PLASMID PROFILE OF ANTIBIOTIC RESISTANT *PSEUDOMONASAERUGINOSA* ISOLATED FROM WOUND INFECTIONS AMONG PATIENTS ATTENDING SOME HOSPITALS IN ZARIA, KADUNA STATE, NIGERIA

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JULY, 2017

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DECLARATION

I declare that the work in this dissertation entitled 'PLASMID PROFILE OF ANTIBIOTIC RESISTANT PSEUDOMONAS AERUGINOSA ISOLATED FROM WOUND INFECTIONS AMONG PATIENTS ATTENDING SOME HOSPITALS IN ZARIA, KADUNA STATE, NIGERIA' has been carried out by mein the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of reference provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution

Zainab Abubakar _____ _____

Name of Student Signature Date

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CERTIFICATION

This dissertation entitled 'PLASMID PROFILE OF ANTIBIOTIC RESISTANT PSEUDOMONAS AERUGINOSA ISOLATED FROM WOUND INFECTIONS AMONG PATIENTS ATTENDING SOME HOSPITALS IN ZARIA; KADUNA STATE, NIGERIA' by ZAINAB ABUBAKAR meets the regulations governing the award of the degree of Masters in Science, Microbiology from Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Wound infections are the major problems in our health care facilities. *Pseudomonas aeruginosa* is the most common pathogen causing wound infections and it is known to possess intrinsic resistance to many antibiotics. Objectives of this study were to; isolate and biochemically characterise *Pseudomonas aeruginosa* from wound samples, determine the antimicrobial susceptibility patterns of the isolates, Characterise the plasmid profile of the resistant isolates and determine risk factors associated with *Pseudomonas aeruginosa* in wound infections. One hundred and fifty (150) wound swab samples were collected from patients attending some hospitals in Zaria, Kaduna State Nigeria. The samples were cultured on cetrimide agar, twelve samples produced green colonies on the agar. The twelve isolates were subjected to biochemical(motility, Simmon citrate, oxidase, indole and triple sugar ion agar tests) and microscopic(Gram stain reaction) analysis. The twelve (12)isolates were positive for *Pseudomonas aeruginosa.* Antibiotic susceptibility test was carried out the twelve (12)isolates using eight different antibiotic discs(Ciprofloxacin, Tetracyclin, Chloramphenicol, Gentamycin, Ampicllin, Amoxicillin-clavulanate, Nitrofurantoin and Erythromycin). Only one (1) isolate was susceptible to the eight (8)antibiotics while the eleven (11) isolates were resistant to; Ampicllin, Amoxicillin-clavulanate, Nitrofurantoin and Erythromycin. Plasmid profile analysis was carried out on the twelve (12) isolates; no plasmid was isolated on the organism that was susceptible to all the antibiotics but in those organisms that were resistant to some antibiotics plasmids were isolated. On 0.8% agarose gel was isolated from twelve wound swab samples; some of the isolates were resistance toChloramphenicol and Erythromycinbut all isolates were completelyresistance toAmpicllin, Amoxicillin-clavulanate, and Nitrofurantoin. The isolates that were resistance to many

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antibiotics harbored plasmids with molecular size of 800bp. Risk factors associated with contamination of wound with *Pseudomonas aeruginosa* are age, sex and location of wound on the body.

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CHAPTER ONE

INTRODUCTION

1.1. Background of the study

The skin is a host to various transient bacteria contaminants. These are found in the openings of the hair follicles, sweat glands and sebaceous glands (Deurden *et al.,* 2007; Omole and Stephen, 2014). Any breach in the skin surface, whether accidental or surgical, provides an open door for bacterial infection (Dettenkofer *et al.,* 2009; Omole and Stephen, 2014). The skin is a protective barrier that shields the body from contamination by viruses, bacteria and toxins (Kirketerp-Moller*et al.,* 2008; Omole and Stephen, 2014).

Damage to the skin compromises the body's ability to fight off infections by providing an unobstructed route into the blood stream(Omole and Stephen, 2014). According to Deurden *et al.* (2007), every individual carries a large resident microbial population on the skin surfaces, and in the openings of the hair follicles, sweat glands and sebaceous glands.

*Pseudomonas aeruginosa*is a non-fermentative Gram negative bacteria widely distributed in nature and can survive on a wide variety of surfaces and in hospital environment (Arora *et al.,* 2011). According to Leila and Mehdi(2014),*Pseudomonas aeruginosa*is a Gram-negative, aerobic, non-fermentative, rod-shaped and motile bacterium that is ubiquitous and very versatile.

*Pseudomonas aeruginosa*is a Gram negative, non sporeforming, straight or slightly curved rod-shaped bacterium that occurs as a single bacterium or in pairs and occasionally in short

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chains. It is widely distributed in nature including soil, water and various types of vegetation throughout the world. Besides that it has also revealed its presence in disinfectants, respiratory equipment, sinks, taps, and mops within the hospital as a biofilm (Murray *et al.*, 2002; Todar, 2011; Kirecci and Kareem, 2014).*Pseudomonas aeruginosa* secretes a variety of pigments, including pyocyanin (blue-green), fluorescein (yellow-green), pyoverdin, and pyorubin (red-brown) (Leila and Mehdi,2014).

Pseudomonas aeruginosa does not produce spore and non-acid-fast. This bacterium uses a unipolar flagellum for motility. Many strains of *Pseudomonas aeruginosa* were initially isolated based on their ability to produce pyocyanin, their typical colony structure when grown on agar media, and their distinctive grapelike odor, which is due to the production of aminoacetophenone (Leila and Mehdi,2014).

Because these bacteria can grow on most surfaces, including medical devices such as catheters, they are an important cause of nosocomial infections. *Pseudomonas aeruginosa* tends to form biofilms, which are complex bacterial communities that adhere to a variety of surfaces, including metals, plastics, and medical implant materials, and tissues. Growth in biofilms promotes bacterial survival; once a biofilm is formed, it is extremely difficult to destroy (Leila and Mehdi,2014).

Pseudomonas aeruginosa is endowed with weak pathogenicpotentials. However, its profound ability to survive oninert materials, minimal nutritional requirement, toleranceto a wide variety of physical conditions and its relativeresistance to several unrelated antimicrobial agents andantiseptics, contributes enormously to its ecological successand its role as an

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effective opportunistic pathogen (Gales*et al.,* 2001). The organism is pathogenic when introducedinto areas devoid of normal defences (Jawetz *et al.,* 1991; Ndip *et al.,* 2005).

Pseudomonas aeruginosa is the most important human pathogen in the Genus*Pseudomonas*. The spectrum of diseases caused by this agent ranges from superficial skin infections to fulminant sepsis. It is the major cause of nosocomial infection. It causes nosocomial urinary tract infections, wound infections and bacteremia. Wound infections due to *Pseudomonas aeruginosa* are particularly troublesome in burn patients. The high rate of sepsis following wound infections is responsible for significant mortality rate (Nester *et al.,* 2001).The role of *Pseudomonas aeruginosa*in a variety of human infections ranging from neonatal sepsis, burn sepsis and acute and chronic lung infections has been documented (Fujitani*et al.,* 2014).*Pseudomonas aeruginosa* found its entry into the hospital environment either through visitors and patients or goods that enter in to hospital. Contact transmission or vehicle transmission is common mode of transmission in hospital (Murray *et al.,* 2002; Todar, 2011; Kirecci and Kareem, 2014).

In burns and other wound infections, *P. aeruginosa* can colour the tissues green from pigments released from bacteria. It is especially dreaded because it often invades the blood stream and cause chills, fever, skin lesions, and shock. The overall effect of *P. aeruginosa* infection of burns and other wounds is to produce tissue damage, prevent healing and increase the risk of septic shock. Most strains of *P. aeruginosa* produce protease that causes localized hemorrhages and tissue necrosis (Nester *et al.,* 2001).

*Pseudomonas aeruginosa*has been increasingly recognized for itsability to cause significant hospital-associated outbreaksof infections, particularly with the emergence of

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multidrugresistant strains. Outbreaks of multidrug-resistant *P. aeruginosa*colonization or infection have been reportedin urology wards, burn unit, hematology/oncologyunits, and adult and neonatal critical care units (Gillespie*et al.,* 2000; Douglas *et al.,* 2001).Drug resistance is an alarming problemworldwide andis spreading rapidly due to overuse, self medication, andnon-therapeutic use of antimicrobials (Slama *et al.*,2005).

Russel and Path (2001) are of the opinion that acquired resistance to antibiotics may arise by cellular mutation or by acquisition of genetic elements in the form of plasmids. According to Akubuenyi *et al.*(2011), the occurrence of strongly selective environments such as hospitals promotes not only the growth of resistant bacteria, but also leads to an increase in the frequency of resistance bacterial genes and genetic elements such as plasmids.

Bacterial resistance to different classes of antibiotics has been reported in *Pseudomonas aeruginosa* isolates from different sources (Thomas*et al.,* 2015). Most of these resistances are acquired and may be caused by production of plasmid mediated beta (β)-lactamase, extended spectrum β-lactamase and metallo β-lactamase (MBL) enzymes (Manchanda and Singh2008). β-lactamases are the most widespread and effective mechanism through which bacteria can become resistant to β-lactam drugs (Thomas*et al.,* 2015).

Antimicrobials act as selectivepressure which allow the growth of resistant bacteriawithin a population and inhibit susceptible bacteria(Levy, 1994;Hayfaa *et al.,* 2013). Drug resistance property in bacteria isusually born in R-plasmids, which can be disseminated todiverse population and regions causing worldwideproblems. R-plasmids from resistant strains of anorganism may transfer to a sensitive counterpart, whichcan show the same drug resistance in the donor strain(Brussow *et al.,* 2004). An increase in the emergence ofmulti-drug resistant

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bacteria in recent years is worrying,though the presence of antibiotic resistant genes onbacterial plasmids has further helped in the transmissionof drug resistance among pathogenicbacteria (Zulkifli *et al.,* 2009).

*P. aeruginosa*is resistant to most of the commonly used antibiotics. Antimicrobials that usually show activity against *Pseudomonas* include aminoglycoside, polymyxin and some penicillins and cephalosporins (Cheesbrough, 2010).Hospital- acquired infections remain a cause of morbidity, extended hospital stay and death of patients (Naeem *et al.,* 2006). Bacterial infections in burn and wound patients are common and are difficult to control (Armour *et al.,* 2007).

According to Chandler (2015), wound is any type of injury to skin; it can be open wound in which the skin is broken or closed wound. Open wound can bleed and run the risk of infections while closed wound can also be dangerous depending on the extent of tissue damage. There are five major categories of wounds namely incision, laceration, abrasion, contusion and puncture.

Wound is a breach in the skin, an exposure to subcutaneous tissue following loss of skin integrity provide a moist, warm and nutritive environment that is condusive for microbial colonization proliferation (Motayo *et al.*,2013). Motayo *et al.* (2013), further explained thatwound can be classified into two; open wound and closed wound. Open wound include incisions, lacerations, puncture wounds, gunshot wounds and abrations. Closed wounds include contusion commonly known as bruises, hematomas crash injury.

A breach in protective skin barrier, reduced immunity and prolonged hospital stay are important factors responsible for infection of wound with opportunistic pathogens especially

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multi-drug resistant (MDR) *Pseudomonas aeruginosa* (Naqvi *et al.*,2005). The presence of dead, denatured tissues and moist environment make the burn wound vulnerable to infection by *P.aeruginosa*(Ikpeme *et al.,* 2013). Nosocomial infection requires more interest and attention because it is responsible for great deal of mobility and mortality among hospitalized patients in addition to unavailability of records, statistics or enough information about the problem and lack of program or approach to control it (Abdel Rahman *et al.,* 2010).

The development of wound infection depends on the integrity and protective function of the skin (Ikpeme *et al.,* 2013). Wound infection isuniversaland the bacterial type varies with geographical location, resident flora of the skin and clothing at site of wound (Oguntibeju and Nwobu, 2004). In recent years, there has been a growing prevalence of Gram negative organisms which have almost replaced *Staphylococcus aureus* in nosocomial infection of Gram negative bacilli, *Pseudomonas aeruginosa*has been of a particular interest(Ikpeme *et al.,* 2013).

A wound can be considered infected if purulent materials drain from it, even without confirmation of positive cultures. Also, many wounds are colonized by bacteria, whether infected or not. Infected wounds may not yield pathogens by culture owing to the fastidious nature of some pathogens, or if the patient has received an antimicrobial therapy (Nwachukwu *et al.*,2009). Many bacterial agents are known to cause wound infections. Initial injury to the skin triggers coagulation and an acute inflammatory response followed by exposure of subcutaneous tissue following loss of skin integrity which provides a moist, warm, and nutritive environment that is conducive to microbial colonization and proliferation (Yah *et al.,* 2010).

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Despite introduction of a wide variety of antimicrobial agents with anti-pseudomonal activity, life-threatening infections caused by *Pseudomonas aeruginosa*contribute to morbidity and mortality in hospitalized patients (Nwachukwu *et al.*,2009).The wound gets infection by either single or multiple organisms. Wound infections are mostly due to nosocomial pathogens that differ from country to country and from one hospital to another within the same region which remains the major source of post-operative morbidity (Akingbade*et al.,* 2012).

The high intrinsic antibiotic resistance of *P. aeruginosa* is due to several mechanisms which include a low outer membrane permeability, the production of an AmpC -lactamase and the presence of numerous genes coding for different multidrug resistance efflux pumps (Akingbade*et al.,* 2012).*P. aeruginosa*develops resistance by various mechanisms like multi-drug resistance efflux pumps, biofilm formation, production of β-lactamases and aminoglycoside modifying enzymes. The risk for acquiring MDR organisms may be related to the number of carriers in the same ward as well as to individual risk factors, such as patient characteristics and in-hospital events (invasive devices and antibiotic treatment) (Carmeli *et al.*,2002; Ahmed *et al.*, 2013).

Plasmids are DNA molecules, but are not indispensable, and therefore, may or may not be present in a cell; when absent, episomes cannot originate de novo and can be acquired only from other strains through different modes of genetic exchanges; when present, plasmids may be found in autonomous (free) or integrated state (integrated in to chromosomes). In other words they may change their state from autonomous to integrated or vice-versa. Such plasmids (as also some temperate phages) have been referred to as episomes, a term which is not popularly used; when present, plasmids may be lost irreversibly, also referred to as plasmid curing (Arif,2007).

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Plasmids carry genes that could be spread by conjugation and transductions while the genome based resistant genes are also spread by replication (Akingbade*et al.,* 2012).They are extra chromosomal double stranded DNA materials, have been found to be useful for pathogens' genetic diversity and prowess as infectious agents (Bennett, 2008; Thomas*et al.,* 2015).

According to Arif (2007), plasmids are pieces of DNA that exist separate from the chromosome. They contain an origin of DNA replication and thus, replicate independent of the chromosome. Most plasmids are small, their sizes ranges from about 0.2 to 4% the size of the bacterial chromosome (In *E. coli* the chromosome is 4639 kilobase pairs). There are families of plasmids whose members have very similar sequences. Plasmids are faithfully transmitted to daughter cells to ensure that they are stably maintained in the population. In addition to an origin for DNA replication, plasmids contain a variety of other genes. These other genes include antibiotic resistance determinant, genes that allow the cell to use a variety of different carbon sources, or genes that are involved in causing diseases.

Methods of the epidemiological investigation of infections caused by *P. aeruginosa* in different parts of the world include, biotyping, serotyping, antibiogram, phage typing, bacteriocin typing, plasmid profile, and more recent techniques like pulsed-field gel electrophoresis and random amplified polymorphic DNA analyses have been used in typing the organism (Herna´ndez *et al.,* 1997; Ndip *et al.,* 2005).

