BIOFILM FORMATION AND ANTIMICROBIAL RESISTANCE IN ISOLATES OF *PSEUDOMONAS AERUGINOSA* FROM BENUE STATE UNIVERSITY TEACHING HOSPITAL MAKURDI, NIGERIA

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DECLARATION

I declare that the work presented in this thesis entitled ″Biofilm formation and antimicrobial resistance in clinical isolates of *Pseudomonas aeruginosa* from Benue State University Teaching Hospital, Makurdi Nigeria″ was carried out by me in the Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria under the supervision of Dr. B.A. Tytler andDr. B.O. Olayinka. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma in this institution or any other.

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CERTIFICATION

This thesis entitled ″BIOFILM FORMATION AND ANTIMICROBIAL RESISTANCE IN CLINICAL ISOLATES OF *PSEUDOMONAS AERUGINOSA* FROM BENUE STATE UNIVERSITY TEACHING HOSPITALMAKURDI NIGERIA″ by Josiah Udokwu INEKE, meet the regulations governing the award of the degree of Master of Science in Pharmaceutical Microbiology of the Ahmadu Bello University, Zaria and is approved for its scientific contribution to knowledge and literary presentation.

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This work is dedicated to almighty God for His exceeding grace upon my life and also to my family for their unflinching support.

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ABSTRACT

Pseudomonas aeruginosa is one of the biofilm-forming bacteria implicated in infections such as, urinary tract infections, medical device infections, middle ear infections, cystic fibrosis, wounds etc. that pose serious threat to patients, resulting in prolong hospital stay, morbidity and high mortality, with ultimate economic burden and retardation of antibiotics effectiveness. This study determined the biofilm production potential and antimicrobial resistance pattern in isolates of *Pseudomonas aeruginosa* from Benue State University Teaching HospitalMakurdi, Nigeria. All suspected *Pseudomonas aeruginosa* isolates from samples submitted to the Medical Microbiology Laboratory Unit of the Hospital within a period of six (6) months from August, 2013 to January 2014, were collected, purified and identified using standard microbiological techniques. The distribution of *Pseudomonas aeruginosa* isolates (n-81) confirmed were 32(39.51%) from ear swab, 24(29.63%) from urine, 17(20.98%) wound swab, and 8(9.88%). Majority of the isolates 54 (67%) were biofilm positive. The prevalence of biofilm production by *Pseudomonas aeruginosa* isolates in the different samples evaluated were as follows: 44.4% (ear swab), 27.8% (wound swab), 18.5% (urine) and 9.9%(blood). *Pseudomonas aeruginosa* isolates evaluated in this study were resistant to the antipseudomonad agents: 73% to Ticarcillin-Clavulanic acid; 32% to Ceftazidine; 28% to Ciprofloxacin; 26% to Amikacin; 20% to Gentamicin; and 1 % to Imipenem. Statistical analysis showed that there was no significant difference in antibiotics resistance pattern of biofilm producers and non-biofilm producing *Pseudomonas aeruginosa* isolates. In this study, 36 (44.4%) of the isolates were multidrug resistant (MDR). Resistance to Ciprofloxacin, an indicator antibiotic for multi-drug resistant (MDR) isolates was twenty eight percent (28%). Screening for quinolone resistant gene qnr in the study showed that 4(21.1%) carriedqnrB quinolone resistant genes.

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ABBREVIATIONS

A.B.U: Ahmadu Bello University ABUTH: Ahmadu Bello University Teaching Hospital

AHL: Acyl Homoserine Lactone AIs: Author Inducers

AST: Antibiotic Susceptibility Testing

BSAC:British Society for Antimicrobial Chemotherapy

BSUTH: Benue State University Teaching Hospital

CDC:Center for Disease Control and Prevention

CEH: Center of Ecology and Hydrology.

CLSI: Clinical and Laboratory Standards Institute CSP: Competence Signal Peptides

CF: Cystic Fibrosis

DNA: Deoxyribose Nucleotide

EDTA: Ethylene Diamine Tetra Acetate

ECDC: European Centre for Disease Prevention and Control

ELISA: Enzyme Linked – Immunosorbent Assay

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FDA: Food and Drug Administration

IUPAC: International Union of Pure and Applied Chemistry

MAR: Multiple Antibiotic Resistances

MDR: Multiple Drug Resistance MFG: Manufacturing

MICs: Minimum Inhibitory Concentrations

MRSA: Methicillin Resistant *Staphylococcus aureus*MSSA: Methicillin Sensitive *Staphylococcus aureus*

NCEZI: National Center for Emerging and Zoonotic Infectious Diseases

NCBI: National Center for Biotechnology Information

NCCLS: National Committee For Clinical Laboratory Standards

OD: Optical Density PCR: Polymerase Chain Reaction.

PCR: Polymerase Chain Reaction

PMNs: Polymorphonucleotides.

Qnr: Quinolone Resistance

QS: Quorum Sensing

RNA: Ribose Oxynucleotide r

TAE: Tris-acetate

TSI: Triple Sugar Iron

UK: United KIngdom

USA: United State of America

USP: United State pharmacopoeia

UTIs: Urinary Tract Infections

UV: Ultra Violet light

WHO: World Health Organisation

µl: Microliters

µm: Microgram.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of study

Biofilm which is an assemblage of microbial cells irreversibly associated with a surface is a prevailing bacterial life style, where the bacteria are usually enclosed in a matrix of polysaccharide material (Costerton, 2003;StanleyandLazazzera2005). Large molecular weight exopolysaccharides are often components of the biofilm matrix (Branda *et al*., 2005). Depending on their locations, biofilms can either be beneficial or detrimental to the environment. For instance, the biofilms found on rocks and pebbles underwater of lakes and ponds are an important food source for many aquatic organisms. Biofilms that develop on the interiors of water pipes might cause clogging and corrosions (Litzler, *et al*., 2007), while those on indwelling medical devices, medical implants materials and tissuesreleases antigens which stimulatethe production of antibodies, that can causeimmune complex damage to surroundingtissues (Wolcot *et al*., 2008*),* or cross-infections in hospital patients (Abreu*et al.*, 2013). Infections resulting from pathogenic biofilms are characterized by a chronic or recurrent nature and are highly resistant to conventional treatments (Hong*et al.*, 2014). Life in a microbial biofilm offers considerable advantages over the planktonic mode of growth to the microorganism. The biofilm provides protection for embedded cells from external stress such as antibacterial agents and human defense mechanisms in cases of biofilms formed in the human body (Coenye *et al.*, 2011). Biofilms has also been reported to impair cutaneous wound healing and reduce topical antibacterial efficiency in infected skin wounds (Davis *et al.*, 2008). Microorganisms in established biofilms can tolerate antimicrobial agents at concentrations of 10 to1000-times than needed to kill genetically equivalent planktonic bacteria, via reduced antimicrobial diffusion through the biofilms to

the bacteria (Wood *et al*., 2006), reduced growth rates,(Keren *et al*., 2004) andplasmids mediated resistance (Molin and Tolker-Nielsen, 2003). Biofilms are also extraordinarily resistant to phagocytosis, making biofilms extremely difficult to eradicate from living hosts, (Lewis, 2001; Wolcot *et al*., 2008) and the bacteria within these biofilms exhibit differences in antibiotic resistance (Bagge, 2004b).Eighty percent (80%) of bacterial infections involve biofilms (Costerton, 2004) and *Pseudomonas aeruginosa* is notorious for biofilm formation (Sauer *et al.* 2004). In fact, *Pseudomonas aeruginosa,* aGram-negative bacterium, rank among the clinically most significant organisms that form biofilms, and one strain PA01, has become the model organisms for studying Gram-negative biofilms(Bollinger*et al*., 2008). *Pseudomonas aeruginosa b*iofilms contribute toits persistence in a variety of diseases (Matthew *et al.*, 2011), as a result of periodic release from the said biofilm foci (Kerksiek, 2008).*Pseudomonas aeruginosa* biofilms have been shown to be an important factor in the pathogenesis of the bacterium in ventilator-associated pneumonia, urinary and peritoneal dialysis catheter infections, bacterial keratitis, otitis externa and burn wound infections (Aleksandra*et al*., 2013). Although *Pseudomonas aeruginosa* colonization usually precedes infections, the exact source and mode of transmission of the pathogen are often unclear because of its ubiquitous presence in the environment. If colonization occurs in critical body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal. *Pseudomonas aeruginosa* is resistant to a number of antimicrobial agents despite improvements in antibiotic therapy, involving multiple classes of antimicrobial agents and ever growing multi-drug resistant (MDR) strain has been reported widely (Agarwal *et al*.,2005;Smith *et al*., 2012). Some of the resistancemechanisms to antimicrobials in *Pseudomonas aeruginosa* arecell wall impermeability, multi-drug efflux pump system, and a chromosomal AmpC beta-lactamase

(Olayinka *et al.*, 2004;Henrichfreise*et al*., 2007). These resistance factors can be transferred through the acquisition of plasmids, trasposons or intergrons leading to adaptive resistance to beta-lactam and aminoglycosides through the production of beta-lactamases, aminoglycosides modifying enzymes and biofirm formation (Farzam *et al*., 2011). Although research on biofilms has surged over the past few decades (Proal, 2008), few of them focus on biofilm formation and antimicrobial resistance, especially in Makurdi, Nigeria. Antibiotic resistance is a major health concern because it increases healthcare costs, causes people to stay in hospital for longer, results in treatment failures, and sometimes death (Stuart *et al.*,2002;ECDC, 2012), it therefore become imperative to carry out this study. This will help to expand the treatment options available to patients with chronic biofilm infections.

1.2 STATEMENT OF RESEARCH PROBLEM

Biofilm provide survival advantages for micro-organisms as resistance to antimicrobials, protection against antiseptics, disinfectants, bacteriophages, host's immune system, among other (Flach*et al*., 2005). *Pseudomonas aeruginosa* is an opportunist bacterium, invasive and toxigenic, a major nosocomial pathogen implicated in endocarditis, meningitis, urinary infections associated with catheters and skin infections, especially in critical intensive care unit patients (Bonomo and Szabo 2006). *Pseudomonas aeruginosa* was the second most common cause of nosocomial pneumonia, the third most common cause of nosocomial urinary tract infections, and the seventh most common cause of nosocomial bacteraemia (NNIS, 2004).All *Pseudomonas aeruginosa* infections are treatable and potentially curable, but due to the fulminant nature of *Pseudomonas aeruginosa* infections seen, in bacteremic pneumonia, sepsis, burn wound infections, and meningitis, extremely high mortality rates are inevitable (Obritsch *et al.*, 2004). *Pseudomonas aeruginosa* is intrinsically resistant to a

number of antimicrobial agents, including multiple classes of antimicrobial agents (Smith *et al*., 2012). The bacteria that forms biofilm, such as *Pseudomonas aeruginosa* experience significant changes in about 800 genes and protein expression, as well as metabolic activity (Sauer *et al.*, 2004), and also resistance to antimicrobial therapy (Anderson*et al.*, 2008). Therefore this study tries to evaluate biofilm production and antimicrobilal susceptibility patterns of isolates of*Pseudomonas aeruginosa* in a University Teaching Hospital.

1.3 JUSTIFICATION

Bacterialbiofilms have been consistently described as being more resistant to biocides and antibiotics than planktonic cells (Bisset *et al*. 2006; Smith and Hunter 2008).Benue State University Teaching Hospital (BSUTH) like any other Teaching Hospital in Nigeria are faced with management of infectious diseases associated with biofilm forming microorganisms like, *Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Staphyloccocus aureus,Haemophilus influenzae*and*Klebsiella spp,* (Drenkard, 2003;Wood, 2009;Saravanan*et al*.,2012; Vuotto*et al*.,2014). *Pseudomonas aeruginosa* an epitome of opportunistic nosocomial pathogen that forms biofilms is reported to cause wide spectrum of infections and leads to substantial morbidity and mortality (Gaynes *et al*., 2005; Rossolini and Mantengoli,2005). It causes between 10% and 20% of infections in most hospitals (Anton and David, 2010). The fatality rate of patients infected with *Pseudomonas aeruginosa* has been reported to be near 50% and mortality of 70% is reported associated with nosocomial pneumonia by *Pseudomonas aeruginosa* (Obritsch *et al.*, 2004). Bassetti and Viscoli, (2008) reported that despite advances in antimicrobial therapy, *Pseudomonas aeruginosa* infection remains associated with high mortality ranging of 18% -61%. *Pseudomonasaeruginosa* has been reported Philip *et al.*, (2005; 2009)as aproblematic drug-

resistant pathogen with multidrug-resistant property and can acquire antibacterial resistance, even during the course of therapy. The resistance of *Pseudomonas aeruginosa* in upper respiratory tract infections (UTIs) against antibiotics was shown to be extremely high (Safar *et al.,* 2009). In a study by Olayinka*et al.,* (2004), *Pseudomonas aeruginosa* was 100% resistant to ampicillin and ampicillin/ cloxacillin, 97.8% to chloramphenicol, ofloxacin 82.6%, perfloxacin 58.7%, streptomycin 23% and 7.7% to gentamycin. Jombo *et al.,* (2008), showed that all the *Pseudomonas aeruginosa* isolates screened were 100% resistant to penicillin, cloxacillin, tetracycline, nitrofurantoin and nalidixic acid. Most of the Studies carried in Nigeria, as seen above focus on antibiotics resistance pattern of planktonic *Pseudomonas aeruginosa*,studies on prevalence of antibiotic resistance among biofilm producing *Pseudomonas aeruginosa* are not common.Hence, the need to evaluate biofirm formation and antibiotic resistance pattern of *Pseudomonas aeruginosa* isolates from clinical samples from BSUTH. This will provide expanded options for management and treatment of *Pseudomonas aeruginosa* infected patients in Benue State.

1.4 AIM OF THE STUDY

The aim of the study is determine the biofilm forming potentials and antibiotic susceptibility pattern of isolates of *Pseudomonas aeruginosa* from Medical Laboratory of Benue State University Teaching Hospital (BSUTH), Makurdi.

1.5 SPECIFIC OBJECTIVES

- To isolate and identify *Pseudomonas aeruginosa* in samples from (BSUTH), Makurdi.
- To determine the biofilm forming potentials of the isolates.
- To quantify the biofilm produced by isolates of *Pseudomonas aeruginosa*.

- To determine the antibiotic susceptibility pattern of the isolates of *Pseudomonas aeruginosa*
- To determine MIC of ciprofloxacin against isolates of *Pseudomonas aeruginosa; and*
- To relate biofilm forming potential to the antibiotic resistance pattern.

1.6 RESEARCH HYPOTHESIS

1.6.1 Null Hypothesis

There is no incidence of biofilm-forming, multidrug-resistant *Pseudomonas aeruginosa in* Benue State University Teaching Hospital, Makurdi.

1.6.2 Alternate Hypothesis

There is an incidence of biofilm-forming, multidrug-resistant *Pseudomonas aeruginosa in* Benue State University Teaching Hospital, Makurdi.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Bacterial biofilms

Biofilm is an assemblage of microbial cells that is irreversibly associated with a surface and usually enclosed in a matrix of polysaccharide material. Biofilm is composed primarily of

microbial cells and extracellular polymeric substance (EPS) (Kokare *et al*., 2009). Biofilm are ubiquitous and nearly every species of microorganisms adhere to surfaces and can contain many different types of microorganisms such as bacteria, archaea, protozoa, fungi, and algea (Lazar, 2003; Marsh, 2006).Van Leeuwenhoek, using his simple microscopes, first observed microorganisms on tooth surfaces and can be credited with the discovery of microbial biofilms (Narasimha, 2013). Biofilm-associated organisms differ from their planktonic (freely suspended) counterparts with respect to the genes that are transcribed (Donlan, 2002; Anderson*et al.,* 2008).Bacteria biofilmsare important clinically because they exhibit recalcitrance to antimicrobial compounds and persistence of infections in spite of sustained host defenses (Hall-stooldey *et al.,* 2009; Lenz *et al.,* 2008). Biofilms have been found to be involved in a wide variety of microbial infections in the body, by one estimate 80% of all infections(Costerton, 2004;Biel, 2010).The bacterial that forms biofilm experience significant changes in gene and protein expression, as well as metabolic activity which confers resistance to antimicrobial therapy (Anderson*et al.,* 2008). Microbial biofilms have been subjected to intense study during the last decade mainly for two reasons. First, it is of basic scientific interest to understand how bacteria form and live in multicellular communities. Second, biofilm formation causes considerable problems in medical and industrial settings, because bacteria in biofilms can resist antibiotic treatment, host immune responses, and biocide treatment (Morten *et al.*, 2010**)**. Biofilm formation is also recognised as causing or exacerbating numerous chronic infections (Hall-Stoodley and Stoodley, 2009). These include periodontitis, medical device-related infections, cystic fibrosis pneumonia, chronic urinary tract infections (UTI), recurrent tonsillitis, chronic rhinosinusitis, chronic otitis media (OM) and chronic wound infections (Hall-Stoodley and Stoodley, 2009). In considering the importance of biofilm in clinical practice, this review

focuses on elucidation of biofilm nature and how its mechanisms contribute to antimicrobial resistance, especially the antipseudomonad agents.

2.2Biofilm Structure

Biofilms are composed primarily of microbial cells and extracellular polymeric substances (EPS) and EPS may account for 50% to 90% of the total organic carbon of biofilms (Wang*et al*., 2015). EPS is considered to be the primary matrix material of the biofilm and vary in chemical and physical properties, but it is primarily composed of polysaccharides (Flemming *et al.*, 2010;Wang*et al*., 2015). The biofilm matrix also consists of polymers secreted by microorganisms within the biofilm, absorbed nutrients and metabolites, and cell lysis products; all major classes of macromolecules (proteins, polysaccharides, and nucleic acids) are present in addition to peptidoglycan, lipids, phospholipids, and other cell components (Sutherland, 2001; Aleksandra, 2013). EPS is also highly hydrated because it can incorporate large amounts of water into its structure (Flemming and Wingender, 2010).

2.2.1 Biofilm Formation

Biofilm formation can be divided into three main stages: early (attachment, intermediate(growth), and mature (detachment), (Stoodley and Dirckx, 2003; Marc*et al*., 2014). During the early stage, planktonic cells swim along a surface often using their flagella mode of movement, they can also be transferred passively with the body fluids. Next, the microorganisms makecontact a surface resulting in the formation of a monolayer of cells, (Figure 2.1). At this stage, the bacteria are still susceptible to antibiotics, for example perioperative antibiotic prophylaxis (Kostakioti *et al*., 2013). The intermediate stage involves irreversible binding to the surface, multiplication of the microorganisms, and the formation of microcolonies (Høiby, 2010). During this stage, the polymer matrix is produced around the microcolonies and generally consists of a mixture of polymeric

compounds, primarily polysaccharides. The third step of biofilm formation is the formation of a mature community with mushroom-shaped microcolonies (Costerton, 2003;Kostakioti *et al*., 2013) (Figure 2.2). During this stage, the biofilm structure can be disrupted, and microbial cells can be liberated and transferred onto another location/surface, causing expansion of the infection (Høiby, 2010).

