ISOLATION AND CHARACTERISATION OF MULTI-DRUG RESISTANT PSEUDOMONAS AERUGINOSA FROM CLINICAL, ENVIRONMENTAL AND POULTRY LITTER SOURCES IN ASHANTI REGION OF GHANA

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DECLARATION

I, Odoi Hayford, hereby declare that the experimental work was carried out at the Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, KNUST, Kumasi, Ghana and the thesis consists entirely of my own work produced towards a Master of Philosophy (Mphil) Degree. Permissible excerpts/references from other sources have been duly acknowledged.

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ABBREVIATIONS

ABC	ATP-binding cassette
ADMER	Antibiotic Drug use Monitoring and Evaluation of Resistance
AmpC	Inducible Cephalosporinases
ATCC	American Type Culture Collection
CDC	Center for disease control
CDDEP	The Centre for Disease Dynamics, Economics and Policy
CRE	Carbapenem resistant *Enterobacteriacae*
DDST	Double disc synergy test
DNA	Deoxyribonucleic acid
EARS-Net	European antibiotic resistance surveillance network
EARSS	European Antimicrobial Resistance Surveillance System
EDTA	Ethylene-diamine-tetraacetic acid
ERIC	Enterobacterial repetitive intergenic consensus sequence
ESBLs	Extended spectrum betalactamase enzyme
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organisation of the United Nations
FAOSTAT	Food and Agriculture Organisation Statistical Database
H	Hour
KPC	Klebsiella Pneumoniae type Carbapeneamses
MATE	Multidrug and toxic compound extrusion
MBLs	Metallo-betalactamase
MDR	Multi-drug resistant
MFS	Major facilitator subfamily
Min	Minute
MLST	Multilocus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
NaoH	Sodium Hydroxide
PACE	Proteobacterial antimicrobial compound efflux
PAL	Peptidoglycan associated lipoprotein

PCR	Polymerase chain reaction
PDR	Pandrug resistant
PIA	Pseudomonas isolation agar
QRDR	Quinolone-resistance determining region
ReAct	Action on Antibiotic Resistance
REP-PCR	Repetitive-element based polymerase chain reaction
RND	Resistance nodulation cell division
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
Sec	Second
SLST	Single locus sequence typing
SMR	Small multidrug regulator
TE	Tris-Ethylenediamine tetra acetic
UPGMA	Unweighted pair group method with arithmetic averages
VRE	Vancomycin-resistant *Enterococcus*
WGS	Whole genome sequencing
WHO	World Health Organisation
XDR	Extensively-drug resistant

ABSTRACT

Antibiotic resistance in bacteria is now a major global health challenge. The increase and indiscriminate use of antibiotics is pivotal in the selection of resistant bacteria strains and the spread of resistance genes and resistance determining factors. The occurrence of *Pseudomonas aeruginosa*, a commonly implicated organism in nosocomial infections as well as poultry diseases has been found to be on the increase in samples in Ghana. This study therefore sought to determine the prevalence, susceptibility pattern, resistance mechanisms, resistance determining factors and the clonal relatedness of *P. aeruginosa* isolates obtained from stool, urine, blood, poultry litter and the environment in the Ashanti Region of Ghana. The *P. aeruginosa* isolates were identified using their biochemical characteristics and genotypically confirmed through PCR amplification of specific outer membrane lipoprotein (*opr*L) genes. Kirby-Bauer disc diffusion method was used to determine the susceptibility of the isolates to commonly used antipseudomonal agents. Plasmid sizes and resistance determining factors present in the isolates were detected using alkaline lysis method and PCR, respectively. Out of 900 samples screened, 87(9.7%) *P. aeruginosa* isolates were obtained. 75% of the *P. aeruginosa* isolates from the various sources were identified to be resistant to more than a single antipseudomonal agent and 38(43.6%) of the isolates were multidrug resistant (resistant to antibiotics from three or more antipseudomonal classes). The most common resistance pattern was observed with ciprofloxacin (62%), gentamicin (69%) and ticarcillin (56%).

High prevalence of extended spectrum β-lactamases (84.2%), metallo- β-lactamases (34.1%) and AmpC inducible cephalosporinases (50%) were observed in the MDR

isolates. However, no strain produced KPC type carbapenemase. Among the MDR strains, 57.8% displayed moderate to very high efflux capacity and 65.7% of the MDR isolates haboured one to five plasmids with sizes ranging from 2.0kb to 116.8kb. While common β- lactamase encoding genes (bla_{SHV}, bla_{TEM}, bla_{CTX-M}, bla_{VIM} and bla_{IMP}) were not detected in any MDR isolates, class 1 integrons were detected in 89.4% of the MDR isolates with 15.7% and 13.1% respectively carrying quinolone resistance gene mutations in gyrA and parC subunits of DNA gyrase and topoisomerase IV. Antibiogram typing was found to be discriminatory (D=0.9502), differentiating the MDR isolates into 24 antibiogram types with 19 distinct susceptibility patterns and 5 antibiogroups. Genotypic relatedness of the strains from the various sources generated through ERIC-PCR identified all the *P. aeruginosa* isolates to belong to two groups at a similarity of 62%. Dendrogram generated using Pearson coefficient as a similarity index and UPGMA as a distance measure revealed 27 *P. aeruginosa* genotypes. All the clinical strains of *P. aeruginosa* were closely related. From this study, there is the possibility of *MDR P. aeruginosa* transfer from the environment to patients as well as among patients in the same hospital. *P. aeruginosa* strains in humans and poultry may develop extensive antipseudomonal resistance which could be disseminated between patients and the environment.

CHAPTER ONE

GENERAL INTRODUCTION

1.0 Introduction

The emergence and dissemination of antibiotic resistant bacteria is considered globally as a threat to antibacterial therapy (WHO, 2014). The revolutionary development of antibacterial agents was envisioned to bring a halt to the clinical difficulties posed by infectious bacteria in the pre-antibiotic era. The use of antibiotics in the treatment of infectious diseases resulted in a drastic decrease in mortality and morbidity (WHO, 2000). With the view that the battle against infectious bacteria had been won, most drug manufacturers refocused their attention on finding remedies to metabolic or non-communicable diseases. However, within a few years after the introduction of penicillin, penicillin resistant strains of *Staphylococcus aureus* began to emerge. Since then, there has been a global surge of antibiotic resistance, resulting in serious global public health concern with economic, social and political implications (WHO, 2014).

Extended spectrum β-lactamases (ESBL) producing and carbapenem resistant *Enterobacteriacae* (CRE), vancomycin-resistant *Enterococcus* (VRE), Methicillin-resistant *Staphylococcus aureus* (MRSA), multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter*, drug-resistant *Campylobacter*, *Shigella*, typhoidal and non-typhoidal *Salmonella* are now widespread globally (Center for Disease Control (CDC), 2013).

Increasing trends of antimicrobial resistance in Gram-negative bacteria has been observed in Europe, illustrating the continuous loss of effective antimicrobial therapy (European Antibiotic Resistance surveillance network (EARS-Net, 2012). Within the African region, the true extent of antibiotic resistance is limited. This is because surveillance of drug

resistance is carried out in a few countries (EARS-Net, 2012). There is also scarcity of accurate and reliable data on antibiotic resistance for common infectious conditions of public health significance such as meningitis, pneumonia and bloodstream infections. The few available data however, indicates that, the African region shares the worldwide trend of increasing drug resistance (WHO, 2014).

Newman *et al.* (2015) reported high prevalence and resistance of common pathogenic bacteria from both the northern and southern sectors of Ghana. In the study, common antibacterial agents like ampicillin, tetracycline, chloramphenicol, trimethoprim and sulfamethoxazole were found to be ineffective in about 80% of the frequently isolated pathogenic bacteria. Most of the isolates were multidrug-resistant (MDR) with over 50% producing β-lactamase. Almost 90% of the isolates sampled produced ESBLS (Newman *et al.*, 2015). There have also been previous reports of high levels of resistant bacterial isolates in both teaching and regional hospitals in Ghana (Newman *et al.,* 2006; Bieranye, 2011).

The global rise in the trends of antibiotic resistant bacteria has resulted in a corresponding increase in the amount and frequency of antibacterial use (Levy and Marshall, 2004). As a result, treatment costs for previously easily treatable infections are now high, due to treatment failures. It is estimated that drug resistant infections could cause 100 million deaths and cost approximately US $100 trillion a year by 2050 (O'Neill, 2014).

The evolution and spread of resistant bacteria can be attributed to both natural phenomena as well as human practices in the area of antibiotic use (WHO, 2015). Antibiotics either provide selective pressure that results in the acquisition of resistance through mutation or

transfer of resistance determining factors such as conjugable plasmids, transposons, integrons and antibiotic resistance genes (Davies and Davies, 2010). Resistance to antibiotics can be intrinsic or acquired. Naturally, genetic determinants of defense mechanisms may originate from bacteria such as antibiotic producing bacteria (Dantas and Sommer, 2014). As a defense against their own antibiotics produced, these bacteria may carry genes responsible for antibiotic resistance. These genes may be integrated into mobile genetic elements such as plasmids, transposons and integrons which could be passed on through horizontal transfer to other bacteria (Dantas and Sommer, 2014). The overuse and misuse of antibiotics in the treatment of human illness, animal husbandry and agriculture leaves residual traces of these antibiotics in the respective environments, enabling the population of bacteria to adapt and acquire resistance (Joanne *et al.*, 2009).

The existence and growing concern of the problem of antibiotic resistance has called for global efforts to protect the few effective antibiotics. Surveillance of antibiotic resistance is an essential part of an effective response to the global threat of antibiotic resistance (Laxminarayan *et al.*, 2013). Surveillance results provide information on the magnitude and the trends of resistance. The World Health Organization's (WHO) Global Action Plan against antimicrobial resistance has also identified surveillance as one of the key pillars to combating this problem (WHO, 2014). In light of this, WHO first attempted in 2013, to assemble information on national antibiotic surveillance in order to present a global picture of the problem. Extensive national and regional programmes have been instituted to monitor antibiotic resistance patterns in high income countries. Thus, in resource-limited countries like Ghana, which are also stricken with a high burden of infectious diseases, the need arises for extensive surveillance of antibiotic use and resistance patterns

of common pathogens. This will augment the global efforts to monitor and curb the problem of antibiotic resistance.

The concerted efforts of the European Antimicrobial Resistance Surveillance System (EARSS), now the European Antimicrobial Resistance Surveillance Network (EARS-Net), the Swedish Strategic Programme for the Rational Use of Antimicrobial Agents and Surveillance of Resistance, and the Action on Antibiotic Resistance (ReAct), through their vision of a world free from fear of untreatable infections, have empowered many countries including Ghana to take up the fight against antibiotic resistance. This is evident in the support offered Ghana through the Antibiotic Drug use Monitoring and Evaluation of Resistance (ADMER) project, for a six month nationwide surveillance of antimicrobial resistance (Newman *et al.,* 2015). Reports from this study indicated occurrence of *Escherichia coli* (27.5%), *Pseudomonas species* (14%), *Staphylococcus aureus* (11.5%), *Enterobacter* species (9.3%), *Citrobacter* (9.1%), *Streptococcus* species (2.3%) and *Salmonella enterica serovar typhi* (0.6%) in about 1,598 clinical samples collected nationwide.

The primary source of most of the collected samples used for the routine surveillance of antibiotic resistance were in and out-patients who presented to the various regional and district hospitals in the country (Newman *et al.,* 2015). Likewise, routine surveillance in most countries employs samples from critically infected patients with less samples taken from the community (WHO, 2014). This presents a limitation to the nationwide prevalence picture and the resistance profiles of the important pathogenic bacteria being monitored.