Plasmid profile analysis (PP) is a method of determining the number and sizes ofplasmids in bacterial isolates(Biljana *et al.*,2008). It examines the total bacterial plasmid content, or subjects plasmids to restriction endonucleases and separates the cleaved plasmid DNA by electrophoresis for analysis. This method is a powerful tool for following the spread of

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antibiotic resistance, because resistance is usually passed between bacteria on plasmids (Akingbade*et al.,* 2012).

Profiling pathogens for their harbored plasmids has been found to be very useful in epidemiological studies, diagnosis and elucidation of mechanisms of drug resistance (Bennett, 2008; Thomas*et al.,* 2015).Combination of simple typing methods as antibiotyping with plasmid profiling may be the cheapest and effective method for tracing source of infection (Ahmed*et al.,* 2013).

Cross-transmission from patient to patient may occur via the hands of the health care staff or through contaminated materials and reagents (DuBois *et al.,* 2001). However, it is believed that *P. aeruginosa* is generally environmentally acquired and that person-to-person spread occurs only rarely (Harbour *et al.,* 2002). As such, contaminated respiratory care equipment, irrigating solutions, catheters, infusions, cosmetics, dilute antiseptics, cleaning liquids, and even soaps have been reported as vehicles of transmission (DuBois *et al.,* 2001).

One of the essential features in the infectious process is the colonization and invasion of burns by *P. aeruginosa* with the elaborate production of virulence enzymes such as elastase, hyaluronidase, collagenase, and lipases that enhance the infectious process. This could highly influence the rate of morbidity and mortality in severe burn patients on prolonged hospitalization (Yah *et al.,* 2010).

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1.2 Statement of Research Problem

Pseudomonas aeruginosa is responsible for about 10% -20% of nosocomial infections as bacteraemia and sepsis in ICU, cystic fibrosis, pneumonia, urinary tract infections, burn infection and wound infection (Carmeli *et al.*,1999; Ahmed *et al.*, 2013).

It has been observed that 28% of healthy people in hospital environment are carriers of *Pseudomonas aeruginosa*(Ikpeme *et al.*, 2013)..

Pseudomonas aeruginosa, is one of the most problematic bacterial pathogens due to its ability to readily develop resistance to many antibiotics using mechanisms like biofilm formation, production of β-lactamases, and multi-drug resistance efflux pumps (Jonathan and Courage, 2015).

Wound infection is one of the health problems that are caused and aggravated by pathogenic organisms in different parts of the body (Malu *et al.*,2010).The wound is considered one of the major health problems in the world, and infection is one of the most frequentand severe complications in patients who have sustained wounds (Zogani *et al.*, 2002).

P. aeruginosa are particularly problematic because the organism is inherently resistant to many drug classes and is able to acquire resistance to all effective antimicrobial drugs (Gad *et al.*, 2007). *Pseudomonas aeruginosa* is an important life threatening nosocomial pathogen which plays a prominent role in wound infections and burn patients (James *et al.*, 2013).

1.3 Justification

Wound infections are major problem in our health care facilities; they are the most common hospital acquired infections resulting into extended length of stay in the hospital with high cost of healthcare (James *et al.*, 2013). *P. aeruginosa* is the most common pathogen causing

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wound infection especially from developing Countries (Akingbade*et al.,* 2012). In developing countries, large numbers of people die of opportunistic nosocomial pathogens, which cause diseases such as wound infections (Malu *et al.*,2010).

P. aeruginosa becomes one of the worst pathogens of human and it is known to possess intrinsic multi-drug resistance (Agarwal *et al.,* 2005). Despite introduction of a wide variety of antimicrobial agents with anti-pseudomonal activity, life-threatening infections caused by *Pseudomonas aeruginosa* contribute to morbidity and mortality in hospitalized patients (Nwachukwu *et al.,* 2009). Therefore, there is need to carry out research on antibiotic resistant *Pseudomonas aeruginosa* from wound in order to determine new prevalence of the organism from wound, the bacterial content, risk factors associated with contamination of wound with the organism and most effective drugs for treatment of the organism.

1.4 Aim of the study

Aim of the study was to characterize the plasmid profile of antibiotic resistant *Pseudomonas aeruginosa* isolated from wound infections in Zaria, Kaduna state Nigeria.

1.5 Objectives of the study

Objectives of the study were to;

1) Isolate and biochemically characterise *Pseudomonas aeruginosa* from wound samples.
2) Determine the antimicrobial susceptibility patterns of the isolates.
3) Characterise the plasmid profile of the resistant isolates.
4) Determine risk factors associated with *Pseudomonas aeruginosa* in wound infections.

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CHAPTER TWO

LITERATURE REVIEW

2.1. Wound

Wound simply means skin lesion, wound is traditionally defined as a break in continuity of any bodily tissue to violence i.e any action by an external agent (Michel and Hermens, 2010).

2.1.1.Wound Healing

Healing is a complex process, but in principle all lesions go through similar steps which include; homeostasis, inflammation, proliferation and remodeling as well as similar cellular and humoral conditions. Surgical sutured incisions do not develop a visible granulating surface (Wlaschek *et al.,* 2005). Burn and surgical intervention(excision and grafting) used to avoid the regular healing sequences (granulation) and to speed up closure (Wlaschek *et al.,* 2005).

Reactive oxygen species (ROS), proteinase and many soluble mediators (secreted by neutrophils which rapidly invade a skin lesion in large number) and cells are crucial for dealing with necrosis, debris and microbial invasion.The humoral compounds need careful regulation, since they are naturally aggressive and corrosive (Wlaschek *et al.,* 2005). Normally for every up grating mechanism down regulating counter acting mechanism exist in the body when this balanceis not adequate, situationof prolonged and persistent inflammation may occur (Kennedy *et al.,* 2010).

According to Astrid (2010), cutaneous wound healing is a dynamic and complex process that involves blood cells, epithelial and connective tissue cells, extracellular matrix and a variety

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of cell mediators. The process is in general consisting of three phases – inflammation, granulation tissue formation, and tissue remodeling. In the first phase, platelets and blood proteins cause the blood to coagulate into a fibrin clot which quickly stops blood loss and also serves as a provisional extracellular matrix. This allows for first line immune system cells such as neutrophils, monocytes and macrophages to migrate into the blood clot directed by different chemoattractants (Astrid,2010).

They remove bacteria, cell debris and foreign particles by phagocytosis but also release several growth factors that influence surrounding tissue cells to initiate production of granulation tissue, which in the second phase replaces the blood clot. Fibroblasts which are attracted into the wound area produce new extracellular matrix that provide a scaffold for further cell proliferation and ingrowth, and new blood vessels grow into the wound in order to supply the cells with oxygen and nutrients. Epidermal cells start to proliferate and migrate into the wound area in a process called reepithelialization. In the last phase, wound contraction occurs by the action of fibroblasts which are also responsible for remodeling of the collagen matrix into mature tissue (Astrid,2010).

2.1.2Non Healing Wounds

There are many cases where the normal wound healing process is disturbed and prolonged; this can lead to the development of a chronic, non-healing wound. The most common examples are pressure ulcers, diabetic ulcers, and venous leg ulcers. (Rickard *et al.,* 2009) Venous leg ulcers are believed to be caused by inadequate function of the valves in lower extremity veins, leading to increased hydrostatic pressure and edema in subcutaneous tissues which increases the risk for ulcer formation (Thomsen *et al.,* 2010).

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The treatment is compression therapy and wound fluid handling in order to restore the venous pressure. Diabetic foot ulcers are resulting from repetitive loading on the neurophatic foot where the tactile sense is impaired. In combination with an inadequate blood perfusion and weakened immune system with low motility of neutrophils, ulcers are prone to occur. In extreme cases non-healing diabetic foot ulcers lead to amputation of the foot or the leg. Treatment consists of off-loading of the foot and improvement of circulation (Sibbald *et al.,* 2005; James *et al.*, 2009).

Pressure ulcers arise from long-term loading on sensitive areas such as shoulders, sacral region and heels on the immobilized patient and are treated with off-loading and movement. Chronic wounds in general are estimated to affect 1 – 2 % of the world's population (Gottrup 2004), and about 1 % is suffering from venous leg ulcers (Gottrup 2004; Trent *et al.*, 2005). Apart from contributing to increased cost in the health care sector, chronic wounds cause pain, decreased quality of life and the risk for further, more dangerous complications in patients. Along with the worldwide increase in diseases like obesity, diabetes and cardiovascular disease, it can be assumed that the number of patients with chronic wounds will continue to grow. Other life style factors that can affect the ability of wound healing are smoking, malnutrition and alcoholism (Bjarnsholt *et al.,* 2008).

The general characteristic of a chronic non-healing wound is a detention in the inflammatory phase of healing and an inability of the wound to progress into the proliferative phase. This features a continuous influx of neutrophils into the wound area, which release free radicals, cytolytic enzymes and inflammatory mediators that cause damage to invading pathogens as well as to host tissue (Sibbald *et al.,* 2005). Other leukocytes present produce inflammatory mediators that affect inflammatory cells as well as fibroblasts, epithelial cells and vascular

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epithelial cells, hence contributing to the detention in the inflammation phase (Sibbald *et al.*, 2005).

Another feature is elevated amounts of matrix metalloproteinases (MMP) enzymes, produced mainly by neutrophils. These enzymes play a key role in normal wound healing for removing dead tissue and later for enabling migration of fibroblasts, keratinocytes and other cells. An excess amount of MMPs is however believed to impair wound healing (Bjarnsholt *et al.*, 2008). The balance between pro-inflammatory substances and their inhibitors that exist in acute wounds has been altered in chronic wounds; resulting in degradation of extra-cellular matrix, impaired cell migration and reduced fibroblast proliferation and collagen synthesis (Stojadinovic *et al.*, 2008).

Progression towards healing is further inhibited by inadequate tissue perfusion caused by arterial or venous insufficiency, blood loss, trauma or edema. Poor blood supply results in hypoxic conditions in tissues which can lead to cell death and tissue necrosis. This provides good growing conditions for pathogenic microorganisms leading to establishment and colonization of bacteria in the host tissue (Sibbald *et al.*, 2005).

2.1.3Influence of micro-organisms in wound

All wounds are *contaminated* by bacteria, meaning that microorganisms are present but not replicating. Wounds become contaminated from endogenous sources such as the gastrointestinal tract, the surrounding skin, the environment or from the healthcare provider (Sibbald *et al.*, 2005).

The wound is said to be *colonized* when bacteria are replicating to a certain number, but are still not impairing the healing process and do not cause host response. If the established

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bacteria are allowed to continue to grow, the microbial load will eventually initiate a host response, thus an inflammatory reaction. *Infection* is when the invading microorganisms are present in high amounts that cause significantly impaired wound healing and toxicity (Stojadinovic *et al.*, 2008).

The classic signs of infection in wounds include swelling, redness, pain, heat, purulence, and impaired function. Chronic wounds can also show symptoms such as low transcutaneous oxygen tension, development of necrotic tissue, foul odor and wound breakdown (Gardner *et al.*, 2001), as well as deterioration and discoloration of granulation tissue and increased friability (Sibbald *et al.*, 2005). As some of the above signs may be absent in chronic wounds

even if the healing process is disturbed, the most important symptom of infection is failure to heal (Schultz 2003; Sibbald *et al.*, 2005).

The concentration above 10^6 colony forming units (CFU)/ml of wound fluid (Sibbald *et al.*, 2005) or above 10^5 bacteria/g tissue (Bjarnsholt *et al.*, 2008) would be a sign of infection. However, the perspective has been altered so that no clear limit in amount of bacteria exists between a colonized wound and an infected wound. Instead, the relationship between the bacterial load and the host resistance determines the outcome (Sibbald *et al.*, 2005; Bjarnsholt *et al.*, 2008; Stojadinovic *et al.*, 2008). This implies that a patient with strong host response can handle a higher bacterial load without resulting in disease compared to a patient with compromised host resistance. Consideration must also be taken into what bacteria that is invading. Bacteria have different capabilities to cause injury to the host, where some species can be present in relatively low amounts and still cause significant harm, while other bacteria can exist in greater number without affecting the wound healing process (Schultz *et al.*, 2003).

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Pathogenic bacteria invading a host are producing several toxic substances that allow them to establish in the host tissue. These substances are called virulence factors and are described in more details below. When the immune system of the host responds to the bacterial invasion, inflammatory cells like neutrophils release cytotoxic enzymes, oxygen radicals and inflammatory mediators which cause further damage to host tissue. This mechanism is also contributing to the non-healing stage of the infected wound (Bjarnsholt *et al.,* 2008).

2.2.*Pseudomonas aeruginosa*

2.2.1. Origin of *P. aeruginosa*

P. aeruginosa is a motile, non-fermenting, Gram-negative organism belonging to the family Pseudomonadaceae. In 1850s, Sédillot observed that a blue-green discharge was frequently present and associated with infection in surgical wound dressings (Lyczak *et al.*,2000). The infectious organism was a rod-shaped and blue-green pigmented bacterium (Alaa, 2015). In 1960s, *P. aeruginosa* emerged as an important human pathogen (Alaa, 2015).

2.2.2. Nomenclature of *Pseudomonas aeruginosa*

Pseudomonas was derived from Greek pseudo means ‘false’ and monas means ‘unit’ while *aeruginosa* was from Latin which means ‘full of blue green copper dust’(Talaro and Talaro 2002).

Pseudomonas are large group of free-living bacteria that live primarily on soil, sea water and fresh water, they also colonise plants and animals and are frequent contaminants in homes and clinical settings (Talaro and Talaro 2002). Pseudomonas is the most important genus in the order Pseudomonadales and family Pseudomonadceae .The genus *Pseudomonas* contains straight or slightly Gram negative curved rods, 0.5 to 0.1 (Willey*et al.,* 2002).

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2.2.3. Scientific Classification of *P. aeruginosa*

Kingdom; Bacteria

Phylum; Proteobacteria

Class; Gammaproteobacteria

Family; Pseudomonadacea

Genus; *Pseudomonas*

Species; *P. aeruginosa*

Binomial Name; *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative,rod shaped bacterium that can cause disease in plants and animals including humans. It is citrate,catalase and oxidase positive. It is found in soil,water,skin flora and manmade environments throughout the world (Astrid,2010). One of the most common pathogens in chronic wounds is *P. aeruginosa*, a very problematic microbe due to its ability to form resistant biofilms (Bjarnsholt *et al.,* 2008; Kirketerp-Moller *et al.,* 2008; Ammons *et al.,* 2009;Thomsen *et al.,* 2010). The presence of these bacteria in wounds seems to impair or even stop the healing process (Bjarnsholt *et al.,* 2008).

Gjodsbol *et al.* (2006) found that 52.2 % of venous leg ulcers were infected with *P. aeruginosa*, and also that these wounds tended to be larger. *P. aeruginosa* is a gram-negative bacteria belonging to the proteobacteria phylum (Madigan and Martinko, 2006), originally found in soil and water but is common everywhere in the environment including hospitals. The metabolism is aerobic and very versatile due to many inducible operons in its genome, allowing for growth on a large variety of carbon compounds. It is an opportunistic pathogen,

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often acquired in hospital environments and is often associated with infections in the respiratory and urinary tracts, in burn damage wounds and in lungs of patients with cystic fibrosis (Madigan and Martinko, 2006). It has been estimated that about ten percent of all hospital-acquired infections are caused by *P. aeruginosa,*and for immune-compromised patients the mortality rate range from 20 to 70 % (Parsons *et al.,* 2007). It has a natural resistance mechanism to many antibiotics because of a *resistance transfer plasmid,* extra genetic material carried in the cells with genes that code for proteins that destroy antibiotic substances (Madigan and Martinko, 2006).

