc.). Bacteria have an average mutation rate of 1 for every 106 to 109 cell divisions, and most of these mutations will be deleterious to the cell[67].

Mutation that aid in antimicrobial resistance usually occur only in a few types of genes; those encoding drug targets, those encoding drug transporters, those encoding regulators that control drug transporters, and those encoding antibiotic-modifying enzymes. In addition, many mutations that confer antimicrobial resistance do so at a certain cost to the organism. For example, in the acquisition of resistance to methicillin in *Staphylococcus aureus*, the growth rate of the bacteria is significantly reduced[68].

One great conundrum of antimicrobial resistance is that the use of these drugs leads to increased resistance. Even the use of small or very low concentrations of antimicrobials (sub-inhibitory) can lead to selection of high-level resistance in successive bacterial generations, it may select for bacteria that are hypermutatable strains (increase the mutation rate), may increase the ability to acquire resistance to other antimicrobial agents, and may promote the movement of mobile genetic elements[69].

2.5 Beta lactamases

The β-lactam drugs are the most widely used group of antimicrobial agents. The members of this group of drug all share a specific core structure which consists of a four-sided β-lactam ring.

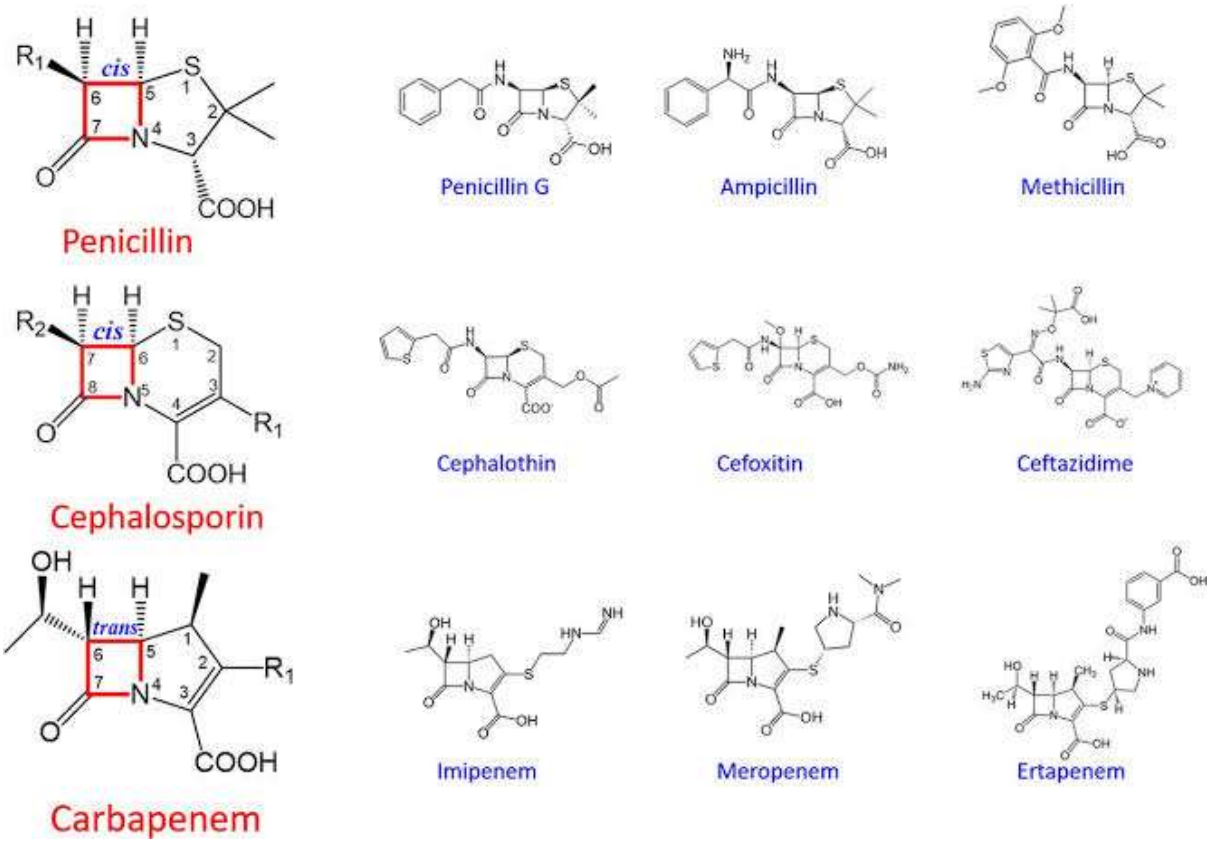

Figure 2; Structures of the Beta Lactam Drugs[167]

2.5.1 Resistance to the Beta lactam Drugs

This occurs through three general mechanisms:

(1) Preventing the interaction between the target PBP and the drug, usually causing modification in the ability of the drug to bind to the PBP (this is mediated by alterations to existing PBPs or acquisition of other PBPs;

(2) The presence of efflux pumps that can extrude β-lactam drugs;

(3) Hydrolysis of the drug by β-lactamase enzymes[70]. The β-lactamases (primarily called penicillinases and cephalosporinases) inactivate β-lactam drugs by hydrolyzing a specific site in the β-lactam ring structure, making the ring to open. The open-ring drugs are not able to bind to their target PBP proteins. The known β-lactamases are wide-spread, and the group contains enzymes that are able to inactivate any of the current β-lactam drugs. . Since the discovery of penicillin 80 years ago, Gram Negative bacteria have become proficient at evading the lethal activity of β-lactam antibiotics, originally through the production of β-lactamases[71]. The production of the enzyme called β-lactamase is the most common resistance mechanism used by gram negative bacteria against β – lactam drugs, and the most important resistance mechanism against penicillin and cephalosporin drugs[72]. The β-lactamase enzymes are classified based on their molecular structure and or functional characteristics. Structurally they are placed into four main categories (A, B, C, or D). Three functional categories exist based on the substrate specificity: the cephalosporinases, the serine β-lactamases, and the metallo (zinc-dependent) β-lactamases. These enzymes may also be commonly known by their enzyme family; such as: the TEM (named after the first patient) family, the SHV (sulfhydryl variable) family, and the CTX (preferentially hydrolyze cefotaxime) family. Gram negative bacteria may produce β-lactamases from all four structural groups. The β-lactamases found in gram positive bacteria are majorly from group A, with few from group B[73]. These enzymes may be innately found on the bacterial chromosome or may be acquired through a plasmid. Many members of the *Enterobacteriaceae* family of gram-negative bacteria possess chromosomal β-lactamase genes. Other gram-negative bacteria that possess these are *Aeromonas spp*., *Acinetobacter* spp., and *Pseudomonas* spp. Plasmid-carried β-lactamase genes are most commonly found in the *Enterobacteriaceae*, but may also be found in some species of gram-positive bacteria such as

Staphylococcus aureus, *Enterococcus faecalis*, and *Enterococcus faecium*[74].

Recently there has been an emergence of β-lactamases that are active against the carbapenems (carbapenemases), and are found primarily in the *Enterobacteriaceae group*. There are two known types of carbapenemases; the *Klebsiella pneumoniae* carbapenemases (KPCs), and those referred to as Carbapenem-Resistant Enterobacteriaceae (CRE) enzymes. The KPCs belong to the serine Class A (functional group 2f) β-lactamases and are resistant to all β-lactam drugs, but may still be affected by β-lactamase inhibitors. In bacteria that are CRE strains the carbapenemases are all metallo-β-lactamases (MBLs) in Class B, functional group 3a, and are capable of hydrolyzing all β-lactam drugs, but are not inactivated by β-lactamase inhibitors. The most widely distributed CREs are the IMP-1 (for imipenem resistance) and VIM-1 (Verona integron encoded MBL) types. A new MBL has recently been discovered, mainly in strains of *E. coli*. It has been designated as NDM-1 (New Delhi MBL). Infections caused by CRE strains have been associated with in-hospital mortality of up to 71%[75].

There has been a lot of emphasis on the development of more effective β-lactamase inhibitor drug combinations, especially in an effort to combat the CRE strains. One newer β-lactamase/drug combination is ceftolozane /tazobactam, which is mainly used against *P. aeruginosa*, and shows promise against gram negative ESBL producing strains. There are also newer β-lactamase inhibitors that do not have a structure similar to the β-lactam drugs. The first one of these to be approved for use is avibactam, and it has been assigned for use with ceftazidime against gram negative bacteria. In addition, avibactam is being tested for use with aztreonam against CREs. Another β-lactamase inhibitor which is non β-lactam structured is vaborbactam. It was approved for use along with meropenem in 2017 against gram negative bacteria causing complicated urinary tract infections (cUTIs). Unfortunately, so far none of the newer combination drugs is designed to combat the CREs directly. The metallo-β-lactamases are recently proving difficult to defeat as these enzymes comprise three main groups that vary greatly in structure and mechanism[76]

2.5 .2 Extended Spectrum Beta- Lactamases (ESBLs)

Extended spectrum beta-lactamase (ESBL) producing organisms are those that hydrolyse the oxyimino beta-lactams and monobactams, but exhibit no effect on the cephamycins and carbapenems[77]. ESBLs are a member of enzymes synthesized by some bacteria capable of hydrolyzing broad-spectrum β-lactam antibiotics like cephalosporins, penicillins and monobactams[78]. The betalactamases inactivating all the penicillins and cephalosporins including the extended spectrum cephalosporins are so called as Extended Spectrum Beta- Lactamases (ESBLs). There are almost 500 different ESBLs itemized, that are mutations of the classical broad-spectrum beta lactamase enzymes, initially named TEM and SHV (TEM-1, TEM-2, SHV-1)[79].