Local surveillance of the resistance profiles and characterization of prevalent resistant bacteria in selection prone areas like animal husbandry, aquaculture and agriculture are vital to the fight against antibiotic resistance (Centre for Disease Dynamics, Economics and Policy (CDDEP), 2015). Wide surveillance studies fail to fully characterize and identify the spread of particular resistant strains of bacteria. However, determining the selection, evolution, source, spread, resistance profile and mechanism of resistance are epidemiologically relevant and key to gaining control of the problem of antibiotic resistance (EARS-Net, 2012). A particular resistance strain that evolves in an environment highly selective of resistance may have its resistance determining factors shared within the surrounding bacteria population. Dissemination of this resistant strain through human contact, food, water, animal waste, wind or any other natural phenomena will ensure acquisition of the resistant traits by commensal pathogenic and non-pathogenic bacteria (Dantas and Sommer, 2014).

Monitoring and characterizing bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* in different environments, makes it possible to compare the prevalence of resistance, and detect possible transfer of resistant bacteria and resistance genes between animals, humans and the environment. It also helps to identify any rising antibiotic resistance selective factors as well as resistance selective environments (Bogaard and Stobberingh, 2000).

Particularly, high prevalence of *Pseudomonas* species in clinical samples is worrying, owing to its intrinsic resistance and the therapeutic challenges it poses. It is ubiquitous in moist environments like water, soils, plants and animals. It can colonize human body sites,

with preference for moist areas such as skin (0-2%), nasal mucosa (0-3.3%), throat (0-6.6%), faecal samples (2.6-24%), ear and perineum (Mena and Gerba, 2009). Among poultry, diseases of Pseudomonas occurs in chickens, ducks, geese and ostriches (Patttison *et al.,* 2008). This bacteria presents a great therapeutic challenge due to the complexity of mechanisms which confer resistance both intrinsically and extrinsically (Lister *et al.,* 2009). Its intrinsic resistance is to a wide range of antibiotics including ampicillin, amoxicillin, ceftriaxone, tetracyclines, trimethoprim, chloramphenicol and ertapenem, with a few antibiotics like piperacillin, ticarcillin, ceftazidime, cefepime, meropenem, imipenem, aztreonam and polymyxin B remaining effective (EUCAST, 2015; Mesaros *et al.*, 2007).

The therapeutic difficulty posed by *P. aeruginosa* is worsened by its ability to develop resistance to multiple classes of antibacterial agents during the course of therapy. This makes selection of antibiotics for management of related infection difficult, doubling the length of hospitalization and the overall cost of patient care (Hancock and Speert, 2000). The prevalence and spread of multidrug resistant strains of this bacteria in flagged areas of high antibiotic use such as animal husbandary and human medicine in the country is of great concern. This study thus seeks to identify and characterize resistance profiles and resistance determining factors in multidrug-resistant strains of *Pseudomonas aeruginosa* from poultry farms, patients and the environment.

1.1 Main objective

To determine the phenotypic and genotypic resistance profiles and resistant determining characteristics in multi-drug resistance *P. aeruginosa* isolated from various sources in the Ashanti region of Ghana.

1.1.1 Specific objectives

- To collect stool, urine and blood samples from hospitals in the Ashanti Region of Ghana.
- To collect poultry litter samples from poultry farms in the Ashanti Region of Ghana.
- To collect environmental samples from sewage, market floors and soil in the Ashanti Region of Ghana.
- To isolate and identify *P. aeruginosa* from collected samples.
- To determine the susceptibility patterns of the *P. aeruginosa* isolates to commonly used antipseudomonal agents.
- To determine the clonal relatedness and possible spread of multi-drug resistant (MDR) *P. aeruginosa* isolates among the various sources.
- To determine the presence and prevalence of extended spectrum β-lactamases (ESBLs), inducible AmpC, metallo-β-lactamases (MBLs) and KPC type carbapenemase enzymes among the isolated MDR *P. aeruginosa.*
- To determine the plasmid profiles of the MDR *P. aeruginosa* isolates.
- To determine the presence and prevalence of some antibiotic resistant genes and integrons in the MDR *P. aeruginosa* isolates.

CHAPTER TWO

LITERATURE REVIEW

2.1 Antibiotic resistance

Non-susceptibility of a microorganism to a previously susceptible therapeutic agent (antibiotic, antifungal, anthelmintic, antiviral or antiparasitic) is referred to as resistance (WHO, 2015). The development of resistance thus potentially compromises the effective therapeutic use of antimicrobial agents (Davies and Davies, 2010). Bacteria may constitutively possess complex molecular mechanisms and structural modifications that encode resistance to a wide range of antibiotics (Dzidic *et al.*, 2008). Extrinsically, genetic variability may also be introduced in the bacteria genome through point mutations and rearrangement of large segments of genetic material resulting in both micro and macro-evolutionary changes (Bockstael and Aerschot, 2009). The mutational changes which may occur are dependent on selection events that take place in the immediate environment of the bacteria. Residual sub-inhibitory concentrations of antibiotics which remain after antibiotic use, may provide selective pressure for the evolution of resistant bacteria (Davies *et al.*, 2006).

There are also reports on pooling antibiotic resistance genes (resistome) (Dantas and Sommer, 2014) which is caused by selective pressure provided by naturally occurring soil antibiotic producing bacteria primarily of the *Streptomyces* genus of the *Actinomycete* phylum. Dissemination of this natural resistome (antibiotic resistance genes) and resistant bacteria in high resistance selective environments gradually changes bacteria susceptibility profiles and ensure persistence of super-resistant strains of both commensal and pathogenic bacteria (Dantas and Sommer, 2014).

2.1.1 Classification of multiple resistance in bacteria

Reduced permeability, increased efflux and direct modification of antibiotics and antibiotic targets introduce resistance in bacteria. According to Magiorakos *et al.* (2011), multi-resistant bacteria may be classified as multidrug-resistant (MDR), extensively drug-resistant (XDR) or pandrug-resistant (PDR). MDR bacteria are non-susceptible to at least one agent in three or more antimicrobial categories. An XDR bacteria are non-susceptible to at least one agent in all but two or fewer antimicrobial categories whereas PDR bacteria are non-susceptible to all agents in all antimicrobial categories (Magiorakos *et al.,* 2011)

2.1.2 Patterns and trends of antibiotic use and their impact on antibiotic resistance

2.1.2.1 Antibiotic use in humans

Antibiotics are mainly used in the treatment of infections in humans and animal husbandry (CDDEP, 2015). In humans, they are used as treatment for infectious diseases and as prophylaxis to prevent infections after both major and minor surgeries (Laxminarayan *et al.*, 2013).

Demand for antibiotics continue to rise particularly in the treatment of sepsis and pneumonia in children (CDDEP, 2015). Globally about 935,000 deaths resulted from pneumonia in children under five in 2013 alone (Liu *et al.,* 2015). On a global scale, the consumption of antibiotics between 2000 and 2010 increased by more than 30% (Boeckel *et al.,* 2014). About 80% of antibiotics are used in communities as compared to 20% use in hospitals. In the community, consumers purchase antibiotics either directly over the counter or with prescriptions from prescribers enabling indiscriminate use of antibiotics and enhancing evolution of drug resistant bacteria (Kotwani and Holloway, 2011).

The development of antibiotic resistant bacteria has been reported in hospitals soon after new antibiotics were introduced (Levy, 1998). Resistance of bacteria to antibiotics is a

natural phenomenon which can be accelerated by inappropriate human antibiotic use practices (Kenneth, 2008). The increasing resistance in bacteria is influenced by prescribing of large doses of broad spectrum antibiotics, non-adherence to prescribed doses and long durations of antibiotic treatments (Pechère, 2001).

Also, non-adherence to established protocols and guidelines surrounding antibiotic use and the availability of antibiotics over the counter in most communities have exacerbated the problem of antibiotic resistance (Lalitha, 2004).

Inadequate dosing, lack of access to antibiotics and substandard antibiotics may however, also play a crucial role in the emergence of multidrug resistant bacteria (Kunin, 1993).

2.1.2.2 Antibiotic use in animal husbandry

In animal husbandry, antibiotics serve therapeutic, prophylactic and metaphylactic purposes as well as in growth promotion (CDDEP, 2015). Evaluation of antibiotic use trends later in the 1940s and 1950s indicated vast non-therapeutic use of antibiotics as growth promoters in food producing animals (Jukes *et al.* 1950). Earlier reports of increased production with antibiotic use in animals fueled this new found use for antibiotics (Ogle, 2013).

About 60 to 80% of all antibiotics used in humans today are used in animal production (Boeckel *et al.,* 2014; Boeckel *et al.,* 2015). Currently, there are about 27 antimicrobial classes that are used in animal husbandry. Only nine (9) of these classes are, however, approved for veterinary use (Pagel and Gautier 2012). Cattle, sheep, poultry and dogs mostly kept by farmers in most countries, including Ghana, are fed antibiotics belonging to varied antibiotic groups such as tetracyclines, β-lactams, chloramphenicol, and

macrolide (Moats, 1986). Chicken and pigs are fed most of the antibiotics used in food animals around the world (CDDEP, 2015).

There is also an undoubtedly significant use of antibiotics in aquaculture (Millen *et al.* 2011). However, the practice of flock or herd treatment of both diseased and healthy farm animals coupled with the routine supplementation of feed and water with antibiotics exposes commensal bacteria to sub-therapeutic amounts of antibiotics over time (Walton, I983).

Residual antibiotic concentrations have been detected in food and food products like meat, egg and milk from African countries like Tanzania, Ghana, Nigeria, Kenya, Sudan and Egypt (Ibrahim *et al.,* 2010; Abavelim, 2014). Majority of Ghanaians are known to rely on beef (66%), chevon (39%), pork (46%) and eggs (77%) as a source of protein (Donkor *et al.*, 2011, Boamah, 2015). Between 3 to 41% of these products have however been found to contain traces or residues of antibiotics such as chloramphenicol and oxytetracycline (Donkor *et al.*, 2011; Abavelim, 2014).

Inappropriate global veterinary antibiotic use and farm management practices account for sub-therapeutic concentrations of antibiotics in circulating food products (CDDEP, 2015). Knowledge deficits in the withdrawal requirements of drugs, health risks associated with antibiotics in food animals, lack of involvement of veterinary officers and inadequate training in animal farming may have resulted to the flaws in farm practices related to farm management and antibiotic use (Donkor *et al.*, 2011).

The poultry industry has seen tremendous growth both locally and globally. This may be attributed to increasing human population which drive an increasing demand for animal protein. Also, the development of feed, slaughter and processing technologies have been

the driving force behind the vast growth in the poultry industry (Food and Agriculture Organisation of the United Nations (FAO), 2014). The most common birds kept in Ghana are chickens, turkeys, ducks, geese and guinea fowls. The total population of chickens had doubled as at 2010 (Food and Agriculture Organisation Statistical Database (FAOSTAT), 2013). Between 2000 and 2007, poultry production increased by more than 80%, with Ghana's overall livestock production being on the rise since 2000 (FAO, 2014). Poultry are mainly distributed in the Brong Ahafo (29.62%), Ashanti (28.07%) and Eastern (10.72%) regions of Ghana (FAO, 2014). Viral, bacterial and parasitic infections are common in poultry (Turkson, 2008; WHO, 2015). Boamah (2015) in a recent study, discovered the incidence of diseases such as Gumboro, Newcastle, Chronic respiratory disease and coccidiosis on most poultry farms in the Ashanti, Greater Accra and Brong Ahafo regions for which poultry farmers relied heavily on antibiotics and other antimicrobial agents to manage. Most of the antibiotic products used by farmers are combined formulations containing multiple antibacterial agents (Boamah *et al.,* 2016).