The strong persistency of *P. aeruginosa* in chronic wounds due to biofilm production makes the bacteria almost impossible to eliminate from a wound with antibiotics, once established. Kirketerp-Moller *et al.* (2008) demonstrated that colonies of *P. aeruginosa* were located deeper inside, underneath the wound bed surface, and were embedded in a matrix consisting mainly of alginate. This was in contrast to the very common wound pathogen *Staphylococcus aureus,* which was found to colonize the wound surface. The different locations probably contribute to the risk of misinterpretation of culture wound swabs, taken only from the wound surface (Kirketerp-Moller *et al.,* 2008).

The exopolysaccharide alginate produced by *P. aeruginosa* contributes to the antibiotic resistance, since the substance has been shown to inhibit diffusion of, and reduce the antimicrobial activity of several antibiotics (Ammons *et al.,* 2009). The non-healing wound is sustained in the healing process by *P. aeruginosa* through the release of toxic substances called virulence factors, explained further in the following paragraph.

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2.2.4. Ecology of *Pseudomonas*

Pseudomonas bacteria can be found in soil marshes, coastal marine habitats, plants' and animals' tissues; generally these bacteria can tolerate a variety of physical conditions some of these species such as *Pseudomonas aeruginosa* are opportunistic pathogens. Cystic fibrosis patients, burn victims, people with cancer and patients who are in intensive care units for extended periods of time are at risk of contacting a disease caused by *Pseudomonas aeruginosa* (Drogon and Boor, 2003).

According to Alaa(2015), *Pseudomonasspecies* are responsible for the spoilage of milk even after pasteurization. They are able to grow and proliferate in refrigerator causing large decrease in the shelf life of dairy products.

2.2.5. Habitat of *Pseudomonas*

Pseudomonas bacteria can be found in many different environments such as soil, water and plant's and animal's tissues; many different species of these bacteria are opportunistic pathogens that affect humans, animals and plants. *Pseudomonas aeruginosa* called the epitome of opportunistic pathogens almost never infects uncompromised tissue; however, it can infect practically any type of compromised defenses (Alaa, 2015).

2.2.6. Metabolism in *Pseudomonas aeruginosa*

Some species of *Pseudomonas* such as *Pseudomonas aeruginosa*, are opportunistic pathogens that secrete extracellular proteases to adhere and invade host issue. More than half of the clinical isolates of *Pseudomonas* bacteria produce pyocyanine, a blue green pigment Alaa(2015).*P. aeruginosa*is the most importantspecies in the family (*Pseudomonadaceae*) and

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possesses the metabolic versatility forwhich pseudomonads are so renowned. Organic growth factors are not required, and itcan use more than thirty organic compounds for growth.

According to Fan (2005), *Pseudomonas aeruginosa*cangrow in distilled water to greater than 10^6 per ml, which is evidence of its minimalnutritional requirements. Its tolerance to a wide variety of physical conditions, includingtemperature, contributes to its ecological success as an opportunistic pathogen.*Pseudomonas aeruginosa*does, however, show a predilection for growth in moistenvironments, a reflection of its natural existence in soil and water. Well known tomineralize aliphatics, *P. aeruginosa*strains attacking aromatic and polyaromatichydrocarbons (PAHs) have also been isolated. The species are typically active denitrifiersand produce effective biosurfactants (rhamnolipids) when growing on hydrophobicsubstrates (Greenberg, 2000).

All anaerobic respiration mechanisms, denitrification is mostfavorable energetically and gives out benign nitrogen (N_2) as the predominant product (Fan, 2005).

Moreover, being among the top three causes of opportunistic human infections(Stover *et al.*,2000), *Pseudomonas aeruginosa*secrets many extracellular virulence factors, such asproteases, hemolysins, exotoxin A, exoenzyme S, and pyocyanin (Fan, 2005). These virulence factors cause tissue damage, pneumonia, nosocomial bloodstream infections, and serious lung infection in the cystic fibrosis patients. In cystic fibrosisairway infection, the formation of biofilm by *P. aeruginosa*confers an inherent antibioticresistance and the bacterium undergoes the phenotype change from nonmucoid(with flagella or type IV pili) to mucoid (alginate capsule) (Fan, 2005).

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In addition to the lossof motility, the phenotype transition causes significant changes in the expression ofvirulence factors. Large amounts of alginate and exopolysaccharides are produced andother virulence factors such as exotoxin A, exoenzyme, elastase, alkaline protease,phospholipase and rhamnolipids are released (Rumbaugh, 1999). The alginate and exopolysaccharideshelp bacteria adhere to the host cells, form the microcolony matrix, evade the hostimmune system response, and protect against phagocytosis, antibodies and antibiotictreatment. The dissolved oxygen level (DO) in the environment plays animportant role in the phenotype change.It has been reported that the bacteria grow fastwithout mucoid under aerobic conditions, but turn mucoid and form biofilm matrix undermicroaerobic or anaerobic conditions in the presence of nitrate (Fan, 2005).

In another studyof biofilm formation of *P. aeruginosa,* the anaerobic biofilm was found 3-fold thickerand 1.8-fold higher in cell viability than the aerobic biofilm (Yoon *et al.*,2002). Monitoring thebacterium's physiological responses at different DO is therefore very important to theunderstanding of its metabolism and respiratory mechanisms.*Pseudomonas aeruginosa*possesses an inducible active transport system forglucose as well as inducible enzymes for the Enter-Duodroff pathway and the oxidativeportion of hexose mono-phosphate-pentose cycle. In *Pseudomonas aeruginosa*glucose and gluconate were activelytransported as the free sugars. Evidence has been found, moreover, which implicatesthe phosphoenopyruvate-phosphotransferase system as the mechanism by which fructoseis transported by *Pseudomonas* species. Thus, with the sole exception of fructose, sugarsare considered to be accumulated by a carrier-mediated active transport system in *P. aeruginosa*(Fan, 2005).

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2.2.7. Culture of*Pseudomonas aeruginosa*

According to Pitt and Simpson (2006), *P. aeruginosa*is the glucose non-fermenting Gram negative rod most often associated with human infection. It has the characteristic grape-like smell of aminoacetophenone. It is a strict aerobe with a growth temperature range of 5-42°C. Most other pseudomonads will not grow at 42°C (with certain exceptions, notably *Burkholderia pseudomallei*). The characteristic blue-green appearance of colonised/infected pus or of an organism culture is due to the mixture of pyocyanin (blue) and pyoverdin (fluorescein, yellow). Production of blue-green pigment is indicative of *P. aeruginosa*. Some strains produce other pigments, such as pyorubin (red) or pyomelanin (brown). Almost all strains are motile by means of a single polar flagellum.

*P. aeruginosa*can produce at least six colonial types after aerobic incubation on nutrient agar for 24hr at 37°C. The most common, type 1, is that of colonies which are large, low, oval, convex and rough, sometimes surrounded by serrated growth. Colonial variation from one type to another does not necessarily indicate the presence of more than one strain. Many strains exhibit metallic iridescence with colonial lysis. This resembles lysis by bacteriophage, but is not associated with phage activity (Pitt and Simpson, 2006).

Colonies isolated on *Pseudomonas* selective medium or blood agar may be presumptively identified by a positive oxidase reaction and characteristic pigment production as '*P. aeruginosa'*. However, some strains of *P. aeruginosa*, particularly the mucoid ones, may not produce pyocyanin, as well as displaying a slow oxidase reaction and may therefore require further tests to confirm identification. Colonies isolated on other selective agars (such as BCC) may be identified by colonial morphology and a commercial identification system. Other species from blood or selective media and strains of *P. aeruginosa*and *B. cepacia*

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complex requiring further characterisation should be identified by a commercial identification system and/or referral to a Reference Laboratory. It should be noted that isolates from cystic fibrosis patients can be atypical/stressed and should be incubated at 30°C or room temperature for 48hrs so that their phenotypic features may reliably be expressed (Pitt and Simpson, 2006).

2.2.8. Causes of Resistance in *P. aeruginosa*

*P. aeruginosa*is intrinsically resistant to antimicrobial agents due to low permeability of its cell wall,it has the genetic capacity to express a wide repertoire of resistance mechanisms.The organism can become resistant through mutation in chromosomal genes which regulate resistance genes and it can acquire additional resistance genes from other organisms via plasmids, transposons and bacteriophages (Lambert, 2002).

2.2.9. Penetration of antibiotics through the cell envelope of *P. aeruginosa*

All of the major classes of antibiotics used to treat *P. aeruginosa* infections have to cross the cell wall to reach their targets. The aminoglycosides (gentamicin,tobramycin, amikacin) inhibit protein synthesis by binding to the 30S subunit of the ribosome. Quinolones (ciprofloxacin) bind to the A subunit of DNA gyrase, which maintains the ordered structure of the chromosome inside the cells. The b-lactams (e.g. piperacillin, ceftazidime, imipenem, meropenem and aztreonam) inhibit the peptidoglycan-assembling transpeptidases located on the outer surface of the cytoplasmic membrane. Finally the polymyxins (colomycin) bind to phospholipids in the cytoplasmic membrane, destroying its barrier function.The innate resistance of *P. aeruginosa* to all classes of antibiotics has generally been attributed to the low permeability of its cell wall. Failure of antibiotics to accumulate within the organism is due to

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a combination of restricted permeability of the outer membrane and the efficient removal of antibiotic molecules that do penetrate by the action of efflux pumps (Lambert, 2002).

2.2.10. The outer membrane of *P. aeruginosa* as a barrier

It was explained by Lambert (2002), that the outer membrane of *P. aeruginosa* presents a significant barrier to the penetration of antibiotics, restricting the rate of penetration of small hydrophilic molecules and excluding larger molecules. Small hydrophilic antibiotics such as the b-lactams and quinolones can only cross the outer membrane by passing through the aqueous channels provided by porin proteins. These are barrel-shaped molecules which span the outer membrane, usually associated as trimers. *P. aeruginosa* produces several different porins, oprF being the major speciespresent in all strains. Although mutants lacking oprF have been reported, loss of oprF has not been found to be a major cause of antibiotic resistance, presumably because such strains have restricted ability to take up hydrophilic nutrients. OprD is a specialized porin which has a specificrole in the uptake of positively charged amino acids such as lysine. Loss of oprD is frequently associated with resistance to imipenem, which requires this porin to cross the outer membrane. Loss of the oprD porin increases the minimum inhibitory concentration from 1–2 to 8–32 mg/L and 17% rate of resistance has been reported during treatment. Interestingly, meropenem is not affected by loss of oprD, indicating that the carbapenems have crossed the outermembrane by different channel (Lambert, 2002).

2.2.11. Changes in Targets of *P. aeruginosa*

This mechanism of resistance results from mutational changes in target enzymes which result in maintenance of their vital role in cell metabolism but resistance to the action of selective inhibition by antibiotics. In *P. aeruginosa* it is most commonly encountered with the

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quinolones through mutation in the gyrA gene encoding the A subunit of the target enzyme, DNA gyrase. Changes in the structure of the ribosome 30S subunit (the aminoglycoside target) influence streptomycin sensitivity but not that of the anti-pseudomonal aminoglycosides. Alteration in the penicillin-binding proteins of *P. aeruginosa* resulting in resistance to b-lactams has been reported (Kipnis *et al.,* 2006).

2.2.12. Virulence factors of *P. aeruginosa*

When pathogenic bacteria are entering host tissues, different substances called virulence factors are released from the bacteria. Virulence factors cause damage to tissues through toxicity and invasiveness and allow for establishment of bacteria. *P. aeruginosa* produce a variety of virulence factors, both extracellular and cell-associated products. (Kipnis *et al.,* 2006). The secreted virulence factors maintain the chronic wound in a persistent inflammatory phase (Kipnis *et al.,* 2006). Exotoxins are secreted which bind to specific sites on the ribosomes in host cells, resulting in inhibited protein synthesis and cell death (Madigan and Martinko, 2006). Several enzymes are produced e.g. proteases and phospholipases that disrupt host cell structures, and adhesins that allow for binding to epithelial cell receptors (Bjarnsholt *et al.*, 2008).

Bjarnsholt *et al.,* (2008) suggest that the virulence factor rhamnolipid plays an important role in chronic *P. aeruginosa* infections, Rhamnolipids destroy neutrophils, resulting in leakage of several different substances with harmful effects on both tissue cells and bacteria. On the outer surface of the bacterial cell are lipopolysaccharides deposited, which contribute to attachment to host cells but also stimulate inflammatory responses (Kipnis *et al.*, 2006). Other cell surface structures such as flagella also allow for adherence to endothelial cell, another

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important virulence factor for *P. aeruginosa* is the toxic blue pigment pyocyanin (Kipnis *et al.,* 2006).

P. aeruginosa have a variety of virulence factors that contribute to its ability to grow in various host environments and cause many different types of infections. These virulence factors include; adhesion, toxins, biofilms, proteases and pigments which disrupts or control host cellular pathways and target the extracellular matrix (Klans *et al.*,2014).

2.2.13. Pyocyanin

Pyocyanin is a blue pigment belonging to the group of phenazine compounds. Phenazines are low-molecular weight, heterocyclic compounds with redox-active properties, produced by *Pseudomonas* species and some other Pseudomonads (Parsons *et al.,* 2007). Pyocyanin is the major phenazine pigment produced by *P. aeruginosa* and has been shown to contribute to its pathogenicity (Parsons *et al.,* 2007). The presence of pyocyanin is easy to detect due to its blue color that turns stationary phase cultures of *P. aeruginosa* green, and is commonly found to stain infected tissues, pus, or dressings (Astrid,2010). Muller *et al.* (2009) examined wound dressings from burn patients infected with *P. aeruginosa* and found that four of seven dressings contained pyocyanin. One important function of the pigment is to assist in iron uptake from the environment, i.e. host tissue (Astrid,2010).

Due to its redox-active properties, pyocyanin can cause reduction and release of iron from transferrin, a protein that transports iron in our bodies. Hence this crucial mineral can be accumulated by the bacteria (Astrid,2010).

The redox-active properties also play an important part for the toxicity of the substance. Pyocyanin can exist in an oxidized, blue form or in a reduced, colorless form, and can easily

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cross cell membranes. When entering a host cell, pyocyanin reacts with NADH and NADPH, leading to reduction of pyocyanin (O'Malley *et al.,* 2004). The reduced form can react with molecular oxygen and give rise to reactive oxygen species like superoxide and hydrogen peroxide (H_2O_2) as was first shown by Hassan and Fridovich in 1980. The created oxygen species impose oxidative stress on host cells which cause cellular damage. One important defense mechanism in host cells to oxygen species is the glutathione redox cycle, where e.g. hydrogen peroxide is reduced to water (O'Malley *et al.,* 2004).

Studies have shown that pyocyanin decreases glutathione levels in host cells resulting in modulation of the defense cycle. (Lau *et al.,* 2004) The result is a depletion of NADH, glutathione and other antioxidants in the host cell and an alteration of the redox status, together with generation of oxygen radicals (Muller, 2002; Lau *et al.,* 2004; Rada *et al.,* 2008).

2.2.14. Invasin

The production of excellular proteases are important for *Pseudomonas aeruginosa*'s virulence, especially in adherence and invation of host cells (Klans *et al.*,2014).