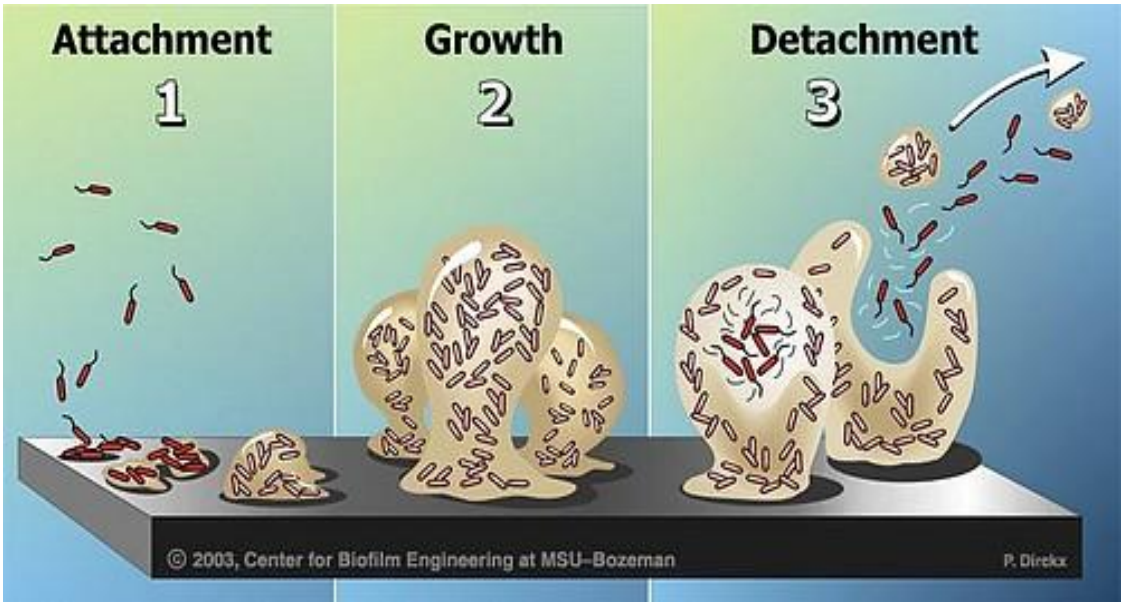


Fig 2.1:Steps in Biofilm Development (Adapted fromStoodley and Dirckx. 2003)

Life in bacteria biofilm is regulated at different stages through diverse mechanisms, among which the best studied, is quorum sensing (QS) (Joint*et al*., 2007). The QS mechanism involves the production, release, and detection of chemical signaling molecules, which permit communication between microbial cells. The QS process regulates gene expression in a cell-density-dependent state (Donlan, 2002; Hooshangi *et al*., 2008). There are three well-defined groups of signaling QS molecules in bacteria: oligopeptides, acyl homoserine lactones (AHLs) and autoinducer-2 (AI-2) (Donlan, 2002; Hooshangi *et al*., 2008). Gram-

positive bacteria predominately use oligopeptides as a communication molecule, and AHLs are specific for Gram-negative bacteria (Reading *et al*, 2006). AI-2 is reported to be a universal signaling molecule that is used for both inter species and intraspecies communication (Donlan, 2002). This form of communication allows the cells to regulate gene production which results in control of certain cell functions (Uedal and Wood, 2009) Mature biofilms contained living bacteria and are structurally, chemically, and physiologically heterogeneous. The principal architectural elements observed by electron microscopy can represent useful morphological clues for identifying bacterial biofilms in vivo (Marc *et al* 2009). Mature biofilms can also harbor large numbers of cells that are inactive and not growing (Erin *et al.*, 2006).

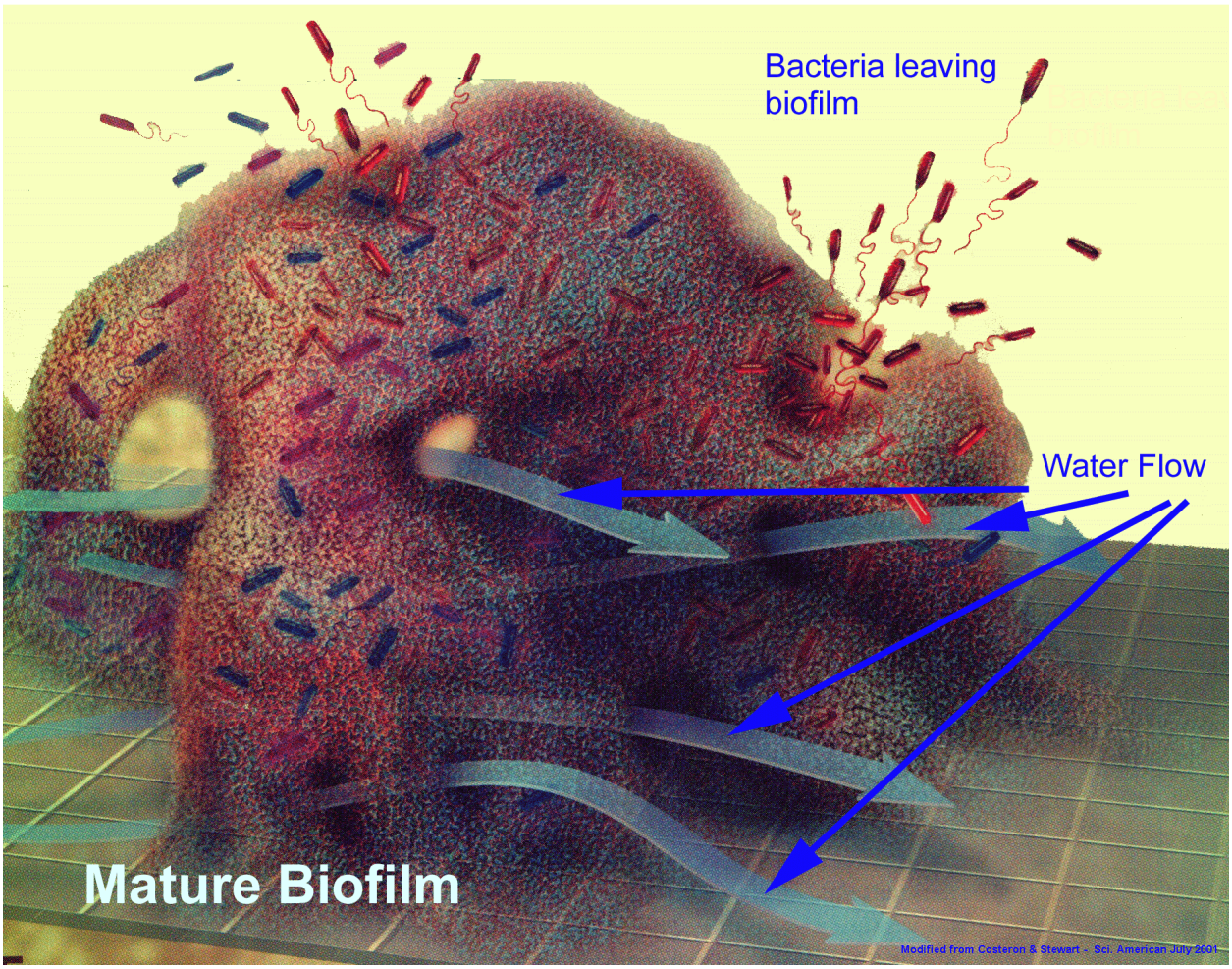


Fig. 2.2: A Mature biofilms (Adapted fromStoodley and Dirckx, 2003)

Pseudomonas aeruginosa a Gram negative organism is a key opportunistic pathogen characterized by its biofilm formation ability and high-level multiple antibiotic resistance (Zhang *et al.*, 2008).

2.3*Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a member of the Gamma Proteobacteria class of Bacteria. It is a Gram-negative, aerobic, non-spore forming, rod-shape bacterium with unipolar motility (Ryan and Ray 2004) and belongs to the bacterial family Pseudomonadaceae (Todar, 2008). *Pseudomonas aeruginosa* was first described as a distinct bacterial species at the end of the nineteenth(19th) century, after the development of sterile culture media by Pasteur. In 1882,

the first scientific study on *Pseudomonas aeruginosa*, entitled "On the blue and green coloration of bandages," was published by a pharmacist named Carle Gessard. *Pseudomonas aeruginosa* has many strains, including *Pseudomonas aeruginosa* strain PA01, *Pseudomonas aeruginosa* PA7, *Pseudomonas aeruginosa* strain UCBPP-PA14, and *Pseudomonas aeruginosa strain* 2192 (NCBI, 2007). Most of these were isolated based on their distinctive grapelike odor of aminoacetophenone, pyocyanin production, and the colonies'structure on agar media *Pseudomonas aeruginosa* normally lives in moist environments anddoes not ferment carbohydrates. It is positive in the indophenols oxidase test, and is Simmon's citrate positive, l-arginine dehydrolase positive, l-lysine decarboxylase negative, and l-ornithine decarboxylase negative (Kiska and Gilligan, 2003). Although classified as an aerobic organism, *Pseudomonas aeruginosa* is considered by many as a facultative anaerobe as it is well adapted to proliferate in conditions of partial or total oxygen depletion. *It*can achieve anaerobic growth with nitrate as a terminal electron acceptor, and in its absence it is also able to ferment arginine by substrate-level phosphorylation (Palmer *et al*., 2007). *Pseudomonas aeruginosa* an ubiquitous microorganismcatabolizes a wide range of organic molecules including organic compounds such as benzoate (Shyamala and Pavani, 2014). It grows on many solid media and at both 37°C and 42°C. *Pseudomonas aeruginosa* has minimal nutrition requirements while being able to use several organic compounds for growth (Todar, 2008).

2.3.1 *Pseudomonas aeruginosa* Habitats

Pseudomonas aeruginosa is typically found in moist environments and can be found in water and soil as well as on fruits, vegetables, and flowers (Kiska and Gilligan, 2003). Hence, those at high risk of serious infections are adviced to exercise caution on the consumption of fruits and uncooked vegetables (Paterson, 2006). Other examples of moist

environments, that can be colonised by *Pseudomonas aeruginosa*solutions include, illicit injectable drugs, and the inner soles of sneakers (Kiska and Gilligan, 2003). In the hospitals include swimming pools, hot tubs, contact-lens environment, where the most serious infections occur, *Pseudomonas* can be spread on the hands of healthcare workers or by equipment that gets contaminated and is not properly cleaned (CDC, 2013). It is found in aqueous solutions used in medical care (for example, irrigation fluids, eye drops, dialysis fluids, and even soaps and disinfectants) may also become contaminated with the organism (Morrison and Wenzel 1984; Paterson, 2006) Pseudomonas *aeruginosa* may also be found in the aerators and traps of sinks, in respiratory therapy equipment, on inadequately cleaned bronchoscopes (Srinivasan *et al* 2003). *Pseudomonas aeruginosa*is rarely found as part of the microbial flora of healthy individuals (Paterson, 2006). In the rare circumstance that colonisation of healthy individuals occurs, the sites of colonization include the gastrointestinal tract and moist body sites such as the throat, nasal mucosa, axillary skin, perineum and chronic wounds(Rossolini and Mantengoli, 2005).

2.3.2 Infections caused by *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa*is an opportunistic human pathogen. It is "opportunistic" because it seldom infects healthy individuals. Instead, it often colonizes immunocompromised patients, like those with cystic fibrosis, cancer, or AIDS (Todar, 2008; CDC, 2013). it can involve the following parts of the body, Respiratory tract: pneumonia (Bjarnsholt *et al*.,2009), Bloodstream;bacteremia, Heart; endocarditis, (Scott *et al*.,2005) ,CNS: meningitis, though rare, (Lu *et al* 1999), Ear ; otitis externa and media, (Sander, 2001; *CDC 2013),* Eye; corneal ulceration, endophthalmitis, (Mendelson *et al*.,1994; Boyle *et al*.,2001; *CDC, 2013*), Bones and Joints; osteomyelitis, (Brouqui*et al*., 1995), and GI tract ; diarrhea, enteritis, enterocolitis, Urinary tract and skin infections (Driscoll *et al.,* 2007). Mortality

rates ranging from 40% to more than 60% have been reported in bacteraemic nosocomial pneumonia and in ventilator associated pneumonia (Rello *et al.*, 1997; CDC, 2013). In a single center review of patients with culture positive,Health care-associated pneumonia(HCAP), *Pseudomonas aeruginosa* was responsible for 25.5% of cases (Micek *et al.*, 2007).*Pseudomonas aeruginosa*is a rare cause of true community acquired pneumonia.*It*is well known as a cause of chronic infection of the lungs and airways in patients with cystic fibrosis. Patients with cystic fibrosis (CF), an autosomal recessive genetic disorder, have chronic cough with episodes of deterioration in respiratory status. *Pseudomonas aeruginosa*, along with Staphylococcus aureus, are responsible for most respiratory infections in patients with cystic fibrosis (Maria *et al*., 2003), Patients with bronchiectasis may also have chronic colonisation with *Pseudomonas aeruginosa*, punctuated by exacerbations of respiratory infection. Patients with neutropenia (typically as a result of chemotherapy) are classically regarded as at high risk of pseudomonal bacteremia. However, the proportion of neutropenic patients who develop *Pseudomonas aeruginosa* bacteremia is reported quite low. Others at risk of bloodstream infection with *Pseudomonas aeruginosa* include those undergoing surgical procedures, those with extensive burns, and patients with urinary tract colonisation with the organism (Todar, 2008). A proportion of cases of post-neurosurgical meningitis are due to *Pseudomonas aeruginosa*, however, the organism is distinctly unusual as a cause of community-acquired meningitis. *Pseudomonas aeruginosa* may cause a rapidly progressive keratitis, which can lead to a loss of vision in the affected eye (Jeng *et al*., 2010). Occasionally, *Pseudomonas aeruginosa* may cause significant bone and joint infections, which may be secondary to bacteremia. *It* is the classic cause of malignant otitis externa, whereby extension of ear infection occurs to cartilage of the ear, the middle ear, and eventually the temporal bone

(Mayers, 2009**)**. Finally, *Pseudomonas aeruginosa*is a rare cause of infective endocarditis and the classic cause of "swimmer's ear" otitis externa due to pseudomonal infection of moist, macerated skin of the external ear canal (Mayers, 2009). Several factors account for the success of *Pseudomonas aeruginosa* to cause infections; among many is the abilities to utilise a broad spectrum of nutrients; grow in hospital drains, sinks and even disinfectant solutions, multiple virulence factors that can cause damage to the host (Matheson *et al.*, 2006; Yates *et al.*,2006; Zulianello *et al.*, 2006). Furthermore, many strains have acquired resistance factors and propensity to form biofilms in infections.

2.3.3 Antibiotic resistance in *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa*shows inherent resistance to antimicrobial agents through a variety of mechanism (Slama, 2008); decreased permeability of the outer membrane, (Kerr and Snelling, 2009), efflux systems which actively pump antibiotics out of the cell (Mauldin *et al.*, 2010) and production of antibiotic-inactivating enzymes (Moore and Flaws, 2011).

Outer membrane permeability

The outer membrane of Gram-negative bacteria is a barrier which prevents large hydrophilic molecules to pass through it. Aminoglycosides and colistin interact with lipopolysaccharides changing the permeability of the membrane in order to pass whereas beta-lactams and quinolones need to diffuse through certain porin channels. Bacteria produce two major classes of porins: general; which allow almost any hydrophilic molecule to pass (Hancock *et al*., 2004) and specific; which have binding sites for certain molecules, allowing them to be oriented and pass in the most energy-efficient way (Tamber *et al*.,2006). Most bacteria possess lots of general porins and relatively few specific ones.

However, the exact opposite occurs for *Pseudomonas aeruginosa* that expresses mainly specific porins (Hancock *et al*., 2004).

Efflux systems

*Pseudomonas aeruginosa*expresses several efflux pumps that expel drugs together with other substances out of the bacterial cell. These pumps consist of three proteins (Slama, 2008): a protein transporter of the cytoplasmatic membrane that uses energy in the form of proton motive force, (Kerr and Snelling, 2009), a periplasmic connective protein, and (Mauldin *et al*., 2010) an outer membrane porin (Hancock *et al*., 2004). Most antibiotics are pumped out by these efflux systems therefore their first two components are named multidrug efflux (Mex) along with a letter (e.g. MexA and MexB) (Lister *et al*., 2009; Strateva and Yordanov, 2009).

Antibiotic-inactivating enzymes

*Pseudomonas aeruginosa*produce chromosomal-encoded and inducible AmpC beta-lactamases. These are cephalosporinases that hydrolyze most beta-lactams. Other endogenous beta- lactamase produced by *Pseudomonas aeruginosa*is the class D oxacillinase PoxB (Girlich *et al.,* 2004; Kong *et al*., 2005).

Plasmid-mediated Quininolone resistance

Pseudomonas aeruginosa'sintrinsic resistance makes it resistant to many antibacterial agents, such that treatment of *Pseudomonas aeruginosa*infections usually present with difficulty. Quinolones; ciprofloxacin are effective antibiotics for the treatment of *Pseudomonas aeruginosa*infections and resistance to quinolones is often a result of chromosomal mutations and by the effect of efflux pumps (Coban *et al*., 2011). The

emergence of plasmid-mediated quinolone resistance (PMQR) has been reported in members of Enterobacteriaceae family. The PMQR genes have be found more frequently in isolates of Enterobacteriaceae than *Pseudomonas aeruginosa* (Ogbolu *et al*., 2011, Rodríguez-Martínez *et al*., 2011, Yang *et al.*, 2008). The genes responsible for this resistance is called qnr (Coban *et al*., 2011) and are carried on mobile DNA molecules called plasmid.Plasmids are *replicons*, capable of replicating autonomously within a suitable host and can be transferred between bacterial hosts through a process known as bacterial conjugation. **Plasmids**are DNA molecules, generally circular, which can replicate in Bacterial, Archaeal and Eukaryotic cells. They take advantage of the cellular environment of the cell but can also carry a rich diversity of genes which can be beneficial for the cell. Some plasmids confer the ability to degrade organic compounds and to fix nitrogen. Other plasmids carry antibiotic resistance genes and their spread in pathogenic bacteria is of great medical significance (CEH, 2013).

2.4 Biofilms in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an important opportunistic pathogen and causative agent of emerging nosocomial infections. It forms biofilm and is a model organism for the study of diverse bacterial mechanisms that contribute to bacterial persistence (Stefan, 2008).These biofilms are complex bacterial communities that adhere to a variety of surfaces, including metals, plastics, medical implant materials and tissues. The biofilms are characterized by "attached for survival" because once they are formed, they are very difficult to destroy. The formation of biofilms facilitates chronic bacterial infections and reduces the efficacy of antimicrobial therapy (Hall-Stoodley and Stoodley, 2009; Parsek, 2003;Matthew*et al.*, 2011). It has been reported that bacterial biofilms may impair cutaneous wound healing and reduce topical antibacterial efficiency in healing or treating infected skin wounds (Davis *et*

al 2008). Thus, this organism causes persistent infections, more as a result of periodic microcolonies released from the biofilm foci (Kerksiek, 2008). The view that the formation of biofilms is mediated by a number of mechanical, biochemical and genetical tools, is similar to physiochemical interactions such as cell surface hydrophobicity (long-range noncovalent interactions, defined as the attraction among apolar or slightly polar cells or other molecules immersed in an aqueous solution), charge, roughness and chemical constitution of the material, which have been studied to mediate bacterial adhesion to the surface during biofilm formation (Lorite *et al*.,2013). Some studies on *Pseudomonas aeruginosa* suggested that adhesion was dependent in pyrolytic carbon surface free energy and roughness (Litzler *et al.*, 2007). The Pili and flagella are generally involved as adhesive structures to help in attachment to the biotic or abiotic surfaces (Gohl *et al.*, 2006; Luke, 2007). Southey-Pillig *et al.*, (2005) reported biofilm formation to be influenced by large-scale changes in protein expression over time. Biofilm formation is said to be under genetic control, the genetics of biofilm formation in *Pseudomonas aeruginosa* and other important human pathogens of otitis media and urinary tract infections have also been documented (Bagge *et al.*, 2004b). The genes that have been identified as crucial to biofilm formation include those that regulate or express surface adhesion proteins, genes encoding pili, flagella or extracellular matrix material, and many regulatory pathway proteins (Bagge *et al.*, 2004b). Also reported is that bacteria within biofilms exhibit differences in the expression of surface molecules, nutrient utilization, virulence factors andantibiotic resistance(Pearson *et al.*, 2006; Zhang and Mah, 2008).