Apart from the consumption of poultry products by majority of the world's population, litter from most poultry farms serve as a major source of manure for food crop cultivation. Ampofo and Clark (2010) reported the use of organic manure such as poultry, pig and cow manure as fertilizers for fish ponds. The use of poultry litter, which may contain traces of antibiotic residues and antibiotic resistant bacteria may spread to wide environments disseminating antibiotic resistance determinants in the environment (Joanne *et al.,* 2009). This may influence the emergence of resistance and acquisition of virulence in previously susceptible or non-virulent strains of bacteria.

The presence of residual sub-therapeutic antibiotic concentrations in agricultural products and the effect on the evolution of antibiotic resistant bacteria has called for global strategies to control the use of antibiotics in animal production. Countries like Denmark, Sweden, United Kingdom and other European countries have therefore banned the use of antibiotics for growth promotion, metaphylaxis and prophylaxis in farm animals (Cogliani *et al.,* 2011; Maron *et al.,* 2013).

2.2 Characteristics of the genus *Pseudomonas*

Pseudomonas is a genus of Gram-negative, aerobic gammaproteo-bacteria that belongs to the family Pseudomonadaceae (Skerman *et al.,* 1980; Euzéby, 1997). They are straight or slightly curved rods measuring 0.5 to 1.0 by 1.5 to 5.0 µm. *Pseudomonas, Azomonas, Cellvibrio, Rhizobacter, Azomonotrichon, Rugamonas*, *Azorhizophilus, Azotobacter, Mesophilobacter* and *Serpens* make up the Pseudomonadaceae family of bacteria (Skerman *et al.,* 1980, Cornelis, 2008). The genus Pseudomonas are ubiquitous in soil, water and on plants. It consists of 191 subspecies belonging to species groups including *P. fluorescens, P. pertucinogena, P. aeruginosa*, *P. chlororaphis, P. putida, P. stutzeri,* and *P. syringae*. Members of the genus are motile with either one or two polar flagella, oxidase positive or negative and catalase producing with some species producing characteristic pigments such as pyocyanin, pyorubin, chlororaphin, oxiphenazin and pyoverdine (Krieg *et al.,* 1984).

Pseudomonas are widely distributed in nature with some species being pathogenic for humans, animals or plants. Most species cannot thrive in acidic conditions (pH 4.5 or lower) and do not require organic growth nutrients. They grow at an optimum temperature of 28°C but have the ability to survive at temperatures between 4°C and 45°C (Krieg *et*

al., 2005). The species *P. aeruginosa* has increasingly gained clinical importance as an opportunistic pathogen with inherent resistance to a wide spectrum of antibiotics (Carmeli *et al.*, 1999).

2.2.1 Characteristics of *Pseudomonas aeruginosa*

The characteristic blue-green pigment (pyocyanin) produced by *P. aeruginosa* has enabled identification of this species since the eighteenth century. In an 1882 publication by Carle Gessard entiltled "On the blue and green colouration of Bandages" (Forkner, 1960), *P. aeruginosa* was isolated from wounds of two patients with bluish-green pus. It is common in moist environments including soil, water, sewage, lakes and surfaces of plants and in the intestinal contents of animals and birds. *Pseudomonas aeruginosa* thrives in diverse environments and possesses an efficient adaptive machinery that enables survival even in toxic environments. It can grow in very simple organic mineral environments (Krieg *et al.*, 2005).

The plasticity and complexity of the large *P. aeruginosa* genome (6.3Mbp) reflects the evolutionary adaptations conferred to this species (Cristina and Eusébio, 2013). This organism can achieve anaerobic growth with nitrate or nitrite or use oxygen as the terminal electron acceptor for respiratory metabolism (Garrity *et al.*, 2005). In the absence of oxygen, nitrate or nitrite, it is able to ferment arginine and pyruvate by substrate-level phosphorylation (Schobert and Dieter, 2010). *P. aeruginosa* grows at an optimum temperature of 37^{o}C but reserves the ability to grow at 42^{o}C but not at 4^{o}C which is distinguishing of the species (Krieg *et al.*, 2005).

Three (3) colony types are produced by *P. aeruginosa* strains; a large smooth flat edged colony with an elevated center ("fried egg" appearance), silver-gray metallic shining patches and a small rough convex colony present on clinical materials or a mucoid colony type often isolated from secretions of the respiratory and urinary tract. It produces characteristic soluble pigments of which the siderophore known as pyoverdine (yellow-green) and the phenazine blue pigment called pyocyanin ("blue pus") are common (Meyer and Abdallah, 1978; Meyer and Hornsperger, 1978). Pigmentation is a striking feature of *P. aeruginosa* but it is not always a dependable feature for identification of a species since some strains do not produce pigments. Other strains of *P. aeruginosa* produce a red phenazine pigment (pyorubin) and a brown melanin pigment (pyomelanin) (Mann, 1969).

P. aeruginosa in spite of its ubiquitous nature seldom produce infections in healthy individuals (Levison, 1977). Over, time it has gained clinical relevance as a major opportunistic pathogen in chronically immune-compromised patients and animals including poultry. It is implicated in burns, wounds, pneumonia, endocarditis, pharyngitis meningitis, urinary tract infections, sepsis and frequently colonizes the lungs of patients with cystic fibrosis and bronchiectasis producing biofilms that enhance multidrug resistance (Rayner *et al.,* 1994).

It produces a variety of virulence factors such as haemolysin, pigments, exotoxin A, lipases, pili, protease enzymes and histamine aiding host colonization and invasion (Krieg *et al.,* 1984). Treatment of *P. aeruginosa* infections is thus limited to a few β-lactam, aminoglycoside, polymyxin and fluoroquinolone antibiotics (Hancock and Speert, 2000).

Pseudomoniasis, an opportunistic *P. aeruginosa* infection is also common in poultry birds like chickens, turkeys, ducks, geese and ostriches where infection in eggs kills embryos. Infections may occur through skin wounds, contaminated vaccines and antibiotic solutions or needles used for injection. The disease may be systemic, affecting multiple organs and tissues or localized in tissues as infraorbital sinus or air sacs producing swelling of the head, wattles, sinuses and joints in poultry birds (Pattison *et al.,* 2008).

The most worrying feature that presents a huge difficulty during therapy is the intrinsic resistance of *P. aeruginosa* (Seol *et al.,* 2002). The presence of chromosomally encoded penicillinases and cephalosporinases, coupled with efficient efflux porins and external biofilm matrix has made most strains of *P. aeruginosa* naturally resistant to a wide spectrum of antibiotics (Ferguson, 2007). *P. aeruginosa* is also known to habour antibiotic resistant plasmids, integrons and transposons and is able to transfer these genes to other species (Opal and Pop-vicas, 2004) .

2.2.2 Identification of *P. aeruginosa*

The identification of *P. aeruginosa* has traditionally been limited to microscopy, culture, biochemical and phenotypic methods (Kidd *et al.,* 2011). However, the vast phenotypic differences that exist in different environmental strains of bacteria of the same species makes only phenotypic identification difficult. To overcome these difficulties, several molecular based detection and identification methods have been developed. These include polymerase chain reaction (PCR) amplification and sequencing of 16S rRNA and some conserved genes (oprI, oprL and housekeeping genes) (Wellinghausen *et al.,* 2005).

The presence of species specific conserved genes (toxA, algD, and 16S rRNA) in the genome of *P. aeruginosa* can be utilized in its identification and classification through polymerase chain reaction (PCR) and sequencing (Kidd *et al.,* 2011). The 16S ribosomal ribonucleic acid (16S rRNA) gene is present and highly conserved in all bacteria and homologous in related species (Fox *et al.,* 1980). Therefore, amplification, sequencing and alignment of 16S rRNA gene sequences have been used to identify and determine the evolutionary relationship between bacteria of closely related or unrelated species (Weisburg *et al.,* 1991).

Matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectroscopy has also found use in the identification of bacteria including *P. aeruginosa*. Intense laser light is used to evaporate the bacteria cell which is subsequently subjected to a strong electrical field (Dworzanski and Snyder, 2005; Belkum *et al.,* 2007). Small generated ions move at high speed and reach a detector before the larger ones. The signals generated are recorded and give rise to complex spectra, characteristic for the molecular content the bacterial cell. When these spectra are compared using appropriate computer software, bacterial types can be distinguished (Du *et al.,* 2002).

Also, routine identification of *P. aeruginosa* can be achieved by amplification of a constitutively produced peptidoglycan associated lipoprotein (oprL) gene which appears to be involved in the structural integrity of *P. aeruginosa* (Abdullahi *et al.*, 2013). This method provides a rapid, efficient and cost effective means of identifying *P. aeruginosa.*

2.3 Typing of bacteria

Recombination events that occur in bacteria introduces genetic diversity in different strains of the same species. A strong link has been established between species diversity and bacteria characteristics such as pathogenicity, antibiotic resistance and overall fitness in bacteria (Brian *et al.,* 2002).

Selective pressure that induces antibiotic resistance in bacteria species may be more common in particular strains of the same species. Horizontal acquisition of resistance markers, such as plasmids and the overall rearrangement of genomic sequences introduces great diversity in a species, enhancing the survival and spread of clonal groups of a particular strain within diverse environments (Brian *et al.,* 2002). Establishing the similarity or relatedness of particular strains within a species thus enhances epidemiological characterization of the source, spread and antigenic profiles of pathogenic bacteria from different environments (Achtman, 2002).

P. aeruginosa as well as other bacteria have varied genomic, antigenic and phenotypic profiles which can be utilized to establish clonal relatedness in bacteria isolates. This has thus been the basis for development of typing techniques such as biotyping, antibiogram typing, serotyping, phage typing, plasmid typing and sequence and fragment based typing methods (Kidd *et al.,* 2011).

High variability and low discriminatory and reproducibility of phenotypic typing methods makes them inadequate in determining relatedness of bacteria species (Sabat *et al.,* 2013). Sequence and fragment based methods, such as whole genome sequencing (WGS), single locus sequence typing (SLST), multilocus sequence typing (MLST), Pulsed field gel

electrophoresis, amplified fragment length polymorphism and repetitive-element based polymerase chain reaction (REP-PCR) introduces high discrimination and reproducibility in bacteria typing (Yildirim, 2011).

Short intergenic repeat sequences exist in the genome of most bacteria including *P. aeruginosa* (Versalovic *et al.,* 1991). Hybridization of these sequences with primers enables PCR amplification of genomic regions between the adjacent repeats, creating a characteristic fingerprint after electrophoresis (Sabat *et al.,* 2013). The families of repeat sequences that are common in bacteria include the enterobacterial repetitive intergenic consensus (ERIC) sequence (Hulton *et al.,* 1991), repetitive extragenic palindromic (REP) sequences (Gilson *et al.,* 1984) and the BOX element sequences (Koeuth *et al.,* 1995). REP-PCR is relatively cheaper, fast and highly discriminatory for many bacteria pathogens (Yildirim, 2011).

2.4 Dissemination of resistant bacteria and antibiotic resistance genes

The natural resistome of bacteria and anthropologic influences on antibiotic use account for the evolution of resistant bacteria (Davies and Davies, 2010). Residual sub-inhibitory concentrations of antibiotics in the environment selects for resistant bacteria strains promoting their survival and subsequent gene transfer and recombination (Joanne *et al.,* 2009). Emergence and spread of resistance in bacteria may be due to mutational events introduced during bacteria replication and vertical transmission of genetic variants through generations in a particular bacteria strain (Harbottle *et al.,* 2007). Accessory genetic elements carrying multiple antibiotic resistance determinants (plasmids, integrons, transposons, antibiotic resistance genes) may also be disseminated horizontally in bacteria leading to wide spread of resistance (Kung *et al.,* 2010).