2.2.15. Biofilm

A biofilm is a protective polysaccharide matrix produced by bacteria. Most ulcers develop a biofilm over time, while biofilm formation in trauma seems to be less common unless they remain open for a long period (Kennedy *et al.,* 2010). According to Percival *et al.* (2010), biofilms are considered to play a major role in preventing wound. Biofilm forms when bacteria adhere to a surface where they start to proliferate into microcolonies. The surface can be biological e.g. living or dead tissue in wounds, non-biological material e.g. medical

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implants, as well as bacterial aggregates. The bacteria produce and embed themselves in an exopolymer matrix, which functions as a shield allowing for attachment and protection from the host responses. The matrix consists mainly of polysaccharides and proteins, and to a lesser extent DNA (Donlan and Costerton, 2002).

Mature biofilm has a complex three-dimensional structure consisting of dense regions with bacterial microcolonies, interspersed with channels carrying water, nutrients and waste products (Lindsay and Von Holy, 2010). The EPS matrix constitutes a physical barrier and slows down diffusion of antibiotics. It also prevents penetration of cells of the immune system and the antimicrobial oxygen species which they produce. The antibiotic resistance of bacteria in biofilm is believed to rely on other causes as well, e.g. a steady state growth mode caused by limitations of oxygen and nutrients (Drenkard, 2003).Yet another protection mechanism is that bacterial cells in biofilms develop a specific phenotype with altered expression patterns of genes and proteins, which could be linked to a higher resistance to antimicrobials (Drenkard, 2003).

More than 80 % of persistent infections involve bacterial biofilms (Bjarnsholt *et al.*, 2008). The strong resistance of bacteria in chronic infections, example given in endocarditis, osteomyelitis and periodontitis, is most likely due to the formation of biofilm (Parsek and Singh, 2003; Drenkard, 2003). Therefore, an expected conclusion is that the healing process of chronic wounds is inhibited by the same reason (Thomsen *et al.*, 2010; Ammons *et al.*, 2009; Kirketerp-Moller *et al.*, 2008; James *et al.*, 2009). The wound bed provides a good location for bacterial cell attachment due to the fibrin network and the nutrient levels in the environment promotes encapsulation in biofilm, resulting in great difficulties for treating the infection with antibiotics (Harrison-Balestra, 2013).

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2.2.16Resistance *Pseudomonas aeruginosa* to Antibiotics

According to Leemreijze (2001), one of the challenges in managing *Pseudomonas aeruginosa*infections is an inherent resistance mechanism, referred to as intrinsic resistance. *P aeruginosa*is naturally resistant to many antibiotic and it is increasingly becoming resistant to many anti-pseudomonal agents, therefore treatment of burn patients infected with *P aeruginosa* becomes difficult due to limited treatment options(Ikpeme *et al.*,2013).

P aeruginosa is naturally resistant to penicillin and most of the B lactam antibiotics (Asghar *et al.*,2013). Carbapenems are antibiotic of choice for treatment of *P aeruginosa* infections, however gradually increasing resistance to carbapenems has become a major concern (Asghar *et al.*,2013).

2.2.17. Mechanism of resistance in *P aeruginosa*

Resistance may be mediated by loss of the Opr D porin, up regulation of multi-drug efflux pumps and production of certain B-lactamases and carbapenemases (Asghar *et al.*,2013). According to Pier (2010), mechanism of aminoglycoside resistance in *P aeruginosa* are due to reduced uptake of aminoglycosides across the outer and cytoplasmic membranes associated with the production of aminoglycoside modifying enzyme. Modification of DNA gyrase is responsible for the development of resistance to quinolones. The use of polymyxins were limited due to their nephrotoxicity.

Other factors that are associated with antibiotic resistance are production of antibiotic degrading enzymes or antibiotic inactivating enzymes, outer membrane to evict the antibiotics and mutation to change the antibiotic targets (Alex *et al.*, 2012). Emergence of multidrug resistance in pathogenicbacteria has created immense clinical problem in thetreatment of

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infectious diseases. Elimination of plasmidDNA mediated antibiotic resistance in pathogenicbacteria is of great practical significance both inchemotherapy of bacteria and in microbial genetics.Elimination of plasmid DNA can be performed usingdifferent chemical materials including plant extracts(Reuter and Sendel, 1994; Hayfaa *et al.*, 2013).One strategy to minimize plasmid transmission ofantibiotic resistance is to eliminate the plasmids. This process is known as‘curing’ and many compounds havebeen shown to be capable of causing this effect (Hayfaa *et al.*,2013).

Curing may occur naturallythrough cell division or by treating the cells with chemicaland physical agents (Hayfaa *et al.*, 2013).The inhibition of conjugational transfer of antibioticresistance plasmid can be exploited to reduce the spreadof antibiotic resistance plasmid in the ecosystem.Inhibition of plasmid replication at various stages, asshown in the “rolling circle” model (replication, partition,and conjugal transfer) may also be the theoretical basisfor the elimination of bacterial virulence in the case ofplasmid mediated pathogenicity and antibiotic resistance (Brussow, 2004).

2.3. Plasmid

Plasmid is defined as double stranded, extrachromosomal genetic element that replicate independently of the host cell chromosome and is stably inherited. Plasmids capable of integration into the chromosome were earlier called episomes. Plasmids differ from chromosomes in being small and coding for genes that are non-essential for the bacterial survival. Absence of plasmids doesn’t killbacterium, but their presence provides additional benefits to the bacterial cell (Sridhar,2012).

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It was reported by Thomas*et al.*(2015), that bacterial plasmids serve as the scaffold on which are assembled arrays of antibiotic resistance genes, by transposition (transposable elements and ISCR mediated transposition) and site-specific recombination mechanisms (integron gene cassettes). The evidence suggests that antibiotic resistance genes in human bacterial pathogens originate from a multitude of bacterial sources, indicating that the genomes of all bacteria can be considered as a single global gene pool into which most, if not all, bacteria can dip for genes necessary for survival. In terms of antibiotic resistance, plasmids serve a central role, as the vehicles for resistance gene capture and their subsequent dissemination (Bennett*et al.*, 2008; Thomas*et al.*, 2015).

2.3.1. Plasmid Size

Plasmids vary in size; smallest plasmid is only 846 bp long and contains only one gene. Some plasmids carry more than 500 genes. The largest plasmid known carries 1,674 genes. Like chromosomes, plasmids are also closed circular and supercoiled; although certain plasmids (Eg. *Borrelia*) are known to be linear. *Borreliaburgdorferi* has at least 17 plasmids, 2 are circular and 9 are linear molecules. A cell may have different plasmids existing together. Copy number refers to the number of copies of a plasmid present in a cell. Larger plasmids are present in smaller numbers (1-2) and small plasmids may be found in high copy numbers (Sridhar,2012).According to Arif (2007); The size of the plasmid varies from ~ 1.0 kb to more than 250 kb. Many derived plasmids can be of variable sizes. Size of the plasmids is an important property for gene cloning experiments. Larger plasmids are often less in number compared to smaller ones.

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2.3.2. Plasmid Nomenclature

Plasmids were named on the basis of phenotypic function expressed e.g. R-factor carries genes for resistance to several antibiotics, F-plasmid mediates fertility, Col E1 plasmid carries gene for colicin E1 protein, Tol plasmid contains genes for toluene degradation and Ti plasmid of *Agrobacterium tumefaciens* induces tumors in some plants (Srivastava *et al.*, 2003).

It was explained by Arif(2007);that a plasmid has been named to give some indication as to the function carried by the plasmid. Hence we have the F factor (fertility factor), which allow transfer of cell's chromosomal DNA from one cell to another, the R factor (resistance factor), which carries a large number of antibiotic resistance genes and ColE1, which produce the antibiotic colicin. For historical reasons, these names have persisted. The convention now is to name a plasmids beginning with the small p, followed by the designation unique to that plasmid. For example, pBR322, the small p indicates that it is a plasmid, B and R are the initials of the persons (Boliver and Rodriguez) who constructed the plasmid and 322 is unique designation number of the plasmids. Naming of many plasmids is still based on the unique function they carry, such as p sym (carrying the symbiotic nitrogen fixing genes), and p Toc (gene responsible for toluene degradation).

2.3.3. Plasmid Structure

Plasmids are covalently closed circular structures consisting of double stranded DNA (Srivastava *et al.*, 2003).

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2.3.4. Properties of Plasmid

Plasmids are tiny, circular extra-chromosomal strands which can be free or integrated into the chromosomes, they can be duplicated and passed to offspring (Talaro and Talaro 2002).Plasmids are not necessary to basic bacterial survival but they can be genetically useful and confer greater versatility or adaptability on the recipient cell (Talaro and Talaro 2002).The plasmid replication is initiated at a unique site called Ori, there are two modes of theta replication and rolling circle (Srivastava *et al.,* 2003).

It was explained by Sridhar (2012), that plasmids are capable of transfer to other cells but not all plasmids are transferable. Only those plasmids that have genes for self transfer can be transferred. Plasmids have two prominent properties; the ability to replicate and to partition themselves between the daughter cells after cell division. Plasmids don't freely float in the cell cytoplasm, instead are membrane bound. Sometimes, a plasmid is said to be "lost" when the progeny cells don't receive the plasmid. The loss of plasmids in a population is sometimes referred to as plasmid segregation. The process of obtaining plasmid-free isolates is termed 'curing'. It can be achieved a) spontaneously as in replica-plated colonies, b) following an enrichment, c) by elevated temperature d) by treatment with chemicals such as acridines, ethidium bromide, sodium dodecyl sulfate (SDS) and novobiocin (Sridhar,2012).

Compatibility refers to the ability of two different plasmids to coexist stably in the same cell. Incompatibility refers to inability of two plasmids with similar replicon or segregation system to coexist in the same cell. Whenever two different plasmids have similar replication or partitioning system, there will be competition between the plasmids and whichever is able to utilize the system better will prevail over the other. All plasmids belong to only one of the approximately 30 compatibility groups (Sridhar,2012).

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2.3.5. Types of Plasmids

According to Sridhar (2012), naturally occurring plasmids are wild plasmid found naturally in bacteria, recombinant plasmidsare altered plasmids introduced into the bacterium for genetic studies whilecryptic plasmids are those that serve no known functions (they may be present for possibleexclusion of plasmids that are incompatible with the resident plasmid).

Integrative plasmids: These are plasmids that can occasionally integrate into chromosome(previously called episomes),Metabolic plasmids carry some genes that help in cells metabolism.

Virulence plasmids carry one or several genes that confer virulence properties on the bacterial cell.Conjugative plasmids are those that are able to mobilize self transfer these tend to be largerbecause they have to accommodate genes coding for self transfer. Conjugative plasmids in Gram-positive bacteria tend to be smaller than those in Gram-negative bacteria (Sridhar,2012). Other types of plasmids include; Mobilizable plasmids are those plasmids that lack genes to initiate self transfer but do encode the functions needed specifically for transfer of their own DNA. The initiation function is provided by other conjugative plasmid present in the same cell.

Suicide plasmids referred to those plasmids which get transferred to another bacterial cell butdoes not replicate further. These are actually mobilizable plasmids. R (resistance) plasmids are large conjugative plasmids that carry one or more antibiotic resistance genes. Resistance plasmid can be conjugative or mobilizable. Usually, R plasmids code for their own replication, and they are known to have mobile genetic elements. Colicin plasmids are small plasmids which encode the genes to synthesize colicins (bacteriocines) and finally F (fertility)

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plasmids are those that have complete gene set to mediate self transfer by conjugation. Cells possessing it (donor) are termed F+ and those lacking it (recipient) are termed F- (Sridhar,2012).

2.3.6. Replication, Transfer And Recombination In Plasmids

According to Arif (2007), a plasmid must contain an origin for DNA replication (*ori*). In the absence of an *ori* site, the plasmid cannot replicate and when the cells divide, one daughter cell would inherit a plasmid molecule and the other would not. At each subsequent cell division, only one daughter cell would receive a plasmid molecule. The scenario arising when a plasmid lacks an origin of replication is the loss of plasmid.

An origin consists of a small stretch of DNA that binds specific proteins and/or RNA molecules. These components must open the double helix of the DNA at or near the origin sequence, provide for the synthesis of a primer for DNA polymerase, and provide for the continued DNA synthesis of each strand of the plasmid DNA molecule. As can be imagined, not every origin of replication functions identically. There are many different ways to accomplish the functions that an origin of replication is responsible for. Each plasmid has its own scheme for replication and many variations have been described (Arif,2007).

Several plasmids encode a protein required for initiation of DNA replication and others do not. This plasmid-encoded initiator protein binds to the *ori* region and helps to form an open complex. Frequently, the host-encoded protein DNA binds to the origin along with the plasmid-encoded initiator protein to facilitate this reaction. In other plasmids, the plasmid encodes several proteins required for initiation of replication and DNA is not required,

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Stillother plasmids use an RNA molecule to make an open complex at the *ori*. This RNA is also used as a primer for DNA polymerase (Arif,2007).

Plasmids also differ in their requirement for different host proteins as well as for RNA polymerase for initiation of the replication. Because plasmids have a mechanism to replicate their DNA and ensure that they are stably maintained in a cell population, they are also known asreplicons. Any DNA molecule that has these two properties, including the cell's chromosome, is a replicon (Arif,2007).

Arif (2007), explained that DNA plasmids replicate in semi-conservative manner. It has been shown that while initiation of replication is controlled by plasmid genes, other stages of replication i.e., polymerization (elongation) and termination are controlled by host genes. When a plasmid is inserted in the bacterial chromosome, bacterial control on plasmid replication is absolute, but when free, the control is less than absolute.

Plasmids also have the ability of transferring themselves from one bacterium to another. This transfer is facilitated by the formation of an appendage known as sex pilus, extending out of the outer membrane of the donor bacterium (bacterial conjugation). The F sex pilus is up to 20nm in length and 8 nm wide. The sex pili produced by different plasmids are distinguished by the response they show for attachment of certain phages. When sex pilus is mechanicallyremoved or is absent due to a genetic mutation, no transfer of plasmid DNA is possible, suggesting that sex pilus is essential for transfer (Arif,2007).

Plasmid DNA may undergo recombination either with another plasmid DNA or within the bacterial chromosome. In both, there are insertion sequences (IS) which are involved in homologous recombination. There are at least 17 such sites in *E. coli* chromosome. Mapping

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of genes in plasmid DNA has also been achieved. Methods employed in mapping are not only genetic but also physical (Arif,2007).

2.4. Plasmid Profile Analysis

Plasmid profile analysis (PP) is the determination of the number and size of plasmids in bacterial isolates (*Biljanaet al.,* 2008). According to Sridhar (2012), plasmid profiling is a technique of isolation of plasmids present in a bacterial cell followed by electrophoresis to obtain plasmid counts and sizes. It appears that epidemical strains of Enterobacteriaceae, bacteria of *Pseudomonas* and *Vibrio* strains, *Staphylococcus* and *Streptococcus* can be identified in this way. However, isolates which are not connected with the outbreak maymanifest epidemical profile. Thus, when using plasmid profile analysis in determining epidemical strain for evaluation of this method, it is necessary to compare epidemiological strainswith non-epidemiological ones isolated in the same period of time (Biljana*et al.,* 2008).

According to Biljana*et al.*(2008) plasmid profile analysis is useful in determining the epidemical strain in outbreaks caused by multiple species: *Escherichia, Klebsiella, Pseudomonas,Serratia,Staphylococcus* and so on. This method allowed successful identification of an epidemical strain *P.aeruginosa* isolated from patient on hemodialysis.By plasmid profile analysis, we can determine a different number of profiles, which depends on geographic location of isolates, time of researching, as well as the origin of strains,that is, whether they are isolated from human, animal or from food (*Biljanaet al.,* 2008).