Majority of gram-negative pathogenic bacteria are responsible for extended spectrum *β*-lactamases (ESBLs) production, which portray resistance to some newer generation of antibiotics[80]. The infections caused by extended-spectrum beta-lactamase (ESBL) producing bacteria, constitute gross problems[81]. Infection caused by extended-spectrum beta-lactamases (ESBL) producing organism is a major problem regarding antibiotic resistance[82]. Members of Gram-negative bacteria (GNB) can hydrolyse many *β*-lactam antibiotics through the production of one or more types of extended-spectrum *β*-lactamases (ESBLs)[83,84]. The existence of Extended Spectrum B-lactamase (ESBL) genes plays an important role in spreading B-lactam antibiotic resistance in the strains that produces these so called enzymes. The resistance of gram-negative bacteria, such as *Pseudomonas aeruginosa*, to different antimicrobial agents, especially B-lactams, has been documented in an increasing manner[85]. Resistance to different classes of antibiotics had been detected among ESBL producers[86, 87]. ESBLs have the ability to hydrolyse most beta-lactam antibiotics such as cephalosporin, penicillin, aztreonam and related oxyimino beta-lactams except cephamycins (cefoxitin) and carbapenems which can solely be inhibited by beta-lactamase inhibitors such as clavulanic acid[88].

ESBLs are most of the time produced by *Klebsiella spp*. and *E. coli* although others like Enterobacter *spp*, *Citrobacter spp*, *Morganella spp*, *Serratia spp*, *Pseudomonas* spp and other gram-negative bacilli are also ESBLs producer[89]. In addition, ESBLs producing isolates exhibit co-resistance to other classes of antibiotics such as aminoglycosides, fluoroquinolones and sulfonamides[89]. Treatment is complicated by the presence of ESBL producing Enterobacteriaceae, because they are often termed multidrug resistant. Thus, infections caused by ESBL producing Enterobacteriaceae are of serious concerns. Many ESBLs are frequently expressed in gram-negative bacteria. They confer resistance to ampicillin, amoxicillin, and other penicillin derivatives, as well as to early but not later-generation cephalosporin[79].

Indiscriminate use of antibiotics, poor hygienic practices in hospitals and lack of monitoring for microbial drug resistance create conditions suitable for the emergence and spread of the ESBLs[90]. Other

factors noted as fuelling the spread of ESBLs in developing economies include extensive self-medication, self-prescribing and non-prescription use of antimicrobials. Poor hygienic conditions even in the hospital environment and very low infection prevention practice[91, 92].

2.5.3 Metallo -Betalactamases

Carbapenems is categorized as broad-spectrum beta-lactam (β - lactam) antibiotics, developed as one of the last resort antibiotics for treatment of serious and life threatening infections caused by wide array of multidrug resistant (MDR) Gram-negative bacterial infections, including extended spectrum- β-lactamase (ESBL)-producing Gram-negative bacteria[93, 94]. The carbapenems were introduced in the late 1980s in response to emergence of ESBL producing Gram-negative bacteria[95]. Carbapenems are considered first-line antimicrobial agents to treat severe cases of *P. aeruginosa* infections[96]. Carbapenemases are versatile b-lactamases, having the capability to hydrolyze carbapenems, cephalosporins, penicillins, and monobactams. Carbapenemases typically belongs to two molecular families, namely, "metallo-carbapenemases" in which activity is inhibited by EDTA, it uses zinc molecule at their active sites, and "serine-based carbapenemases" in which activity is not inhibited by EDTA rather used serine residues at their active sites and inactivated through β-lactamase inhibitors like tazobactam and clavulanic acid[97]. One of the most common mechanisms of carbapenems resistance is production of metallo-β-lactamases (MBLs) [98]. MBL enzymes are also able to hydrolyze penicillin and cephalosporins[99].

B-Lactamase enzymes are classified based on two main properties: functional and molecular characteristics. Functional classification was proposed by a scientist "Bush" in 1988, who classified β-lactamases into four functional groups namely, groups 1–4. Carbapenems fall under the 2f subgroup and group 3[100]. Later on, another scientist, Rasmussen, suggested that group 3 can be further divided into three functional subgroups on the basis of substrate specificity[101].The molecular classification was proposed by scientist "Frere" and colleagues, who classified carbapenemases into class A, class B, and class D. Class A carbapenemases require a serine active site at position number 70 in numbering system, this falls under the group 2f, and have the ability to hydrolyse Ambler carbapenems, penicillin, aztreonam, and cephalosporins[102].

2.5.3.1 Classification of Enzymes in Common bacteria

Class A SME, NMC, KPC, IMI, GES All *Enterobacteriaceae*, not usually *P. aeruginosa*

Class B VIM, SPM, GIM, IMP *Acinetobacter* species, *P. aeruginosa*, *Enterobacteriaceae*

Subclass B1 VIM-2, IMP-1, SPM-1, CcrAand BcII

Subclass B2 Sfh-1, CphA

Subclass B3 Gob-1, FEZ-1, CAU-1 & L1

Class D OXA *Acinetobacter* species.

Twelve different types of MBLs (VIM, IMP, SPM, GIM, NDM, DIM, AIM, SIM, KHM, SMB, TMB, and FIM) have been identified so far[103]. These genes are usually encoded by mobile genetic elements, of which are plasmids, transposons, and integrons, that allow them to disseminate horizontally among Gram-negative bacteria, posing a global challenge for all countries[103,104].

One of the most common mechanisms of carbapenem resistance is production of metallo-β-lactamases [(MBLs), including Verona Integron-encoded Metallo-beta-lactamase (VIM), Imipenemase (IMP), São Paulo metalo-beta-lactamase (SPM), German imipenemase (GIM),

New Delhi metallo-beta-lactamase (NDM), Dutch imipenemase (DIM), AIM, Seoul imipenemase (SIM), KHM, *Serratia* metallo-β-lactamase (SMB), Tripoli metallo-β-lactamase (TMB), and Florence imipenemase (FIM)][105]. Metallo-β-lactamases constitute a worrisome group of enzymes; this is because they present a broad-spectrum characteristic, being able to hydrolyze not only penicillins, but also the latest generation of cephalosporins and carbapenems, which constitute at present the last resort antibiotics[106].

This resistance has been attributed to a number or combination of different mechanisms. These include the production of carbapenemase enzymes, modification of outer membrane permeability and up-regulation of efflux system[107]. Most notably among these mechanisms is the production of carbapenemases, which are group of broad spectrum β-lactamase enzymes which has hydrolytic activities against all cephalosporins, carbapenems and penicillins[107]. The enzymes are functionally divided into those possessing serine group at their active site (Ambler classes Aand D) and those possessing divalent metal ion(s)at their active site (Ambler Class B or the metallo-β-lactamase)[95].

Metallo-beta-lactamases (MBLs) are known to efficiently hydrolyse carbapenems and other β-lactams (except monobactams) and are not inhibited by the clinically available β-lactamase inhibitors[107]. These enzymes are most often detected in *Klebsiella* spp. and *Escherichia coli*[94]. The rapid global spread of this bacteria and accompanied virulence is facilitated by the presence of most carbapenemase genes on

ambulatory genetic elements[107]. This is responsible for clonal transfer among bacteria of the same species and horizontal acquisition among bacteria of different species. In spite of this, carbapenem-resistant *P. aeruginosa* (CRPA) has been increasing in recent years, which is associated with high mortality, fatality, long hospital stays, and increased costs[108,109.] Since then, bacteria resistant to carbapenems has emerged and spread in the healthcare settings worldwide[110, 111,112]. This constitutes immediate threat to public health with attendant morbidity and mortality and thus requiring urgent and aggressive action[113]. In the United States, CR-GNB causes approximately 9300 infections per year and up to half of all CR-GN Bloodstream infections resulting in mortality [113]. A higher likelihood of death (more than 40% deaths) has been observed in patients with carbapenem-resistant infections compared with patients infected with carbapenems susceptible organisms[114, 115]. Moreover, longer duration of hospital stays and consequently increased healthcare cost is associated with CR-GNB infections [116]. In agreement with global trend, the prevalence of CR-GNB in Nigeria is increasing [117,118]. The resistance of clinical isolates to carbapenems has increased by about ten-folds within the space of a decade from 4.1% in 2007 to 40.3 % in 2017[117,118]. Molecular detection methods have also shown high circulation of carbapenemase genes in many Nigerian hospital[119, 120].

Though, emergence of carbapenems resistance has been mostly linked to prior carbapenems exposure, its occurrence among patients with no known medical history of its usage has however been documented in several countries including Nigeria[120]. This is similar to the emergence of colistin resistant bacteria among colistin naive subjects[121]. Because of the threat constituted by these organisms, prompt detection of patients with a CR-GNB infection is necessary not only for patient treatment decision and guiding infection control but also for epidemiological surveillance efforts fostered at limiting its spread[116]. The mortality rate associated with MBL producers is reported to be from 18 to 67%[122].

2.6 Incidence of Antibiotic Resistance

Antibiotic resistance was reported to occur when a drug has lost its ability to inhibit bacterial growth effectively. Bacteria become 'resistant' and continue to multiply in the presence of therapeutic levels of the antibiotics[123].When antibiotics were first introduced in the 1900's, it was thought that the war against microorganisms had been won. It was soon discovered however, that the microorganisms were capable of developing resistance to many of the drugs that were used. Apparently most pathogenic microorganisms have the capability of developing resistance to at least some antibacterial agents[124]. For several years, Antibiotics were dispensed to treat infections caused by bacteria and other microorganisms. After their discovery in 1928, antibiotics rapidly grew in number and potency, causing doctors and scientists to almost entirely disregard the challenge of treating bacterial diseases[125]. However, much has changed ever since then, as bacterial resistance now reduces the efficacy of antimicrobial agents[126]. Fleming was the first who cautioned about the potential resistance to penicillin if used too little or for a too short period of treatment[127].

The misuse and overuse of antibiotics have resulted in a continuous evolution of bacteria resistant to the drugs that were able to control them at instance. Bacterial resistance was first demonstrated when penicillin was administered during clinical trials. Initial cultures of *Penicillium* were contaminated with *Escherichia coli*, which produced an enzyme that degraded penicillin. In the second clinical trial in 1943, one out of the 15 patients died from a *streptococcal* infection after *E. coli* had become resistant to the antibiotic[128]. Soon after, a description of penicillinase-producing strains of *Staphylococcus aureus* was published far back in 1944, and scientists learned that bacteria could become resistant to penicillin[129]. Other bacteria have shown antibiotic resistance. For example, clinicians have tried to control the spread of methicillin-resistant *S. aureus* (MRSA) bacteria since it was first identified in the 1960s. *Haemophilus influenza* and *Neisseria gonorrhoeae* became resistant to penicillin in the mid-1970s. Even vancomycin, often the antibiotic of last resort, got into a dilemma; in year 2002, a vancomycin-resistant *S. aureus* (VRSA) isolate was recovered from a hospitalised patient in Michigan. The resistant determinant may have been acquired through the exchange of genetic material from a vancomycin resistant enterococcus[130].