Inter-connections existing between people, animals and the environment (Figure 2.1) make it easier for antibiotic resistant bacteria and resistance determinants to circulate between species of bacteria in the same or different environments (Dantas and Sommer, 2014). Thus, a resistant bacterial strain living in soil could travel through run-off water and get passed onto humans through drinking water or recreational swimming. Also, resistant bacteria can be transferred from animals and plants to humans through the food chain (Roe and Pillai, 2003; Soonthornchaikul and Garelick, 2009), direct or indirect contact with livestock or animal health workers (Levy *et al.*, 1976) and through environments contaminated with manure from animal waste.

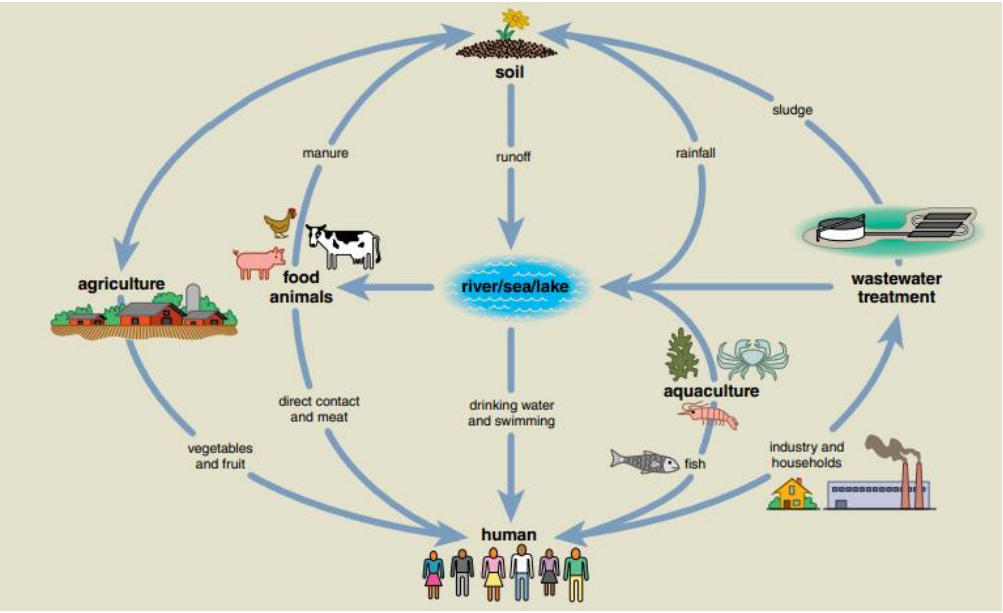


Figure 2.1: Interconnections between people, animals and the environment enabling spread of resistance bacteria and resistance genes (Adapted from Dantas and Sommer, 2014)

The entry of antibiotic resistant bacteria into soil and water via manure application yields a potentially significant reservoir of antibiotic resistance genes (Joanne *et al.*, 2009) which could be disseminated in different populations of bacteria.

Genetic mechanisms involved in lateral exchange of antibiotic resistance genes within the bacteria population may include: conjugative transfer (e.g. via plasmids, transposons, and integrons), transduction by bacteriophage and transformation (Madhavan *et al.*, 2011).

Conjugative horizontal transfer of resistance determinants involves transfer of chromosomal or plasmid DNA between a donor and recipient bacterial cell through a pili bridge (Mazodier and Davis, 1991). The frequency of conjugation is influenced by cell densities and the immediate environment since physical cell to cell contact is required (Joanne *et al.*, 2009). According to Salyers *et al.* (1995) and Hausner and Wuertz (1999) high frequencies of conjugation events were recorded in hydrated environments of the gastrointestinal tract and biofilms respectively. The presence of sub-inhibitory concentrations of antibiotics may stimulate plasmid and transposon mediated conjugal transfer of resistance genes (Barr *et al*., 1986; Ohlsen *et al.* 2003).

Bacteriophages are common in all natural environments (Weinbauer and Rassoulzadegan, 2004) including marine, swamps and lakes. Bacteriophages can also mediate transfer of genetic material by transduction. They package DNA from host bacteria genome into phage capsid during replication (Brüssow *et al.*, 2004; Miller, 2001). The foreign DNA may thus be incorporated by homologous recombination into the host genome (Figure 2.2). Jiang and Paul (1998) reported that in marine environment where abundant phage populations occur, transduction could be a significant mechanism of horizontal gene transfer.

Also, exogenous DNA molecules can be transported and integrated into the DNA of a recipient in a process called transformation (Fiedler and Wirth, 1988). This process

requires cells that are genetically competent or cells that can take up foreign DNA without intracellular restriction by nucleases (Figure 2.2). Pseudomonas, Bacillus, Micrococcus and Vibrio among other genera have been shown to exhibit natural competence in soil environments (Demanèche *et al.*, 2001; Levy and Miller, 1989). Natural salt (e.g., Ca^{2+}) concentrations in fresh water, manure and soil can induce competence in bacteria (Cérèmonie *et al.*, 2004; Lorenz and Wackernagel, 1994) and enhance uptake of foreign genetic material.

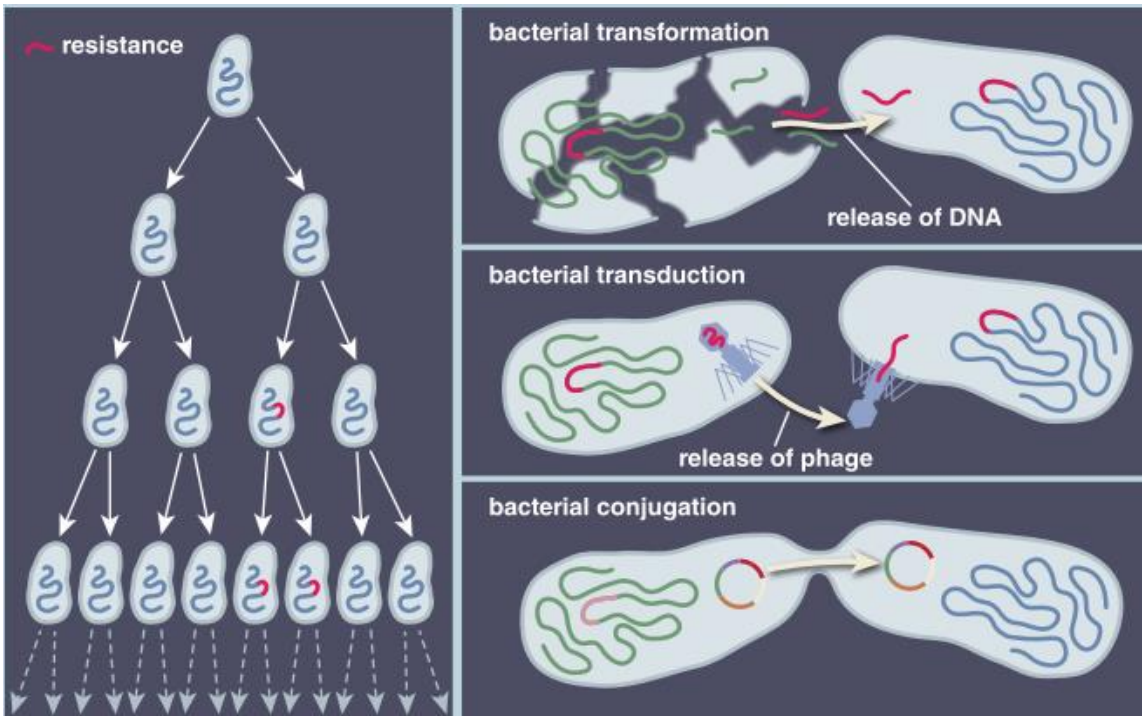


Figure 2.2: Horizontal and vertical transfer of resistance genes in bacteria (Adapted from Dantas and Sommer, 2014)

2.4.1 Elements of horizontal gene transfer-mobile genetic elements

Many antibiotic resistance genes that result from mutational events are haboured on mobile genetic elements such as transposons, integrons or plasmids and can be transferred readily between members of the same species or bacteria of diverse genera (Joanne *et al.*,

2009). This may result in the transfer of resistance machinery to bacteria of the same or varied species.

2.4.1.1 Plasmids

Extrachromosomal covalently closed circular double stranded DNA known as plasmids may exist in the cell of most Gram-negative and Gram-positive bacteria (Grinsted and Bennett, 1988). Genetic variability introduced in the bacterial genome through mutational events also occur on plasmids which introduces genes that encode antibiotic resistance. Plasmids also function in regulating cellular metabolic processes as well as virulence characteristics (Wang *et al.*, 2006). The continued maintenance of appreciable copy numbers is enabled by the ability of plasmids to autonomously replicate (Bennett, 2008). Sizes of plasmids may vary ranging from 1.0 kb to over 200 kb (Thomas and Summers, 2008).

Conjugative plasmids have genes needed for transfer which makes their sizes larger than non-conjugative plasmids (Opal and Pop-Vicas, 2004). Multiple plasmid copies may exist in the cell (Denyer *et al.*, 2004). After plasmid transfer however, a copy of the plasmid is always retained in the parent cell. Small plasmids that lack an inherent conjugative machinery may share the conjugative apparatus of other conjugative plasmids (Opal and Pop-Vicas, 2004).

2.4.1.2 Transposable genetic elements

Short 'jumping' sequences of DNA carrying functional genes (transposons) or existing as insertion sequences (Mahillon and Chandler, 1998) are essential for resistance evolution on plasmids and chromosomal DNA (Masterton, 2003). These sequences of genetic

material lack autonomous replication and they are disseminated as part of the chromosomal DNA, plasmids or through bacteriophage carriage after homologous or non-homologous recombination using specialized recombination systems (Salyers, 1985). Conjugative transposons can also move from one bacterium to another without being inserted into plasmids or chromosomal DNA.

2.4.1.3 DNA integration elements

Integrons may be present on transposons, plasmids or chromosomal DNA. They are regions encoding an integrase gene (int) together with an attachment (attI) site where genes can be integrated by site specific recombination mediated by the integrase enzyme (Recchia and Hall, 1995). Genes are thus inserted into the attI site or remain vacant upon deletion. These insertion or deletion units are gene cassettes mostly carrying a single gene together with a short sequence of 59-base element essential for site specific recombination (Achtman, 2002). The gene cassettes integrated into integrons are mostly antibiotic resistance genes (Bennett, 2008). These cassettes provide resistance to most classes of antibiotics including β-lactams, aminoglycosides, chloramphenicol, trimethoprim, rifampicn, erythromycin, quinolones and antiseptics of the quaternary ammonium–compound family (Mazel, 2006; Fluit *et al.,* 2004).

More than 130 different cassettes harboring various antibiotic resistance genes have been identified in mobile integrons (Fluit *et al.,* 2004). The presence of an efficient promoter at the 5'end regulates expression of newly inserted genes with the frequency of transcription diminishing as the distance between the promoter and the antibiotic resistant gene cassette increases. Five different classes of mobile integrons have been described

based on the homology of the integrase gene but only the first three have been involved in multidrug-resistance (Cambray, *et al.,* 2010).