2.5 Resistance of *Pseudomonas aeruginosa biofilms*

Bacteria growing in biofilms are much more resistant to antibiotics and disinfectants than are planktonic growing cells of the same isolate (Niels *et al*., 2001). *Pseudomonas*

aeruginosa, a key opportunistic pathogen is characterized by its biofilm formation ability and high-level multiple antibiotic resistance (Zang *et al.*, 2013). Biofilm resistance has been related to several factors (Hall-Stoodley and Stoodley 2009; Høiby et *al.*, 2010; Corbin *et al.*,2011;Jorge*et al.* 2012) such as thethe development of oxidative stress caused by an imbalance between the formation of reactive oxygen species (ROS) and the antioxidant system which increases mutability in biofilms (Alugupalli *et al.*, 1995;Driffield*et al.*,2008); nutrient depleted zones throughout biofilms due to oxygen and nutrient gradients, which cause bacteria to enter into a stationary phase-like dormancy and not be affected by antimicrobials (Altschul *et al.*,1990), the higher frequency of mutation and horizontal gene transmission found in biofilms compared to planktonic bacteria, explains the rapid development of antibiotic resistance in biofilms (Altman *et al.*, 2006).the appearance of nutrient depleted zones throughout biofilms due to oxygen and nutrient gradients, which cause bacteria to enter into a stationary phase-like dormancy and not be affected by antimicrobials (Altschul *et al.*, 1990), and the delay of antibiotic penetration into the matrix of the biofilm, which contains polymers that bind to antibiotics and hinder their action, and antibiotic-degrading enzymes that deactivate them (Fernando and Jose, 2013).

2.5.1 Low oxygen concentration and slow growth

Growth, protein synthesis and metabolic activity is stratified in biofilms, and it has shown that the oxygen concentration may be high at the surface but low in the centre of the biofilm where anaerobic conditions may be present. Such that the high level of activity at the surface and a low level and slow or no growth in the centre, is one of the explanations for the reduced susceptibility of biofilms to antibiotics (Werner *et al.*,2004; Keren *et al.*, 2004). Slow in situ growth rates of *Pseudomonas aeruginosa* biofilms have been measured andantibiotics where activeonly against dividing *Pseudomonas aeruginosa* cells, but

inefficient at eradicating biofilm infections (Yang*et al*., 2008).

2.5.2 Mutators: Frequency of mutation and horizontal gene transmission in biofilm.

The mutation frequency of biofilm-growing bacteria is significantly increased compared with planktonically growing isogenic bacteria (Driffield *et al*., 2008) and there is also increased horizontal gene transmission in biofilms (Molin and Tolker-Nielsen, 2003). These physiological conditions may explain why biofilm-growing bacteria easily become multidrug resistant by means of traditional resistance mechanisms against β-lactam antibiotics, aminoglycosides and fluoroquinolones, which are detected by routine susceptibility testing in the clinical microbiology laboratory where planktonic bacterial growth is investigated. Thus, bacterial cells in biofilms may simultaneously produce enzymes that degrade antibiotics and the presence of a hypermutable bacterial subpopulation, and the presence of high percentages of hypermutable *Pseudomonas aeruginosa* isolates associated with antibiotic resistance has actually been reported (Oliver *et al*., 2000; Fengjun *et al*.,2013). The hypermutable phenotype of *Pseudomonas aeruginosa* isolates is reported to be due to alterations in genes of the DNA repair systems of either the mismatch repair system (MMR), which involves *mutS*, *mutL* and *uvrD*, or the DNA oxidative lesions repair system , which involves *mutT*, *mutY* and *mutM (*Oliver *et al*.,2002*;*Mandsberg *et al*.,2009). It has been shown that mutations in either of the two systems determine the emergence of antibiotic-resistant isolates, especially due to selection of isolates expressing multidrug efflux pumps (Mandsberg *et al*., 2009; Macia *et al*., 2005). The hypermutability of bacteria in biofilms promotes the emergence of mutations conferring antibiotic resistance. Development of resistance to all classes of antibiotics during biofilm formation has been reported world wide(Ciofu *et al*., 2003; Shadia and

Aeron, 2014). Resistance to β-lactam antibiotics occurs due to mutations in the regulatory genes of β-lactamase production leading to the occurrence of isolates with stable or partially stable derepressed production of AmpC β-lactamase (Ciofu, 2003; Fengjun *et al*., 2013). Resistance to ciprofloxacin by *Pseudomonas aeruginosa* isolates was shown to be mediated by mutations in *gyrA* and alterations in two efflux systems (MexCD-OprJ and MexEF-OprN), and resistance to tobramycin was due to overexpression of the MexXY-OprM multidrug efflux pump (Islam *et al*., 2009).

2.5.3 Chromosomal β-lactamase and biofilm matrix components

Overproduction of chromosomally encoded AmpC cephalosporinase is considered the main mechanism of resistance of biofilm forming *Pseudomonas aeruginosa* isolates to β-lactam antibiotics in cystic fibrosis (CF) (Gabriel *et al*., 2014). The role of this β-lactamase phenotype is especially important for resistance to β-lactam antibiotics. Bacteria expressing a high level of chromosomal β-lactamase growing in biofilms showed reduced concentration of β-lactam antibiotics owing to accumulation of the enzyme in the polysaccharide matrix (Ciofu*et al*., 2005; Gabriel *et al*., 2014).

2.5.4Biofilm matrix

The biofilm matrix can act as a barrier to delay the diffusion of antibiotics into biofilms (Shadia and Aeron, 2014), because antibiotics may either react chemically with biofilm matrix components or attach to anionic polysaccharides. If the time required for an antibiotic to penetrate biofilms is longer than the duration of antibiotic treatment, the slower penetration will explain the antibiotic resistance (Stewart*et al*., 2009). Antibiotics have been shown to readily penetrate biofilms in some cases, but poorly in others depending on particular antibiotics and biofilms. The binding of the positively charged aminoglycosides to the negatively charged biofilm matrix polymers of *P. aeruginosa* will

delay the penetration of aminoglycosides (Bjarnsholt *et al*., 2009) while the penetration of fluoroquinolones occurs immediately and without delay (Fengjun *et el*.,2014)The penetration of oxacillin and cefotaxime (β-lactams), and vancomycin and teicoplanin (glycopeptides) is significantly reduced through *Staphylococcus aureus* biofilms, whereas that of amikacin (aminoglycoside), and rifampicin and ciprofloxacin (fluoroquinolones) was unaffected (Singh *et al*.,2010)The antimicrobial activity of antibiotics will resume in any of the following cases: the biofilm matrix becomes saturated due to the full adsorption/reaction of antibiotic molecules; the time required for an antibiotic to penetrate biofilms is shorter than the duration of antibiotic treatment; and the replenishment of biofilm matrix proceeds at a rate slower than the adsorption/reaction/diffusion of antibiotic molecules(Fengjun *et el*.,2014).

2.5.5 High cell density and quorum sensing (QS)

Quorum sensing influenced the development of high cell density biofilm, which has been shown to determine the tolerance of *Pseudomonas aeruginosa* biofilms to antibiotic therapy. Quorum sensing also influenced the innate inflammatory response dominated by polymorphonuclear leukocytes (PMNs) (Bjarnsholt *et al*., 2005). The connection between QS and biofilms has been named sociomicrobiology (Parsek and Greenberg, 2005).

2.6Antipseudomal Agents

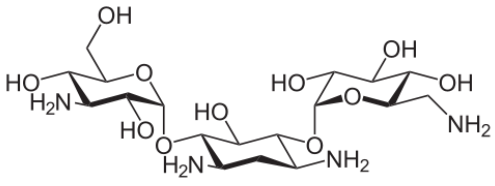
To create a standardized international terminology with which to describe resistance profiles in bacteria often responsible for healthcare-associated infections and prone to multidrug resistance. A group of international experts came together through a joint initiative by the European Centre for Disease Preventionand Control (ECDC) and the Centers for Disease Control and Prevention (CDC). Epidemiologically significant antimicrobial categories were constructed for each bacterium. Also Lists of antimicrobial

categories proposed for antimicrobial susceptibility testing were created using documents and breakpoints fromthe Clinical Laboratory Standards Institute (CLSI), the European Committee on Antimicrobial Susceptibility Testing (EUCAST) andthe United States Food and Drug Administration (FDA) (Magiorakos *et al*.,2011).They include Aminoglycosides (Gentamicin, Amikacin, Tobramycin, but not Kanamycin), broad-spectrum cephalosporins (cefoperazone, cefsulodin, ceftazidime, cefepime, and cefpirome), extended-spectrum penicillins (ticarcillin, piperacillin, and azlocillin), monobactams (aztreonam), carbapenems (imipenem), and quinolones (ciprofloxacin). The addition of beta-lactamase inhibitors to extended-spectrum penicillins has expanded the antibacterial spectra of these agents (clavulanate-ticarcillin and tazobactam). Though, since the early 1980s, many of them have been shown to posses potent "antipseudomonal" antibiotics (Korvick and Yu, 1991).

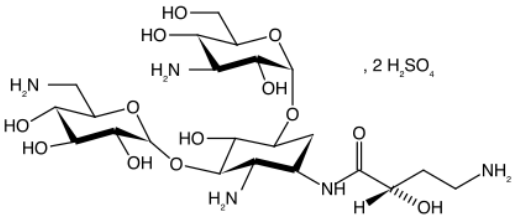
2.6.1 Aminoglycosides

Aminoglycoside is a medicinal and bacteriologic category of traditional Gram-negative antibacterial therapeutic agents that inhibit protein synthesis and contain multifunctional hydrophilic sugars that possess several amino and hydroxy functionalities (Lakshmi *et al*.,2000). Aminoglycoside antibiotics display bactericidal activity against Gram-negative aerobes and some anaerobic bacilli where resistance has not yet arisen, but generally not against Gram-positive and anaerobic Gram-negative bacteria (Levison, 2012).The first-in-class aminoglycoside antibiotic is streptomycin derived from *Streptomyces griseus*, the earliest modern agent used against tuberculosis, and an example that lacks the common 2-deoxystreptamine moiety present in many other class members. Other examples include the deoxystreptamine-containing agents kanamycin, tobramycin, gentamicin, andneomycin.Aminoglycosides that are derived from bacteria of the *Streptomycesgenus* are named with the suffix mycin, examples Erythromycin, Tobramycin, Kanamycin (Fig.2.3),

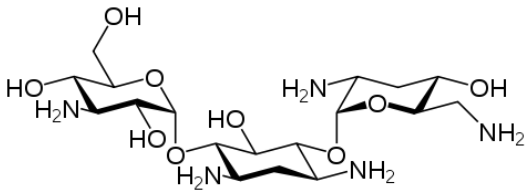
whereas those that are derived from *Micromonospora* are named with the *suffix micin*, examples includes Gentamin, Amikacin etc. (Kroppenstedt *et al.*, 2005; Dewick ,2009). Examples includes



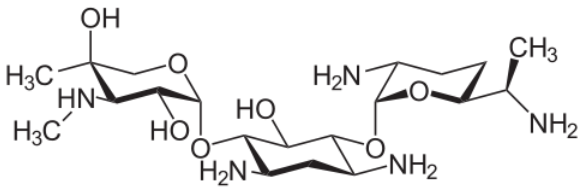
Kanamicin



Amikacin



Tobromycin



Gentamicin

Figure 2.3: Chemical structure of some Aminoglycosides

Aminoglycosides mechanisms of actions

Aminoglycosides display concentration-dependent bactericidal activity against "most gram-negative aerobic and facultative anaerobic bacilli" apart from some bacilli and methicillin-resistant staphylococci, but not against gram-negative anaerobes and most Gram-positive bacteria (Levison, 2012). These activities are attributed to a primary mode of action as protein synthesis inhibitors. The inhibition of protein synthesis is mediated through aminoglycosides' energy-dependent, sometimes irreversible binding, to the cytosolic,

membrane-associated bacterial ribosome(Mingeot *et al.*, 1999; Gad *et al.*, 2011). Aminoglycoside presence in the cytosol generally perturbs peptide elongation at the 30Sribosomal subunit, giving rise to inaccurate mRNA translation and so biosynthesis of proteins is truncated.

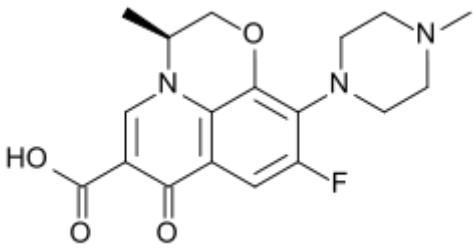
Mechanisms of bacterial resistance to Aminoglycosides

Bacteria may be resistant to aminoglycosides because of failure of the antibiotic to penetrate intracellularly, low affinity of the drug for the bacterial ribosome (by alterating the 30S ribosomal subunity or methylation of the aminoglycoside binding site), drug inactivation by moditying enzymes acquired by conjugative transfer of resistance plasmids. These enzymes phosphorylate, adenylate, or acetylate specific hydroxdyl an amino groups, prevents binding to ribosomes. Amikacin is less susceptible to aminoglycoside-modifying enzymes because of protective side chains and therefore still useful when resistance to gentamicin or tobramycin develops (Miller *et al.*, 1995; Llano-Sotelo *et al.,* 2002).

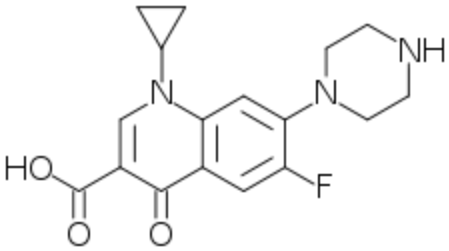
2.6.2 Quinolones: (Ciprofloxacin, levofloxacin, but not moxifloxacin).

Quinolones are synthetic antimicrobials structurally related to the heterobicyclic aromatic compound quinoline, named from the oily substance obtained after the alkaline distillation of quinine (Andersson and MacGowan, 2003). They include two main groups (non-fluorinated quinolones and fluoroquinolones) of drugs that differ in structure, activity, pharmacokinetics and spectrum of indications for use. Quinolones are divided into four generations: I generation (Nalidixic acid, Oxolinic acid, Pipemidic acid); II generation (Lomefloxacin, Norfloxacin, Ofloxacin, Pefloxacin, Ciprofloxacin); III generation (Levofloxacin, Sparfloxacin); IV generation (Moxifloxacin). Nalidixic acid a first generation of quinolones was introduced in 1962 for treatment of urinary tract infections in humans. Nalidixic acid was discovered by George Lesher and co-workers in a distillate

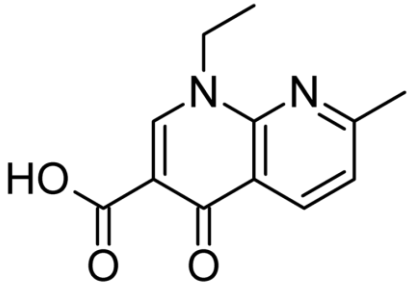
during an attempt at chloroquine synthesis (Wentland *et al.*, 1993). Quinolones exert their antibacterial effect by preventing bacterial DNA from unwinding and duplicating (Hooper, 2001). Fluoroquinolones are broad-spectrum antibiotics (effective for both Gram-negative and Gram-positive bacteria) that play an important role in treatment of serious bacterial infections, especially hospital-acquired infections and others in which resistance to older antibacterial classes is suspected (Juno *et al.*,2013). The basic pharmacophore, or active structure, of the fluoroquinolone class is based upon the quinoline ring system. The addition of the fluorineatom at C6 distinguishes the successive-generation fluoroquinolones from the first-generation quinolones. This C6 fluorine atom has since been demonstrated to not be required for the antibacterial activity of this class (Chang, 1997). The majority of quinolones in clinical use belong to the subset fluoroquinolones, which have a fluorineatom attached to the central ring system, typically at the 6-position or C-7 position (Asif, 2014).



Ciprofloxacin



Levofloxacin.



Nalidixic acid

Figure 2.4: Chemical structure of some quinolones (Adapted from Bryan Derksen,2007)

Quinolones of mechanism of actions

Quinolones exert bactericidal effect. They inhibit two critical enzymes of microbial cells - DNA gyrase and topoisomerase IV to violate the synthesis of DNA. First and second generation fluoroquinolones selectively inhibit the topoisomerase II ligase domain, leaving the two nuclease domains intact. This modification, coupled with the constant action of the topoisomerase II in the bacterial cell, leads to DNA fragmentation via the nucleasic activity of the intact enzyme domains. Third and fourth generation fluoroquinolones are more selective for topoisomerase IV ligase domain, and thus have enhanced gram positive coverage. For many gram-negative bacteria, DNA gyrase is the target, whereas topoisomerase IV is the target for many gram-positive bacteria. Some compounds in this class have been shown to inhibit the synthesis of mitochondrial DNA (Bergan *et al.*, 1988; Hooper, 1999).

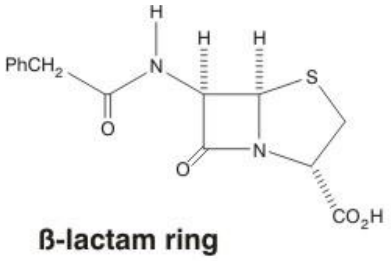
Mechanisms of bacterial resistance to Quinolones

Resistance to quinolones typically arises as a result of alterations in the target enzymes (DNA gyrase and topoisomerase IV) and of changes in drug entry and efflux. Mutations are selected first in the more susceptible target: DNA gyrase, in gram-negative bacteria, or topoisomerase IV, in gram-positive bacteria. Additional mutations in the next most susceptible target, as well as in genes controlling drug accumulation, augment resistance further, so that the most-resistant isolates have mutations in several genes. Resistance to quinolones can also be mediated by plasmids that produce the Qnr protein, which protects the quinolone targets from inhibition (Jacoby, 2005).

2.6.3 Cephalosporins: (Ceftazidime, cefepime, cefoperazone, cefpirome, ceftobiprole;

cefuroxime, ceftriaxone, cefotaxime)

The cephalosporins are a family of bactericidal antibiotics structurally related to penicillin which were first derived from the fungus, *Cephalosporum acremonium*. Their basic structure is similar to penicillin with a thiazolidine and beta-lactam ring, which has a variable side chain. They have a broader activity than the standard penicillins. Five generations of cephalosporins have been developed with varying antibacterial activity. Cephalosporins are indicated for infections with susceptible organisms (Petri, 2011). First-generation cephalosporins are active predominantly against Gram-positive bacteria, and successive generations have increased activity against Gram-negative bacteria, albeit often with reduced activity against Gram-positive organisms (Amitava, 2012).