Resistant bacteria are known to replicate despite in the presence of the antibiotics in use[131]. Researchers now know that antibiotic-resistant genes existed long before humans began developing and using antibiotics[128]. Bacteria that create antibiotics are protected by genes that make them resistant to the antibiotics they produce. Some bacteria that do not produce antibiotics also have resistant genes[132]. Antibiotic resistance can occur as a natural selection process where nature empowers all bacteria with some measure of low-level resistance[133].The emergence of antimicrobial resistance was observed not long after the introduction of new antimicrobial compounds [134].Infections generally becomes difficult to treat because of the pathogens with increasing antibiotic resistance[135]. The indiscriminate use of antibiotics has also lead to the increase in multi-drug resistant

organisms (MDRO) emanating[136]. In the present antibiotics era, infections have become the leading cause of morbidity in patients of surgery, trauma, wounds and others[137]. It is therefore of utmost importance to know the pathogens causing the infections and its antibiotic susceptibility for proper management of the patients[138]. Non-judicial use of antibiotic is highlighted to be responsible for making microbe's resistant[139]. Unfortunately, improper stewardship of antimicrobial agents has helped lead to the tremendous resistance issue that we now have. Factors that have contributed to the growing resistance problem include: increased consumption of antimicrobial drugs, both by humans and animals; and improper prescribing of antimicrobial therapy. Overuse of many common antimicrobials agents by physicians may occur because the choice of drug is usually based on a combination of low cost and low toxicity[140].

In fact, resistance was documented even before the beginning of the usage of the antibiotics in fighting the infection[141]. In the developing world, almost all the antibiotics are available over the counter and can be bought without any medical prescription which is one of the main important factors causing the resistance. Resistance rates vary across countries because of differences in antimicrobial agent usage and systems put in place for the prevention of antimicrobial resistant bacteria. In addition to resistance rates, modes of resistance also differ among countries and even among cities within the same country[142]. In developing countries, the increase in resistance including third- and fourth-generation drugs like cephalosporin, penicillin and fluoroquinolones may be due to their extended use and purchase directly from the drug store without doctors' prescription as self-medication is the usual and common practice[143]. Therefore, if the resistance to the antibiotics needs to be controlled, the only way shall be to educate the patients and the general public on a continual basis[144]. Unfortunately, improper stewardship of antimicrobial agents has helped lead to the tremendous resistance issue that we now face. Factors that have contributed to the growing resistance problem include: increased consumption of antimicrobial drugs, both by humans and animals; and improper prescribing of antimicrobial therapy. Over prescription of many common antimicrobials agents by physicians may occur because the choice of drug is based on a combination of low cost and low toxicity[136]. There may be also improper prescribing of antimicrobials drugs, such as the prior prescription of a broad-spectrum drug that is unnecessary, or ultimately found to be ineffective for the organism(s) causing the infection[137]. The danger is that excessive use of antibiotics in humans leads to emergence of resistant organisms[145, 146]. In addition, prior use of antimicrobial drugs puts a patient at risk for infection with a drug resistant organism, and those patients with the highest exposure. The increasing incidence of antibiotic resistant bacteria is a concern both to the clinicians and the patients due to obvious consequences such as treatment failures, prolonged patients' stay in hospital and nosocomial infections. The choice of the first antibiotic therapy in emergency wards in hospitals is mostly not based on patient-specific microbial culture and susceptibility test result[147]. Rapid emergence of multidrug-resistant bacteria poses a serious threat to public health round the globe due to the limited treatment options and lukewarm discovery of new classes of antibiotics[148, 149].

Antibiotics have also found uses in agriculture and are often the same or similar to antibiotic compounds used clinically[150]; this over-usage could possibly also invite drug resistance. The food chain can be considered as the main route of transmission of antibiotic-resistant bacteria between animal and human populations[151]. In some developed countries, animals receive some measure of antibiotics in their food, water, or parenterally which may be responsible for carrying microbe resistance to that specific antibiotic[150]. For example, the use of antibiotics in cattle feed as growth promoters have caused increase antibiotic resistance[151]. Recent evidence suggests that poultry or pork might be a possible source of quinolone resistant-Escherichia coli in the rural villages in Barcelona, where one fourth of children were found to be faecal carriers of these organisms. However, these kids have never been exposed to quinolones in any way[152]. There has been evidence for the transfer of resistance genes between members of the human microbiota, as well as from livestock-associated bacteria to human-associated bacteria[153].

For many years antibiotics have been used for treating or preventing disease in raising food animals. The animal feed often contains antibiotics in divers' amount that range from below therapeutic levels to full therapeutic levels, and the antibiotics used come from most of the antimicrobial classes used in humans. There is evidence to support the idea that feeding antibiotics to animals may result in increased development of antimicrobial resistant organisms, and that those resistant organisms may be transferred to the humans who consume those animals [154,155]. The antimicrobial resistance patterns seen in the animals reflect the varieties and measures of antibiotics given to the animals. The transmission of antimicrobial resistance from the animals to humans may occur in different ways; with the direct oral route being the commonest (includes eating meat plus ingestion of

faeces in contaminated food or water). Another common route is from direct contact with the animals by humans[155].

Currently, the multifarious causes of resistance constitute many factors including improper use and regulations, lack of awareness, deviations from normal antibiotic usage and the uses of antibiotics as a growth promoter in livestock as well as in poultry for infection control[156]. Basically, the reason behind the resistance emergence is the improper and excessive use of antimicrobials. The powerful tool of antibiotic resistance include infection control standards, sanitation system, drug quality, water hygiene systems, diagnostics and therapeutics, and migration or travel quarantine. Genetic mutations coupled with exchange of genetic material between organisms play a key role in the distribution of antibiotic resistance[157].

2.6.1 Antibiotic Resistance as a Global Threat

Antimicrobial resistance (AMR) has become the global public health problem in its many ramifications. Inappropriate antimicrobial use like as the misuse and abuse in human and veterinary medicine has remained the leading cause to pose a significant public health threat in many countries of the world[158]. No matter how, antimicrobial agents have tremendously played a significant role in reducing the spread of infectious diseases since their discovery, measurable mortality and morbidity rate both in humans and veterinary medicine remained high. The post-antibiotic era, which is accompanied by AMR, creates a likely vicinity to microorganisms to gain resistance gene that throws human to a few lists of drugs[159]. Antimicrobial microbial resistance (AMR) is a fundamentally important global public health problem that has remained the leading consequence to escalate clinical and socio-economic aspects of human beings worldwide. Extended spectrum β-lactamases (ESBLs) have been reported to be a common cause of hospital-acquired infections and can have severe clinical implications with a corresponding multiple antibiotic resistances[158, 159]. With the antibiotic resistant bacteria on the rise, infection is again becoming a concern for modern day medicine. In January 2013 Dame Sally Davies, the chief medical officer in the UK, mentioned the threat from infections resistant to frontline antibiotics was so serious that the issue should be added to the UK government's national risk register of civil emergencies[160]. The World Economic Forum has added antibiotic resistance as one of its Global Risks in its 2013 Report [161], also in May 2014, in the journal of Nature, Woodhouse and Farrar have called for a coordinated and global effort to act on the problem[162]. In response to the rising concern and calls for international action the World Health Organisation (WHO) produced a report; 'Antimicrobial Resistance: Global Report on Surveillance'. According to them 'the problem is so serious that it is a threat to the achievements of modern medicine. A post-antibiotic era in which common infections and minor injuries can cause death is a very real possibility for the 21st Century'[163]. The prevalence of drug resistant bacteria may not only vary from countries to countries but also from institutions to institutions and this can be partially explained by the difference in local antibiotic prescribing habits and difference in effectiveness of infection control program in different health institutes. The global burden of antibiotic resistance is heightening continuously; preferably it mounts up the pressure on veterinary medicine and on human. The WHO made a landmark by promoting and declaring AMR as a global health concern. The agenda of global health concerns are at the developmental stages, for example, a book named *The Evolving Threat of Antimicrobial Resistance: Options for Action* is a precious addition to the archive[160]. Currently, the world is experiencing dramatic pre-antibiotic era, and many of the untreated infection emerge on a large-scale; clinicians often encounter many patients with such infections that normally reported as multidrug resistant (MDR) bacteria by many laboratories and not responding to already available therapeutics. It has been estimated that yearly about two million people acquire vulnerable infections just because of these antibiotic-resistant pathogens, and as a result of this, about 23,000 people were officially reported dead according to Centre for Disease Control and Prevention (CDC) [161].

In a historical perspective, antibiotic resistance is a frightening and compelling concern. New types of antibiotic-resistant bacteria are taking control of ancient drugs. We may be entering the post-antibiotic era, because of increased persistence, spread, and the emergence of superbugs. It has been reported that annually, in the USA, about 99,000 deaths are caused by antibiotic-resistant pathogen-related hospital-acquired infections[162]. While in the United States of America, the annual death rate is about 50,000 caused by two usual Hospital acquired infections (HAIs), known as sepsis and pneumonia, which cost around $8 billion to the economy of the USA. The patients infected with bacterial strains that are resistant to antibiotics must stay in the hospital minimum for 13 days, which adds to 8 million days annually. An annual report of the cost of economy loss with regard to a productivity loss of around $35 billion has been documented[164]. Antimicrobial resistance (AMR) has become the global public health problem in its many aspects. Inappropriate antimicrobial use such as the misuse and abuse in human and veterinary medicine

has remained the leading cause to pose a significant public health threat in many countries of the world[165, 166].

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Chapter Three
Methodology

3.1 Study Area

The study was carried out in the Department of Medical Microbiology and Parasitology of the University College Hospital (UCH) Queen Elisabeth road, Orita mefa, Ibadan. This is a tertiary Teaching hospital located in the South-west region of Nigeria. University College Hospital, Ibadan is a Federal teaching hospital in Ibadan, Nigeria, attached to the University of Ibadan[1].