2.5 Mechanism of multi-drug resistance in *P. aeruginosa*

Micro and macro-evolutionary changes that occurs in the bacteria genome through point mutations, duplications, inversions, insertions, deletions and transpositions of large segments of genetic material and their later clonal spread may lead to expression of varied genotypes and phenotypes in wild type bacteria strains (Achtman, 2002). These genetic variants may develop characteristic virulence mechanisms, resistance profiles and varied cellular and metabolic regulatory processes which ensures survival of the bacteria species (Lupski, 1987). Thus selected strains may acquire modifications influencing cytoplasmic membrane permeability, cause derepressed or overexpressed efflux pump activity as well as varied genetic variants of degradative cellular enzymes with different substrate specificities. These changes may cause decreased membrane permeability, enzymatic alteration or protection, alteration or overproduction of target site, bypass of inhibited processes and binding of antibiotics (Blair *et al.,* 2014).

2.5.1 Decreased membrane permeability

Bacteria membranes provide a selective permeability barrier to toxic cellular compounds. Decreased outer and inner membrane permeability to unaltered antibiotics decreases the concentration of the antibiotic in the cell leading to intrinsic resistance in bacteria (Nikaido, 1985). The thick layer of peptidoglycan in Gram-positive bacteria and the lipid bilayer of Gram-negative bacteria which are composed principally of lipopolysaccharides

provide a barrier to cellular entry of hydrophobic and hydrophilic antibiotic molecules (Schwarz and Nobel, 1999) (Figure 2.3).

Also, non-specific (general) and specific porin proteins and energy dependent transporters that form part of the outer membrane of Gram-negative bacteria enable passage of hydrophilic molecules across the membrane (Nikaido and Vaara, 1985). Emergence of mutants with loss of specific porins and defective electron transport systems cause resistance to substrate antibiotics (Carmeli *et al.,* 1999). The role of porins in bacteria are diverse and not limited to influx. Some bacteria porins may act as receptors for bacteriophages, bacteriocins and immune system elements.

Porin proteins in *P. aeruginosa* belong to three major families. The oprD family of specific porins, the OprM family of efflux porins and the TonB-dependent gated porins (Ratkai, 2011). OprD family of porins have specific roles in the uptake of positively charged molecules like carbapenems and lysine (Rodriguez-Martinez *et al.*, 2009; Wang *et al.,* 2010). Mutations in the OprD gene can cause loss or reduced expression of the OprD porin protein and consequent resistance to imipenem which crosses the outer membrane through this porin (Trias and Nikaido, 1990).

Aminoglycosides and colistin however do not utilize porin permeation to enter the cell but bind to the lipopolysaccharide layer surrounding the outer membrane. Aminoglycoside resistance therefore may be due to overexpression of OprH protein which protects the lipopolysaccharide and prevents it from binding to antibiotics (Ratkai, 2011). Also, the secretion of an anionic exopolysaccharide biofilm matrix by *P. aeruginosa* can reduce the

permeability of the cell to antibiotic classes such as aminoglycosides, β-lactams and polymyxins and contribute to multidrug resistance (Drenkard and Ausubel, 2002).

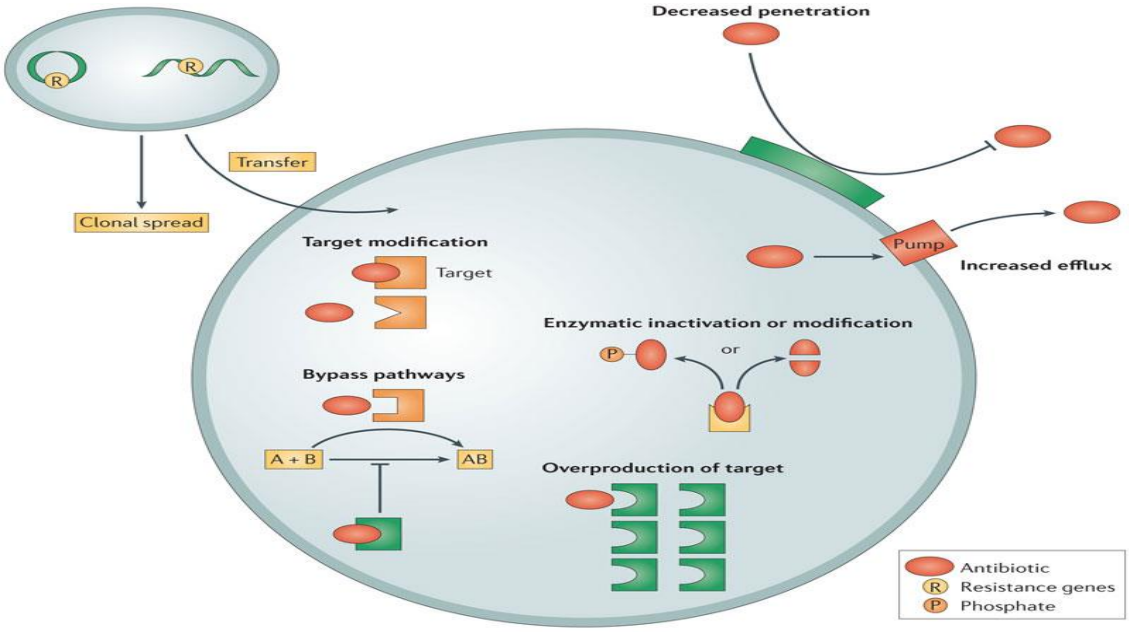


Figure 2.3: Mechanism of antibiotic resistance in bacteria (Adapted from Lewis, 2013)

2.5.2 Efflux system

Efflux pumps in bacteria function as cytoplasmic membrane transport systems, excreting toxic compounds from the cell and maintaining cellular homeostasis (Qinghu *et al.*, 2006). For an antibiotic to act, it must first reach its target either within or outside of the bacterial cell (Walsh, 2000). Activity of the antibiotic is dependent on the cellular concentration of the antibiotic. Hyperexpression of cytoplasmic membrane efflux pump proteins with or without loss or repression of porin proteins can cause multidrug resistance in bacteria (Blair *et al.*, 2014).

Efflux systems are found very useful in antibiotic producing bacteria since they keep the concentration of the produced antibiotic low in the cell. Five (5) protein families of efflux pumps have been described to contribute to multidrug resistance in bacteria, acting either by coupling drug efflux to a counterflow of proteins or through an energy dependent active transport of the antibiotic from the cell (Van Bambeke *et al.*, 2000). These include the multidrug and toxic compound extrusion (MATE), small multidrug regulator (SMR), resistance nodulation cell division (RND) families and major facilitator subfamily (MFS). The energy dependent ATP-driven pumps are the ATP-binding cassette (ABC) family of efflux pumps (Hassan *et al.*, 2015).

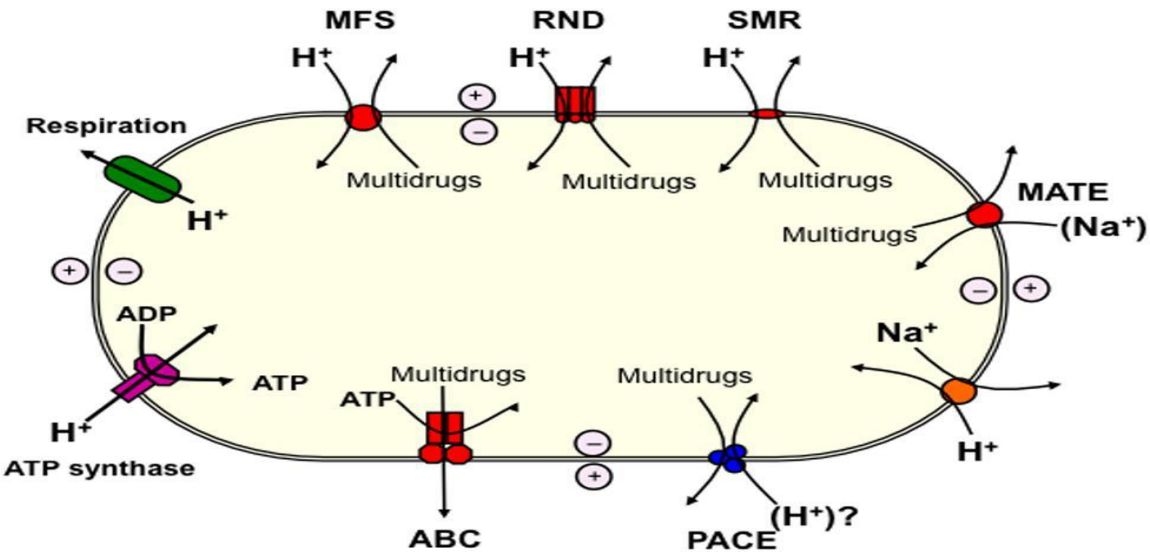


Figure 2.4: Schematic diagram showing multidrug efflux pumps in bacteria and the basis for their energization. Adapted from Hassan *et al.*, 2015. Major facilitator superfamily (MFS), Resistance nodulation division (RND), Small multidrug resistance (SMR) super families, Multidrug and toxic compound extrusion (MATE) family, ATP-binding cassette (ABC) superfamily, Proteobacterial antimicrobial compound efflux (PACE) family.

Resistance nodulation cell division (RND) family of transporters are the most common in *P. aeruginosa* and mediate extrusion of a wide range of antibiotics from the bacteria cell (Poole, 2007). Twelve RND efflux system protein sequences have been identified but only

seven (7) have been characterized: MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM, MexJK- OprM, MexGHI-PrmD and MexVW-OprM (Poole, 2004).

Table 2.1: Efflux pump system of *P. aeruginosa* and their substrates

Efflux system	Efflux pump family	Gene location	Substrates	References
MexAB-OprM	RND	Chromosome	FQ, AMG, BL(MEM, TIC), TET, TGC,CHL	Poole, 2005
MexCD-OprJ	RND	Plasmid	FQ, BL (MEM, TIC), TET, TGC, CHL, ERY, ROX	Poole, 2005
MexEF-OprN	RND	Chromosome	FQ, BL(MEM, TIC), TET, TGC, CHL	Kohler *et al.*, 2001
MexXY-OprM	RND	Chromosome	FQ, AMG, BL (MEM, TIC, FEP),TET, TGC, CHL	Poole, 2005
AmrAB-OprA	RND	Chromosome	AMG	Westbrock *et al.*, 2004
PmpM	MATE	ND	FQ	Su *et al*, 2005
Mef(A)	MFS	Chromosome	MC	Pozzi *et al.*, 2004
$ErmE_{PAF}$	SMR	ND	AMG	Li *et al.* 2003

AMG: Aminoglycoside; BL: β-lactam; CHL: Chloramphenicol; ERY: Erythromycin; FEP: Cefepime; FQ: Fluorquinolone; MATE: Multidrug and toxic compound extrusion; MC: Macrolides; MEM: Meropenem; MFS: Major facilitator superfamily; ND: Not determined; RND: Resistance-nodulation division; ROX: Roxythromicin; SMR: Small multidrug resistance; TET: Tetracycline; TGC: Tigecycline; TIC: Ticarcillin.

MATE, SMR and MFS family of efflux pumps have also been identified to contribute to *P. aeruginosa* efflux (Figure 2.4) ((Meletis and Bagkeri, 2013). Mutational changes occurring in the regulatory genes of the efflux pump operons may cause overexpression

of pump proteins or changes in substrate specificity enhancing increased multidrug resistance (Piddock, 2006).

2.5.3 Target site modification

Resistance to antibiotics can also develop if changes occur at the binding site of the antibiotic (Lambert, 2005). Over-production, protection or structural alteration of the target site to an antibiotic can alter the binding affinity of the antibiotic and lead to resistance (Blair *et al.,* 2014). Changes that occur in antibiotic targets and prevent antibiotic binding but still enable the target to carry out its normal function in the cell can confer resistance to antibiotics.