This analysis is performed by digestion of plasmid with restrictive endonucleases. Techniques used in plasmid analysis could be direct or indirect. The direct techniques include (i) DNA chains hybridization with calculation of homology degree (ii) direct electronomicroscopic

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heteroduplex analysis of small plasmids (nonhomological DNA would appear to be a simple loop), (iii) discovering of DNA sequences by Southern-blot hybridization with radioactive DNA probes. The indirect techniques are (i) agarose gel electrophoresis and (i) restriction endonuclease analysis. Comparison of strains, based on theircontents of plasmids or on restrictive profiles of plasmid DNA, is very useful in the case that a bacterium contains plasmids. However, when a bacterium does not contain plasmids, this method gives us very little benefit (Biljana*et al.*, 2008).

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CHAPTER THREE

MATERIALS AND METHODS

3.1 Ethical approval

Ethical approval was obtained from the ministry of health Kaduna state before commencement of this study. Consent was obtained from patients, for young patients it was obtained from their parents.

3.2 Study design

The study was a hospital based, cross sectional and experimental research.

3.3 Study area

The study was conducted in Zaria local government, Kaduna state Nigeria.

3.4 Study population

The study population comprised of patients of all ages with all types of wound from all parts of the body attending Gambo Sawaba General Hospital, Kofar Gayan, Zaria, Zaria clinic memorial hospital, Tudun Wada, Zaria and Saint Luke's Anglican Hospital, Wusasa, Zaria.

3.5 Sample size

A prevalence of 11% as reported from a study conducted in Zaria (Garba *et al.*, 2012). Was used to determine the sample size, using equation below, as described by Srmukaddan and Gerad, (2006),at 95% confidence interval.

$N = Z^2PQ/L^2$

Where n= sample size

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Z= standard normal distribution at 95% confidence interval (1.96)

P= prevalence (11%) (Garba *et al.*,2012)

L= allowable error, which will be taken as 5% = 0.05

Q= 1-P; 1-0.11= 0.89

Substituting….

$n= (1.96)^2 \times 0.11 \times 0.89/ (0.05)^2$

=150.44

Therefore a total of 150 wound swab samples were collected for this study.

3.6 Inclusion and Exclusion criteria

Inclusion criteria were patients of all ages with wound infections attending the selected hospitals that consented; those that did not consent were excluded.

3.7 Data collection

All consenting patients were given self designed questionnaire which aimed at obtaining socio-demographic data as well as clinical information related to the wound infections.

3.8 Sample collection

Sample collection was carried out by the nurses of the various hospitals. Sterile swab sticks were used to collect pus by swabbing the surfaces of the wounds, special care was taken to avoid contamination of the specimen with commensal organisms from the skin (Cheesbrough, 2010). The specimens were then transferred to the Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University,Zaria for analysis.

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3.9.Isolation and Identification of *Pseudomonas aeruginosa*

3.9.1.Culture technique

Samples were cultured on cetrimide agar and incubated aerobically at 37^0C for 24h (Cheesbrough, 2010).

3.9.2.Phenotypic characterization

Preliminary identification of the isolates was performed based on colonial characteristics on cetrimide agar as described by (Cheesbrough, 2010).

3.9.3.Microscopic examination

The isolated colonies were Gram stained to ascertain their Gram reaction. Dried and heat fixed smears of the isolates were placed on the staining rack and covered with crystal violet for 30 seconds and rinsed with water then Lugols iodine was added for 30 seconds. The smears were rinsed with distilled water followed by decolorization using acetone 2 seconds and rinsed with water again; they were then covered with neutral red for 60 seconds and rinsed with water. The slides were wiped with cotton wool and placed on staining rack. The Gram stained smears were then examined microscopically using 100X oil immersion objectives. The isolates were sub cultured on nutrient agar slant for biochemical tests (Welley*et al.,* 2002).

3.10.Biochemical tests;

Citrate test, oxidase test, motility test and triple sugar iron agar were carried out to confirm *Pseudomonas aeruginosa*as follows

3.10.1.Citrate test

Based on ability of an organism to use citrate as its sole source of carbon, each of the stored organisms on the nutrient agar slant was inoculated on the surface of the Simmon citrate

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medium and incubated for 24h at 37^{o}C. Simmon citrate agar slants contain sodium citrate as source of carbon, ammonium ion as a source of nitrogen and bromothymol blue was used as pH indicator. When the bacteria oxidize citrate, they remove it from the medium and liberatecarbondioxide. The carbondioxide combine with sodium (supplied by sodium citrate) and the water to form sodium carbonate – an alkaline product. This raises the pH and turns the pH indicator to blue colour which represents a positive citrate test; absence of a color change is negative citrate test. Citrate –negative cultures will not grow on the medium (Welley*et al.,* 2002).

3.10.2.Oxidase test

The test is used in identification of organism that produces the enzyme cytochrome oxidase. A piece of filter paper was placed in a clean petri dish and 3 drops of freshly prepared oxidase reagent was added. A piece of stick was used to remove a loop of the test organism and smeared on the filter paper(Cheesbrough, 2010).

3.10.3.Motility test

A straight wire was used to inoculate the test organism into the medium by stabbing the medium and avoidance of the wire from reaching the bottom of the tube and incubated at 37^{0}C for 24h. (Welley*et al.,* 2002).

3.10.4.Triple sugar iron agar

The organisms were inoculated in triple sugar iron agar and incubated aerobically at 37^{o}C for 24h. No fermentation was observed(Welley*et al.,* 2002).

3.10.5.Indole test

It is based on the ability of the organism to metabolize tryptophan; the organism was each grown in 5ml peptone water and incubated for 24h. After incubation, the reagents (Kovac's reagent) were added and shaken gently. A red colour in the reagent layer above the broth

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within 1 minute indicates a positive test while absence of red layer indicates negative test. (Cheesbrough, 2010).

3.11.Antibiotic Susceptibility Test

The antibiotic susceptibility test of the isolates was performed according to clinical and laboratory standard institute (2015) using Kirby- Bauer disc diffusion test on Mueller-Hilton agar. The biochemically confirmed twelve isolates of *Pseudomonas aeruginosa* were taken from overnight culture plate and a colony from each isolates was touched with a sterile wire loop and streaked onto nutrient agar and inoculated at 37^0C for 18hr.4 well isolated colonies of each isolate were transferred into a tube containing 5mls of tryptic soy broth and allowed to stand until the cell suspensions achieved turbidity that is equivalent to 0.5 McFarland standards. Sterile swab sticks were used to inoculate each suspension on surface of pre-dried Mueller-Hilton agar and allowed to stand for 5mins. Eight different antibioticsCiprofloxacin (5µg), Tetracycline (30µg), Chloramphenicol (30µg), Gentamicin (10µg), Ampicillin (30µg), Amoxicillin-clavulanate (30µg), Nitrofurantoin (300µg), and Erythromycin (15µg), were placed individually using sterile forceps and then gently press down onto the agar. The plates were inverted and incubated at 37^0C for 18hr after which the zone of inhibition was measuredin millimeters with a ruler.

3.11. 1.Recording and interpreting results

The zones of growth inhibition were compared with the zone-size interpreted based on standard provided by Clinical and Laboratory Standard Institute (CLSI, 2015). Antimicrobial testing result recorded as resistant, intermediate and sensitive according to zone diameter interpretive standards provided by CLSI,(2015).

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3.12.Plasmid Profile Analysis

Plasmid Isolation and Profiling: Plasmid isolation was done using a commercial plasmid isolation kit(Plasmid Miniprep Kit, Zymogen Co. Ltd. UK) according to the manufacturer instructions.

Gel Electrophoresis: Electrophoresis of the DNA was carried out on a 0.8% agarose gel (Akingbade*et al.*, 2012).

3.12.1 Plasmid Isolation

Six hundred (600) µl of the isolate culture grown in lysis buffer (LB) was added to a 1.5ml microcentrifuge tube.One hundred(100) µl of 7X lysis buffer (blue) was added and mixed by inverting the tube 5 times. After 2 minutes, 350 µl of cold Neutralization buffer (yellow) was added and mixed thoroughly. The solution was centrifuged at 16,000 x g for 4 minutes. Nine hundred(900) µl of the supernatant was transferred in to zymo-spin^{TM} IIN column. The column was placed into collection tubeand centrifuged for 15 seconds. The flow-through was discarded and the column was placed back into the same collection tube. Two hundred(200) µl of Endo-Wash Buffer was added to the column and centrifugedfor 30 seconds. Four hundred(400) µlof zyppy^{TM} Wash Buffer was added to the column and centrifuged for 1 minute. The column was transferred into a clean 1.5 ml microcentrifuge tube, 30 µl of zippy^{TM} Elution Buffer was added directly to the column matrix and was allowed to stand for 1 minute at room temperature. The solution was centrifuged for 30 seconds to elute the plasmid DNA.

3.12.2 Gel Electrophoresis

One point five (1.5) g of agarose powder was weighed in 500 ml conical flask. One hundred (100) ml of TBE buffer was added and dissolved in a microwave oven. It was brought out and placed on bench to cool to about 45^0C. Casting trays were assembled and comb

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positioned.Five(5) µlof ethidium bromide was added to the agarose and mixed gently. It was poured onto the casting tray and allowed to solidify. Combs were carefully removed. The tray was placed inside the running tank. The tank was filled to required volume and connected to a power pack. The gel was run at 75V for 45 minutes. The gel was documented in gel documentation unit and saved in a computer.

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CHAPTER FOUR

RESULTS

4.1. Culture, Microscopy And Biochemical Identification Of The Isolates

Twelve samples produced blue green pigment on cetrimide agar. The isolates were rod in shape and pink in colour when viewed under microscope after Gram staining.For biochemical tests motility, simmon citrate,oxidase and triple sugar iron agar; for motility, the twelve isolates moved away from inoculation point. For citrate all the isolates changed citrate media from green to blue. For oxidase test deep purple precipitate was produced as a result of mixing the isolates and oxidase reagent. In triple sugar iron agar both butt andslant changed to red colour.

4.2. PrevalenceOf *Pseudomonas aeruginosa* From Wound Infections InZaria Local Government

A total of hundred and fifty (150) wound swab samples were collected from three different hospitals in Zaria local government and examined by culture, microscopically and biochemically.Twelve (8%) of the total samples examinedwere positive for*Pseudomonas aeruginosa*(Fig. 4.1).

4.3.Prevalence Of *Pseudomonasaeruginosa* In Sample Population

The one hundred and fifty wound swab samples collected from three hospitals: Gambo Sawaba General Hospital, Kofan Gayan, Zaria (GSGH), Zaria Clinic, Tudun Wada, Zaria(ZC), and Saint Luke's Anglican Hospital (SLAH), Wusasa, Zaria. The highest prevalence rate of 13.3% was found inSLAN, followed by 8% in GSGH while ZC had 0% (Fig. 4.2). The chi square (χ^2) was 1.449 and P value was 0.4846 indicating that it is statistically not significant because P value is above 0.05.

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4.4.Prevalence Of *Pseudomonas aeruginosa* In Wound Infection In Relation To Age Group Of Patients Tested

Figure 4.3 shows the prevalence of *Pseudomonas aeruginosa*from wound infections in relation to age group. Age group 21-30 years had the highest prevalence of 14% followed by 31-40 years with prevalence of 12% and the least prevalence 0%was observed among age group 41-50 years and 51-60 years. Age group 1-10 had prevalence of 5%while 60 years and above had 6% prevalence. The chi square (χ^2) was 5.006 and P value was 0.5430 indicating that it is statistically not significant because P value is above 0.05.

4.5. Prevalence Of *Pseudomonas aeruginosa* From Wound Infection By Sex

Prevalence of*Pseudomonas aeruginosa* from wound infections by sex is presented in Figure 4.4. A total of sixty seven wound swab samples were collected from females,*Pseudomonas aeruginosa* was isolated from 3 (4.5%) samples.Eighty three samples were collected from males of which*Pseudomonas aeruginosa*was isolated from 9 (10.8%) samples. The chi square (χ^2) was 2.041 and P value was 0.1531 indicating that it is statistically not significant because P value is above 0.05.

4.6. Prevalence Of *Pseudomonas aeruginosa* From Wound Infections By Occupation

Prevalence of *Pseudomonas aeruginosa* from wound infections by occupation is presented in Table4.1. The highest prevalence was observed among business people with prevalence rate of 11.8% followed by students with prevalence rate of 10.3%, civil servant had the prevalence rate of 6.7% the least prevalence 4.2% was seen among house wives. The chi square (χ^2) was 1.964 and P value was 0.5799 indicating that it is statistically not significant.

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4.7. Distribution Of *Pseudomonas aeruginosa* From Wound Infections In Relation To Medical History Of The Patient

Figure 4.5 shows the distribution of *Pseudomonas aeruginosa* in wound infections in relation to medical history of the patient. The highest prevalence rate of9% was found among patients with no any medical history while patients with hypertension and diabetes as well as those with drug allergy had prevalence of 0%. The chi square (χ^2) was 1.588 and P value was 0.6621 indicating that it is statistically not significant.

4.8. Distribution Of *Pseudomonas aeruginosa* From Wound Infections In Relation To Cause Of The Wound

Table 4.2 shows the distribution of *Pseudomonas aeruginosa* in wound infection in relation to cause of the wound. Accident patients had the highest prevalence of 13.6%, surgery patients had 10.8%, patients with boil had 5.3% and the least prevalence 0% was observed in burnt and bite patients. The chi square (χ^2) was 5.384 and P value was 0.2501 indicating that it is statistically not significant.

4.9. Prevalence Of *Pseudomonas aeruginosa* From Wound Infections By Location Of The Wound On The Body

Prevalence of *Pseudomonas aeruginosa* in wound infection by location of the wound is presented in Table 4.3. Patients with wound located on their hands had highest prevalence of 13.6%, those with wound located on their abdomen had 9.1%, those with wound located on their back had 10%, and those with wound located on their legs had 8.8%, while the least prevalence 0% was seen on those with it located on their heads. The chi square (χ^2) was 3.387 and P value was 0.4953 indicating that it is statistically not significant.

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4.10. Prevalence Of *Pseudomonasaeruginosa* From Wound Infections In Relation To Antibiotic Usage

Figure 4.6 shows the Prevalence of *Pseudomonas aeruginosa* in wound infection in relation to antibiotic usage.The highest prevalence of 8.6% was observed in those that used antibiotic while those that do not use antibiotic had least prevalence of 0%. The chi square (χ^2) was 1.032 and P value was 0.3096 indicating that it is statistically not significant.

4.11. Antibiotic Susceptibility Result

The antibacterial susceptibility results of the twelve isolates are summarized in table 4.4.The result indicates that the eleven isolates were highly resistance to Ampicilin, Nitrofuratoin and Amoxycillin clavulanate (91.7%), ten of them were resistance Chloramphenicol (83.3%), and nine were resistance to Erythromycin (75%) but the twelve isolates were completely susceptible to Ciprofloxacin, Tetracyclin and Gentamicin.

4.12 The Result of Agarose Gel Electrophoresis Of The Isolated Plasmids

Plate 1 shows the result of agarose gel electrophoresis of the isolatedplasmids, it indicates that no any plasmid was isolated in the first organism while the rest of eleven organisms harbored plasmid with molecular size of 800bp for each isolate.