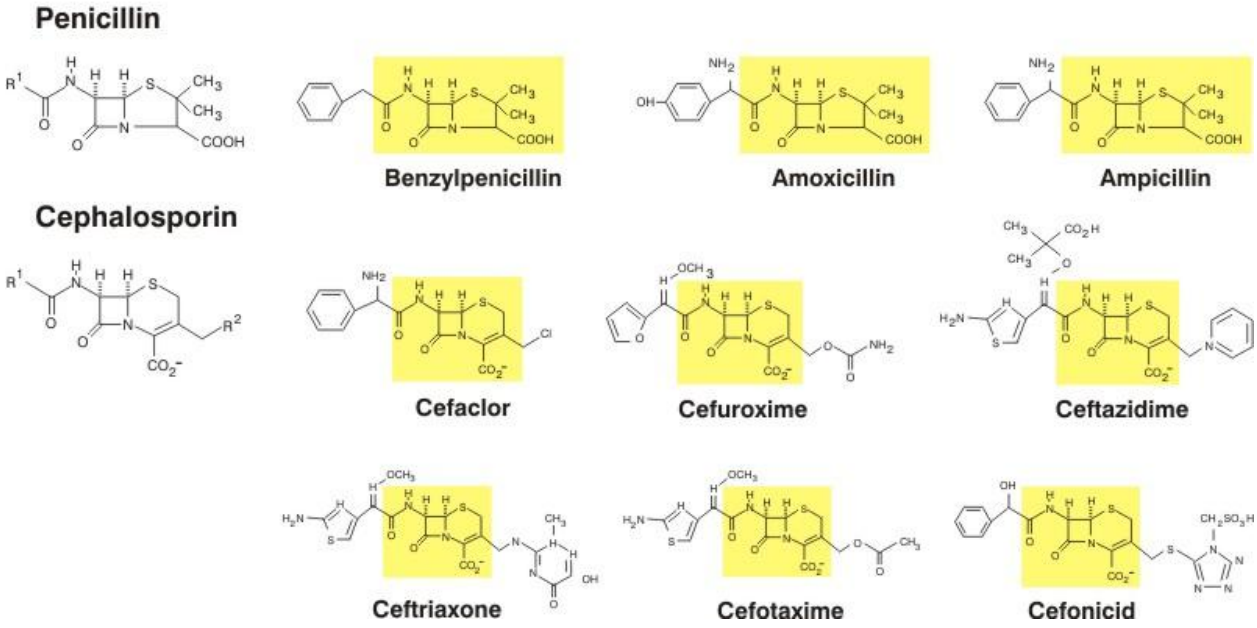


Figure 2.5: Chemical structures for 7-aminocephalosporanic acid (7-ACA), penicillin, the cephalosporin nucleus (Nelson and Grumach, 2006).

Cephalosporins Mechanism of actions

Cephalosporins are bactericidal and have the same mode of action as other beta-lactam antibiotics (such as penicillins) but are less susceptible to penicillinases. Cephalosporins disrupt the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as penicillin-binding proteins (PBPs). PBPs bind to the D-Ala-D-Ala at the end of muropeptides (peptidoglycan precursors) to crosslink the peptidoglycan. Beta-lactam antibiotics mimic the D-Ala-D-Ala site, thereby irreversibly inhibiting PBP crosslinking of peptidoglycan. Theyarrests bacterial growth by binding to 1 or more penicillin-binding proteins, thereby, in turn, inhibiting final transpeptidation step of peptidoglycan synthesis in bacterial cell-wall synthesis and inhibiting cell-wall biosynthesis, causing cell lysis particularly in rapidly growing organisms (Bruntonet *al*.,2007).

Mechanism of bacterial Resistance to Cephalosporins

Cephalosporins, like other beta-lactams, bind to the bacterial penicillin-binding proteins (PBPs), i.e the D-ala-D-ala trans-, carboxy- and endo-peptidases responsible for catalysing the cross-linking of newly formed peptidoglycan. Resistance arises when the PBPs, particularly the transpeptidases-are modified, or when they are protected by beta-lactamases or 'permeability barriers'. Target-mediated cephalosporin resistance can involve either reduced affinity of an existing PBP component, or the acquisition of a supplementary beta-lactam-insensitive PBP. Beta-lactamases are produced widely by bacteria and may be determined by chromosomal or plasmid DNA (Kumarasamy *et al*., 2010). Plasmid-

mediated beta-lactamases (PMBLs) have also been implicated as causes of resistance, and other cephalosporinases have been described. Point mutations of specific amino acids of well-recognized PMBLs (e.g., TEM-1 and SHV-1) have also produced enzymes capable of attacking a wider spectrum of beta-lactam agents (Donati *et al*., 2014).

2.6.4 Antipseudomonal Penicillins: carboxypenicillins carbenicillin and ticarcillin

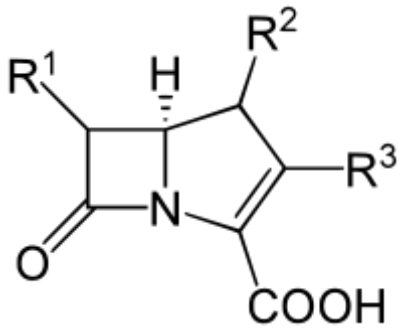
Antipseudomonal penicillins are antimicrobial agents, which are used to treat pseudomonal infections. They have the activity of penicillins and aminopenicillins, and additional activity against *Pseudomonas, Enterococcus and Klebsiella spp*. Antipseudomonal penicillins are usually given with beta-lactamase inhibitors because like other penicillins they are susceptible to hydrolysis by beta-lactamases and therefore are not consistently active against *Staphylococcus spp*, some gram-negative rods and certain beta-lactamse producing gram-negative anaerobes). Thes group of Penicillins alsobind to the bacterial Penicillin Binding Proteins (PBPs), inhibiting the cross-linking of formed peptidoglycan and eventually prevent bacterial cell-wall biosynthesis.

Mechanism of bacterial Resistance to antipseudomonals penicillins.

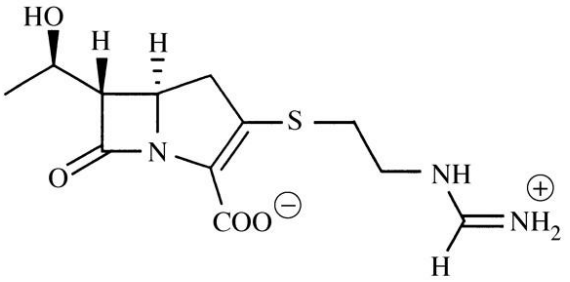
Fundamentally, the mechanisms of resistance resistance to penicillins including the antipseudomonal penicillinsare enzymatic degradation of antibiotics drugs, alteration of bacterial proteins that are antimicrobial targets and changes in membrane permeability to antibiotics. The most common important mechanism of resistance is hydrolysis. The antibacterial resistance can be by bacteria chromosome or plasmid mediated. Organisms that are not actively multiplying or do not have a cell wall are not susceptible. These penicillins when given with aminoglycosides work effectively and avoid development of resistance strains of bacteria (Livermore andWoodford, 2006; Henry, 2007).

2.6.5 Carbapenem: meropenem, imipenem, doripenem.

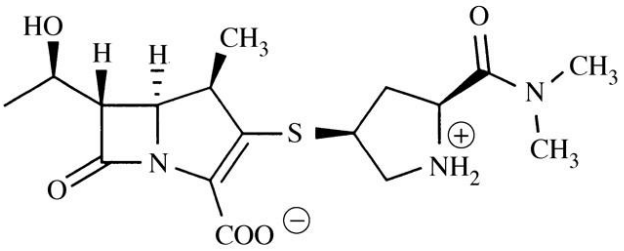
Carbapenems are a class of β-Lactam antibiotics with a broad spectrum of antibacterial activity. They have a structure that renders them highly resistant to most β-lactamases(Livermore, 2002). Carbapenem antibiotics were originally developed from the carbapenem thienamycin, a naturally derived product of *Streptomyces cattleya*. Carbapenems are one of the antibiotics of last resort for many bacterial infections, such as *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (Smith, 2010). Alarm has been raised over the spread of drug resistance to carbapenem antibiotics among these coliforms, due to production of carbapenemases, including the New Delhi metallo-β-lactamase, NDM-1 (CDC, 2010;McKenna, 2013). There are currently no new antibiotics in development to combat bacteria resistant to carbapenems, and worldwide spread of the resistance gene is considered a potential nightmare scenario (Pennington, 2010). The following drugs belong to the carbapenem class and are approved for use by health authorities; Imipenem, Meropenem, Ertapenem,Doripenem. These agents have the broadest antibacterial spectrum compared to other β-lactam classes such as penicillins and cephalosporins, Carbapenems circumvent β-lactamase by binding it with high affinity and acylating the enzyme, rendering it inactive(Hamilton, 2003).**Figure2.6below shows** Chemical structures of imipenem, meropenem and ertapenem.



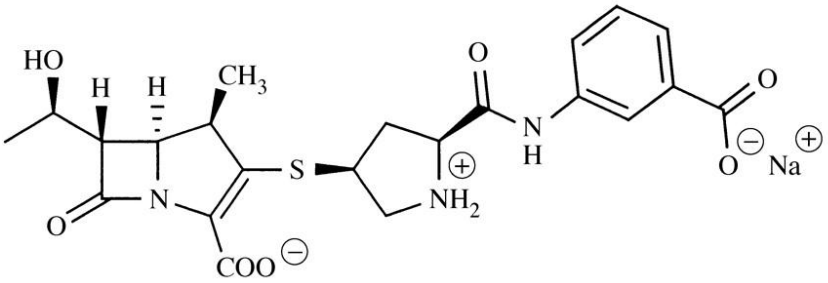
Structure of the carbapenem backbone



Imipenem
Plasma protein binding = 20%
Molecular weight:317.37



Meropenem
Plasma protein binding = 2%
Molecular weight: 437.52



Ertapenem
Plasma protein binding = ~ 94%
Molecular weight: 497.50

Fig.2.7:Chemical structures of imipenem, meropenem and ertapenemAdapted (Adapted from British Society for Antimicrobial Chemotherapy, 2003)

Mechanism of bacterial Resistance to carbapenem

Strains of carbapenem-resistant enteric bacteria have been isolated from patients having received recent medical care in Pakistan, Bangladesh, and India (Roberts, 2010). These strains carry a enzyme called New Delhi metallo-β-lactamase(NDM-1) that is responsible for the production of a metallo-β-lactamase enzyme that hydrolyses carbapenem

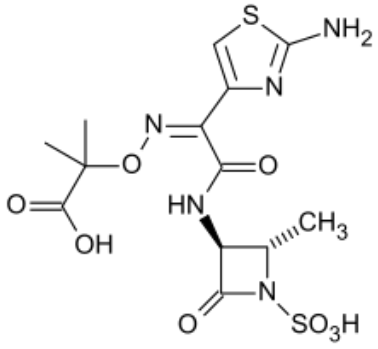
(McKenna, 2013). Imipenem resistance in *Pseudomonas aeruginosa* is shown to be associated with loss of the porin OprD combined with activity of chromosomal beta-lactamase (AmpC), while overexpression of multidrug efflux pumps is considered to confer meropenem resistance (El Amin *et al*.,2005).

2.6.6 Monobactams (Aztreonam)

Aztreonam is a synthetic monocyclic beta-lactamantibiotic (a monobactam), with the nucleus based on a simpler monobactam isolated from *Chromobacterium violaceum*. It was approved by the U.S. Food and Drug Administration (FDA) in 1986, (MHRA, 2015)

Aztreonam Mechanism of action

Aztreonam is a bactericidal antibiotic, which interferes with the synthesis of the bacterial cell wall (Georgopapadakou *et al.*, 1982), the mechanism being similar to that of penicillins and cephalosporins. It binds preferentially to the Penicillin binding protein-3 (PBP-3) of gram negative bacteria and causes lysis and death. There is poor affinity of Aztreonam for the PBP's of gram positive and anaerobic bacteria, which accounts for its narrow spectrum of activity (Seema and Geeta, 2004).



Aztreonam

Figure2.8: Chemical structures of Aztreonam

Mechanisms of bacterial Resistance to Aztreonam.

Resistance to aztreonam is primarily through hydrolysis by beta-lactamase, alteration of

penicillin-binding proteins (PBPs), and decreased permeability (Squibb, 2013Jacob *et al.*, 2009).

2.7 Test Methods in Detecting Antimicrobial Resistance

There are several antimicrobial susceptibility testing methods available today, and each one has their respective advantages and disadvantages. They all have one and the same goal, which is to provide a reliable prediction of whether an infection caused by a bacterial isolate will respond therapeutically to a particular antibiotic treatment. This data may be utilized as guidelines for chemotherapy, or at the population level as indicators of emergence and spread of resistance based on passive or active surveillance. Some examples of antibiotic resistance detecting methods from include the followings(CLSI; 2012):

2.7.1. Disk-diffusion method

The disk diffusion method is probably the most widely used method for determining antimicrobial resistance .A growth medium, usually MuellerHinton agar is first evenly seeded throughout the plate with the isolate of interest that has been diluted to a standard concentration (approximately 1 to 2 x 10^8 colony forming units per ml). Commercially prepared disks, each of which are pre -impregnated with a standard concentration of a particular antibiotic, are then evenly dispensed and lightly pressed onto the agar surface. The test antibiotic immediately begins to diffuse outward from the disks, creating a gradient of antibiotic concentration in the agar such that the highest concentration is found close to the disk with decreasing concentrations further away from the disk. After an overnight incubation, the bacterial growth around each disc is observed.If the test isolate is susceptible to a particular antibiotic, a clear area of "no growth" will be observed around that particular disk. The zone around an antibiotic disk that has no growth is referred to as

the zone of inhibition since this approximates the minimum antibiotic concentration sufficient to prevent growth of the test isolate. This zone is then measured in mm and compared to a standard interpretation chart used to categorize the isolate as susceptible, intermediately susceptible or resistant.

2.7.2. E-test

The Etest gradient technology is based on a combination of the concepts of dilution and diffusion principles for susceptibility testing. As with other dilution methods, Etest directly quantifies antimicrobial susceptibility in terms of discrete MIC values. However, in using a predefined, stable and continuous antibiotic concentration gradient, Etest MIC values can be more precise and reproducible than results obtained from conventional procedures based on discontinuous two-fold serial dilutions.Etest is a thin, inert and non-porous plastic strip. One side of the strip (A) carries the MIC reading scale in μg/mL and a two or three-letter code on the handle to designate the identity of the antibiotic (bioMérieux ,2012).

2.7.3. Automated methods

The automated methods are intended to reduce technical errors and lengthy preparation times. Most automated antimicrobial susceptibility testing systems provide automated ino culation, reading and interpretation.These systems have the advantage of being rapid (some results can be generated within hours) and convenient, but one major limitation for most laboratories is the cost entailed in initial purchase, operation and maint enance of the machinery. Some examples of these include: Vitek System (bioMerieux, France), Walk - Away System (Dade International, Sacramento, Calif.), Sensititre ARIS (Trek Diagnostic Systems, East Grinstead, UK), Avantage Test System (Abbott Laboratories ,Irving, Texas), Micronaut (Merlin, Bornheim -Hesel, Germany), Phoenix (BD Biosciences, Maryland) and many more.

2.7.4. Mechanism-specific tests such as beta-lactamase detection test and chromogenic cephalosporin test.

Beta lactamase detection can be accomplished using an assay such as the chromogenic cephalosporinase test (Cefinase disk by BD Microbiology Systems, Cockeysville, MD and BBL DrySlide Nitrocefin, Becton Dickinson, Sparks, MD) and detection for chloramphenicol modifying enzyme chloramphenicol acetyltransferase (CAT) may utilize commercial colorimetric assays such as a CAT reagent kit (Remel, Lenexa, Kansas)

2.7.5. Genotypic methods

Some of the most common molecular techniques utilized for antimicrobial resistance detection are as follows:

2.7.6 Polymerase chain reaction (PCR)

PCR is one of the most commonly used molecular techniques for detecting certain DNA sequences of interest. This involves several cycles of denaturation of sample DNA, annealing of specific primers to the target sequence (if present), and the extension of this sequence as facilitated by a thermostable polymerase leading to replication of a duplicate DNA sequence, in an exponential manner, to a point which will be visibly detectable by gel electrophoresis with the aid of a DNA -intercalating chemical which fluoresces under UV light.

2.7.7 DNA hybridization.

This is based on the fact that the DNA pyrimidines (cytosine and thymidine) specifically pair up with purines (guanine and adenine; or uracil for RNA). Therefore, a labeled probe

with a known specific sequence can pair up with opened or denatured DNA from the test sample, as long as their sequences complement each other. If this “hybridization” occurs, the probe labels this with a detectable radioactive isotope, antigenic substrate, enzyme or chemiluminescent compound. Whereas if no target sequence is present or the isolate does not have the specific gene of interest, no attachment of probes will occur, and therefore no signals will be detected.

2.8.8 **Modifications of PCR and DNA hybridization.**

With these basic principles, several modifications have been introduced which further improve the sensitivity and specificity of these standard procedures. Examples of such developmentswere the use of 5’ - fluorescence-labeled oligonucleotides, the development of molecular beacons, development of DNA arrays and DNA chips, among many others.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

Autoclave, incubator, hot air oven, microtiter pippette refrigerator, PCR thermocycler (Techne TC-312), gel electrophoresis machine (Max Fill Scie-plas. Model HU10 serial no 5237), comb, laminar air flow cabinet (PCR-8 recirculating laminar flow pre station Labcaire product 220/240v) and microscope, freezer, bunsen burner, spectrophotometer (Eppendorf Biophotometer 8,5mm, Lichtstrahihohe), UV illiminator (Vilberb Lourmat TFX-35-M serial no V02 8104), centrifuge (Eppendorf centrifuge 5417R), micropipette, microwave oven (HINARI Life Style 800watts model MX310TCSL), Electronic weighing balance (QT 600), Touch plate Super Mixer, (CAT No 1291,Lab-line instrument Incorporated, USA).

3.1.2 Glass wares

Beakers (Pyrex, England), Conical flask (Pyrex, England), Test-tubes (Pyrex, England), Measuring cylinder (Pyrex, England), bottles, universal bottles, glass slides and Petri-dishes (Pyrex, England), sterile flat-bottomed 96 –well polystyrene microtitre plate (Linbro Scientic, Inc. Hamden, Conn 06517, US), measuring cylinder(Pyrex, England).

3.1.3 Reagents

Crystal violet (May and Baker Ltd. Dagenham, England), Lugol‘s iodine (May and Baker Ltd. Dagenham, England), neutral red (May and Baker Ltd. Dagenham, England), oil immersion (BDH 60 Chemicals Ltd. Poole, England), Oxidase reagent (Liofilchems.r.l. Bacteriology products, Roseto, Italy), Dettol (ReckitBenckiser Ltd., Nigeria), Ethanol (BDH Chemical Ltd., England), Acetic acid (BDH Chemical Ltd., England),Acridine orange (BD-Diagnostics,Taiwan), TAE, Tris buffer, KOH, NaOH, Ethidium bromide (Sigma chemical Ltd., England),), Glycerol, Glacia acetic acid, 6X loading dye, Lysis solution (Fermentas, UK), Neutralizing solution (Fermentas, UK), 96% Ethanol (BDH

Chemical Ltd., England), Wash solution (Fermentas, UK), Elution buffer (Fermentas UK), Tris-Acetate EDTA(TAE) (Fermentas, UK).

3.1.4 Laboratory Media

The following laboratorymedia were used in this study: MacConkey Agar (Oxoid Ltd., England), Nutrient Agar (Oxoid Ltd., England), Nutrient Broth (Fluka Spain), Triple sugar Iron (Biotech Laboratory Ltd., UK), Muller Hilton Agar (Oxoid Ltd., England),Cetrimide agar (HiMedia Laboratories,india), Peptone Water (Fluka Spain), Brain Heart Infusion broth(Oxoid, UK), Luria and Bertani broth (HiMedia Laboratories,india), ,Agarose Gel (Schwarz/Mann, England), EDTA (TAE) (Sigma chemical Ltd., England), Ethydium bromide dye (Sigma chemical Ltd., England), Tris-HCl (Sigma chemical Ltd., England), Re-suspension solution (Fermentas, UK).