3.2 Ethical Approval

This was sought from the Ethics and Research Committee of the University of Ibadan /University College Hospital, UCH, Ibadan, Oyo state, Nigeria, for this study with full approval granted with number UI/EC/21/0135 (see appendix1)

3.3 Sample Size

This was obtained using the Cochran (1977) formula which reads thus:

$N_{\circ} = Z^2PQ \div e^2$, where

N = required sample size

P = expected prevalence rate with reference from similar previous study.

For this purpose, the work done by Kabita et al[2] was used with a Prevalence of 56.95%.≈ 0.6.

e = margin of sample error at 5% (standard value of 0.05)

Z = confidence level =1.96

Q = the desired level of precision

$N_{\circ} = 1.96^2 \times 0.6 \times 0.4 \div (0.05^2)$

$= 1.96 \times 1.96 \times 0.6 \times 0.4 \div 00025$

$= 368.7$

≈ 370 Wound samples.

3.4 Samples Collection

Exudates from wound were collected with a sterile swab stick from the in-patients and out patients unit within the Hospital.

3.5 Identification of Isolates Using Biochemical Characterization

The following procedures were carried out following standard methods:

3.5.1 Gram Staining

Gram staining reaction was carried out to distinguish the organisms into Gram positive and Gram-negative isolates according to their ability to retain the primary stain (Crystal violet) used during the procedure.

A minute quantity of the isolates colonies was carefully transferred from the Petri dish on to a sterile slide previously placed with a loopful of normal saline. The culture was spread evenly and gently with an inoculation loop to make a small circular thin film and then air-dried and fixed over a gentle flame. Crystal violet stain was added over the fixed culture and allowed to stand for 10-60 seconds. Enough mordant (iodine solution) was added to cover the fixed culture and allowed to stand for another 10-60 seconds. The slide was rinsed with running water, thereafter, carefully cleansed to wipe off the excess water from its surface. A few drops of acetone solution (a decolourizer) were added such that the solution trickled down the slide. After optimal decolonization, the slide was again rinsed with water and neutral red solution (a counterstain) was applied for 40-60 seconds. After washing with water, the slide was air-dried and then viewed under the X 100 objective. Gram positive organisms retain the purple colour of the primary stain while the Gram negative takes the colour of the counter stain (pink or red).

3.5.2 Oxidase Test:

This test was carried out with all the Gram-negative isolates to distinguish *Pseudomonas aeruginosa* from other *Enterobacteriaceae*. Two drops of oxidase reagent were placed on a filter paper on a slide, a colony of the test organism was collected using a glass slide, and smeared across the emulsified filter paper, a purple colour shows on the portion of the filter paper where the test organism was placed. The oxidase reagent is usually controlled before use by testing against a known pseudomonas; a purple colour is positive test.

3.5.3 Indole Test:

About 3ml of sterile peptone water was placed in a bijou bottle, after which the isolates were inoculated in these bottles and incubated at 35-37°C for up to 24 hrs. Indole ring formation was tested by adding 0.5ml of Kovac's reagent with a gentle shake. A red ring formation is positive for indole production. Absence of red ring is a negative test.

3.5.4 Coagulase Test:

This distinguishes pathogenic *Staphylococcus aureus* (positive test) from non-pathogenic Staphylococcus epidermidis (negative test). A clean microscope glass slide was divided into two sections (A and B) using a grease pencil. Section A was "test" and Section B "control". A small drop of normal saline was placed on each section and then a small amount of the isolates was picked from the Petri plate using sterilized cooled inoculating loop and emulsified on section A to make a smooth smear. A drop of plasma was placed on section A, presence of agglutination shows the production of coagulase enzyme this is a positive test**.** No agglutinations were observed in the section B(control) on the slide.

3.6 Bacterial preservation

The isolates were stored in Tryptose Soy Broth (TSB) (Oxoid Ltd., Basingstoke, United Kingdom and preserved at − 70 °C and were recovered by re-suspension whenever needed for use.

3.7 Antibiotics Susceptibility Testing to Beta Lactam Drugs

Antimicrobial susceptibility testing was carried out using the Kirby-Bauer disc diffusion method on Mueller Hinton agar and the resulting zone of inhibition were expressed as susceptible, intermediate or resistant according to clinical and laboratory standards institute guideline (CLSI)[3]. The organisms were tested against different antibiotics and commonly used discs were : Piperacillin/tazobactam (100/10 µg) amikacin (30 µg), Augmentin (20µg amoxicillin/10 µg clavulanic acid), ceftazidime (30 µg),ceftriaxone (30 µg), ciprofloxacin (5 µg), colistin (10 µg),gentamicin (10 µg), ertapenem (10 µg), levofloxacin (5 µg),meropenem (10 µg), Erythromycin (15g),Vancomycin , clindamycin, Cefuroxime and Tigercycline(µg)

3.8 Determination of Beta Lactamase Production

This was done using the starch iodide acidimetric method of beta lactamase detection[4]

A solution of 0.2% soluble starch and 1% potassium penicillin G for injection was made by adding 0.1 gram of soluble starch to 45ml of sterile de ionized water and was heated to properly dissolve then allowed to cool. Five (5) millilitre of potassium penicillin G (10U/ml) was then added to attain a final concentration of 10 U/ml.

Filter paper (Whattman No 3) was cut into strip and immersed in the starch – penicillin solution; this was drained and allowed to air dried for about 2hours at room temperature. The strip was saturated with Gram's iodine, pouring off the excess iodine and placing it on dry absorbent paper towel. The strip was smeared with heavy inoculums rubbing it on the surface of .the paper in a circular manner to cover about 5mm diameter. A positive and negative control were done alongside using a positive strain of *staphylococcus aureus* ATCC 12981 and negative beta lactamase strain of *Escherichia coli* ATCC 12241 obtained from the department of medical microbiology and parasitology, University college hospital Ibadan.

A positive beta lactamase test is indicated by the decolourisation of the purple colour of the starch – iodide paper to white. A negative result shows no decolourisation.

3.9 Extended –Spectrum Beta- Lactamases (ESBL) Detection

3.9.1 Screening for Potential ESBL-Producing Isolate.

The microorganism that has a zone of inhibition (zoi) of less than22mm for ceftazidime are considered potential Esbl producers and shall be considered for confirmatory tests[3].

3.9.2 Confirmation of ESBLs Producing Isolate

This was done using Double Disc Synergy Test (DDST) [5]. 20 ml of Mueller Hinton agar was prepared and dispensed aseptically into a Petri dish and was allowed to solidify. The plates were seeded with test organisms pre-adjusted to 0.5 McFarland turbidity standards. A disc of (amoxicillin 20 µg and clavulanic acid 10 µg) was placed at the centre of the Petri dish in between two cephalosporin antibiotics (ceftazidime 30 µg and ceftriaxone 30 µg) placed 30 mm apart. It was incubated at 37°C for 18-24 hrs after which the various Inhibition Zone Diameters (IZDs) were measured. Enhanced zone of inhibition between any of the beta-lactam discs and the centre disc was recorded. In this study, an enhanced zone of inhibition between any of the third-generation cephalosporin antibiotic discs and the amoxicillin clavulanic disc was confirmation of ESBL production[6].

3.10 Metallo-Beta-Lactamase (MBL) Detection

This is to test for MBL Production. The isolates were subjected for MBL detection when it is resistant to carbapenems.

3.10.1 MBL Confirmation by Combination Disk (Cd) Method

The isolates showing resistance to carbapenems were subjected to confirmation for MBL production by combined disc assay using carbapenems and carbapenems/ethylene diaminetetraacetate discs[3].

Two Ertapenem (ETP) disks (10 µg) were used. In one of them, 10 µL of 0.1 mol/L (292 µg) anhydrous ethylenediaminetetraacetic acid (EDTA) was added. Then the two disks were placed 25 mm apart (centre to centre). An increase in zone diameter of > 4 mm around the ETP-EDTA disk compared to that of the ETP disk alone was considered positive for an MBL[3] production.

3.11 Detection of Plasmids in Resistant Isolates

Plasmids were isolated using the QIAGEN Plasmid Purification mini kit.

A. Isolation of Plasmid DNA

1. Harvest and Lysis of bacteria: 1.5 ml of broth containing the resistant isolate was transferred into 1.5 ml centrifuge tubes to be centrifuged at 13000 rpm for 1 min.The supernatant were discarded.
2. The cells were suspended in 200 µl of Resuspension solution. It was pipetted up and down.
3. About 200µl of Lysis Solution were added, inverted gently to mix. Allowed to Clear for about 5 min.
4. Preparation of clear lysate: About 350µl of Neutralization solution (S3) was added to the lysate then Inverted to allow it to mix for about 4-6 times.
5. Centrifuged at 13,000 rpm for 10 mins.

6. Preparation of binding column: In a collection tube about 500 µl Column Preparation Solution were added to the binding column.
7. Allowed to spin at 13,000rpm for 1 min. Discard flow through.
8. Binding plasmid DNA to column: the cleared lysate were transferred to the binding Column.
9. Centrifugation done at 13,000 rpm for 1 min. 2
10. This was washed to remove contaminants: About 750 µl of Wash solution was added to column and allowed to spin for 1 min.
11. This was allowed to spin for 1 min to dry the column (dry by speed vacuum).
12. Elution of purified Plasmid DNA: The column were transferred to a fresh collection Tube. About 70µl of Elution Solution were added. Spin for 1 min at 13,000 rpm. The eluate contains plasmid DNA. Store the plasmid DNA at -20°C or -80°C.

A Preparation of Agarose Gel For Electrophoresis Of The Isolated Plasmid Dna 1A 0.8% Agarose solution in 1X TBE was prepared..
2. The agarose was poured into Gel casting trays allowed to pre-set along with combs. The Agarose gel was allowed to polymerize and then the combs were removed without breaking the wells. The gel were Submerged into the horizontal electrophoresis tank containing 1X TBE buffer.
B. Electrophoresis of the isolated plasmid DNA:
1. About 5µl of the (eluate) plasmid DNA were taken and 2 l of Sample buffer was added.
2. About 7 µl was loaded into each well along with a marker DNA.
3. Electrophoresis was ran at constant voltage and the DNA was allowed to run in 1x TBE.
4. The gel was removed and placed in a solution of Ethidium Bromide for staining DNA For about 30min. The gel were removed with gloves, rinse with water and placed in the Gel Documentation system / Trans illuminator and visualize the DNA bands in the UV light.
5. DNA concentration was quantified from the standard (ladder) used.
Gel Integrity The integrity of the extracted plasmid was checked on a 1% Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1% agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60°C and stained with 3µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (2 l) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 10µl of each PCR product and loaded into the wells after the 100-3000bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of the molecular weight ladder that was ran alongside experimental samples in the gel.