β-lactam antibiotics act by binding to penicillin binding protein (PBPs) and disrupting the cell wall integrity in bacteria (Katayama, 2000). Mutations that occur in genes encoding general penicillin binding proteins (PBPS) can lead to production of alternate PBPs which regulate cell wall formation but have no or decreased binding affinity for β-lactam antibiotics leading to resistance (Lambert, 2002). In methicillin resistant *S. aureus* (MRSA) for instance, the acquisition of a staphylococcal cassette chromosome mec (SCCmec) habouring a mecA gene encodes a β-lactam insensitive protein PBP2a, which enables occurrence of cell wall biosynthesis despite inhibition of the native PBP by the antibiotic (Blair *et al.,* 2014).

Target modifications resulting from substitutions in the quinolone resistance determining regions of gyrA and parC genes of DNA gyrase and topoisomerase IV confer resistance to fluoroquinolones (Jacoby, 2005). The expressed mutation reduces the binding of quinolones to the enzyme-DNA complex through the production of petapeptide repeat

proteins (PRPs) which bind the enzyme-quinolone complex, releasing the enzyme to complete its activity (Vetting *et al.,* 2011). In addition, protection of antibiotic target sites such as the 16SrRNA and 23SrRNA through methylation by methyltransferases and ribosomal methylases can increase resistance to some antibiotics like macrolides, chloramphenicol, aminoglycosides and lincosamides (Sigmund *et al.,* 1984).

2.5.4 Enzymatic inactivation

Some enzymes produced in bacteria can degrade or modify antibiotics rendering them ineffective during therapy (Yoneyama, 2006). These enzymes either recognize and hydrolyze susceptible bonds in the antibiotic or cause structural changes to the antibiotic impairing target binding (Opal and Pop-Vicas, 2004).

The amide bond of the β-lactam ring present in β-lactam antibiotics (penicillins, cephalosporins, carbapenems) is susceptible to hydrolysis by a wide range of enzymes classified as beta-lactamases (Livermore, 1995; Abraham and Chain, 1940). These enzymes have been grouped originally based on their substrate specificities, biochemical properties, sequence similarities, gene location and strains of bacteria and samples habouring them (Ferguson, 2007). The application of isoelectric focusing has enhanced characterization of many enzymes (Jacoby, 2006). Selective pressure that induces mutation in the loci of most β-lactamase encoding genes has driven the evolution of varied genetic variants of β-lactamases that hydrolyze a wide range of substrates conferring multidrug resistance to bacteria (Petrosino *et al.,* 1998).

Two classification schemes for beta-lactamases have been proposed. Ambler classifies β-lactamases as class A, B, C or D based upon their amino acid sequence (Ambler, 1980).

Alternatively, Bush–Jacoby–Medeiros have also grouped beta-lactamase into class 1, 2a, 2b, 2be, 2br, 2c, 2d, 2f, 3 and 4 based on their functional groups dictated by their substrate profile and susceptibility to β-lactamase inhibitors (Table 2.1) (Bush *et al.*, 1995). Class A, C, and D β-lactamases hydrolyze the β-lactam ring through a catalytic serine residue at their active site, whereas class B enzymes (metallo-β-lactamases) use zinc (Zn^{2+}) to break the amide bond. β-lactamases thus may be cephalosporinases, narrow and extended spectrum beta-lactamases, metallo-beta-lactamases, oxacillinases or carbapenemases with various genetic variants (Opal and Pop-Vicas, 2004).

All species of *P. aeruginosa* possess the AmpC gene of the inducible β-lactamase. These chromosomally encoded enzymes confer resistance to penicillins, narrow spectrum cephalosporins, oxyimino β-lactams and cephamycins (Lodge *et al.*, 1990). Induction of the enzyme alone, however, may not account for resistance but overexpression due to spontaneous changes in the regulatory gene may enhance phenotypic expression of resistance by AmpC β-lactamases. PER, TEM, SHV, CTX-M and OXA derived β-lactamases and their genetic variants however confer multi resistance to a wide range of β-lactam antibiotics (Poole *et al.*, 2011).

Carbapenemases not only hydrolyse carbapenems such as imipenem and meropenem but also exert hydrolytic activity on broad-spectrum penicillins, oxymino-cephalosporins and cephamycins (Livermore, 1987). The KPC- enzyme type of Ambler class A, (IMP, VIM, SPM, GIM and SIM) (Villagas, 2007), metallo-β-lactamases of class B (Meletis and Bagkeri, 2013) and OXA carbapenemases of class D (Lister *et al.*, 2009) have all been

identified in *P. aeruginosa* and could be spread on mobile genetic elements to strains in different environments.

Aminoglycosides are an essential component of antipseudomonal chemotherapy. They kill bacteria by binding to the 16S rRNA, inhibiting protein synthesis and also disrupt the integrity of the cell membrane (Gilbert *et al.*, 2003). The activity of aminoglycoside antibiotics can be prevented by enzymes that chemically modify specific sites on the antibiotic (Ramirez and Tolmasky, 2010). These enzyme either transfer acetyl groups from actylcoA or adenyl and phosphate groups from ATP to the antibiotics effecting chemical changes to the drug, and rendering it ineffective (Walsh, 2003).

Aminoglycoside modifying enzymes (AME) are often plasmid or chromosomally encoded enabling their possible dissemination through conjugation, transformation or transduction (Opal and Pop-Vicas, 2004). Aminoglycoside O-Phosphotransferases (APHs), Aminoglycoside N-Acetyltransferases (AACs) and Aminoglycoside O-Nucleotidyltransferases (ANTs) phosphorylate, acetylate or adenylate some amino or hydroxyl groups on the antibiotic (Ramirez and Tolmasky, 2010). The nomenclature of the enzyme indicates the enzyme type, the site of the antibiotic it affects (in parenthesis) and the different enzymes that have the same effect at a specific site (in roman numerals) (Figure 2.5). Aminoglycoside N-Acetyltransferases (AACs) is the largest group of AMEs and the most common in *P. aeruginosa.*

Table 2.2: Molecular classes of β-lactamases found in *P. aeruginosa* and their substrate specificities

Bush Jacoby Medeiros	Ambler molecular class	Enzyme type	Genetic variants found in *P. aeruginosa*	Main substrates	References
1	C	Cephalosporinase	AmpC	Cephalosporins	Meletis and Bagkeri, 2013 Opal and Pop-vicas, 2004
2a	A	Penicillinase		Penicillins	
2b	A	Broad spectrum	TEM-1, -2, -90 , -110 and SHV-1	Penicillins, cephalosporins	
2be	A	Extended spectrum	PER-1 and -2;VEB-1, -2 and -3;TEM-4, -21, -24,-42 and -116; SHV-2a, -5 and -12;GES-1, -2, -5,-8 (IBC-2) and -9;LBT 802 CTX-M-1, CTX-M-2 and CTX-M-43;BEL	Penicillins, narrow and extended spectrum cephalosporins, monobactams	
2br	A	Inhibitor-resistant		Penicillins	Meletis and Bagkeri, 2013 Opal and Pop-vicas, 2004
2c	A	Carbenicillinase	PSE-1 (CARB-2), PSE-4 (CARB-1), CARB-3, CARB-4, CARB-like and AER-1	Penicillin, carbenicillin, extended spectrum	
2d	D	Oxacillinase	Narrow spectrum: LCR-1, NPS-1, OXA-1–5, -7, -9, -10, -12, -13, -20, -21, -30 and -46 Extended-spectrum: OXA-11, -14–19, -28, -31, -32, -35, -45, -53 and -161 Carbapenemase – naturally occurring: OXA-50 Carbapenemase – acquired: OXA-24/40	Penicillins, oxacillin. There are also variants with extended-spectrum activity	
2e	A	Cephalosporinase		Cephalosporins	
2f	A	Carbapenemase	KPC-2 and KPC-5	Penicillins, cephalosporins, monobactams, carbapenems	Meletis and Bagkeri, 2013 Strateva and Yordanov, 2009
3	B	Carbapenemase	IMP-1, -4, -6, -7, -9, -10, -12, -13, -15, -16, -18 and -22 VIM-1, -2, -3, -4, -5, -7, -8, -11, -13, -15, -16, -17 and -18 SPM-1 GIM-1 AIM-1, NDM-1	Penicillins, cephalosporins, carbapenems	Meletis and Bagkeri, 2013 Opal and Pop-vicas, 2004
4	ND	Penicillinase		Penicillins	

These enzymes acetylate the 1, 3, 6' and 2' amino groups and inactivate antibiotics such as gentamycin, tobramycin, kanamycin and amikacin (Shaw *et al.,* 1993).

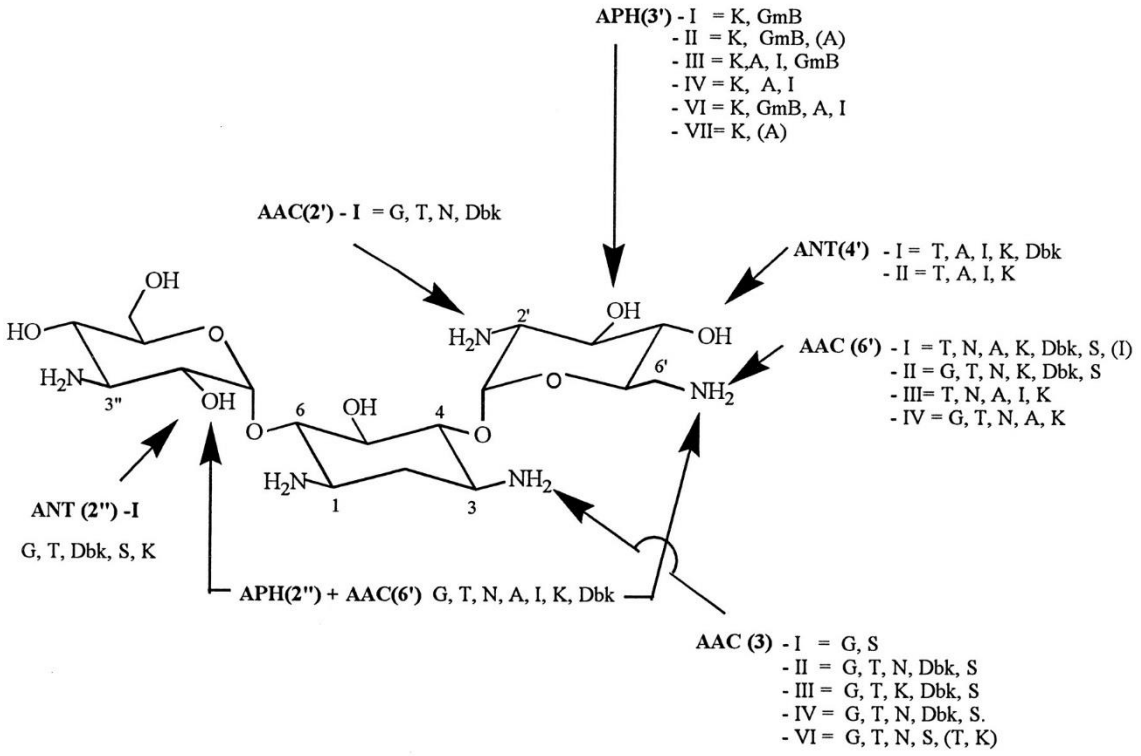


Figure 2.5: Sites of action and antibiotic substrate specificities of aminoglycoside modifying enzymes. A, amikacin; Dbk, dibekacin; G, gentamicin; I, isepamicin Km, kanamycin; Lv, lividomycin; N, netilmicin; S, sisomicin; T, tobramycin. (Avaiable at www.lookfordiagnosis .com/mesh_info. php?term= aminoglycosides&lang=1). Accessed March 2016