3.1.5 Susceptibility Discs

Table 3.0 Antibiotics discs and their strength

AntibioticsDisc Strength (µg)	
Penicillins	
Ticarcilin-Clavulanic acid (TIM)	30

Cephalosporins	
Ceftazidine (CAZ)	10
Fluoroquinolones	
Ciprofloxacin (CIP)	5
Carbapenems	
Imipenems (IPM)	10
Monobactams	
Azetreoam (ATM)	30
Amino-glycosides	
Gentamicin (CN)	10
Amikacin (AK)	30

All the discs were made by Oxoid Ltd., England

M.I.C.Evaluator Strips (Oxoid Ltd, England)

3.2 Methods

3.2.1 Preparation of Media

All the media as listed in section 3.1.4 were prepared according to manufacturer's instruction and sterilized using autoclave at $121^{O}C$ for 15 minutes and dispensed appropriately into bottles or petri dishes prior to use.

3.2.2 Collection of Isolates

All suspected *Pseudomonas aeruginosa* isolates from samples submitted to the Medical Microbiology Laboratory of the Benue State University Teaching Hospital, Makurdi, Nigeria, over a six month period from August, 2013 to January, 2014, were collected, purified and subcultured in cetrimide agar, and identified by standard microbiological procedures, (Cheesbrough, 2006). These isolates were inoculated onto agar slant and then transported from Medical Microbiology Laboratory of the Benue State University Teaching Hospital, Makurdi, to the Pharmaceutical Microbiology Laboratory, Zaria in a container with ice packs for analysis.

3.2.4 Purification of Isolates

The isolates were purified by single colony isolation: Isolates were inoculated into nutrient broth and incubated at 37°C for 18hours and then streaked on sterile nutrient agar and incubated at 37°C for 18hrs to obtain the single colony.

3.2.5 Identification of the isolates

Pseudomonas aeruginosa isolates were characterised and identified using a combination of colonial morphology, Gram stain characteristics, smell, pyocyanin production, oxidative-fermentation test and oxidase tests using standard microbiological procedures (Cheesbrough, 2006).

Gram Staining

To determine the morphology of the organisms and classify the organism into Gram positive and Gram negative, Gram staining according to Cheesbrough, (2006) was carried out;Using a single colony, a smear was made by emulsifying the colony in sterile distilled water and a thin preparation was made on a labeled clean slide. After making the smear, it was allowed to air dry and the slides pass over heat to fix the organism so as to prevent wash off. This was followed by staining with crystal violet for 1 minute and rapidly washed

with clean water. After which Lugol iodine was used to cover the smear for 1 minute and washed off with clean water. The stain was rapidly decolorise with ethanol and washed off immediately with clean water and counter stained with neutral red for two minutes, then rinsed off with clean water, the smear was allowed to air dry and examined under a microscope after adding oil immersion.

Growth on Selective Media

The suspected *Pseudomonas aeruginosa* isolates were inoculated on selective media and incubated for 24 hours at 37^{o}C. The color and morphology of the colonies were observed and noted. MacConkey agar differentiates the lactose fermenters and the non-lactose fermenters (e.g *Pseudomonas aeruginosa*). Furthermore, cetrimide agar was used to differentiate *Pseudomonas aeruginosa* isolates as yellow greenish colored colonies.

3.3 Biochemical Tests

The Biochemical Tests were carried out according to the methods described by Cheesbrough (2006) and Chakraborty and Nishith (2008).

Oxidase Test

The oxidase test is a test used in microbiology to determine if a bacterium produces certain cytochrome c oxidases enzymes. The method describe by (Cheesbrough, 2006) was used.

A piece of filter paper was soaked with a few drops of oxidase reagent. A colony of the test organism is then smeared on the filter paper. The filter paper will turn deep purple colour if the organism was oxidase-producing, due oxidation of phenylenediamine.

Carbonhydrate fermentation in Triple Sugar Iron Agar (TSI)

This test identifies carbonhydrate fermenters and H_2S producers among the Enterobacteriaceae.Slopes of medium in test tubes were prepared as recommended by the manufacturer. Then a straight sterile wire was first used to streak the slope with the

organisms and then the bottom. The tubes were incubated at 37°C for 24 hours. Glucose fermenters are identified when tube bottom turns yellow; lactose/sucrose fermenter turns tube slant yellow; H_2Sproducers' forms black spot on the surface of the slope; no fermentation when no color changes for both at the bottom and the slant (Chakraborty and Nishik, 2008).

3.4 Biofilm Production Assay

Method described by Christenen *et al* (1985),modified by O'Toole, **(**2011) was used.The isolates were grown overnight for 18hrs at 37°C in Brain Heart Infusion Broth supplemented with 2% glucose. The cultures were diluted 1:100 in Brain Heart Infusion Broth and 150µL of suspension was used to inoculate sterile flat-bottomed 96 –well polystyrene microtitre plate and incubated for 48hrs at 37°C, after which the suspensions were poured off and the well gently washed three times with 300 µL of distilled water and dried in an inverted position. The dried wells were stained with 300 µL of 1.0% crystal violet solution in water for 45 minutes. The excess stain was decanted off and the wells washed 3 times with 300 µL of distilled water and allowed to dry.A positive result was defined by the presence of a layer of stained material which adheres to the inner wall of the microtiter plate wells.

Quantitative Biofilm Assay

Method described by Christenen *et al* (1985),modified by O'Toole, **(**2011) was used.The quantitative analysis of biofilm production was performed by adding 200 µL of ethanol – acetic acid (95:5 vol/vol) to destain the wells. A 100 µL measure from each well was transferred to a new flat bottom microtiter plate and the optical density (OD) of the crystal violet present in the destaining solution was measured at 545nm using a microtiter plate reader (ELISA Machine B.Bran Science Company, England). Each assay was performed in

triplicate .The control was uninnoculated media to determine background OD. The mean OD_{545} value from the control wells were substracted from the mean OD_{545} value of the test wells which gives the amount of the biofilm produced.

3.5 Antibiotic Susceptibility Testing

The antibiotic susceptibility pattern of the *Pseudomonas aeruginosa* isolates was determined using the Modified Kirby-Bauer Disc Agar Diffusion method as describe in EUCAST Guidelines 2013.

3.5.1 Standardization of inoculum

Overnight cultures of the test organisms were diluted in 5ml of sterile normal saline in transparent test tubes bottles. Using a sterile wire loop, discrete colonies of similar appearance from solid media were emulsified into the sterile 5ml normal saline. The turbidlty of the suspension was compared to the turbidity of the standard (0.5 Mcfarland). The concentration of the organism should be approximately1.5 x 10^5 – 10^6 cfu/ml (Mcfarland, 1907; Samie*et al*. 2005).

3.5.2 Antibiotic Susceptibility Testing

Susceptibility testing was performed by Modified Kirby-Bauer Disc Agar Diffusion techniques.Mueller-Hinton agarwas prepared accordance to the manufacturer's instructions. Under aseptic condition, a standard inoculum adjusted turbidity equal to that of a 0.5 McFarland turbidity standard was used to inoculate Mueller-Hinton agar plates by swabbing them with a sterile cotton swab. After drying for about 15 min, the antibiotic disks were placed on the surfaces of inoculated and the plates were incubated at 37°C for 18hrs, the diameters of the zones of growth inhibition were measured and compared with EUCAST, 2013Interpretive Charts to determine organism as susceptible, intermediate or resistant to the antimicrobial agent.

3.6 Determination of Minimum Inhibitory Concentration (MIC) of Antibiotic

The MIC was determined using M.I.C.E. strips method (EUCAST Guidelines, 2013), with the following indicator antibiotic; Ciprofloxacin (5µg), Ticarcillin-clavulanic acid (75-10µg), Imipenem (10µg).Using standard inoculums adjusted to 0.5 McFarland swabbed onto to surfaces of Mueller-Hinton agar, the nylon strips were placed on the agar with antimicrobial side down on the bacterial lawn after allowing plates to dry for about 5 minutes. The plates were incubated at 37°C for 24 hours, and then the MIC was determined by noting the point where ellipsoid inhibition zone crosses the strip.

3.7 Plasmid curing experiment

Plasmid curing was carried out to determine the presence of plasmids by employing the acridine orange treatment as previously described by (Iroha*et al.*, 2008; Esimone*et al*, 2010).The sub-inhibitory concentrations of Acridine orange were determined since plasmid curing takes place at sub-inhibitory concentrations of the respective agent. Using a sterile syringe 1ml of standard inoculums (0.5McFarland turbidity) were added to seven 50 fold dilutions of acridine orange from stock solution of 5000µg/ml (least dilution was 39.06µg/ml) in nutrient broth and incubated at 37°C for 24hrs. Cultures containing the highest concentration of acridine orange in which growth is clearly visible were selected, subcultured in nutrient broth, grown overnight at 37°C for 18 hours and susceptibility of for ciprofloxacin was carried out asdone earlier. The cells were tested for plasmid curing by subjecting them to further antibiogram studies and the Presence or absence of plasmids was identified by evaluating loss of antibiotic resistance from antibiotic susceptibility testing pre and post acridine orange treatment (Iroha*et al.*, 2008).

3.8 Molecular Characterization of the quinolone Resistant Genes in *Pseudomonas aeruginosa* isolates

The presence of resistant genes was investigated by testing the resistant isolates for the presence of plasmids, followed by characterization of the gene by agarose gel - electrophoresis.

3.8.1 Genomic DNA Extraction

Bacterial Cell Preparation

Bacterial cells that were resistant to ciprofloxacin were prepared according to the method described by Duby (2009). Pure colonies from cetrimide agar were picked and inoculated in 5ml Luria Bertani (LB) broth and incubated at 37°C for 18 hours.

DNA Extraction

Genomic DNA was isolated using a ZR Fungal/Bacterial DNA purification kit (Zymo Research Corporation)according to manufacturer's instructions. Bacterial cells from the overnight growth were harvested by centrifugation at 4°C; 800rpm (6800xg) in a microcentrifuge for 2 minutes in an Eppendorff tube, the step was repeated for higher yield of cells. The supernatant was discarded and cells harvested. The harvested cells pellets were dislodged and 200µl of deionized water was added and mixed thoroughly by vortexing. Exactly 400µl of the lysis solution was transferred to Zymo-Spin™ IV Spin Filter in a Collection Tube and centrifuge at 7,000 rpm for 1 min.1200µlof DNA Binding Buffers was added to the filtrate in the collection Tube from the preceding Step. Next, 800µl of the mixture from the step above was transferred to Zymo spin IIC column in a new collection tube and centrifuge at 10000 xg for 1minute. The flow through from the above step in the collection tube was discarded and the step repeated. 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin Column in a new collection tube and centrifuge at 10,000 x *g* for 1 min., and then washed with 500 µl Bacterial DNA Wash Buffer.The column was transfered to a clean 1.5 ml microcentrifuge tube and 100 µl DNA Elution Buffer added directly to the column matrix and centrifuged at 10,000 x *g* for 1 minute to elute the DNA (Lephoto and Gray, 2013).

3.8.2 Polymerase Chain Reaction (PCR) Amplification of target gene

Amplification of antibiotic resistant gene was carried out usingZymo Research PCR protocol, after an extensive optimization of the reaction to ensure a better amplification. The following procedures were carried out. The thin –walled PCR tubes were marked and the following components added to each isolates in a single reaction of 50µ in the PCR tube: 25µl of Dream Taq PCR master mix, 1.0µl of forward primer, 1.0µl of reverse primer, 7.0µl of template DNA (genomic DNA), and 16µl nuclease-free water to make up a total volume of 50µl. The samples were then spinned and PCR performed using the conditions as stated by Zymo ResearchCorporation, Table 3.1 and Table 3.2.

The PCR conditions for the primers (qnrA, qnrB, qnrC and qnrS) are shown in Tables 3.1 and 3.2.

Table 3.1 PCR condition for Primers: qnrS and qnrB

Steps	Temperatutre °C	Time	Numbers of cycles
Initial denature	95	5 minutes	1
Denaturation	95	30 secs	30
Annealing	58	30 secs	1
Extension	72	30 secs	1
Final extension	72	7 minutes	1
Final hold	4		

Total time is 1hour 50minutes

Table 3.2 PCR condition for Primers : qnrC and qnrA

Step	Temperatutre °C	Time	Numbers of cycles

Initial denature	95	5 minutes	1
Denaturation	95	30 secs	35
Annealing	60	30 secs	1
Extension	72	30 secs	1
Final extension	72	7 minutes	1
Final hold	4		

Total time is 1 hour 46 minutes

3.8.3 Agarose Gel Electrophoresis

The DNA extract was subjected to agarose gel electrophoresis.The agarose gel was prepared by adding 1.0gm of agarose in ten times(10x) concentration of Tris acetate ethylene diamine tetra acetate (2ml 10x TAE) buffer and 98ml distilled water in a 250ml beaker flask and heated until the agarose dissolved. Then 5µl of ethidium bromide was added to the dissolved agarose solution as dye and mix. The gel was then poured onto a mini horizontal gel electrophoresis tank with the casting combs label red at the base to ensure easy view of the well while filling the PCR product. It was then allow to set and solidify. The combs were then carefully removed after the gel had completely solidified, one time concentration (1x) electrophoresis buffer was then added to the reservoir until the buffer just cover the agarose gel. Exactly 5µl of gel tracking dye (bromophenol blue) was added to 15µl of each sample with gentle mixing and loaded onto the wells of the gel. The mini horizontal electrophoresis gel set up was then covered and the electrodes connected running from cathode (-) to anode (+). Electrophoresis was carried out at 75mV for 30

minutes to allow easy separation of sample, based on molecular weight. At completion of the electrophoresis, the gel was removed and viewed under a Trans-illuminator UV light of wavelength 302nm to visualize the DNA bands. The bands pattern of the target gene was then photographed with a polaroid camera.

Primers used in the study

The primers for the quinolone resistance gene targeted in this study were obtained from Zymo Research Corporation United Kingdom. Details of the expected amplicon sizes and the sequences are shown in Table 3.3.

Table 3.3: The primers for the genes and their References

Resistance genes	Amplicon size (bp)	Oligosequence	Reference
qnrA	492	F:5´-GGATGCCAGTTTCGAGGA-3´. R : 5´-TGCCAGGCACAGATCTTG-3´	Hendrisen*etal*,2009
qnrB	264	F : 5´GGMATHGAAATTCGCCACTG-3´ R : 5´TTTGCYGYYCGCCAGTCGAA-3´	Cattoir*et al.*,2007
gnrC	447	F : 5´GGGTTGTACATTTATTGAATC-3´ R :5´TCCACTTTACGAGGTTCT-3´	Wang *et al.*,2009
gnrS	466	F : 5´TCGACGTGCTAACTTGCG-3´ R :5GATCTAAACCGGTCGAGTTCGG-3´	Cavaco*etal*,2009

CHAPTER FOUR

4.0 RESULTS

4.1 Disribution of collected and purified isolates

A total of ninety (90) susceptedPseudomonas aeruginosa isolates from samples submitted to the BSUTH Medical Micobiology Laboratory were colleted over the period of this study. The distribution of the Laboratory suspected isolates collected is as follows: 25(27.78%) from urine, 18(20%) wound swab, 33(36.67%) ear swab and 14(15.56%) from blood (Tab.4.1). After the isolates from clinical sampleswere purified and identified using standard microbiology methods (Cheesbrough, 2006) at the Pharmaceutical Microbiology Laboratory in Zaria, 81 isolates were confirmed as *Pseudomonas aeruginosa* isolates with the following distribution, 24(29.63%) from urine, 17(20.99%) from wound swab, 32(39.51%) from ear swab and 8(9.88%) from blood (Table 4.1 and Figure 4.1).

Table1 4.1: Distribution of suspected*Pseudomonas aeruginosa* isolates collected by Sample

Sample Sites	No (%) N=90
Urine	25(27.78%)
Wound swab	18(20%)
Ear swab	33(36.67%)
Blood	14(15.56%)
Total	90(100%)

Table1 4.2: Distribution of *Pseudomonas aeruginosa* isolates by sample

Sample Sites	No (%) N=81
Urine	24(29.63)
Wound swab	17(20.99)
Ear swab	32(39.51)
Blood	8(9.88)
Total	81(100)

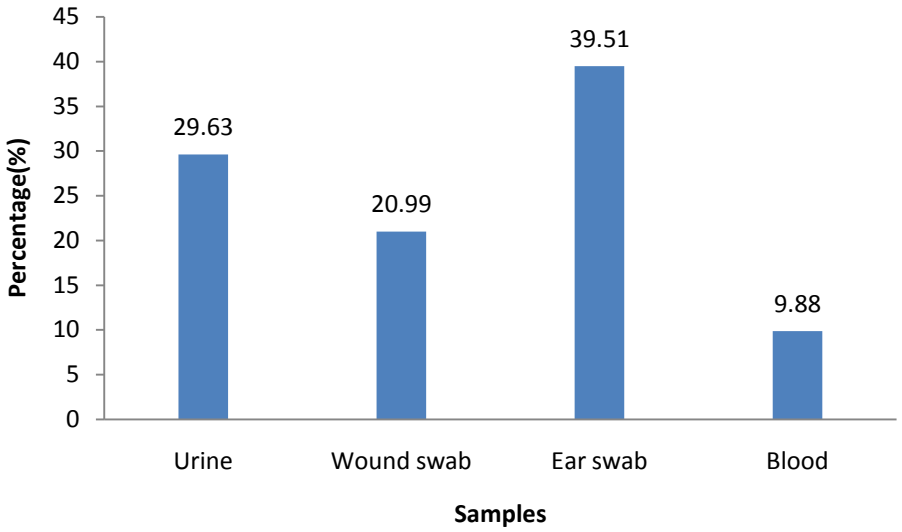


Fig. 4.1: **Percentage occurrence of confirmed *Pseudomonas aeruginosa* (n=81) isolates from the clinical samples**.

4.2 *Pseudomonas aeruginosaBiofilm* production Assay.

A total of 81 *Pseudomonas aeruginosa* isolates were tested for biofilm production, out of which 54 (66.67 %) were positive and 27(33.33 %) were biofilm negative.

4.2.1 Out of the 54 biofilm positive *Pseudomonas aeruginosa* isolates 24(44.4) were from the ear swab (Fig 4.2)

4.2.3Quantitative biofilm production assay

Biofilm production classification was based on method describe by Magesh *et al.*, (2013). Isolates were classified base on the optical density (OD) obtained by subtracting the mean OD_{545} value of control wells from the mean OD_{545} value of the test wells; OD ≤ 0 as non-biofilm producers, OD≥0≤0.04 as weak, OD ≥0.04≤ 0.09 as moderate and OD≥ 0.09≤0.6 as strong biofilm producers.

The quantitative classification of biofilm production in *Pseudomonas aeruginosa* isolates shows that 27(33.33%) were non-producers; 28(34.57%) were weak biofilm producers; 12(14.81%) were moderate producers and 14(17.28%) were strong biofilm producers (Fig.4.3).