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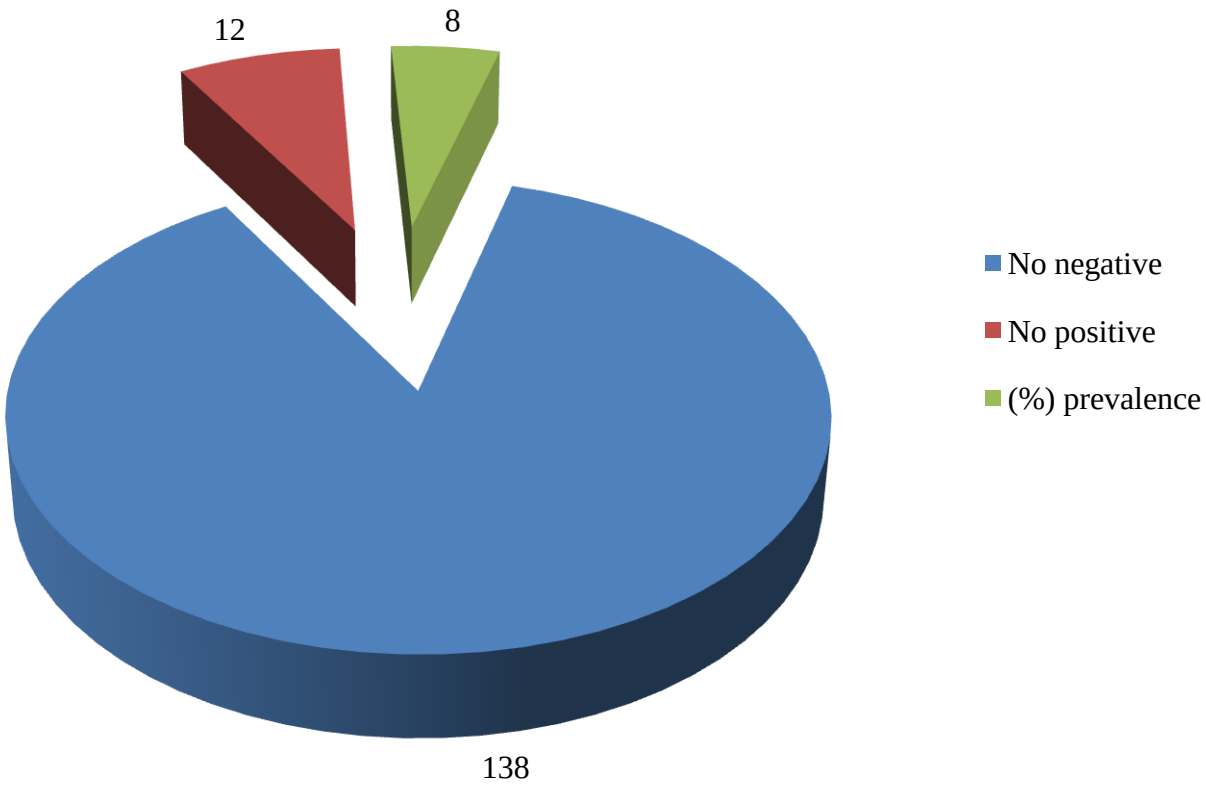
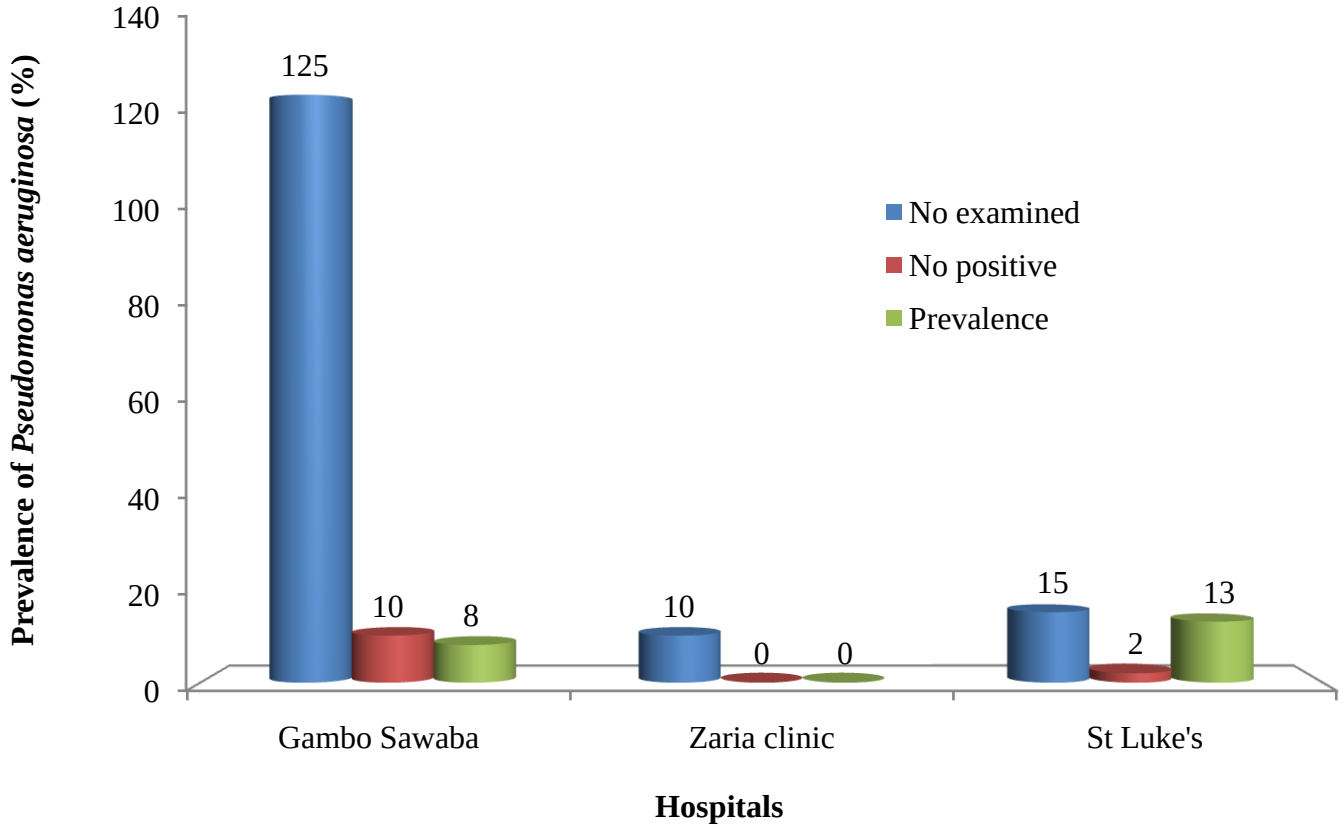


Figure 4.1: Prevalence of *P. aeruginosa* from Wound in Zaria

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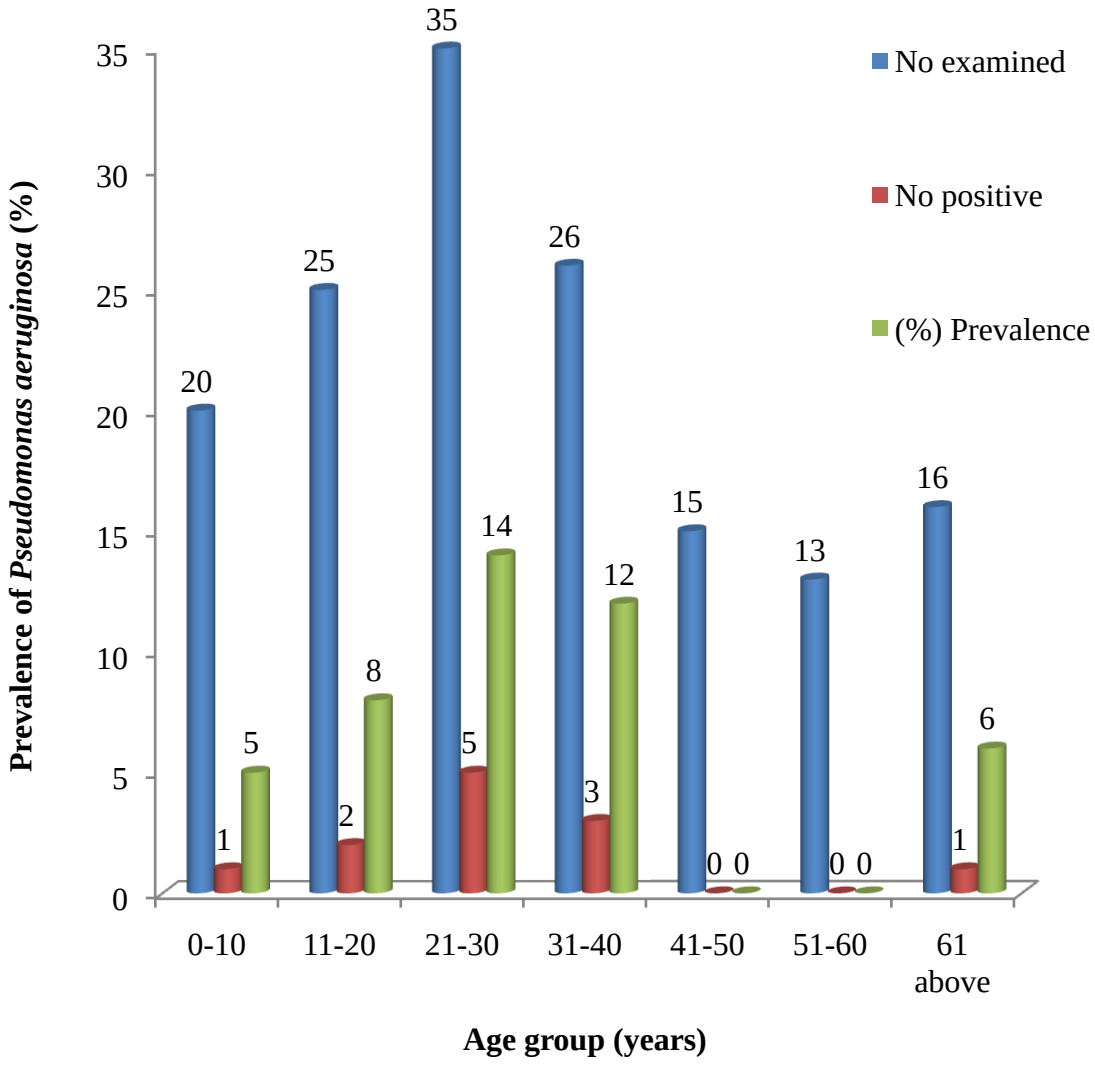
chisquare (χ^2) = 1.449

P value = 0.4846

Df = 2

Figure 4.2:Prevalence of*P. aeruginosa*fromwound infections by sampling sites

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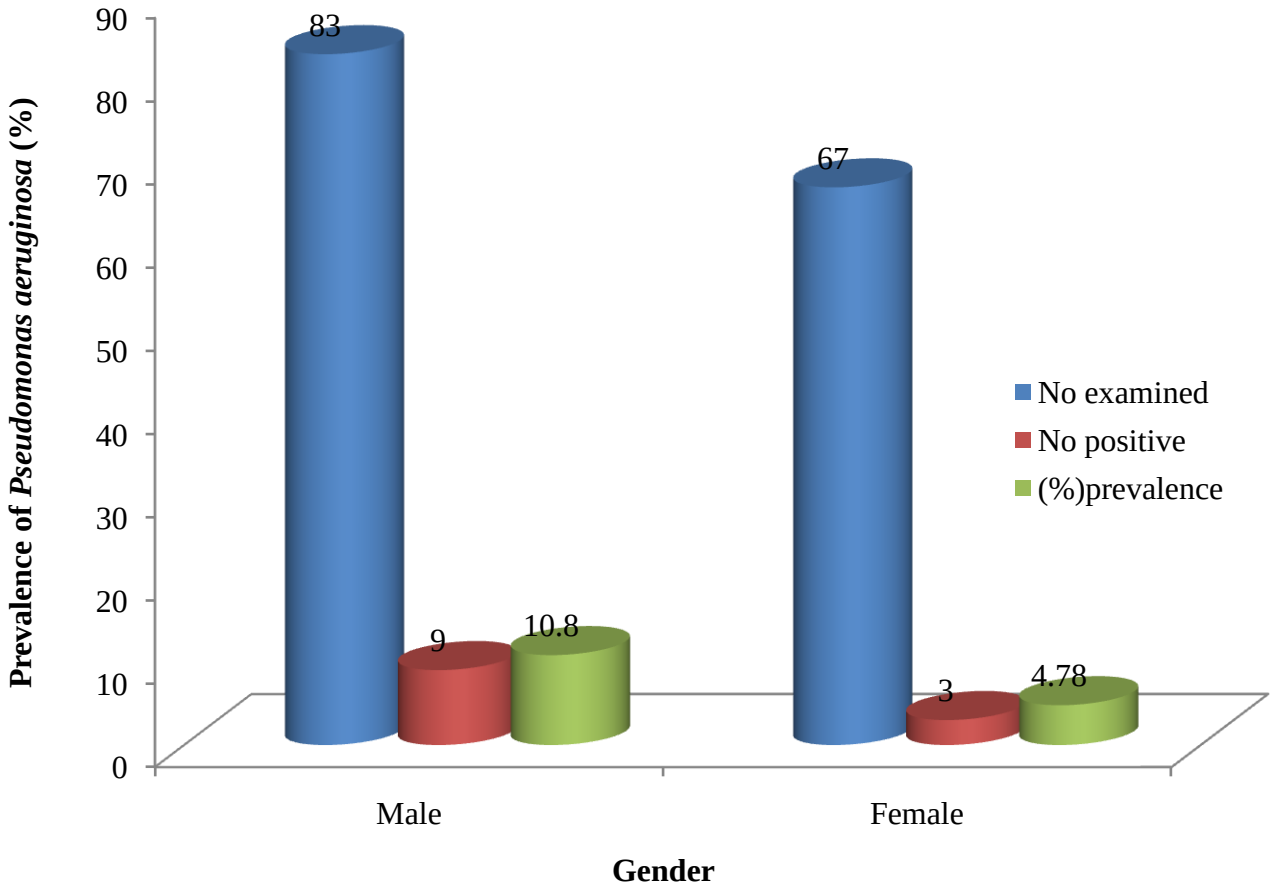
chi square (χ^2) = 5.006

P value = 0.5430

Df = 6

Figure 4.3: Prevalence of *Pseudomonas aeruginosa* from wound in relation to age groups

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chi square (χ^2) = 2.041

P value = 0.1531

Df = 1

Figure 4.4: Prevalence of *Pseudomonas aeruginosa* from wound in relation to sex

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Table 4.1: Prevalence of *Pseudomonas aeruginosa* from wound in relation to occupation