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

Table 3.1: Instruments and equipment

Instruments /Equipment/Materials	Manufacturer/Company/Place
Thermal cycler (Multi Gene II)	Labnet International Inc
Thermal cycler (GeneAmp PCR system 2700)	Applied Biosystems,Singapore
Transilluminator	Fotodyne, Hartland, USA
Vortex mixer	Scientific industries, New York, USA
Thermostatically controlled water bath	New Brunswick, Edison, USA
Autoclave	Systec, Wettenberg, Switzerland
Weighing balance	Ohaus corporation, NJ, USA
Laminar air flow cabinet Model T22472	Skan AG, Allschwill, Switzerland
Heraeus oven	Thermofisher Scientific, Waltham, USA
Gel power supply	Biorad, California, USA
Electrophoretic Gel tank	Biorad, California, USA
Garmin Etrex	Kansas, USA
Disk dispenser	Oxoid Ltd, Basingstoke, UK
Centrifuge 5415C	Eppendorf, Hamburg, Germany
Dry bath incubator	Light Labs, Dallas, USA
Incubator (Gallenkamp Plus II)	Sanyo Corporation, UK
Pipette	Trek diagnostics, Ohio, USA
McFfarland Densitometer	Grant-bio , Shepreth-Cambridgeshire, UK
Water bath	Polyscience, USA

Table 3.2: Culture media

Medium	Manufacturer/Company/Place
Pseudomonas isolation agar	Alpha Biosciences, Maryland, USA
Columbia blood agar base	Oxoid Ltd, Basingstoke, UK
Nutrient agar	Oxoid Ltd, Basingstoke, UK
Lauria Bertani broth	Oxoid Ltd, Basingstoke, UK
MacConkey agar	Oxoid Ltd, Basingstoke, UK
Casein soy bean digest broth	Oxoid Ltd, Basingstoke, UK
Cetrimide agar	Oxoid Ltd, Basingstoke, UK
Mueller Hinton agar	Oxoid Ltd, Basingstoke, UK
Nutrient broth	Oxoid Ltd, Basingstoke, UK
Sheep blood	Department of Veterinary Disease Biology, University of Copenhagen (KU)

Table 3.3: Reagents

Item	Manufacturer/Company/Place
Phenylboronic acid	Sigma Aldrich, Copenhagen, Denmark
Sodium dodecyl sulphate	Sigma Aldrich, Copenhagen, Denmark
Glycerol	Surechem, UK
Tris base	Sigma Aldrich, Copenhagen, Denmark
Petri dishes	Corring, New York, USA
PCR plastics	Lab Box, Barcelona, Spain
Sterile cotton swabs	Eppendorf, Hamburg, Germany
Eppendorf tubes	Lab Box, Barcelona, Spain
Beakers	Lab Box, Barcelona, Spain
Conical flasks	Lab Box, Barcelona, Spain
Pipette tips	Lab Box, Barcelona, Spain
Glass bottles	Lab Box, Barcelona, Spain
Lysis buffer	Department of Veterinary Disease Biology, University of Copenhagen (KU)
TE buffer 50:10	Department of Veterinary Disease Biology, University of Copenhagen (KU)
Phenol:Chloroform:Isoamylalcohol (25:24:1)	Ambion, Copenhagen, Denmark
0.5 McFarland Standard	Thermo Scientific, Copenhagen, Denmark
Gel red	Biorad, California, USA
Loading dye	Biorad, California, USA
Ladder	Promega, Madison, USA
Agarose	Sigma Aldrich, Copenhagen, Denmark
Ethidium bromide	Sigma Aldrich, Copenhagen, Denmark
Cotton swabs	Lab box, Barcelona, Spain
EDTA	Sigma Aldrich, Copenhagen, Denmark BDH Laboratory Supplies, England
Glacial acetic acid	BDH Laboratory Supplies, England
N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride	Sigma Aldrich, Copenhagen, Denmark
Hydrochloric acid	Surechem, UK
Crystal Violet	Surechem, UK
Sodium Hydroxide	Surechem, UK
Tris-EDTA buffer solution	Fluka Analytical, Sigma Aldrich
Nuclease free water(Molecular Biology grade)	Fishher Scientific, New Jersey
Magnesium Chloride	Promega, Madison, USA
Master mix	Promega, Madison, USA

Table 3.4: Reference bacterial strains

Control strains	Study	Source
Escherichia. coli R39	Plasmid characterization	Department of Veterinary Disease Biology, University of Copenhagen (KU), Copenhagen, Denmark
Escherichia. coli V517	Plasmid characterization	Department of Veterinary Disease Biology, University of Copenhagen (KU), Copenhagen, Denmark
Pseudomonas aeruginosa ATCC 27853	Biochemical identification	Department of Applied and Theoretical Science, KNUST, Kumasi, Ghana
Staphylococcus aureus ATCC 25923	Biochemical identification	Department of Applied and Theoretical Science, KNUST, Kumasi, Ghana
Escherichia coli ATCC 25922	Biochemical identification	Department of Applied and Theoretical Science, KNUST, Kumasi, Ghana
Streptococcus pyogenes (clinical)	Biochemical identification	Department of Applied and Theoretical Science, KNUST, Kumasi, Ghana

Table 3.5: Antibiotic sensitivity disks

Antibiotic disk (µg/ml)	Manufacturer/Company/Place
Piperacillin (PIP-100µg)	Oxoid Ltd, Basingstoke, UK
Ticarcillin (TIC-75 µg)	Oxoid Ltd, Basingstoke, UK
Ceftazidime (CAZ-30 µg)	Oxoid Ltd, Basingstoke, UK
Cefipime (FEP-30 µg)	Oxoid Ltd, Basingstoke, UK
Aztreonam (ATM-30 µg)	Oxoid Ltd, Basingstoke, UK
Imipenem (IPM-10 µg)	Oxoid Ltd, Basingstoke, UK
Meropenem (MEM-10 µg)	Oxoid Ltd, Basingstoke, UK
Ciprofloxacin (CIP-5 µg)	Oxoid Ltd, Basingstoke, UK
Gentamycin (CN-10 µg)	Oxoid Ltd, Basingstoke, UK
Levofloxacin (LEV-5 µg)	Oxoid Ltd, Basingstoke, UK
Ticarcillin/Clavulanic acid (TIM-85µg)	Oxoid Ltd, Basingstoke, UK
Piperacillin-Tazobactam (TZP-100/10 µg)	Oxoid Ltd, Basingstoke, UK
Cefotaxime (CTX-30ug)	Oxoid Ltd, Basingstoke, UK
Ceftriaxone (CRO-30µg)	Oxoid Ltd, Basingstoke, UK

Table 3.6: PCR Primers (Inqaba Biotec, Pretoria, South Africa) used for genotypic identification and detection or resistance genes in *P. aeruginosa*

Primer	Primer sequence (5'-3')	Amplicon size (bp)	Annealing temperature	Homology	Reference/Accession number
OprL-F OprL-R	ATG GAA ATG CTG AAA TTC GGC CTT CTT CAG CTC GAC GCG ACG	504	64ºC	Pseudomonas identification	De Vos *et al.*, 1997
ERIC-1 ERIC-2	ATG TAA GCT CCT GGG GAT TCA C AAG TAA GTG ACT GGG GTG AGC G	Variable	53˚C	Repetitive-element-based (ERIC) PCR	Versalovic *et al.*,1991
5'CS 3'CS	GGCATCCAAGC GCAAG AAG CAG ACT TGA CCT GA	Variable	59.5˚C	Conserved region of class 1 integron	Severino and Magalhaes, 2002
Int2-F Int2-R	CACGGATATGCGACAAAAAGGT GTAGCAAACGAGTGACGAAATG	788	60˚C	Class 2 integron	Mazel *et al.*, 2000
aac(3)-IV -F aac(3)-IV-R	GTGTGCTGCTGGTCCACAGC AGTTGACCCAGGGCTGTCGC	627	50˚C	Aminoglycoside acetyltransferase	Harel *et al*., 1991
SHV 1 SHV 2	GGG TTA TTC TTA TTT GTC GC TTA GCG TTG CCA GTG CTC	900	56˚C	SHV ESBL	Ratkai, 2011
TEM1-F TEM1-R	ATG AGT ATT CAA CAT TTC CG CTG ACA GTT ACC AAT GCT TA	867	58˚C	TEM ESBL	Ratkai, 2011
CTMX-F CTMX -R	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC G	593	60˚C	CTMX ESBL	Monstein *et al.*, 2007
VIM-F VIM -R	ATG GTG TTT GGT CGC ATA TC TGG GCC ATT CAG CCA GAT C	261	51˚C	VIM MBL	Ratkai, 2011
IMP -F IMP- R	CTA CCG CAG AGT CTT TG AAC CAG TTT TGC CTT ACC AT	600	55˚C	IMP MBL	Ratkai, 2011
gyrA -F gyrA-R	TTATGCCATGAGCGAGCTGGGCAACGAC AACCGTTGACCAGCAGGTTGGGAATC	360	57˚C	Quinolone resistance gene	Kureishi *et al.*, 1994
gyrB-F gyrB-R	TGCGGTGGAACAGG AGATGGGCAAGTAC CTGGCGGAAGAAGAAGGTCAAC AGCAGG GT	483	60˚C	Quinolone resistance gene	Hocquet *et al.*, 2003
parC-F parC-R	ATGAGCGAACTGGGGCTGGA ATGGCGGCGAAGGACTTGGGA	209	70˚C	Quinolone resistance gene	Hocquet *et al.*, 2003

3.2 Methods

3.2.1 Ethical approval

Approval for conduct of the study was obtained from the Ethical Review Committee of the Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi. Written consent was also sought from the management of the various hospitals and poultry farms from which samples were obtained. The nature and purpose of the study was explained to participants and a written and verbal consent was sought from patients before samples were collected.

3.2.2 Study sites and subjects

Two hospitals (South Suntreso Government Hospital and KNUST Hospital), one hundred and thirty seven (137) poultry farms, the Kumasi central market and Ayigya community, all in the Kumasi metropolis were selected for the study. Kumasi central market was selected because most agricultural products from neighboring towns were sold in large quantities in this market.

3.2.3 Sample collection

3.2.3.1 Collection of samples from patients

The subjects of the study were patients who were suspected of having bacterial infections and were requested to undergo medical diagnosis at the microbiological laboratories. Stool, urine and blood samples from 375 patients were randomly collected from the South Suntreso Government Hospital and KNUST Hospital.

3.2.3.2 Collection of environmental samples

Two hundred and sixty environmental samples were collected from drains, sewages, market floors and tables and community based latrines from Ayigya and Kumasi central market. Samples were collected into sterile containers containing trypticase soya bean digest broth (Oxoid).

3.2.3.3 Collection of samples from poultry farms

A total of 274 poultry litter samples were collected from 137 poultry farms in the Ashanti region of Ghana. One gram of poultry litter was collected from the different sites within a pen and mixed together in sterile sample containers containing trypticase soya bean digest broth (Oxoid).