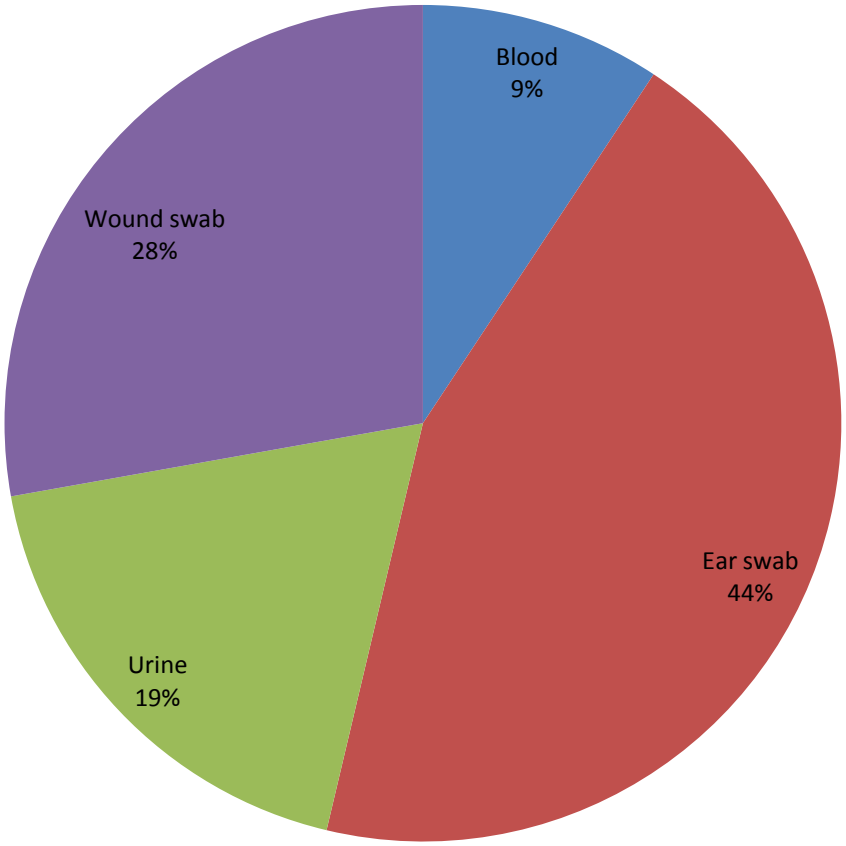


Fig4.2: **Distribution of biofilm-positive *Pseudomonas aeruginosa* isolates by sample source**

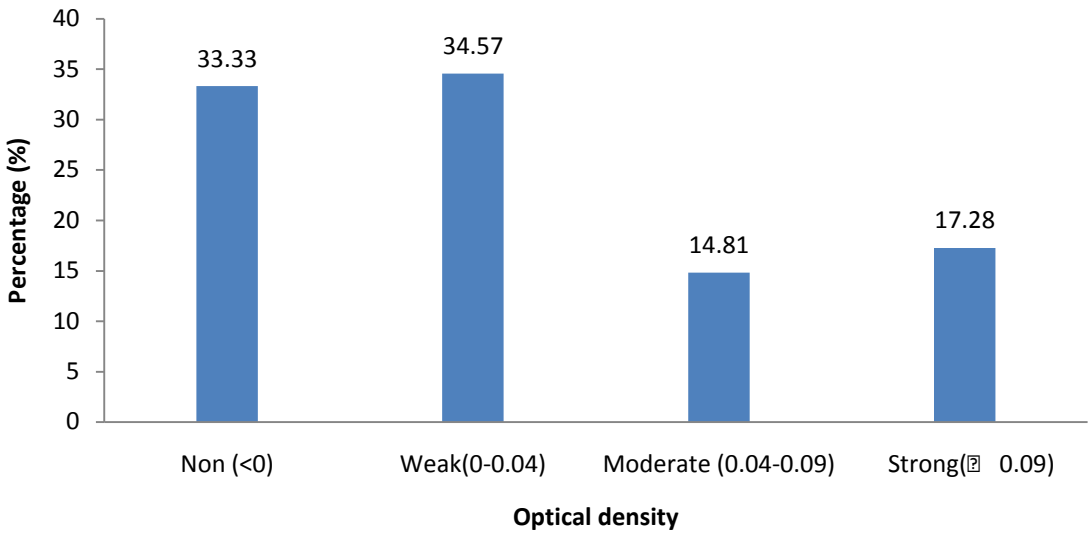


Fig.4.3: Quantitative classification of biofilm production by the clinical isolates of *Pseudomonas aeruginosa* based on OD range

4.3Antibiotics Susceptibility Testing of the isolates

Results of the antibiotic susceptibility testing shows that the Pseudomonas earuginosa isolates were generally resistant to Ticarcillin+Clavulanic acid (72.8%).The percentage susceptibility to the antibiotics tested is shown in Figure 4.4.

4.4 Determination of Multiple Antibiotic Resistance Index (MARI)

The MARI which is used for tracking source of bacteria with high risk of antibiotics over use, shows high percentage occurrence of 47.22% for MARI of 0.4 (Table 4.3).

4.5Antibiotic Resistance in Biofilm-positive and Biofilm-negative *Pseudomonas aeruginosa* isolates.

There was no significant difference antibiotic resistance in Biofilm forming and non-forming *Pseudomonas aeruginosa* isolates (Fig.4.5).

Furhermore, the calculated Pearson‘s product moment correlation coefficient(**r)** to show correlation in antibiotics resistance of *Pseudomonas aeruginosa* isolates to biofilm production potential was is -0.1106 while table value (P @ 0.05) is 0.2573, indicating, no significant correlation.

4.6 Multi-drug Resistance (MDR) Profile of the *Pseudomonas aeruginosa* isolates

The MDR *Pseudomonas aeruginosa* isolates was 44.4% of the 81 isolates. The resistance pattern is shown below in Table 4.4

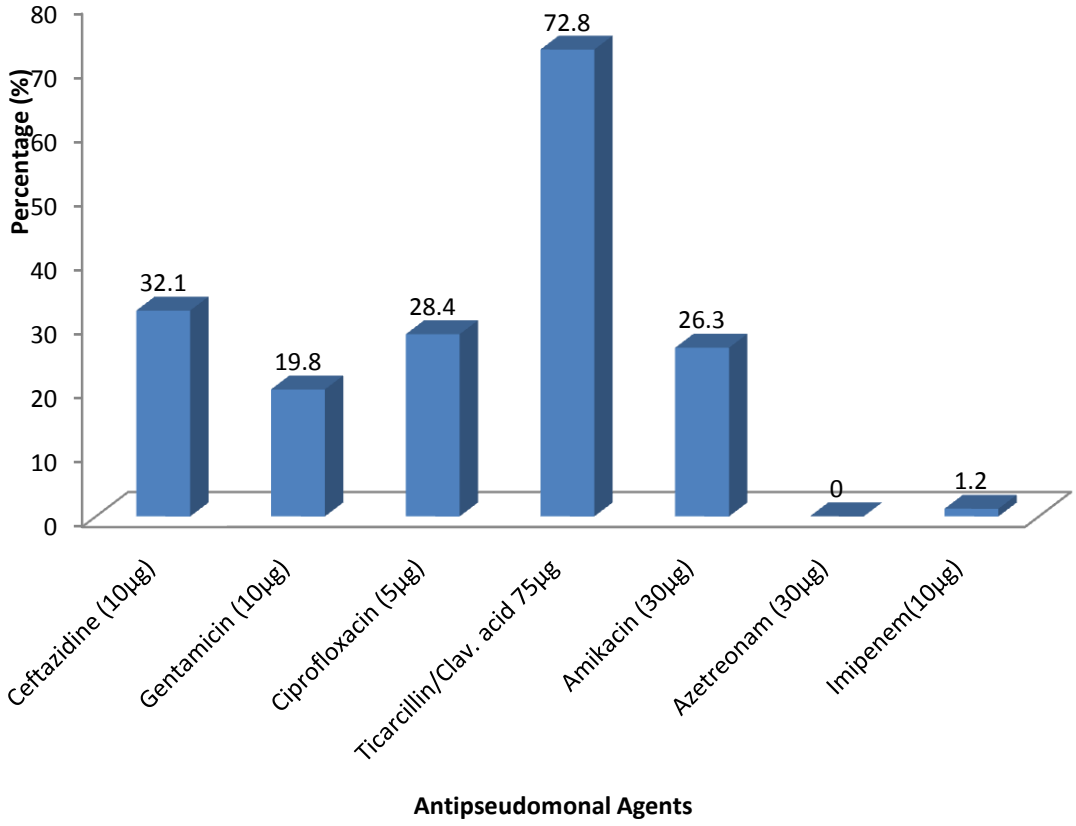


Fig. 4.4: Antibiotic susceptibility of *Pseudomonas aeruginosa*Isolates(n=81) from clinical samples of BSUTH, Makurdi

Table 4.3: Multiple Antibiotic Resistance Index (MARI) and the Percentage

occurrence amongthe Isolates

MAR Index	No.of Isolates	Percentage Occurrence (%)
0.3	15	44.67
0.4	4	11.11
0.6	17	47.22

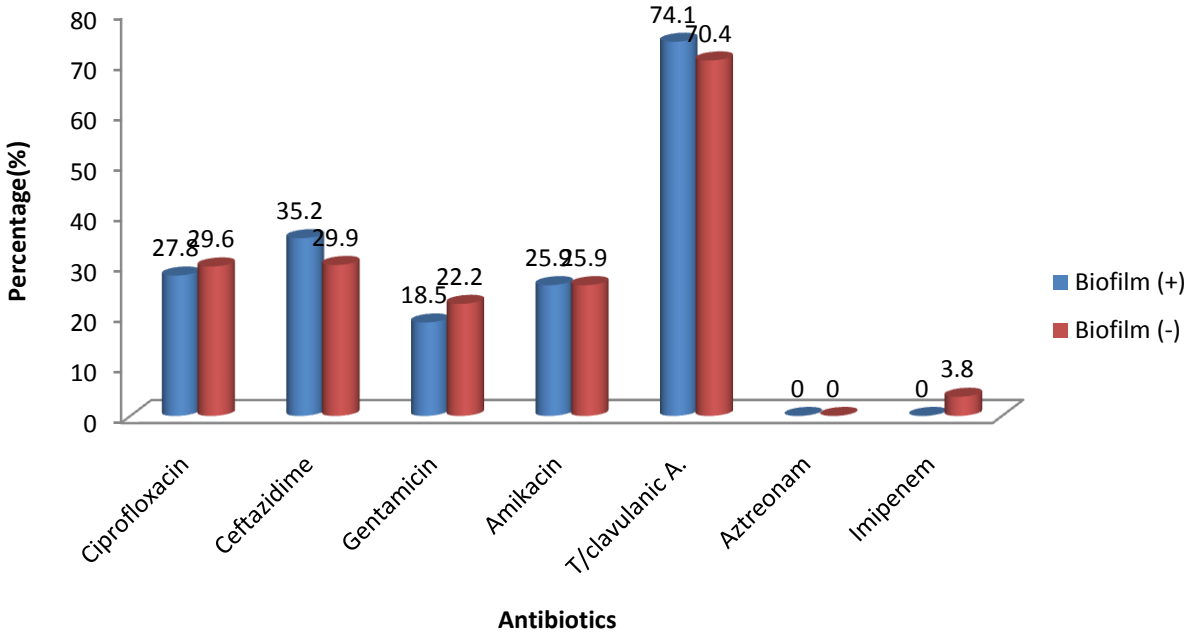


Fig.4.5: Antibiotic resistance pattern in biofilm positive and biofilm negative *Pseudomonas aeruginosa isolates*

TABLE 4.4: Resistance phenotypes of MDR *Pseudomonas aeruginosa* isolates

No of isolates (%)	Resistance pattern
1(1.5)	TIM,CAZ,AK
1(1.5)	TIM,CAZ,A TM,AK,
2(2.9)	TIM, CIP,CAZ
2(2.9)	TIM,CIP,CAZ,AK
1(1.9)	TIM,CN,AK
1(1.5)	TIM,CN,CAZ,AK
11(15.9)	TIM,CN,CIP,AK
2(2.9)	TIM,CN,CIP,CAZ

4.7 Minimum Inhibitory Concentration (MIC) of ciprofloxacin

MIC breakpoints for ciprofloxacin against *Pseudomonas aeruginosa*are MIC of ≤ 0.5

μg/mL (susceptible) and 1 μg/mL(resistant) (EUCAST, 2013).

The MIC of ten ciprofloxacin resistant *Pseudomonas aeruginosa* isolates showed 50% isolates with MIC value of 5μg/mL, 30% had 25μg/mL, while 20% had MIC value of 12μg/mL (Table 4.5). The 10ciprofloxacinresistant*Pseudomonas aeruginosa* isolates were selected based on the least diameter of zone of inhibition.

4.8Plasmid mediated Quinolone Resistant

Varied concentration of Acridine Orange was used to determine the sub-MIC of acridine orange to cure plasmids in 19 ciprofloxacin resistant *Pseudomonas aeruginosa* isolates.

Results of the Antibiotics Susceptibility Testing (AST) for Ciprofloxacin before curing, was compared to the AST after curing treatment showed only 4(21.05%) became sensitive to ciprofloxacin after curing, indicating that the resistannce determinant was probably on a mobile genetic element like plasmids. While 15(78.95%) remained resistant to ciprofloxacin, indicating that the resistant determinants were probably not plasmid mediated (not on mobile genetic elements) (Table 4.6).

TABLE 4.5: D**istribution of MIC values of ciprofloxacin resistant *Pseudomonas aeruginosa* isolates (n=10)**

MIC	Isolates (%)
5 μg/mL	5(50)
12 μg/mL	2(20)
25 μg/mL	2(30)

(Ciprofloxacin MIC test strip 0.002 - 32 μg/mL)

Table 4.6: Diameter of Zone of Inhibition pre and post-curing and antibiotic susceptibility (AST) of ciprofloxacin resistant *Pseudomonas aeruginosa* isolates

Isolates No	Zone of Inhibition diameter	AST

	Pre-curing	post-curing	After curing
39	0	0	R
32	0	0	R
195	13	17	R
12	13	16	R
11	0	0	R
229	13	23	**S**
10	13	13	R
28	0	26	**S**
179	0	0	R
34	12	11	R
219	13	18	R
102	14	25	**S**
45	0	0	R
24	0	0	R
18	0	0	R
37	17	18	R
178	0	0	R
44	15	16	R
23	15	31	**S**

4.9 MOLECULAR CHARACTERIZATION OF CIPROFLOXACIN RESISTANT *PSEUDOMONAS AERUGINOSA*ISOLATES

Pseudomonas aeruginosa isolates that were phenotypically resistant to ciprofloxacin from Antibiotic Susceptibility Testing according to EUCAST, (2013) were selected for molecular

study.

4.91 The result of genomic DNA extraction on Agarose Gel Electrophoresis is shown in Plate.4.1.

4.92 In the detect presence of plasmid mediated resistnce genes (qnr), the result of 1.0% Agarose Gel Electrophoresis of Amplified Quinolone Resistant Isolates Primer (*qnrB*) with DNA ladder of 100bp is shown in Plate 4.2.

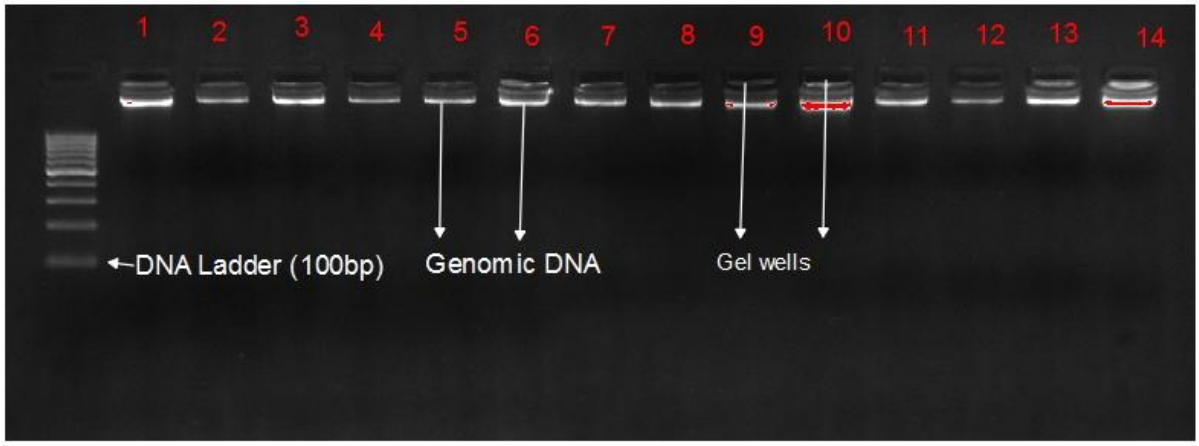


Plate 4.1 Genomic DNA extraction on Agarose Gel Electrophoresis

The result of the 1.0% (w/v) agarose/ethidium bromide gel electrophoresis of the resistant *Pseudomonas aeruginosa* genomic DNA extract, analyzed with DNA ladder of 100bp is shown in plate 4.1.The casting comb lanes bearing the resistant isolates were labeled as follows;

the DNA ladder lane,

Lane 1: Ciprofloxacin resistant clinincal isolate 39

Lane 2: Ciprofloxacin resistant clinincal isolate 32

Lane 3: Ciprofloxacin resistant clinincal isolate 195

Lane 4: Ciprofloxacin resistant clinincal isolate 12

Lane 5: Ciprofloxacin resistant clinincal isolate 11

Lane 6: Ciprofloxacin resistant clinincal isolate 229

Lane 7: Ciprofloxacin resistant clinincal isolate 10

Lane 8: Ciprofloxacin resistant clinincal isolate 28

Lane 9: Ciprofloxacin resistant clinincal isolate 179

Lane 10: Ciprofloxacin resistant clinincal isolate 34

Lane 11: Ciprofloxacin resistant clinincal isolate 219

Lane12: Ciprofloxacin resistant clinincal isolate 102

Lane 13: Ciprofloxacin resistant clinincal isolate 45

Lane 14: Ciprofloxacin resistant clinincal isolate 24.

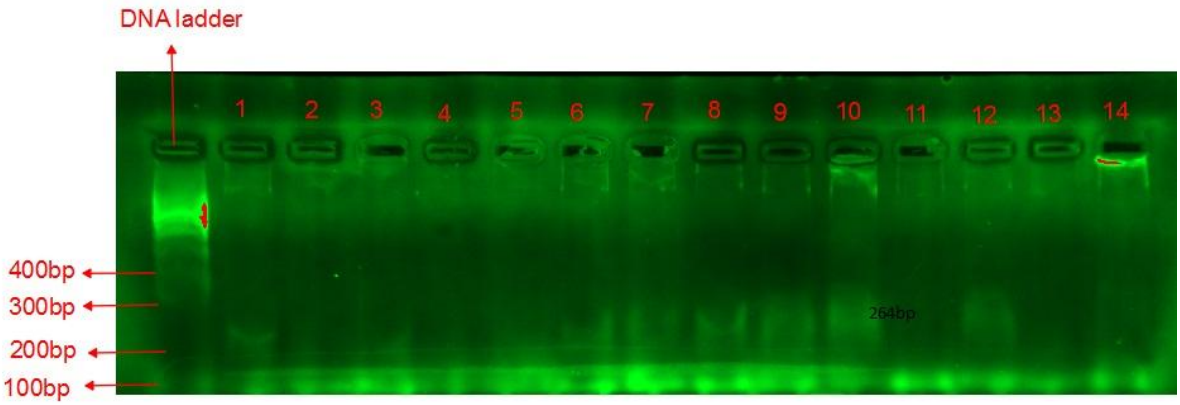


Plate 4.2: 1.0% Agarose Gel Electrophoresis of Amplified product of Genomic DNA with qurB primers

FROM THE LEFT TO THE RIGHT BANDS:

Lane 1:100bp DNA ladder compose of DNA fragments in base pairs of 1000,900,800,700,600, 500,400,300.200 and 100bp

Lane 2, lane 6, 8,9 and 10 showed band corresponding to 264bp. Lane 3, 4, 5, 6, 8, 10, 12-14 showed only primer fronts.