3.12 Plasmid Curing

Plasmid curing experiment was carried out according to the standard procedures using sodium dodecyl sulphate (SDS) as the curing agent[7]. The isolates were incubated on nutrient broth at 37˚C for 24 hrs. This overnight culture was inoculated into 4.5 ml sterile nutrient broth; 0.5 ml of SDS was added. This was incubated for 48 hrs at 37˚C. Thereafter, 0.5 ml of the broth was added into 4.5 ml sterile nutrient broth. Incubation followed for another 24 hrs at 37˚C.

3.13 Post Curing Antibiotics Susceptibility Testing

Antimicrobial susceptibility test was again carried out. The isolates to which plasmid curing has been done were then subjected to another rounds of Antibiotics susceptibility testing.

Results of antimicrobial resistance were interpreted as either plasmid or chromosomal mediated, depending on if resistance was lost or not.

3.14 Quality Control

All prepared media were checked for sterility for 24 hrs. *E. coli* ATCC 12241 was used as a quality control strain for antibacterial susceptibility testing. The *E. coli* strain was also used as a negative control in the screening and phenotypic confirmatory tests of ESBL-producing Gram-negative rods.

3.15 Statistical Analysis

Data was presented as frequencies and/or percentages. Statistical analysis was performed using SPSS software (version 20). Proportions and the actual number of ESBL and MBL producing isolates were used to describe frequency outputs for categorical variables. The data were presented in tables and graphs.

Endnotes

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Chapter Four

Results and Discussions of Findings

4.1 Results

Wound samples were collected from different wards and it was found that the highest influx of wound samples was gotten from the surgical outpatient (SOP) ward of the hospital complex followed by GOP (General outpatient) while the least came from the ENT, SW4 and E2. The ENT is an outpatient ward, while the SW4 and E2 are inpatient wards in the hospital (Table 1).

Results showed that out of the 370 wound samples, 253 showed positive growth (68.4%) while 117 yielded no growth (31.6%) (Table 2). The positive growth varied in the number of identified isolates. The following were the organism identified: *Pseudomonas aeruginosa, Klebsiella pneumoniae, Klebsiella oxytoca, Enterobacter cloacae, Staphylococcus aureus, Coagulase Negative staphylococcus, Methicillin Resistant Staphylococcus aureus, Candida species ,Stenotophomonas maltophilia, Escherichia coli, Providentia stuarti, proteus mirabilis, proteus vulgaris, Enterobacter cloacae, Acinetobacter baumanii, and Klebsiella aerugenes* (Table 3). A total of 114 of the isolated organisms were Gram positive, coagulase positive and were confirmed *Staphylococcus aureus. Staphylococcus aureus* had the highest score of the entire organism (33.6%) followed by a Gram negative and oxidase positive organism confirming it as *Pseudomonas* aeruginosa *(*17.4%) and next in magnitude was also a Gram negative organism, indole negative *Klebsiella pneumoniae (*16.8%).

Table 2: Number of wound samples obtained from different wards in UCH complex, Ibadan

Name of wards	Wound samples obtained
General outpatient (GOP)	20
South west 4 (SW4)	1
EAST2 (E2)	1
ROC	6
Intensive care unit (ICU)	10
West 3	1
North west1 (NW)1	13
Accident and Emergency (A&E)	9
Surgical out patient (SOP)	92
EAST1 E1	5
Chief Tony Anenih Geriatric center (CTAGC)	8
SDM	2
South East3	4
West west 3 WWE3	3
Gynae	13
South west 1 SW1	10
North west2 Nw2	11
Haem Day care unit HDCU	4
West 4	3
East3	2
ENt	1
Gynae emerg	4
Private suites	6
ROW	6
SW3	7
NW3	5
Staff clinic	10
W3	6
SE1	12
W1	5
Total	**370**

Bench work 2021

Table 3: Growth Culture of Wound Samples Collected From, UCH complex, Ibadan

Wound Sample Culture	Number of samples	Percentage Number
Positive Growth	253	68.4%
No Growth	117	31.6%
Total samples	**370**	**100%**

Bench work 2021

Table 4: identified organisms isolated from wound samples collected from, UCH complex, Ibadan

Organism name	Number isolated	Percentage
Stenotophomonas maltophilia	1	
Staphylococcus aureus	114	
Pseudomonas aeruginosa	59	
Providential stuarti	1	
Proteus vulgaris	3	
Proteus mirabilis	27	
Morganella morgani	1	

Methicillin resistant staph aureus	2
Klebsiella pneumoniae	57
Klebsiella oxytoca	19
Klebsiella aerogenes	1
Eschericia coli	34
Enterobacter cloacae	3
Coagulase negative staph	10
Candida spp	5
Acinetobabcter haemolyticus	1
Acinetobacter baumanii	**1**

Bench work 2021

Table 5: Percentage Resistance Pattern of the Isolates

	GEN	CIP	AUG	FOX	ERY	CRO	CAZ	CXM	TZP	MEM	AK	TGC	CT	VA	DA
A.baumanii	0	100	100	0	0	100	0	0	0	0	0	0	0	0	0
A.haemolyticus	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0
Candida spp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CoN Staphylococcus	40	50	10	20	40	0	0	0	0	0	0	0	0	0	20
E. cloaca	50	50	70	40	0	100	65	100	0	35	0	0	0	0	0
E.coli	40	65	55	35	0	60	9	70	0	0	1	0	0	0	0
K.aerogenes	0	0	0	0	0	0	100	0	100	100	100	0	0	0	0
K.oxytoca	40	47	46	35	100	60	31	80	15	25	65	50	0	0	0
K.pneumoniae	47	38	57	60	0	60	48	63	35	25	25	25	0	0	0
MRSA	100	100	100	100	100	0	0	0	0	0	0	0	0	0	0
M.morganii	0	0	100	100	0	0	0	0	0	0	0	0	0	0	0
P.mirabilis	40	31	26	15	0	50	38	70	20	15	15	0	0	0	0
P.vulgaris	0	0	0	0	0	0	35	0	0	0	50	0	0	0	0
P.stuartii	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0
P.aeruginosa	35	30	35	50	0	50	37	100	13	13	7	11	0	0	0
S.aureus	30	45	30	30	25	55	54	35	35	0	34	0	0	8	15
S.maltophilia	0	0	100	100	0	100	100	0	0	100	100	0	0	0	0

Bench work 2021

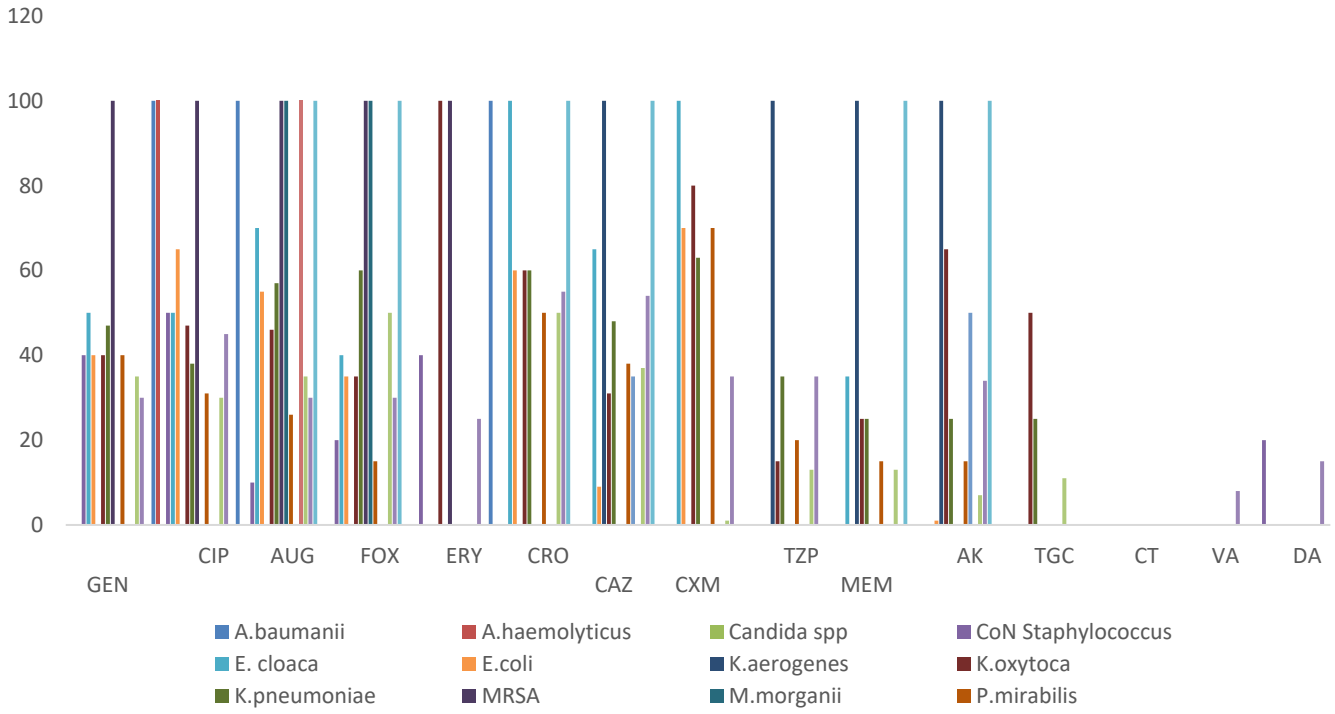

Figure 3**: Percentage Resistance Pattern of the IsolatesBench work 2021**

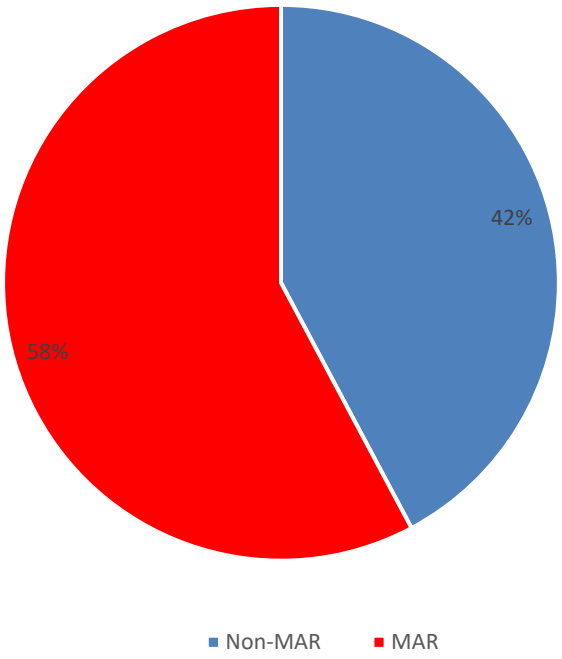

Figure 4: The percentage distribution of Multiple Antibiotic Resistance (MAR) among isolates