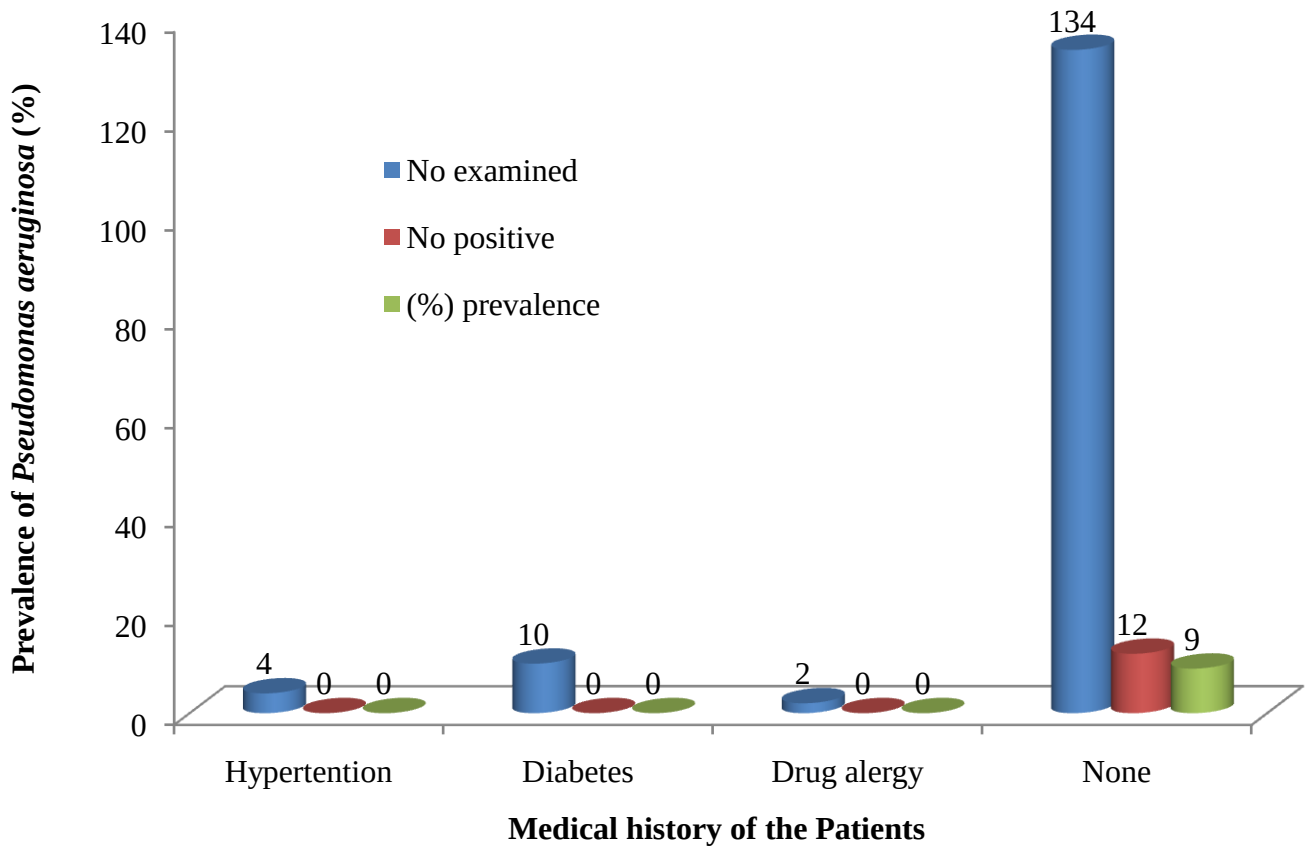
Occupation	No screened	No positive	prevalence (%)
Students	39	4	10.3
House wife	47	2	4.3
Civil servant	30	2	6.7
Business person	34	4	11.8
Total	150	12	8

Chi Square(χ^2) = 1.964

P Value = 0.5799

Df = 3

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chi square (χ^2) = 1.588

P value = 0.6621

Df = 3

Figure 4.5: Prevalence of *Pseudomonas aeruginosa* from wound in relation to medical history of the patients

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Table 4.2: **Prevalence of *Pseudomonas aeruginosa* from wound in relation to cause of the wound**

Cause of the wound	No examined	No positive	Prevalence (%)
Burn	20	0	0
Accident	44	6	13.6
Surgery	37	4	10.8
Boil	38	2	5.3
Bite	11	0	0
Total	150	12	8

chi square (χ^2) = 5.384

P value = 0.2501

Df = 4

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Table 4.3: **Prevalence of Pseudomonas aeruginosa from wound in relation to site of the wound**

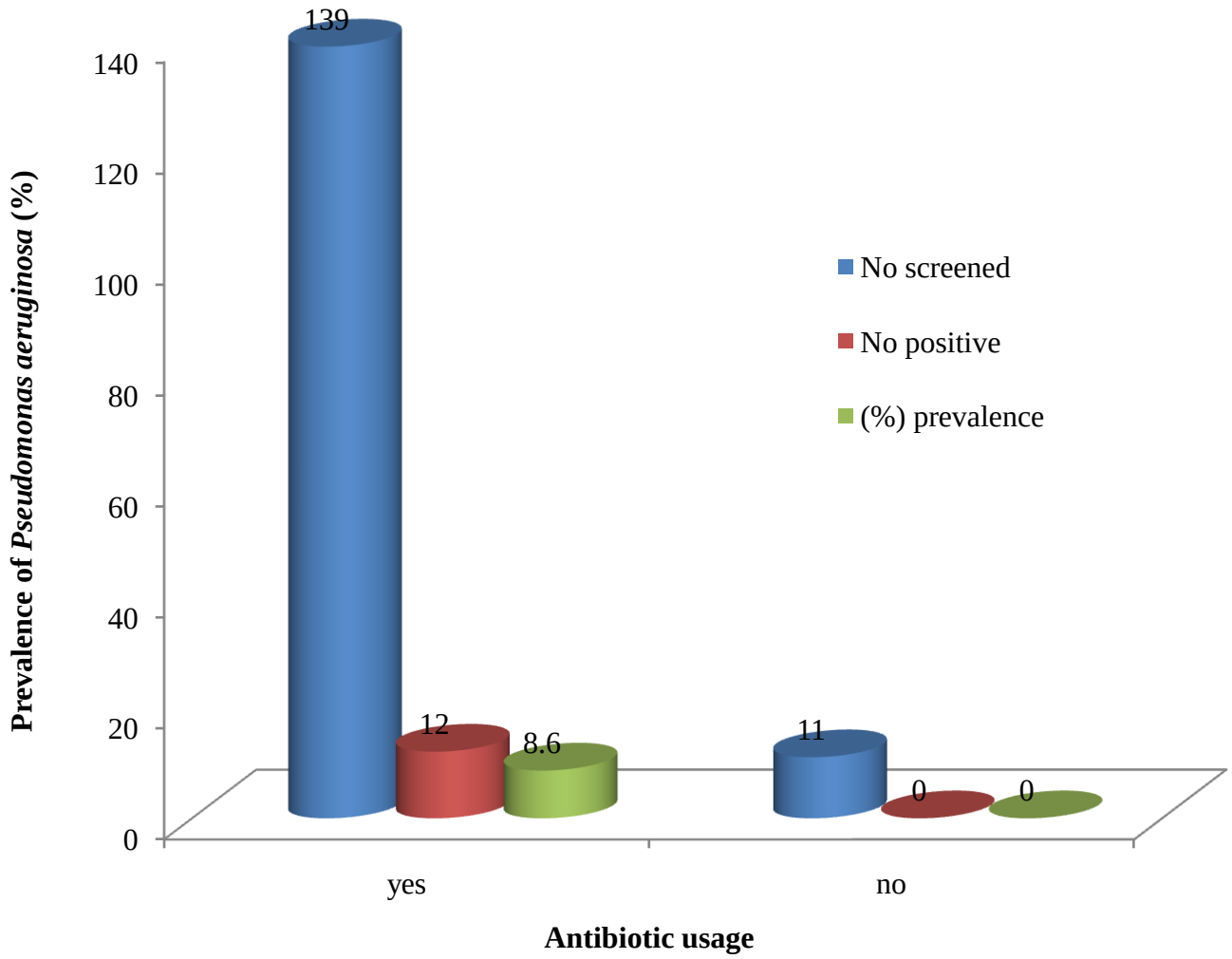
Site of the wound	No examined	No positive	Prevalence (%)
Leg	68	6	8.8
Hand	22	3	13.6
Head	28	0	0
Abdomen	22	2	9.0
Back	10	1	10.0
Total	150	12	8

chi square (χ^2) = 3.387

P value = 0.4953

Df = 4

.



chi square (χ^2) = 1.032

P value = 0.3096

Df = 1

Figure 4.6: Prevalence of *Pseudomonas aeruginosa* from wound in relation to antibiotic usage

.

Table 4.4: Antibiotic susceptibility test result

Antibiotics	Concentration	(n=12) Sensitivity (%)	Intermediate (%)	Resistance (%)
Ciprofloxacin	(5 µg)	12(100)	0	0
Tetracyclin	(30 µg)	12(100)	0	0
Chloramphenicol	(30 µg)	1(8.3)	1(8.3)	10(83.3)
Gentamicin	(10 µg)	12(100)	0	0
Ampicillin	(30 µg)	1(8.3)	0	11(91.7)
Amoxicillin-clavulanate	(30 µg)	1(8.3)	0	11(91.7)
Nitrofurantoin	(10 µg)	1(8.3)	0	11(91.7)
Erythromycin	(15 µg)	1(8.3)	2(16.7)	9(75)

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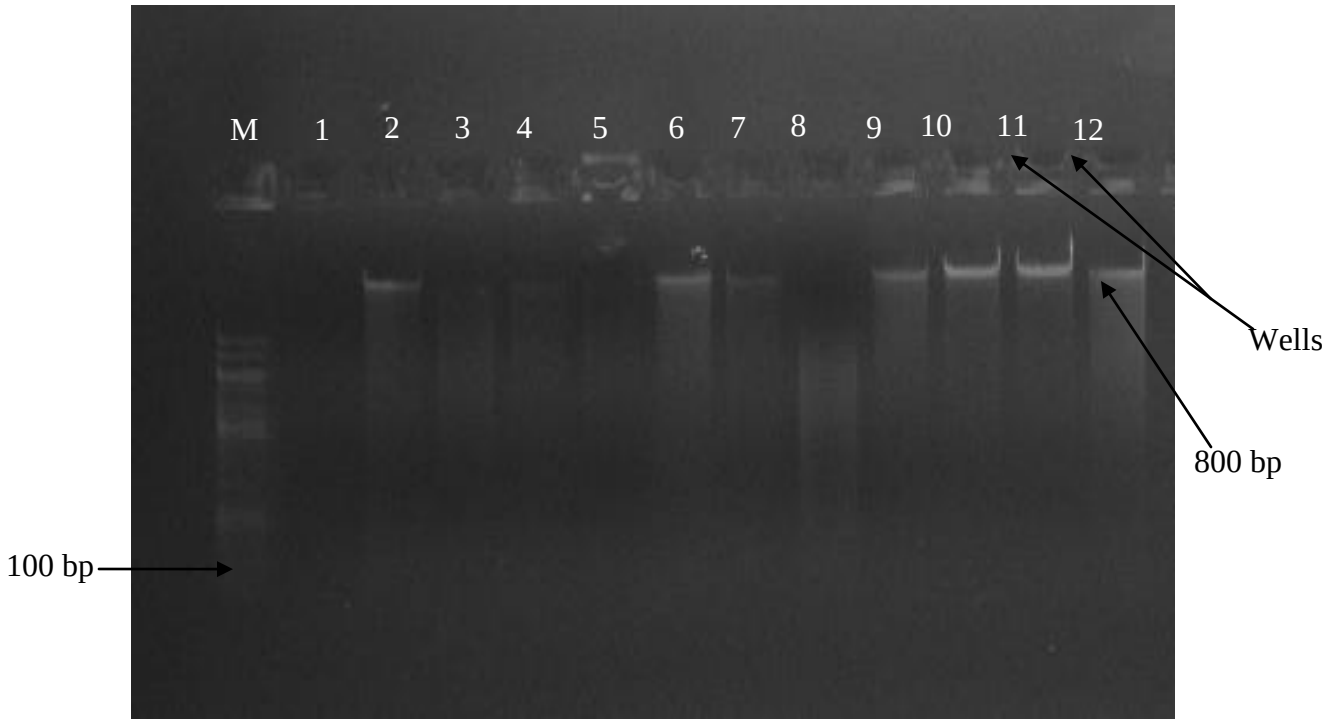


Plate 1: Plasmid profiles of the antibiotic resistant *Pseudomonas aeruginosa isolates*

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CHAPTER FIVE

DISCUSSION

*Pseudomonas aeruginosa*is one of the pathogens commonly isolated from wound infections. Treating wounds contaminated with this organism is difficult due to its resistance to many antibiotics.The prominent and significant role of *Pseudomonas aeruginosa* in mortality and morbidity among various nosocomial infections has made many researchersto study its antibiotic sensitivity pattern.The organism is naturally resistant to several commonly used antibiotics by producing several enzymes as well as change in outer membrane permeability, multi–drug efflux mechanism.Orsi *et al.*(1995); Sule *et al.*(2002); Meenakumari *et al.*(2011) explained that *P. aeruginosa*is a common cause of infections in wound and burns contacted from the hospital environment or from patients own normal flora, and these infections could lead to the longer hospital stays, high treatment costs and mortality rate.

Increasing resistance to different antibiotics especiallyamong nosocomial pathogens has been reported worldwideand become important therapeutic challenge in thetreatment of disease (Jones *et al.,* 2002; Orrett, 2004).This fact agrees with the position of WHO which pointedout that the widespread use of antibiotics both outside andinside of medicine is playing a significant role in theemergence of resistant bacteria by developing severalresistance mechanisms such as production of Betalactamaseenzyme that destroy these antibiotics (WHO,2002).

The major problem of the emergence of resistant bacteriais due to overuse and misuse of antibiotics bypatients (Goossens *et al.,* 2005; Iduh*et al.,* 2015), and may be related to indiscriminate use ofantibiotics without laboratorydiagnosis.

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This study shows that the prevalence of *Pseudomonas aeruginosa*from wound infections in Zaria was 12(8%). The low prevalence could be attributed to their improved hygienic practices, geographical location and the patients reduced the habit of drug misuse. This finding is in contrast with the study conducted by Smith *et al.*(2012) from Southwest Nigeria which shows that the prevalence rate of *Pseudomonas aeruginosa*isolated from wound infections was16.3%.

The research was also in contrast with similar studies carried out by Anupurba *et al.*(2010), who revealed that *P. aeruginosa*was isolated in 32% of isolates. Prevalence of 19.56% was reported in Southern Technical University, Shatrah Technical Institute, Iraq by Al-Zaidi (2016). The differences in the prevalence of *Pseudomonas aeruginosa*in wound infections in these studies could be attributed to differences in geographical locations.

Highest prevalence of *Pseudomonas aeruginosa* in wound infections was obtained in Saint Luke's Hospital Wusasa compared to Gambo Sawaba General Hospital Kofan Gayan, Zaria and Zaria Clinic Tudun Wada, Zaria. This could be due to differences in geographical locations Wusasa has more trees any time leaves fall and decay in the soil, it will increase nutrient content of the soil therefore organisms will be abundant in area.

The highest prevalence of wound was observed among age group of 21-30 years this could be attributed to the fact that they are more active, therefore they can easily come down with wound and could lead to the colonization of their wounds by the organism because *Pseudomonas aeruginosa* is ubiquitous. This finding is in contrast with research conducted by Kericci and Kareem (2014) who reported that most of the patients (45.33 %) aged between 41 - 60 years had *P. aeruginosa* as contaminants of their wound infections. It also disagrees with

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the study conducted byAl-Zaidi (2016), from Southern Technical University, Shatrah Technical Institute, Iraq who reported that *P. aeruginosa* is more among young patients (ages 5 to 25 years).

The occurrence of wound is higher in males than in females, this is because most females in Zaria local government arehouse wives therefore their activities are restricted to their homes so their chances to come down with wound and the wound to get contamination with *Pseudomonas aeruginosa*is low. While males hardly stay in one place as such they have more chance to come down with wound and the wound is easier to get contamination with this organism because *Pseudomonas aeruginosa* is widely distributed in different areas. However, there is no significant statistical association with this parameter.This is in agreement with the findings of Kericci and Kareem (2014) who reported that*P. aeruginosa*are more common in males (57%) compared to females (43%). The study is also comparable with the result of Al-Zaidi (2016), from Southern Technical University, Shatrah Technical Institute, Iraq who reported that *P. aeruginosa*are morein the males (55.6%), than(38.9%) in the female.

Highest prevalence of wound based on occupation in this study was obtained among business people, this could be attributed to their habit of travelling to different places in order to buy and sell items. In this process, there is high chance for them to be involved in different types of accidents which will cause them to come down with wound. So also business people had highest prevalence of *Pseudomonas aeruginosa* in wound, the reason behind this is associatedwith their tradition of selling and buying items and these items are mostly contaminated with soil and water like yam, tomatoes, lettuce among others. *Pseudomonas aeruginosa*are naturally abundant in soil and water therefore there is high probability for them

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to contaminate their wound with the organism. However this relationship is not statistically associated.