CHAPTER FIVE

5.0 DISCUSSION

Pseudomonasaeruginosa has been associated with infections of the blood, ear, eye, post-surgery and patients with wounds from surgery or burns (CDC, 2014; NCEZID, 2014). In this study, the isolation rate of *Pseudomonas aeruginosa* was 5.4% in all the clinical samples over the six month period of sample collection. Eighty one (81) isolates of *Pseudomonas aeruginosa* were obtained from a total of 1492 clinical samples submitted to

the Microbiology laboratory of Benue State University Teaching Hospital, Makurdi. *Pseudomonas aeruginosa* were isolated from all the sample sources in different proportion with highest prevalence being from ear swab 32(39.51%) and the lowest 8(9.87%) from blood. *Pseudomonas aeruginosa* has been isolated from various clinical samples, Ogbolu *et al.,* (2009) isolated majority of their isolates from wounds specimens 39%), followed by ear swabs 30.2%, catheter tips 15.1%, urine 7.5%, aspirate 3.8% and urethral 1.9%. Olayinka *et al.*, (2004) in Zaria, isolated 30.2% from ear swab, 8.6% from urine and 1.1 % from blood while Ozumba, (2003) at Enugu isolated 46% *Pseudomonas aeruginosa* from urine, Garba *et al.,* (2012) in Zaria isolated 11% from wound while Hossein *et al.,* (2012) in India reported 5% from blood. This distribution is in line with documentary evidence that *Pseudomonasaeruginosa* is a clinically significant opportunistic pathogen that colonizes more than 50% of humans, often causing nosocomial infections (Selina *et al.*, 2014). Ithas been reported as a predominant cause of nosocomial pneumonia in ventilated patients (Nicotra *et al.,* 1995), and bacterial persistence in cystic fibrosis (Drenkard and Ausubel, 2002). *Pseudomonas aeruginosa* is the third leading cause of hospital-acquired urinary tract infections (Shaw, 2005) and has also been implicated in bacteraemia and septicaemia as a common complication in patients with immunodeficiency (Krcmery *et al.*, 2006; Marra *et al.,* 2006), as well as the cause of devastating inner ear infections (Wise *et al.,* 1969; Ehrlih *et al*.,2002).Biofilm production has been reported in strains of *Pseudomonas aeruginosa* associated with the infection of biomedical devices (Donlan and Costerton, 2002) and other infections (Sanchez *et al.*, 2013). An evaluation of the biofilm production in the 81 *Pseudomonas aeruginosa* isolates tested showed that majority of the isolates, (54) 67% were biofilm producers (Table 7). This result is comparable to the observations of Nagaveni *et al.*, (2010) detected non- biofilm producing isolates of*Pseudomonas*

aeruginosa, though Donlan and Costerton, (2002) stated all strains of *Pseudomonas aeruginosa* isolatesas biofilm producers. According to Nagaveni *et al.*, (2010), the observed difference could be due to a strong dependence on growth conditions for biofilm formation in *Pseudomonas aeruginosa*. Biofilms protect bacterial like *Pseudomonas aeruginosa,* against offensive like antibiotic and host defense system (Coenye *et al.*, 2011). Nagaveni *et al.*, (2010) reported that *Pseudomonas aeruginosa* forms biofilm readily, which may be the most important reason why the infections cannot be effectively treated and cured. Furthermore, in this study, the highest proportion of *Pseudomonas aeruginosa* that produce biofilm were isolated from ear swab 24(44.4%) and the least was from blood 5(9.3%). Juman *et al*, (2015) reported a 55(57.30%) isolate rate for biofilm producers from ear swab and reasoned along with Claudia *et al*., (2013) that the dominance of biofilm producers from the ear swab may be due to favourable pH growth condition of the ear canal for an enzyme (luxS) expression involved in the *S*-adenosylhomocysteine pathway that synthesizes autoinducer 2 (AI-2), a molecule involved in bacterial quorum sensing and biofilm formation. Also Hall-stoodly *et al.*, (2006) reported that the high rate of biofilm formation among the isolates from the ear might be because of absence of growth medium in otitis media lead to biofilm formation. It has been stated that majority of ear infections are caused by biofilm bacteria such that in many cases recurrent disease stems not from re-infection as was previously thought and which forms the basis for conventional treatment, but from a persistent biofilm (Ehrlich and Christopher 2002). *Pseudomonas aeruginosa* isolates from urine, wound, blood also produced biofilm and are reported in related infections; Sara, (2010) reported that biofilms in urinary tract infections is responsible for persistent infections, causing relapses and acute prostatitis, Davis *et al.*, (2008) reported that biofilmsimpairs cutaneous wound healing and reduce topical antibacterial efficiency

and Parsek, (2003) reported that biofilm provides a source of infection of the bloodstream that persists even during antibiotic treatment.

The introduction of the antipseudomonads, based on therapeutically relevant antimicrobial groups, has improved substantially the prognosis of *Pseudomonas aeruginosa* infections (Selina *et al.*, 2014). The following antipseudomonads shown by many studies to be active against *Pseudomonas aeruginosa* were used in this study: Ciprofloxacin 5µg (CIP), Gentamicin 10µg (CN), Amikacin 10µg (AK), Ceftazidine 10µg (CAZ), Ticarcillin/Clavulanic acid 75µg (TIM), Azetreonam 30µg (ATM) and Imipenem 10µg (IPM). *Pseudomonas aeruginosa* isolates in this study were resistant to almost all the antipseudomonads antibiotics tested (table 4.3). The highest resistance was observed against Ticarcillin/Clavulanic acid. Though the isolates were from different clinical specimen, they exhibited similar resistance trends to Ticarcillin/Clavulanic acid. Isolates from wounds swab and urine samples were both observed to be 70% resistant while isolates from ear swab and blood were observed to be 75% resistant to Ticarcillin/Clavulanic. High resistance to Ticarcillin/Clavulanic has also been observed by Odumosu *et al.*, (2012) at Ibadan who reported 87% resistant rate and Anuradha *et al*, (2007) reported 96% resistance. The high resistance to Ticarcillin/Clavulanic acid might be due to the potential of clavulanic acid to induce expression of chromosomal cephalosporinase which can antagonize antibacterial activity of ticarcillin (Anuradha *et al*, 2007). The resistance expressed by *Pseudomonas aeruginosa* isolates to Ceftazidine (32.1%) in this study were observed to be higher than the 20.6% reported by Aibinu *et al.*, (2007) (20.6 %) and 22% reported by Olayinka *et al.*, (2009) in Zaria (22%), but lower than the 50% resistance level reported by Okesola and Oni (2012). The resistance level of *Pseudomonas aeruginosa* to gentamicin in this study is comparable to the 33.5% reported by Okesola and Oni (2012)

but many other studies have reported higher resistance rates of 41% (Odumosu *et al.*, 2012), 45.4% (Garba *et al*, 2012) and 55.5% (Ozumba,2003).

The resistance to these antipseudomonads aminoglycosides may be due to drug inactivation by chromosomal and plasmid-encoded modifying enzymes, defects in uptake and accumulation of aminoglycosides in the bacterial cytoplasm (impermeability resistance), (**Miller *et al*., 1994; Miller *et al.*, 1995; Goossens,** 2003). Furthmore, the low resistance of *Pseudomonas aeruginosa* to Amikacin reported in this work is similar to the 25% reported by Chander and Raza (2013), 22% by Odumosu *et al.*, (2012) and the 21.7% Ogbolu *et al.*, (2008). The resistance level to Ciprofloxacin by the isolates in this work is comparable to the 35% report by Odumosu *et al.*, (2012) but lower than the 75% reported by Chander and Raza (2013). Increase development of resistance to this flouroquinolones may be due to factors, such as increased use of flouroquinolones due to availability of low priced generics flouroquinolones, self-medication (O'Connor *et al*., 2001) and possible misuse (John, 2001). This result of this study is line with report that there is increase incidence of resistance to flouroquinolones (Agrawal *et al*., 2009). Resistance level of 1.2% expressed by the clinical isolates against Imipenem in this study is comparable to the 1.9% report by Odumosu *et al.*, (2012) at Ibadan. This implies that imipenem remain a highly potent antipseudomonal agent in the study areas in Nigeria. None of the clinical isolates of *Pseudomonas aeruginosa* in this study showed resistance against Aztroenam.This result is in contrast to the 16.0% resistance level reported by Emmanuel *et al.*, (2013) and 36.1% reported Aibinu *et al.*, (2013). The lack of resistance to imipenem, a carbepenem is probably because the agent is not commonly used in this environment, being inaccessible as a result of the high cost (Kristina, 2007). .

Our result showed that 44.4% of the isolates were multidrug resistant (MDR). This level of

resistance is in line with the report by Mesaros *et al.*, (2007) and Nagaveni *et al.*, (2010) that *Pseudomonas aeruginosa* has high resistance with different mechanisms against wide range of structurally and functionally unrelated antimicrobial agents, such as penicillin, aminoglycosides like gentamicin, third generation Cephalosporins, Carbepenems like Imipenem and Meropenem, Fluroquinolones like Ciprofloxacin, Norfloxcin, Tetracycline etc. The incidence of MDR *Pseudomonas aeruginosa* in this study support the observation by Olusanya (2000), that in addition to the increasing trend of antibiotics resistance, the current spread of multidrug resistant bacterial pathogens has added a new dimension to the problem of infections.

Multiple antibiotic resistances index (MARI) has been shown to be a valid method for antibiotics resistant bacteria source tracking (Osundiya *et al.*, 2013). Out of the 81 *Pseudomonas aeruginosa* isolates tested for antibiotics susceptibility, 44.67% of the isolates had MARI of ≥0.3, and 47.22% had MARI of ≥0.3. These indices showed that the isolates in the study area are likely exposed to antibiotic misuse (John, 2001). The high MAR index indicates a serious need for antibiotics stewardship program in BSUTH, Makurdi.

According to Sauer *et al.*, (2002) and Anderson *et al.*, (2008), biofilm formation is accompanied by significant changes in gene and protein expression, which confers resistance to antimicrobial agents. Analysis of antibiotic resistance of biofilm positive and biofilm negative clinical isolates of *Pseudomonas aeruginosa* show no significant difference in the resistance profile, even among the MDR isolates. Further statistical analysis using Pearson‘s product moment correlation coefficient (r) equallyshowed no significant difference in antibiotics resistance profile of biofilm positive and biofilm negative clinical isolates of *Pseudomonas aeruginosa.* Thus the null hypothesis was not

rejected. Therefore the ability of *Pseudomonas aeruginosa* pathogen to form biofilm does not necessary affect its resistance to antibiotics.

The highest MIC value by ciprofloxacin resistant *Pseudomonas aeruginosa* isolates was of ≤ 25µg/ml, while the least was ≥5µg/ml. This shows that these isolates have high resistant property to ciprofloxacin and may be due to its increased use in the environment because low price generics (Zelenitsky *et al*., 2003).

The result of the Plasmid curing showed that 4(21.05%) of the ciprofloxacin resistant isolates demonstrated change in resistance to ciprofloxacin, indicating that the resistance determinant were probably plasmids mediated. However the presence of transferable quinolones resistance was not demonstrated by conjugation in this study. In this study ciprofloxacin resistant *Pseudomonas aeruginosa* isolates haboured qnrB 4(21.1%) quinolone resistant gene, though Cayci *et al*., (2014) reported detection of the qnrB resistant determinant genes in *Pseudomonas florescens* and *Pseudomonas putia* but none in *Pseudomonas* aeruginosa.This influence the rate at which multiple resistant bacteria constitute a global problems (Obrien, 1997).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND REECOMMENDATION

6.1 SUMMARY

This study showed that eighty one (81) *Pseudomonas aeruginosa* isolates were isolated from 1492 clinical samples from Medical Laboratory of BSUTH within the six (6) month period of the study at a recovery rate of 5.4%. The isolates were mainly from ear swab (39.51%), urine (29.63%) and wound swab (20.99%).

Majority of *Pseudomonas aeruginosa* isolates formed biofilm 54(66.70%). The highest proportion of *Pseudomonas aeruginosa* isolates forming biofilm were from ear swab (44.4%). Most of the isolates were resistant to ticarcillin/clavulanic acid (72.80%) and ceftazidime (32.10%). Isolates were generally susceptible to imipenem (98.8%) and gentamicin (80.20%). Majority of the *Pseudomonas aeruginosa* isolates were multidrud-resistant (44.44%), being resistant to at least one agent in three or more classes of

antimicrobial agents. Isolates with MAR index ≥ 0.3 were 44.4%, indicating they probably originated from an environment where antibiotics are often used.

The highest Minimum inhibitory Concentration (MIC) of 25µg/ml was by (30%) of the ciprofloxacin -multidrug resistant *Pseudomonas aeruginosa* isolates. Correlation of antibiotic resistance pattern in biofilm positive and biofilm negative was not significant. In this study ciprofloxacin resistant *Pseudomonas aeruginosa* isolates haboured qnrB 4(21.1%) quinolone resistant gene, though Cayci *et al*., (2014) reported detection of the qnrB resistant determinant genes in *Pseudomonas florescens* and *Pseudomonas putia* but none in *Pseudomonas* aeruginosa.

6.2 CONCLUSION

From the findings in this study *Pseudomonas aeruginosa* was associated with infections in clinical samples from BSUTH. Majority of *Pseudomonas aeruginosa* isolates (66.70%) in this study formed biofilm, with most being from the ear swab (44.4%). *Pseudomonas aeruginosa* isolates in this study were resistance to antimicrobial agent used. Most resistance was against ticarcillin-clavulanic acid. The Mult-drug Resistant *Pseudomonas aeruginosa* isolates were also identified (44.44%). There was no significant difference in antibiotic resistance pattern in biofilm positive and biofilm negative clinical isolates of *Pseudomonas aeruginosa*.

6.3 RECOMMENDATION

This study had further establish that *Pseudomonas aeruginosa* isa prominent causative agent of nosocomial infections, therefore it is recommended that proper infection control measure be put in place in our hospital especially where this study was carried out. Now that biofilm producing *Pseudomonas aeruginosa* isolates from clinical samples are part of

our community, the need to institute an efficient surveillance strategies, should be encourage by policy makers to avoid the incidence of high mortality and morbidity associated with chronic infection of biofilms. The following measure should be undertaken:

- Efficient biofilm screening mechanism should be put in place in our Hospitals.
- Training on how to screen biofilm bacterial should be encourage
- Training on efficient strategy on antibiotics treatment of potential and actual biofilm infections should be encourage
- Adjust antibiotics base on results of culture and sensitivity testing
- Prophylactic antibiotics prior to invasive procedures should be considered
- Provide education on infection control and wound care to patients
- Proper hygiene should be maintained in our hospitals

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APPENDICES

Appendix I

Incidence of *Pseudomonas aeruginosa* isolates collected from Clinical Samples in BSUTH, Makurdi

	Urine		w/swab		Ear swab		blood	
	Total sample/mcs	+ P.aeru.	Total sample	+ P.aeru.	Total sample	+P.aeru.	Total sample	+P.aeru.
Sept	247	4	8	1	1	1		
Oct	252	7	10	3	8	6	30	3
Nov	75	0	2	0	3	2	16	1
Dec	219	3	16	7	12	7	26	3
Jan	275	5	11	2	14	9	26	2
Feb	224	3	13	4	10	8	14	2

Total	**1292**	**25**	**50**	**18**	**48**	**33**	102	14

Appendix II:

BIOCHEMICAL TESTS

S/No	ISO. No	OXIDASE TESTS	TSIA	CIT. TEST	INFERENCE
1	162	+	-	+	*Pseudomonas aeruginosa*
2	6	+	-	+	*Pseudomonas aeruginosa*
3	26	+	-	+	*Pseudomonas aeruginosa*
4	450	+	-	+	*Pseudomonas aeruginosa*
5	617	+	-	+	*Pseudomonas aeruginosa*
6	1	+	-	+	*Pseudomonas aeruginosa*
7	39	+	-	+	*Pseudomonas aeruginosa*
8	27	+	-	+	*Pseudomonas aeruginosa*
9	55	+	-	+	*Pseudomonas aeruginosa*
10	9	+	-	+	*Pseudomonas aeruginosa*
11	43	+	-	+	*Pseudomonas aeruginosa*
12	30	+	-	+	*Pseudomonas aeruginosa*
13	32	+	-	+	*Pseudomonas aeruginosa*
14	17	+	-	+	*Pseudomonas aeruginosa*
15	35	+	-	+	*Pseudomonas aeruginosa*
16	5	+	-	+	*Pseudomonas aeruginosa*
17	558	+	-	+	*Pseudomonas aeruginosa*

18	33	+	-	+	*Pseudomonas aeruginosa*
19	11	+	-	+	*Pseudomonas aeruginosa*
20	7	+	-	+	*Pseudomonas aeruginosa*
21	175	+	-	+	*Pseudomonas aeruginosa*
22	12	+	-	+	*Pseudomonas aeruginosa*
23	19	+	-	+	*Pseudomonas aeruginosa*
24	444	+	-	+	*Pseudomonas aeruginosa*
25	198	+	-	+	*Pseudomonas aeruginosa*

S/No	ISO. No	OXIDASE TESTS	TSIA	CIT. TEST	INFERENCE
26	46	+	-	+	*Pseudomonas aeruginosa*
27	38	+	-	+	*Pseudomonas aeruginosa*
28	2	+	-	+	*Pseudomonas aeruginosa*
29	229	+	-	+	*Pseudomonas aeruginosa*
30	0J	+	-	+	*Pseudomonas aeruginosa*
31	10	+	-	+	*Pseudomonas aeruginosa*
32	13	+	-	+	*Pseudomonas aeruginosa*
33	25	+	-	+	*Pseudomonas aeruginosa*
34	28	+	-	+	*Pseudomonas aeruginosa*
35	37	+	-	+	*Pseudomonas aeruginosa*
36	179	+	-	+	*Pseudomonas aeruginosa*
	911				
37		+	-	+	*Pseudomonas aeruginosa*
38	34	+	-	+	*Pseudomonas aeruginosa*
39	29	+	-	+	*Pseudomonas aeruginosa*
40	219	+	-	+	*Pseudomonas aeruginosa*
41	102	+	-	+	*Pseudomonas aeruginosa*
42	646	+	-	+	*Pseudomonas aeruginosa*
43	45	+	-	+	*Pseudomonas aeruginosa*
44	24	+	-	+	*Pseudomonas aeruginosa*

45	18	+	-	+	*Pseudomonas aeruginosa*
46	23	+	-	+	*Pseudomonas aeruginosa*
47	152	+	-	+	*Pseudomonas aeruginosa*
48	467	+	-	+	*Pseudomonas aeruginosa*
49	72	+	-	+	*Pseudomonas aeruginosa*
50	22	+	-	+	*Pseudomonas aeruginosa*
51	196	+	-	+	*Pseudomonas aeruginosa*