Table 6: Percentage distribution of multiple of antibiotic resistance organisms

Organisms	MAR (%)	Non-MAR (%)	Total
Acinetobacter baumanii	1 (100)	0 (0)	1 (100)
Acinetobacter haemolyticus	0 (0)	1 (100)	1 (100)
Candida spp	0 (0)	5(100)	5(100)
Coagulase Negative Staphylococcus	5 (50)	5 (50)	10(100)
Enterobacter cloacae	2 (66.7)	1 (33.3)	3(100)
Escherichia coli	24 (70.6)	10 (29.4)	34(100)
Klebsiella aerogenes	1 (100)	0 (0)	1(100)
Klebsiella oxytoca	14 (73.7)	5 (26.3)	19(100)
Klebsiella pneumoniae	42 (73.7)	15 (26.3)	57(100)
Methicillin Resistant Staphylococcus aureus	2 (100)	0 (0)	2(100)
Morganella morganii	1 (100)	0 (0)	1(100)
Proteus mirabilis	14 (51.9)	13 (48.1)	27(100)
Proteus vulgaris	1 (33.3)	2 (66.7)	3(100)
Providencia stuartii	0 (0)	1 (100)	1(100)
Pseudomonas aeruginosa	27 (45.8)	32 (54.2)	59(100)
Staphylococcus aureus	58 (50.9)	56 (49.1)	114(100)
Stenotophomonas maltophilia	1 (100)	0 (0)	1(100)

Multiple Antibiotics Resistance (MAR) Index of ≥ 0.2 was taken as a significant MAR;
Bench work 2021

In the total isolates, 55.8% were Gram negative bacteria and 37.2% were gram positive bacteria. *Staphylococcus aureus* had 90.5 % of the entire gram positive with coagulase

negative *staphyloccus aureus* (CONS) taking 7.9% and Methicillin resistant *staphylococcus aureus* (MRSA) had the least 1.6%.

The antimicrobial susceptibility testing results are reported as been susceptible (S) or resistant (R) according to the zone of inhibition (zoi) observed on the Mueller Hinton agar. This result showed varied degree of resistance and susceptibility as shown in (Table 4, Figures 1). The organisms demonstrated varied resistance pattern to the different antibiotics used. The most effective first line antibiotics for *Staphylococcus aureus* were Augmentin and Cefoxitin followed by Clindamycin and Gentamicin but the *S. aureus* showed highest resistance to Ciprofloxacin followed by Augmentin. Amongst the Gram-negative organism, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli* had a high susceptibility rate to Meropenem which is a second line antibiotic while the resistance pattern varied, *Pseudomonas aeruginosa* had a highest resistance to Ceftazidime, *Klebsiella pneumoniae* to Ciprofloxacin and *Escherichia coli* had high resistance to both Ciprofloxacin and Augmentin.

From among all the isolates, only 42% demonstrated multiple antibiotic resistance (Figure 2). The organisms which included *Escherichia coli, Klebsiella oxytoca, Klebsiella pneumoniae, Pseudomonas mirabilis, Pseudomonas aeruginosa* and *Staphylococcus aureus* demonstrated high index of multiple antibiotic resistance (MAR) (Table 5). Multiple Antibiotics Resistance (MAR) Index of ≥ 0.2 was taken as a significant MAR.

Beta-lactamase testing was accessed on all multi drug resistant isolates (gram positive and gram negative) using the starch iodide paper test. All Gram-negative isolates were positive except five of the isolates which showed negative results, so also all the Gram positive MAR isolates were negative except three of the isolates which showed positivity. ESBL testing results revealed that among all the isolates with resistance to ceftazidime, only 5% produced ESBL while others (95%) were negative for ESBL production (Figure 3). The MBL testing showed that all the isolates (86%) with resistance to carbapenems were negative except some few (14%) ones which were positive to MBL testing (Figure 4).

Plasmid Isolation and Curing

Two identified MAR organisms with beta-lactamase production, *Klebsiella oxytoca* (ESBL positive) and *Enterobacter cloacae* (MBL positive), were selected for this study and analysed for the detection of plasmid. Their plasmid analysis showed that they both possessed high molecular weight plasmid 1200bp and 1886bp respectively (Figure 7). Thereafter, plasmid curing was carried out and Figure 8 revealed the absence of plasmids in these organisms after plasmid curing. These plasmid-cured organisms when subjected to antimicrobial susceptibility testing

following standard procedures as outlined by CLSI were found to still maintain the same resistance pattern to the antibiotics as when they were not cured. Meaning that their resistances were not plasmid-mediated.

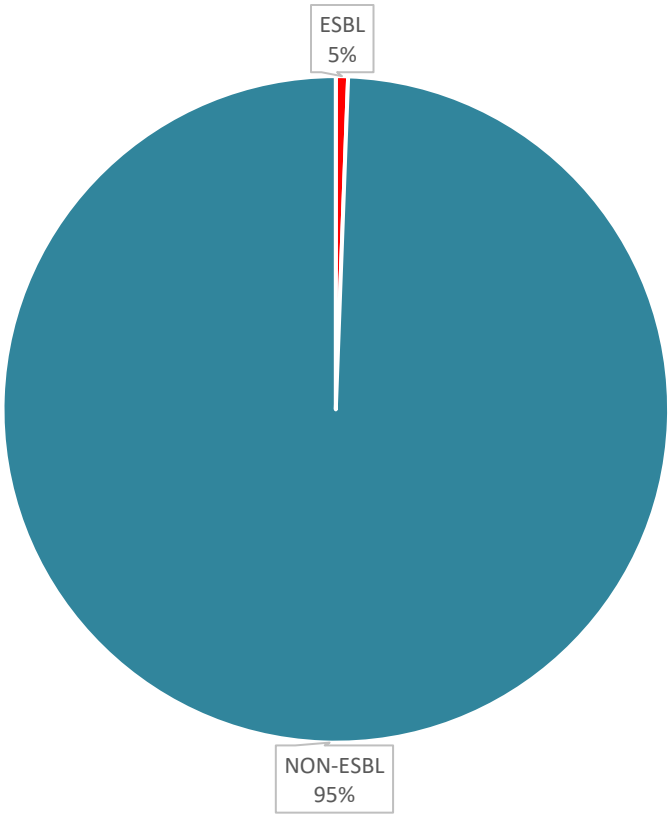

Figure 5. The percentage of ESBL producing organisms in this study

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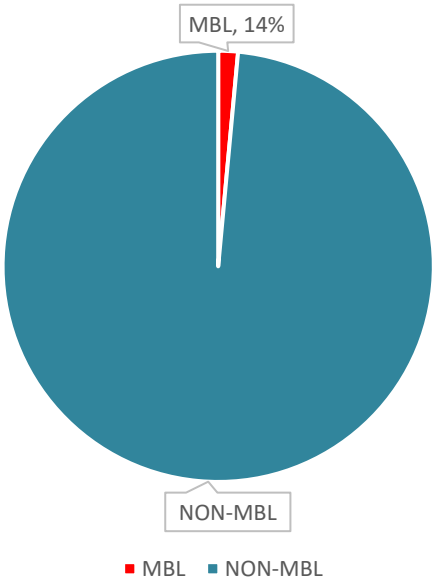

Figure 6. The percentage of MBL producing organisms in this study

Bench work 2021

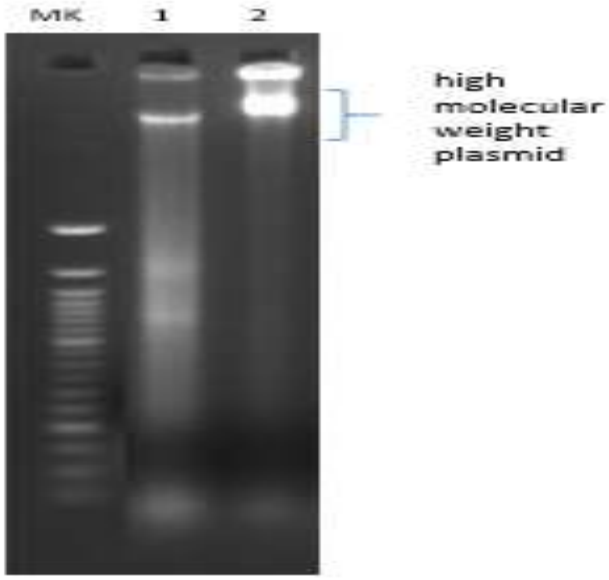

Figure 7 Agarose gels showing the presence of high molecular weight Plasmid
Lane 1 *: Klebsiella oxytoca* (ESBL positive) (1886 bp)and Lane 2: *Enterobacter cloacae* (MBL positive)(1200bp), Mk- Molecular weight

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Figure 8: Agarose gel showing the absence of Plasmid after curing

Bench work 2021

Table 7: Post Plasmid curing sensitivity result

		Antibiotics							
		CAZ	CRO	TZP	AUG	FOX	GEN	CIP	MER
ESBL	*Klebsiella oxytoca*	R	R	R	R	R	S	S	S
MBL	*Enterobacter cloacae*	R	R	R	R	R	S	S	R

key	
CAZ	Ceftazidime
CRO	Ceftriazone
TZP	Piperacilin Tazobactam
AUG	Augmentin
FOX	Cefoxitin
GEN	Gentamicin
CIP	Ciprofloxacin
MER	Meropenem
ESBL	Extended Spectrum Beta Lactamase
MBL	Metallo-beta Lactamase

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4 .2 Discussion

Out of 370 samples in this study 256 (68.5%) showed culture positivity which is in accordance with the study done by Jain *et al*[1] in 2014 which showed a culture positivity of (62%) ,at another time much later than 2014, chaudhary *et al* in 2017 carried out a study also on wound samples revealed a culture positivity of (77.6%) which was quite higher than the one obtained in this study and another study done by Bastola *et al*. showed (48.6%) culture positivity which was lower than in this study[2,3].