There is no statistical association in the prevalence of wound in relation to past history of the wound; highest prevalence was observed among patients with no past history. Zero prevalence of *Pseudomonas aeruginosa*in wound was seen among patients with hypertension, diabetes and drug alergy. This could be attributed to their conscious about their condition so they tried to improve their hygiene practices as well as avoiding drug misuse.

The occurrence of wound based on cause of the wounds in this research indicated that accidents caused the highest number; this could be attributed to the occupation and the tradition of the population in the study area who are mainly business people. Their kind of business involved travelling to different areas to buy and sell items which could lead them toinvolve in various types of accidents as such they come down with wounds in different parts of their bodies. The contamination of wound with *Pseudomonas aeruginosa*is also highest among accident patients; this could due to the fact that, at the time of the accidents, the victims usually rolled on the ground and in this process they can easily pick up the organism the because*Pseudomonas aeruginosa* can resist harsh condition and it is ubiquitous so it can be found in that location at that time. The organism can also colonize their wounds from the people who assisted them in moving away from the point of the accident and from those that help them to visit hospitals because *Pseudomonas aeruginosa*can be found on the body of healthy people. However, there is no statistical association with this cause.

The commonest part of the body where wound occur is the leg, this could be due to legs are the longest parts of the body; therefore there is high chance of wound to occur there. But the

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prevalence of *Pseudomonas aeruginosa* from wound in relation to the location on the body indicated that hand had the highest prevalence; this could be attributed to thehighest activities of the hand especially picking things from the ground but there is no statistical significant association with this.

The prevalence of wound based on antibiotic usage in this research indicated no statistical association; all of the examined patients with wound were under drug usage, some of them engaged themselves in drug misuse so there is high possibility of delaying the wound healing due to modification of the organism to become resistant to the antibiotic.

The organisms isolated showed highest resistance to Ampicillin 11(91.7%), Amoxiciilin-clavulanate 11(91.7%) and Nitrofurantoin 11(91.7%), higher resistance to Chloramphenicol 10(83.3%), high resistance to Erythromicin 9(75%), and complete susceptible to Ciprofloxacin 12(100%), Tetracyclin 12(100%) and Gentamicin 12(100%). It can be concluded that Ciprofloxacin, Tetracyclin and Gentamicin are drugs of choice for treating wounds contaminated with *Pseudomonas aeruginosa.* The reason behind the resistance of the organism to those antibiotics could be attributed to presence of β lactamase enzymes which make it to resist B lactan antibiotics and also the organism is known to produce biofilm and it has low cell wall permeability to antibiotics. This result is similar to the study conducted by Akinbage *et. at.* (2012) from South West Nigeria according to their study; *Pseudomonas aeruginosa* had highest resistance to amoxicillin (92.7%), ampicillin (90%), cloxacillin (88.2%), cotrimoxazole (77.3%), erythromycin (72.7%), tetracycline (70.9%), streptomycin (65.5%) and ofloxacin (60%).

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Li *et al.* (1994); Akinbage *et. al.* (2012) reported that *Pseudomonas aeruginosa* is naturally resistant to β-lactams, including broad plasmidspectrum cephalosporins, quinolones, chloramphenicol and tetracyclines, mainly because of the very low permeability of their cell wall but in this present study there was a good susceptibility to some of the groups of antibiotics.

High susceptibility activities against *P. aeruginosa* were recorded in antibiotics such as ceftazidime (80.0%), gentamycin (73.6%) and levoxin (69.1%) by Van Eldere (2003), he also reported that ceftazidime and gentamycin are good antibiotics against *Pseudomonas aeruginosa*.

According to Jonathan and Courage (2015), *Pseudomonas aeruginosa* Isolated from Some Hospital Patients in Benin City, Nigeriahas 13(40.6%) multi-drug resistance phenotype. Resistance to the tested antibiotics was in the following decreasing order: Nalidixic acid and Ceporex (92.3%), Ampicillin (76.9%), Augmentin and Gentamicin (46.2%), Septrin (38.5%), Ciprofloxacin and Reflacine (30.8%), and Streptomycin (15.4%).

Al-Zaidi (2016), reported that the antibiotic susceptibility testing profile of *P. aeruginosa* shows 100% of isolates of clinical samples and hospital environment were resistant to ticarcillin and ceftazidime. Al-Zaidi (2016), also reported that the resistance of *P. aeruginosa* against the Beta-lactam antibiotics was higher than that of non Beta-lactams, this result can be attributed to the hyper production of Beta lactamase through the resistance genes and mutational processes this is in agreement with the finding of Lister *et al.* (2009); Okon*et al.* (2009).

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The result of plasmid profile shows that the eleven (91.7%) isolates out of twelve harbored plasmids with molecular sizes of 800bp. Only one isolate does not harbor plasmid and this isolate is susceptible to all antibiotics. The isolates that harbored plasmids were completely resistant to Ampicillin, Amoxiciilin-clavulanate, Nitrofurantoin, Chloramphenicol and Erythromicin. *P. aeruginosa* is known to harbor plasmid that enables it to resist the effect of antibiotics. This finding is similar to a research conducted by Akingbade *et. al.* (2012) from South West, Nigeria who reported that all the isolates that had plasmids were resistant to amoxicillin, ampicillin, cloxacillin, cotrimoxazole, erythromycin and tetracycline. They also reported that the sizes of the plasmids among *P. aeruginosa* isolates ranged from 662bp to 830bp which also is in agreement with this study.

A study conducted by Jonathan and Courage (2015), reported that eleven (85%) resistant isolates of *Pseudomonas aeruginosa* from Some Hospital Patients in Benin City, Nigeria harbored plasmids with bands ranging from 150bp to 300bp. Another study from University of Benin Teaching Hospital, Benin City, Nigeria reported by Yah *et al.*,(2010) also demonstrated thatisolates from burn wound samples in UBTH harbored multi drug resistance conjugative plasmids, which may confer resistance to some of these other antibiotics. They were also highly transferable with a frequency range of $2x10^{-2}$ to $6x10^{-4}$. Some of the isolates had plasmids bands that ranged from <0.55kbp to >1.14kbp. (Yah,2010)

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CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The prevalence of *Pseudomonas aeruginosa*from wound infections in some hospitals in Zaria was (8%). Antibiotic susceptibility patterns of *Pseudomonas aeruginosa*from wound infections showed that all isolates showed highest resistance to Ampicillin 11(91.7%), Amoxiciilin-clavulanate 11(91.7%) and Nitrofurantoin 11(91.7%), higher resistance to Chloramphenicol 10(83.3%), high resistance to Erythromicin 9(75%), and complete susceptible to Ciprofloxacin 12(100%), Tetracyclin 12(100%) and Gentamicin 12(100%). The plasmid profile results revealed that the organisms that are resistant to many antibiotic harbored plasmid and the sizes of all isolated plasmids are 800bp. Risk factors associated with *Pseudomonas aeruginosa*from wound infections are gender, age and location.

6.2 Recommendations

The use of gloves and wearing of gowns, extra attention to hospital hygiene and Proper hand washing can reduce plasmid antibiotics mediated resistance among isolates.

Quick and efficient antibiotherapy should be done toeffectively reduce wound infections.

Appropriate use of antimicrobial agents coupled with proper susceptibility test results prior to the prescription of antibiotics should be donein order to reduce delay of wound healing.

Antibiotics chemotherapy can be replaced byimmunization of those at risk.

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APPENDICES

DEPARTMENT OF MICROBIOLOGY,

FACULTY OF LIFE SCIENCES

AHMADU BELLO UNIVERSIITY, ZARIA.

RESEARCH QUESTIONAIRE

PLASMID PROFILE OF ANTIBIOTIC RESISTANT *PSEUDOMONAS AERUGINOSA* ISOLATED FROM WOUND INFECTIONS AMOUNG PATIENTS ATTENDING SOME HOPITALS IN ZARIA, NIGERIA.

1) Age……..
2) Sex a) Male b) Female
3) Hospital……
4) Occupation a) Student b) Housewife c) Businessman/ Businesswoman d) Civil servant
5) Medical history a) Hypertension b) Diabetes c) Drug alergy d) None
6) Cause of the wound a) Burn b) Accident c) Surgery d) Boil e) Bite
7) Site of the wound a) Leg b) Hand c) Head d) Abdomen e) Back
8) Use of antibiotics a) Yes b) No

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Statistical analysis

Prevalence Of*Pseudomonas aeruginosa* From Wound Infections In Relation To Study Population

Hospitals	number positve	number negative	total	Observed positive	Expected positive	Observed negative	Expected negative
GamboSawaba	10	115	125	10	10	115	115
Zaria clinic	0	10	10	0	0.8	10	9.2
St Luke's	2	13	15	2	1.2	13	13.8
total	12	`138	150				

$\chi^2 = 1.449$

Degree of freedom = 2

P value = 0.4846

.

Prevalence Of*Pseudomonas aeruginosa* From Wound Infections In Relation To Age

Age group	Number positive	Number negative	Total	Observed positive	Expected positive	Observed negative	Expected negative
0 - 10	1	19	20	1	1.6	19	18.4
11 - 20	2	23	25	2	2	23	23.0
21 - 30	5	30	35	5	2.8	30	32.2
31 - 40	3	23	26	3	2.1	23	23.9
41 - 50	0	15	15	0	1.2	15	13.8
51 - 60	0	13	13	0	1.0	13	12.0
61-above	1	15	16	1	1.3	15	14.7
Total	12	138	150				

$\chi^2 = 5.006$

Degree of freedom = 6

P value = 0.5430

.

Prevalence Of*Pseudomonas aeruginosa* From Wound Infections In Relation To Sex

Sex	Number Positive	Number negative	Total
male	9	74	83
female	3	64	67
total	12	138	150

$\chi^2 = 2.041$

Degree of freedom = 1

P value = 0.1531

.

Prevalence Of*Pseudomonas aeruginosa* From Wound Infections In Relation To Occupation

Occupation	Number positive	Number negative	Total	Observed positive	Expected positive	Observed negative	Expected negative
Students	4	35	39	4	3.12	35	35.88
House wife	2	45	47	2	3.76	45	43.24
C. servant	2	28	30	2	2.4	28	27.6
B. People	4	30	34	4	2.72	30	31.28
Total	12	138	150				

C = Civil

.

B = Business

$\chi^2 = 1.964$

Degree of freedom = 3

P value = 0.5799

Prevalence Of*Pseudomonas Aeruginosa* From Wound Infections In Relation To Medical History

Medical history	Number positive	Number negative	Total	Observed positive	Expected positive	Observed negative	Expected negative
hypertension	0	4	4	0	0.32	4	3.68
diabetes	0	10	10	0	0.8	10	9.2
drug alergy	0	2	2	0	0.16	2	1.84
none	12	122	134	12	10.72	122	123.28
total	12	138	150				

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$\chi^2 = 1.588$

Degree of freedom = 3

P value = 0.6621

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Prevalence Of*Pseudomonas aeruginosa* From Wound Infections In Relation To Cause Of The Wound

Cause of the wound	Number positive	Number negative	Total	Observed positive	Expected positive	Observed negative	Expected negative
burn	0	20	20	0	1.6	20	18.4
accident	6	38	44	6	3.52	38	40.48
surgery	4	33	37	4	2.96	33	34.04
boil	2	36	38	2	3.04	36	34.96
bite	0	11	11	0	0.88	11	10.12
total	12	138	150				

$\chi^2 = 5.384$

Degree of freedom = 4

P value = 0.2501

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Prevalence Of*Pseudomonas aeruginosa* From Wound Infections In Relation To Site Of The Wound

Site of the wound	Number positive	Number negative	Total	Observed positive	Expected positive	Observed negative	Expected negative
leg	6	62	68	6	5.44	62	62.5
hand	3	19	22	3	1.76	19	20.2
head	0	2.24	28	0	2.24	28	25.76
abdomen	2	20	22	2	1.76	20	20.24
back	1	9	10	1	0.8	9	9.2
total							

$\chi^2 = 3.387$

Degree of freedom = 4

P value = 0.4953

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Prevalence Of*Pseudomonas aeruginosa* From Wound Infections In Relation To Antibiotic Usage

Use of antibiotics	Number positive	Number negative	Total
yes	12	127	139
no	0	11	11
total	12	138	150

$\chi^2 = 1.032$

Degree of freedom = 1

P value = 0.3096

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MINISTRY OF HEALTH AND HUMAN SERVICES
KADUNA STATE OF NIGERIA

ADDRESS: Independence Way, P.M.B. 2014, Kaduna.

MOH/ADM/744/VOL.1/337 — 19th OCT, 2015

NOTICE OF APPROVAL AFTER FULL COMMITTEE REVIEW

ISOLATION, CHARACTERZATION AND ANTIBIOTIC SUSCEPTIBILITY OF PSEUDOMONAS AERUGINOSA OBTAINED FROM WOUND INFECTIONS AMONG PATIENTS ATTENDING A.B.U TEACHING HOSPITAL ZARIA,NIGERIA.

Name of Principal Investigator:	ABUBAKAR ZAINAB
Address of Principal Investigator:	Dept. Of Microbiology, Ahmadu Bello University Zaria, Kaduna State.
Date of receipt of Application	3th Aug, 2015
Date of Ethical Approval	29th Sept., 2015

This is to inform you that the Research described in the submitted Protocol, the Consent forms, advertisements and other participant information materials have been reviewed and given full approval by the Health Research Ethics Committee (HREC).

If there is delay in starting the research or any change, inform the HREC so that the dates of approval can be adjusted accordingly.

However, Researcher is kindly requested to submit a copy of his/her findings to the State Ministry of Health, please.

TUMAN DANIEL ALI ESQ
SECRETARY
FOR: CHAIRMAN

KADUNA ... MINISTRY OF HEALTH
OPE... REGISTRY
... CHED
SIGN... 12/10/15

Phone No.: 08184078693 Website:ttp://www/moh.kd.gov.ng. Email: health@kdsg.gov.ng.

DEPARTMENT OF MICROBIOLOGY
SCHOOL OF POSTGRADUATE STUDIES
AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

UMFORMED CONSENT FORM (ICF)

Serial No.:**……..** ***Hospital No.:*****…… *Age:*……..** ***Phone No.:***
……

This Informed Consent Form is Basically for collection of wound samples. We are inviting you to participate in this research work titled "Isolation, Characterization and antibiotic susceptibility pattern of ***Pseudomonas aeruginosa*** **from wound infections among patients attending some hospitals in Zaria, Nigeria.** The research will involve the collection of wounds samples. The results obtained thereby may be used in any way to improve the understanding and management of disease in our community.

Risk: There is no risk associated with the sample collection.

Benefits: for those that are found positive will be refer to proper channel for treatment.

CERTIFICATE QF CONSENT

I…….. ……of……..

hereby consent to participate in this study. The full procedures of the test/study to me by the investigator. I understand that that stool samples will be collected. I therefore give this consent voluntarily without being subjected to any pressure.

Name. of Participant.....

Signature of Participant/thumb print..... Date……

Statement by Witness:

I have witnessed the accurate reading of the consent form and detail explanation of the study to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name. of Participant.....

Signature of Participant/thumb print..... Date……

Statement by the Researcher/Person Taking Consent

I confirm that sufficient information, including about risks and benefits, to make an informed decision have been fully explained to the participant. The participant was given an opportunity to ask questions about the study; and all the questions asked by participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

Name of Researcher……..

Signature…… Date……