S/No	ISO. No	OXIDASE TESTS	TSIA	CIT. TEST	INFERENCE
52	773	+	-	+	*Pseudomonas aeruginosa*
53	111	+	-	+	*Pseudomonas aeruginosa*
54	101	+	-	+	*Pseudomonas aeruginosa*
55	73	+	-	+	*Pseudomonas aeruginosa*
56	839	+	-	+	*Pseudomonas aeruginosa*
57	37	+	-	+	*Pseudomonas aeruginosa*
58	3	+	-	+	*Pseudomonas aeruginosa*
59	261	+	-	+	*Pseudomonas aeruginosa*
60	114	+	-	+	*Pseudomonas aeruginosa*
61	13	+	-	+	*Pseudomonas aeruginosa*
62	15	+	-	+	*Pseudomonas aeruginosa*
63	14	+	-	+	*Pseudomonas aeruginosa*
64	461	+	-	+	*Pseudomonas aeruginosa*
65	680	+	-	+	*Pseudomonas aeruginosa*
66	4	+	-	+	*Pseudomonas aeruginosa*
67	36	+	-	+	*Pseudomonas aeruginosa*
68	682	+	-	+	*Pseudomonas aeruginosa*

69	178	+	-	+	*Pseudomonas aeruginosa*
70	275	+	-	+	*Pseudomonas aeruginosa*
71	868	+	-	+	*Pseudomonas aeruginosa*
72	354	+	-	+	*Pseudomonas aeruginosa*
73	44	+	-	+	*Pseudomonas aeruginosa*

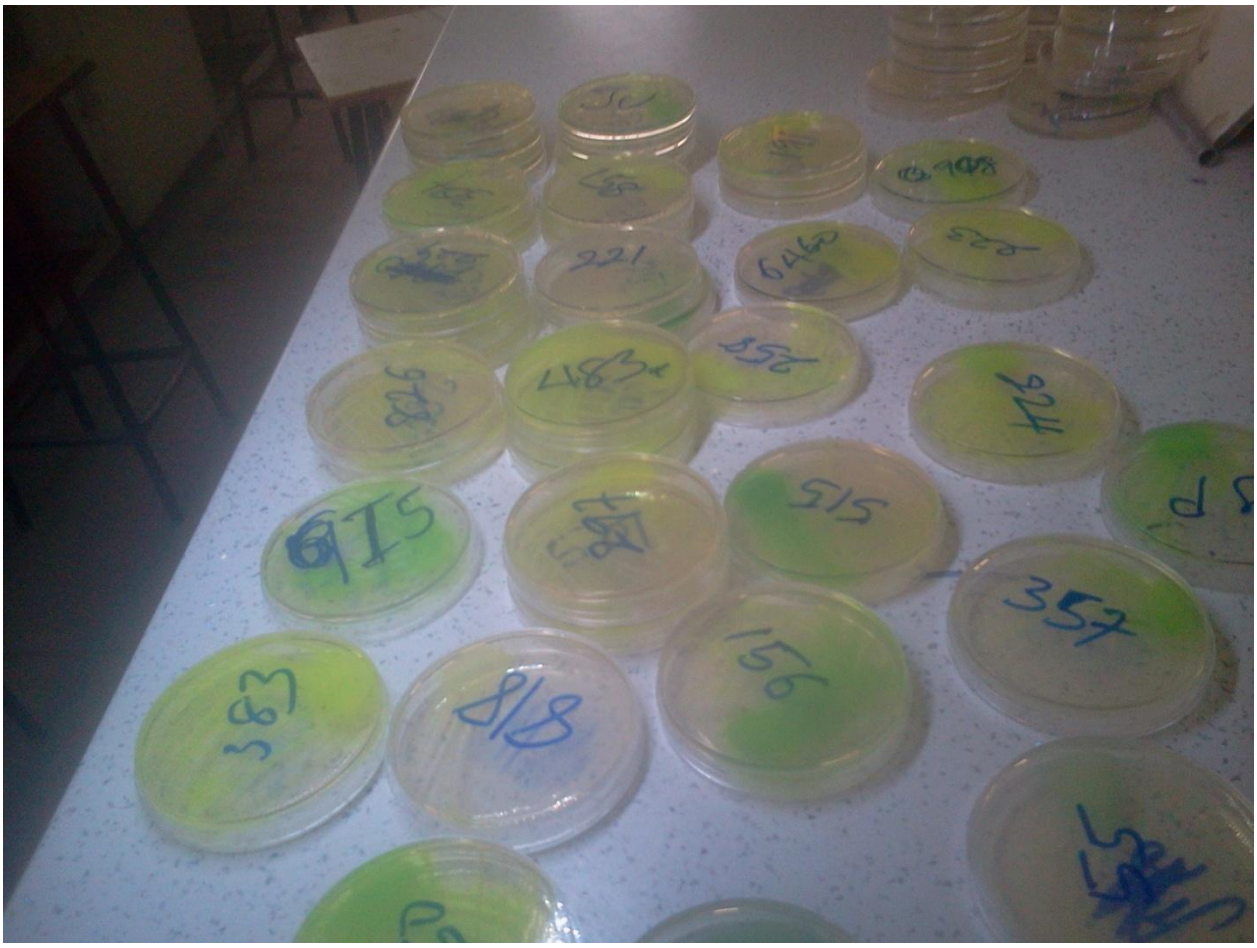
S/No	ISO. No	OXIDASE TESTS	TSIA	CIT. TEST	INFERENCE
74	42	+	-	+	*Pseudomonas aeruginosa*
75	40	+	-	+	*Pseudomonas aeruginosa*
76	185	+	-	+	*Pseudomonas aeruginosa*
77	8	+	-	+	*Pseudomonas aeruginosa*
78	684	+	-	+	*Pseudomonas aeruginosa*
79	232	+	-	+	*Pseudomonas aeruginosa*
80	195	+	-	+	*Pseudomonas aeruginosa*
81	41	+	-	+	*Pseudomonas aeruginosa*
82			-		*Pseudomonas aeruginosa*
			-		*Pseudomonas aeruginosa*

All isolates that showed greenish yellow pigmentation on cetrimide agar where noted as *Pseudomonas aeruginosa*

KEY: +; POSITIVE, - ; NEGATIVE,

Appendix III

Pictorial Presentation of Isolates on Cetrimide Agar



Appendix IV: **Absorbance optical density results Biofilm Production and its classification**

ISO. No	Biofilm Quantification	Biofilm Production Classification
162	0.058333 ± 0.1	3
6	-0.00253 ± 0.1	1
26	0.015667 ± 0.1	2
450	0.005 ± 0.1	2
617	-0.00633 ± 0.1	1
1	-0.01567 ± 0.1	1
39	-0.008 ± 0.1	1
27	-0.003 ± 0.1	1
55	-0.02233 ± 0.1	1
9	-0.02167 ± 0.1	1
43	-0.003 ± 0.1	1
30	0.037667 ± 0.1	2
32	0.000333 ± 0.1	2
17	-0.01367 ± 0.1	1
35	0.016 ± 0.1	2
5	-0.01567 ± 0.1	1
558	0.025667 ± 0.1	2
33	-0.00233 ± 0.1	1
11	-0.00967 ± 0.1	1
7	0.047667 ± 0.1	3
175	-0.015 ± 0.1	1

12	-0.02333 ± 0.1	1
19	0.0118 ± 0.1	2
444	0.107333 ± 0.1	4
198	0.607667 ± 0.1	4
46	0.136667 ± 0.1	4
38	0.007667 ± 0.1	2

ISO. No	Biofilm Quantification	Biofilm Production Classification
2	0.018667 ± 0.1	2
229	0.045333 ± 0.1	3
0J	0.004667 ± 0.1	2
10	-0.00467 ± 0.1	1
13	0.085333 ± 0.1	3
25	0.127333 ± 0.1	4
28	0.005333 ± 0.1	2
37	0.136333 ± 0.1	4
179	0.376 ± 0.1	3
911	0.093667 ± 0.1	4
34	0.054333 ± 0.1	3
29	0.025333 ± 0.1	2
219	0.037667 ± 0.1	2
102	0.021667 ± 0.1	2
646	0.023667 ± 0.1	2
45	0.016667 ± 0.1	2
24	0.012333 ± 0.1	2
18	0.023667 ± 0.1	2
23	0.071333 ± 0.1	3
152	0.078667 ± 0.1	3
467	-0.00967 ± 0.1	1
72	0.106333 ± 0.1	4

22	0.069333 ± 0.1	3
196	0.102333 ± 0.1	4
773	0.015667 ± 0.1	2
111	0.015333 ± 0.1	2
101	0.091 ± 0.1	3

ISO. No	Biofilm Quantification	Biofilm Production Classification
839	0.027333 ± 0.1	2
37	0.074867 ± 0.1	3
3	0.209 ± 0.1	4
261	0.587 ± 0.1	4
114	0.127 ± 0.1	4
13	0.002333 ± 0.1	2
15	0.015333 ± 0.1	2
14	0.034333 ± 0.1	2
461	-0.003 ± 0.1	1
680	-0.011 ± 0.1	1
4	0.079667 ± 0.1	3
36	0.235667 ± 0.1	4
682	0.093 ± 0.1	4
178	-0.00033 ± 0.1	1
275	-0.01967 ± 0.1	1
868	0.005667 ± 0.1	2
354	-0.02833 ± 0.1	1
44	-0.02267 ± 0.1	1
42	0.001333 ± 0.1	2
40	0.037667 ± 0.1	3
185	-0.024 ± 0.1	1
8	-0.021 ± 0.1	1
684	-0.01867 ± 0.1	1
232	-0.02067 ± 0.1	1
195	-0.01133 ± 0.1	1
41	0.009667 ± 0.1	4

Key: Non-biofilm = 1, Weak biofilm = 2, Moderate biofilm =3 and Strong biofilm = 4

Appendix V

Biofilm Production Classification

NON (≤0)	WEAK (0-0.04)	MODERATE (0.04-0.09)	STRONG (0.09-0.6)
27	28	12	14

Appendix VI

Table 3: Zone Diameter Interpretative Standard Using European Committee on Antimicrobial Susceptibility Testing (EUCAST,2013)

S/No.	Antibiotics	Zone Diameter(mm)		
		Resistance	Intermediate	Sensitive
1	Ceftazidine (10µg)	≤ 16	-	≥16
2	Gentamicin (10µg)	≤15	-	≥15
3	Ciprofloxacin (5µg)	≤22	23-25	≥25
4	Ticarcillin+ Clavulanic acid(75µg)	≤17	-	≥17
5	Amikacin (30µg)	≤15	16-18	≥18
6	Azetreonam (30µg)	≤16	16-50	≥50
7	Imipenem(10µg)	≤17	17-20	≥20

AppendixVII:

ZONE OF INHIBITION EXHIBITED BY CLINICAL ISOLATES OF *Pseudomonas aeruginosa* USING OXOID ANTIBIOTIC DISKS

S/No	ISO. No	TIM 75µg	CN 10 µg	CIP 5µg	CAZ 10 µg	IPM 10 µg	ATM 30 µg	AK 30 µg
1	162	13	21	29	10	32	22	18
2	6	18	22	30	20	23	24	14
3	26	NZ	18	15	19	20	24	16
4	04	20	21	28	18	28	22	19
5	617	16	24	30	20	28	22	15
6	01	10	21	28	14	22	27	18
7	39	13	NZ	NZ	20	23	20	14
8	27	18	21	30	20	27	22	19
9	55	15	20	30	22	23	22	18
10	09	17	21	30	22	25	23	20
11	43	15	18	22	20	25	25	14
12	30	20	28	24	18	27	25	21
13	32	NZ	NZ	NZ	12	21	18	16
14	17	18	20	19	R	22	20	18
15	35	18	20	30	20	23	22	19
16	05	10	18	18	12	15	20	12
17	558	11	19	13	12	24	20	14
18	033	19	20	19	8	28	24	14
19	011	10	NZ	NZ	18	20	20	16

20	07	8	20	13	8	26	19	18
21	175	15	21	30	21	25	22	18
22	012	11	18	13	R	24	17	18
23	019	16	23	31	20	22	25	19
24	444	11	21	28	20	25	27	20
25	198	16	22	29	23	20	21	19
26	46	13	19	27	24	24	20	15
27	38	8	19	28	15	26	23	17
28	02	12	20	25	23	23	24	17
29	229	23	28	13	19	20	22	22
30	0J	14	21	31	21	30	21	16
31	10	NZ	8	13	22	21	20	13
32	13	NZ	20	28	16	24	24	18
33	25	18	20	26	11	26	21	18
34	28	NZ	NZ	NZ	10	26	21	15
35	37	NZ	21	27	13	25	19	19
36	179	NZ	NZ	NZ	20	21	20	13
37	911	15	20	28	20	23	20	15
38	34	15	13	12	20	25	24	13
39	29	25	25	25	23	23	16	19
40	219	15	R	13	18	24	20	14
41	102	8	13	14	10	20	18	12

42	646	12	19	22	13	19	21	17
43	45	R	R	R	8	22	24	14
44	24	10	R	R	12	23	20	13
45	18	R	R	R	15	19	18	12
46	23	16	18	15	16	20	21	18
47	152	14	17	18	14	25	20	13
48	467	8	R	R	13	21	23	14
49	72	16	21	24	17	23	20	18
50	22	12	20	25	12	22	14	18
51	196	17	24	28	19	24	21	19
52	773	17	18	24	20	26	24	18
53	111	15	20	26	24	25	21	17
54	101	18	21	27	17	22	19	18
55	73	15	19	21	16	20	22	18
56	839	16	18	20	13	22	26	18
57	37	18	18	17	16	24	16	13
58	3	16	22	28	20	25	18	15
59	261	15	19	21	20	23	19	12
60	114	16	22	28	19	24	21	13
61	13	13	14	19	25	16	20	14
62	15	16	17	30	21	24	23	15
63	14	18	20	20	20	23	23	13
64	461	17	17	25	17	20	19	14

65	680	15	20	29	16	22	18	13
66	04	NZ	27	31	20	21	20	15
67	36	17	20	27	20	24	25	15
68	682	15	21	28	18	20	20	13
69	178	NZ	NZ	NZ	18	22	16	12
70	275	16	22	27	19	27	19	14
71	868	14	19	23	16	25	20	14
72	354	14	16	20	19	20	20	12
73	44	18	25	15	23	R	18	13
74	42	18	20	28	23	23	20	13
75	40	18	20	30	22	22	21	13
76	185	20	20	21	10	23	24	15
77	08	14	20	20	17	20	19	14
78	684	10	20	23	15	22	20	14
79	232	18	20	25	17	26	27	18
80	195	8	20	21	16	22	14	12
81	41	15	17	21	17	23	20	12

Appendix VIII

Antibiotic Susceptibility in Relation to Biofilm production

	NON	WEAK	MODERATE	STRONG

BIOFILM ISOLATE	27			28			12			14		
RESISTANCE PATTERN	S	R	I	S	R	I	S	R	I	S	R	I
TIM	9(33%)	18(67%)	0	8(29%)	20(71%)	0	5(42%)	8(58%)	-	4(29%)	10(71%)	-
CN	21(78%)	6(22%)	0	19(68%)	9(32%)	0	10(83)	2(17%)	-	11(79%)	3(21%)	-
CIP	18(67%)	9(33%)	0	17(61%)	11(39%)	0	8(67%)	4(33%)	-	13(93%)	1(7%)	-
CAZ	19(70%)	8(30%)	0	16(57%)	12(43%)	0	8(67%)	4(33%)	-	11(78%)	3(22%)	-
IPM	26(96%)	1(4%)	0	28(1)	0	0	12(1)	0	0	14(1)	0	0
ATM	0	1(4%)	26(96%)	0	2(7%)	26(93%)	0	1(8%)	11(92%)	0	0	14(1)
AK	20(74%)	7(26%)	0	11(39%)	10(35%)	7(26%)	9(75%)	3(25%)	0	12(85%)	2(25%	0

Appendix IX

Summary of percentage Antibiotic Susceptibility of *Pseudomonas aeruginosa*isolates from clinical samples of BSUTH, Makurdi, Nigeria to some antimicrobial agents.

S/No.	Antibiotics	Resistance (%)	Intermediate (%)	Sensitive (%)
1	Ceftazidine (10µg)	33	-	67
2	Gentamicin (10µg)	30	-	70
3	Ciprofloxacin (5µg)		30	-
70				
4	Ticarcillin+ Clavulanic acid(75 µg)	73	-	27
5	Amikacin (30µg)	27	18	73
6	Azetreonam (30µg)	4	96	0
7	Imipenem(10µg)	99	-	1

Appendix X

Multiple Antibiotic Resistance Index and the Percentage Occurrence of *Pseudomonas aeruginosa*Isolates from clinical samples of BSUTH, Makurdi, Nigeria to some antimicrobial agents.

MAR Index Occurrence (%)	No. of *Pseudomonas aeruginosa* Isolates	Percentage
0	12	15
0.1	32	40
0.2	0	0
0.3	16	20
0.4	4	5
0.5	0	0
0.6	13	16
0.7	4	5

AppendixXI

Criteria for defining MDR, XDR and PDR in *Pseudomonas aeruginosa*

MDR: non-susceptible to ≥1 agent in ≥3 antimicrobial categories.

XDR: non-susceptible to ≥1 agent in all but ≤2 categories.

PDR: non-susceptible to all antimicrobial agents listed.

Correlation between biofilm formation and resistance of *Pseudomonas aeruginosa* isolates.

Using Pearson‘s product moment correlation coefficient (PEARSON **r btw -1.0 and +1.0**)

The formula for the Pearson product moment correlation coefficient, r, is:

$$r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

Data from the the biofilm forming *Pseudomonas aeruginosa*isolates were computed with the numbers of resistance to antibiotics used in the study.

N = 42

∑XY = 0.718966

∑X = 3.303632

∑Y = 10.42857

∑Y*Y = 4.142859

∑X*X = 0.799619583

Putting in these values into the formula given above

r = - 0.11065

Also with Microsoft Excel **r** = - 0.11065

To test whether the relationship -0.11065 is significant or not, using degree of freedom (df) formula for correlation of N-2. In this result df is 42-2 =40

From the Pearson r table at significant level of 0.05 and the df of 40, the critical value is 0.2573.

Since the computed value (-0.11065) is less than the table value (0.2573), the relationship is not significant, I accept the null hypothesis and reject the alternative.

Appendix XII

Minimum inhibitory concentration (MIC)of ciprofloxacin against ciprofloxacin resistant clinical isolates of *Pseudomonas aeruginosa* resistant to ciprofloxacin

MIC	Isolates No
5 µg/mL	37
5 µg/mL	178
5µg/mL	24
25 µg/mL	32
12 µg/mL	34
5µg/mL	18
5µg/mL	179
25 µg/mL	23
12µg/mL	11
25 µg/mL	28

(MIC test strip ciprofloxacin 0.002 - 32µg/µL).

Appendix XIII

Sub-MIC of Acridine Orange against ciprofloxacin resistant clinical isolates of *Pseudomonas aeruginosa*

Isolates No	Sub MIC(µg/ml)
39	312.5
32	625
195	625
12	156.25
11	625
229	312.5
10	312.5
28	312.5
179	625
34	312.5
219	1250
102	312.5
45	312.5
24	312.5
18	156.25
37	1250
178	156.25
44	312.5
23	156.25

Appendix XIV

Zone of Inhibition (mm) of resistant Organism by Ciprofloxacin Pre and Post Acridine

Orange Treatment.

Isolates No	pre-zone	Post-zone	Resistance	Resistance category
39	0	0	R	MDR
32	0	0	R	MDR
558	13	17	R	MDR
12	13	16	R	MDR
11	0	0	R	NIL
229	13	23	S	NIL
10	13	13	R	MDR
28	0	26	S	MDR
179	0	0	R	MDR
34	12	11	R	MDR
219	13	18	R	MDR
102	14	25	S	MDR
45	0	0	R	MDR
24	0	0	R	MDR
18	0	0	R	MDR
37	17	18	R	NIL
178	0	0	R	MDR
44	15	16	R	NIL
23	15	31	S	NIL

Appendix XV

Pictorial Presentation of E-test for MIC determination