In this study, 74.3% of growth positive samples revealed mono-microbial growth(single isolate) which was a bit lower than the one reported by Mama M *et al* [4] in their findings in 2014 that has (91.6%).The poly microbial growth in Mama *et al* study was about(26.5%) which is quite higher than the one obtained in this study, Similarly Acharya J *et al*[5] showed (10.5%) mixed bacterial growth which in tandem with this study. Various studies carried out on wound infection had shown a higher rate of mono-microbial infection than poly-microbial infection (Karki[6]; Komolafe *et al*[7])

The most common bacterial isolates were *staphylococcus aureus* followed by *Pseudomonas aeruginosa* and then *Klebsiella. pneumoniae*. The most predominance of *S. aureus* followed by *pseudomonas* is supported by the study of Chaudhary *et al*[2] in 2017. Kabita *et al* [8] in 2020 in their work on infected wounds in Nepal established that the most common bacterial isolates were *Escherichia coli* followed by *Staphylococcus aureus* which is not the same as obtained in this work. *Pseudomonas aeruginosa* was the highest gram negative bacteria isolated in this study followed by *Klebsiella pneumoniae* and then *Escherichia coli* and it is similar to the work done by Mulugeta K .Azene and Bayeh .A Beyene[9].

This study showed that the most susceptible first-line antibiotic for *S. aureus* was Cefoxitin followed by augmentin and clindamycin this was in contrast to the findings of Mabrouk *et al* in 2016 that obtained an 100% resistant rate to cefoxitin against *S. aureus.*

Resistance to the selected antimicrobials was very high. The overall multiple drug resistance of the isolates in this study was (58%), which was a little bit lower to 66.7% and 68.3% from the study done by Kabitha *et al*[8] and Raza *et al*[10]. But the study did by Pirvanescu H *et al*[11]. obtained only (27.6%) MDR. High resistance of the isolates to antibiotics may be due to practicing self-medication or unavailability of guideline regarding the selection of drugs thereby which lead to inappropriate use of antibiotics [12].

In a study from Uganda, the ESBL producing Gram-negative bacteria in wound swab was 100%, Kateregg *et al*.[13,] this value is quite higher than the 5% obtained in this study. Varying prevalence figures of MBLs have been reported in Nigeria, Yusuf[14] and co-authors while working on some clinical isolates reported 16.7% in *K. pneumoniae*, 16% in Proteus and 13.5% in *E.coli*. They also observed MBL producers in both hospital and community

isolate[15]. Many metallo-betalactamase genes constitute reservoirs in the environment. During the course of evolution, some environmental bacteria (B. cereus, B. anthracis) produced metallo-enzymes to protect themselves against beta-lactams produced naturally by some soild wellingbacteria (Streptomyces sp.) or fungi. The dissemination of carbapenemase genes therefore proceeds in two directions: environmental sources may provide the genetic source of the enzyme, and clinical strains may spread these enzymes both within hospital and into the environment. In the past, all ESBLs were sensitive to carbapenem such as imipenem or meropenem. It was actually used to define ESBLs. Carpabenems were the last resort for infections caused by ESBLs. This is changing as revealed in this study. Though a very low prevalence figure of ESBL resistant to the carbapenem, imipenem was observed, it is emerging. The control of spread is now or never because cumulative experience with ESBL supports the idea that once the prevalence goes beyond a critical level, their eradication from bacterial communities is nearly impossible. Bearing the frightening properties of metallo-enzymes in mind, it is important that a great effort should be made in pursuance of preventive medicine.

This study reveals that the isolates were still maintaining the resistance pattern as when not cured which is in agreement with the work by Costa *et al*[16] in 2014 where the isolate resistance is said to be chromosomally mediated after plasmid curing. Reboucas *et al*[17] in their study reported a rather different dimension where two of the five isolates become totally susceptible to all the antibiotics they were resistant to indicating the resistance was plasmid mediated.

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Chapter Five

Conclusions and Recommendations

List of contribution to knowledge in this study

This study had showcased the various organisms causing wound infections in the University College Hospital as a tertiary hospital, out of these organism some strains are somehow rare and they are not in the majority example of such includes *Stenotophomonas maltophilia, Klebsiella aerogenes, Acinetobacter baumannii.* The most predominant organism however was found to be gram positive followed by the gram negatives.

The antibiotics usually prescribed for most wound infections have been observed becoming increasingly resistant when sensitivity tests were carried out in this study.

The extent to the continual prescription of second and third line group of Antibiotics is also made known as seen in some of the data generated in this study.

This study had established the existence of beta lactamase producing capacity in the isolates obtained from wound infections in the University College Hospital Ibadan (UCH).

The multiple resistance index (MAR) for each of the organism was also highlighted giving an idea of their virulence capabilities

Conclusions

In this study (68.4%) samples Showed culture positivity. Staphylococcus aureus was the common causative agent of wound infection in UCH. Bacteria showed more than 50% resistance towards commonly used first line antibiotic like Cefuroxime, Ceftriaxone, Ciprofloxacin and gentamicin which is a matter of concern. MDR rate was moderately high (58%) and we encountered (1.6%) MRSA, (14%) MBL and (5%) ESBL producer organisms. It is necessary for every medical practitioner to have better knowledge about causative agent of wound infections and their sensitivity pattern to control the wound infection and to minimize the rate of mortality, morbidity and prolonged hospital stay due to wound infections. A proper control of antibiotic usage will prevent the emergence of resistant strains of bacteria.

Gram-positive bacteria were found to be more predominant compared to Gram-negative bacteriain wound infections in this study. Staphylococcus aureus was one of the major pathogens responsible for causing wound infections followed by *pseudomonas aeruginosa*. Besides these,other organisms most frequently encountered in this study were *K.pneumoniae*, *Escherichia coli*., *Acinetobacter baumani*, *Enterobacter cloacae*, CoNS, MRSA, *P. vulgaris*, *P. mirabilis*, *S.maltophilia* , and *K. oxytoca.* Most of the organisms were resistant to ciprofloxacin, augmentin, cefoxitin and ceftazidime. Meropenem has the highest susceptibility rate especially among the gram negative organisms.

Recommendations

It is therefore recommended that individuals and stake holders in implementation of health practices should be made aware of the menance of non-prescription, misuse of antibiotics or self-prescription of these drugs from man to man and as a result a proper advocacy on right and proper administration of drugs in all dosage forms should begin on a high pedestal.

Suggestion for further studies

This will include the detection of other types of beta lactamase apart from ESBL and MBL. In order to advance more and more in learning to the discovery and to better contribute to knowledge , the use of molecular tools to identify and characterise the organism is encouraged.

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NOTICE OF FULL APPROVAL AFTER FULL COMMITTEE REVIEW

Re: Beta Lactamase production and resistance pattern of wound isolates in University College Hospital patients Ibadan

UI/UCH Ethics Committee assigned number: UI/EC/21/0135
Name of Principal Investigator: **Grace I. Soyinka**
Address of Principal Investigator: Department of Biological Sciences
Lead City University

Date of receipt of valid application: 15/04/2021

Date of meeting when final determination on ethical approval was made: N /A

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

This approval dates from **30/07/2021 to 29/07/2022.** If there is delay in starting the research, please inform the UI/UCH Ethics Committee so that the dates of approval can be adjusted accordingly. Note that no participant accrual or activity related to this research may be conducted outside of these dates. *All informed consent forms used in this study must carry the* UI/UCH EC *assigned number and duration of* UI/UCH EC *approval of the study.* It is expected that you submit your annual report as well as an annual request for the project renewal to the UI/UCH EC at least four weeks before the expiration of this approval in order to avoid disruption of your research.

The National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the tenets of the Code including ensuring that all adverse events are reported promptly to the UI/UCH EC. *No changes are permitted in the research without prior approval by the* UI/UCH EC *except in circumstances outlined in the Code. The* UI/UCH EC *reserves the right to conduct compliance visit to your research site without previous notification.*

UI/UCH EC APPROVED COLLEGE OF MEDICINE U.I.

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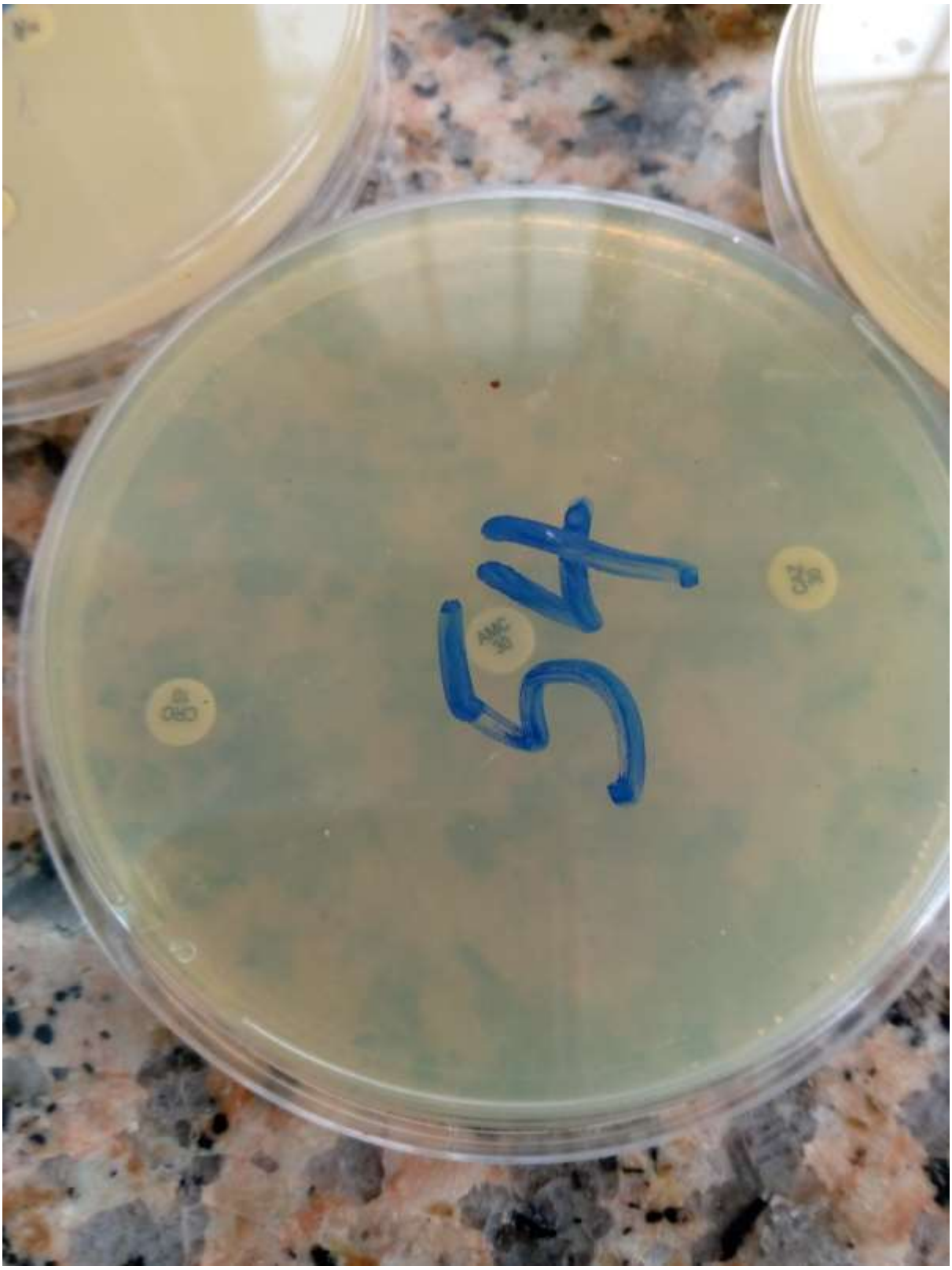
Research Units • Genetics & Bioethics • Malaria • Environmental Sciences • Epidemiology Research & Service • Behavioural & Social Sciences • Pharmaceutical Sciences • Cancer Research & Services • HIV/AIDS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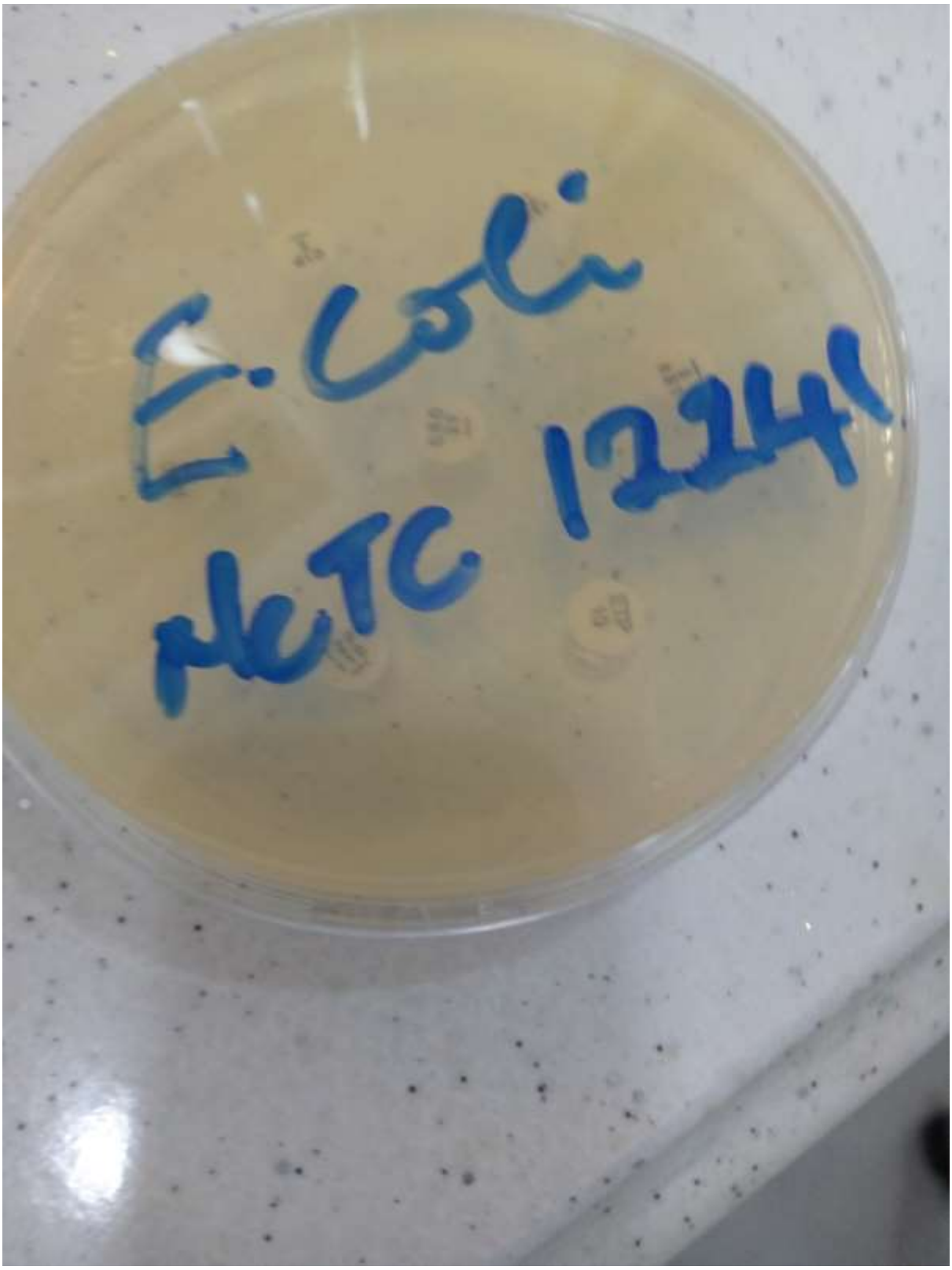
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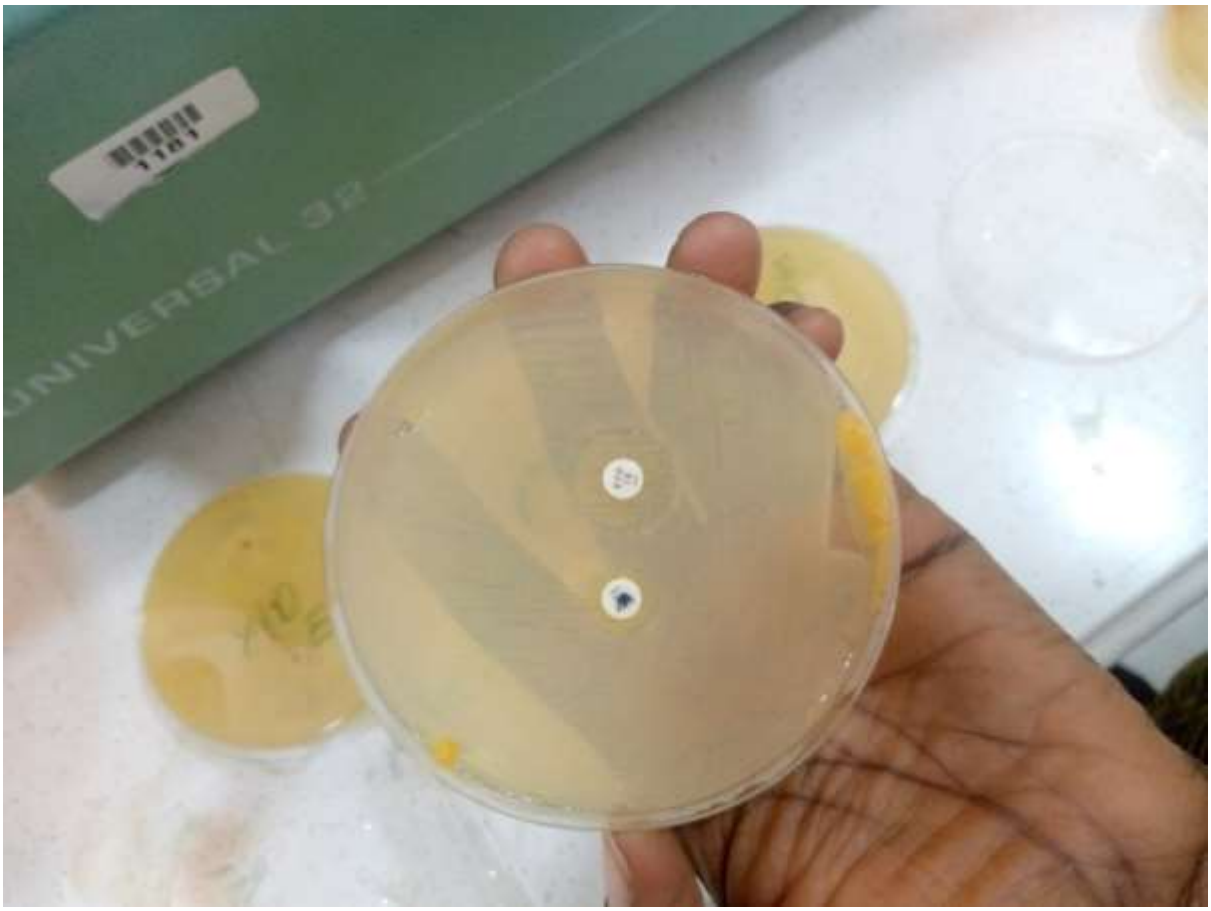

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This is to certify that the thesis by SOYINKA, Grace Iyabo with the Matric Number LCU/PG/001278 in the Department of BIOLOGICAL SCIENCES, Faculty of Basic Medical and Applied Sciences, Lead City University Ibadan, is in full compliance with the approved university format and style.

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