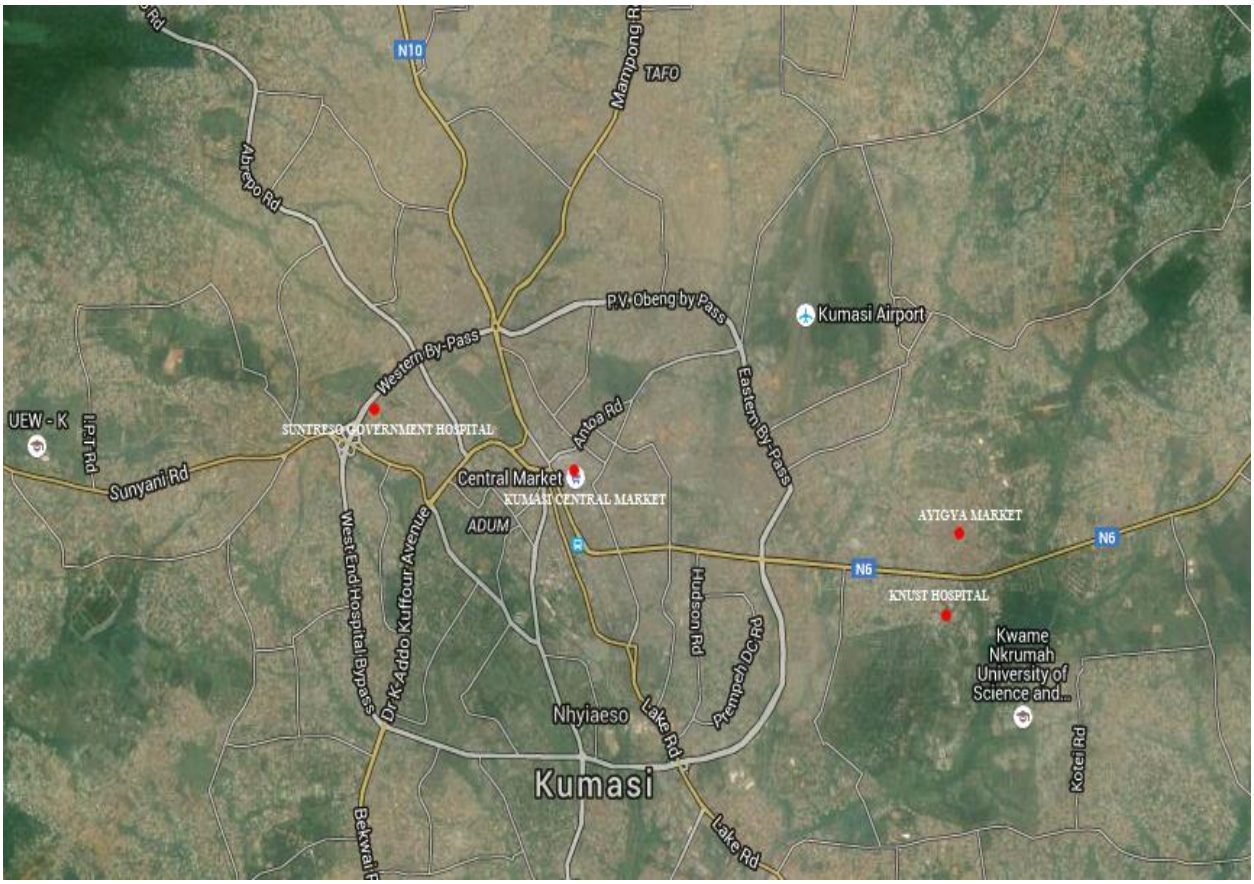


Figure 3.1: Distribution of hospitals and markets in Kumasi where clinical and environmental samples were obtained. =Sampling locations

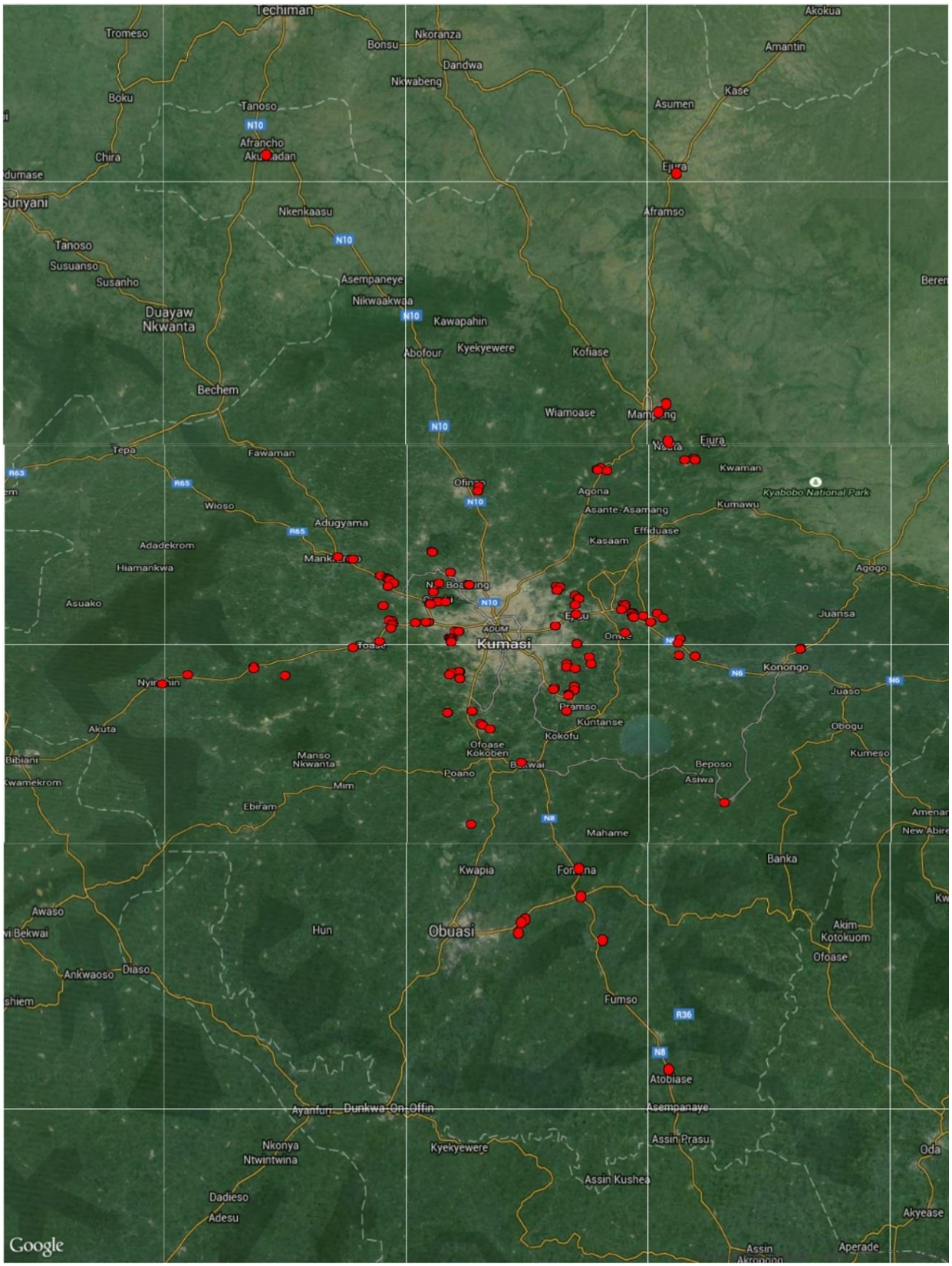


Figure 3.2: Distribution of towns in the Ashanti region of Ghana where poultry farms were sampled. =Sampling location

3.2.4 Preparation of culture media

Pseudomonas isolation agar, Mueller-Hinton agar, cetrimide agar, MacConkey agar, trypticase soy broth, nutrient broth, nutrient agar and Columbia blood agar base were all prepared according to the manufacturers' protocol as stated in Appendix I.

3.2.5. Maintenance of isolated and reference bacterial strains

Glycerol broth was used for storage of isolated *P. aeruginosa* and reference *P. aeruginosa* ATCC 27853, *E.coli* R39 and *E. coli* V517 strains. Colonies of reference strains cultured on nutrient agar were fished into 30% glycerol broth and frozen at -80^{o}C. One loopful of stored samples were inoculated in 5 mL nutrient broth and incubated at 37^{o}C for 24 h to revive the cells.

3.2.6 Isolation of *Pseudomonas aeruginosa*

Five milliliters (5 mL) of trypticase soy broth was added to the various samples collected and incubated at 37^{o}C for 24 h. Ten microliters (10 µL) of the revived bacteria was subcultured on cetrimide agar and incubated at 37^{o}C for 24 h. Bacterial colonies obtained after incubation were fished into 1 mL nutrient broth and incubated at 37^{o}C for 24 h.

3.2.7 Identification of *Pseudomonas aeruginosa* isolates

3.2.7.1 Morphological characteristics

The presumptive *P. aeruginosa* isolates were streaked on 20 mL nutrient agar and incubated at 37^{o}C for 24 h. The nature, cell and colony morphology of the isolates were observed and recorded.

3.2.7.2 Temperature profiles

P. aeruginosa remains viable at temperatures as high as 42^{o}C but loses its viability when cultured at 4^{o}C (Cowan *et al.*, 2004). This property of *P. aeruginosa* provides a distinguishing feature which

aids preliminary identification of the species. One loopful of isolated colonies were subcultured in casein soya bean digest broth and incubated at 4^{o}C and 42^{o}C for 24 h.

3.2.7.3 Gram staining

A loopful of an overnight culture was air-dried and heat fixed on a glass slide. Ammonium oxalate crystal violet solution was applied as primary stain for 30 sec. Excess stain was washed off with a gentle stream of water. Gram's iodine was applied for 30 sec and rinsed off with water. The iodine, added as a mordant forms a crystal violet-iodine complex. The stain was then washed with 95% ethanol and then stained with safranin as secondary stain for 1 min. The counter stain was then washed with water for 5 sec and viewed under the microscope. Gram-negative cells are decolourised upon addition of ethanol but pick up the reddish pink colour of the counter stain whiles Gram-positive bacteria retained the purple primary stain when viewed under the microscope. Reference bacteria include *Pseudomonas aeruginosa* ATCC 27853 (Gram-negative) and *Staphylococcus aureus* ATCC 25923 (Gram-positive).

3.2.7.4 Biochemical identification of *Pseudomonas aeruginosa*

3.2.7.4.1 Catalase activity

A loopful of *P. aeruginosa* culture was emulsified with a loopful of 3%v/v hydrogen peroxide. Effervescence caused by liberation of free oxygen as gas bubbles, indicated a positive result (Cowan *et al.,* 2004). Reference bacteria include *Staphylococcus aureus* ATCC 25923 (catalase positive) and *Streptococcus pyogenes* ATCC 19615 (catalase negative).

3.2.7.4.2 Oxidase activity

Bacterial cultures were smeared across filter papers impregnated with a 1% (w/v) aqueous solution of N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride. The formation of a purple

colour within 5 to 10 sec indicated oxidase positive cultures (Cowan *et al.,* 2004). Reference bacteria were *Pseudomonas aeruginosa* ATCC 27853 (oxidase positive) and *Escherichia coli* ATCC 25922 (oxidase negative).

3.2.7.4.3 Haemolysin production

A loopful of the inoculum of the isolates was streaked on a plate of 20 mL Columbia blood agar base infused with 5% horse blood. The plates were incubated at 37ºC for 24 h. The presence of a clear colourless zone due to haemolysis of red cells indicated haemolysin production (β-haemolysis) (Cowan *et al.,* 2004). *Streptococcus pyogenes* (β-haemolysis) was used as the reference strain.

3.2.7.4.4 Pigment production

Characteristic pigments produced by *P. aeruginosa* was studied by streaking isolates on a plate of 20 mL Pseudomonas isolation agar and incubated at 37ºC. The production of fluorescein (yellow), pyocyanin (blue-green), pyorubin (red) and pyomelanin (brown) by the isolates were detected and recorded after 24 h.

3.2.7.5 Confirmation of *P. aeruginosa* by polymerase chain reaction (PCR)

3.2.7.5.1 Extraction of bacteria DNA

DNA from bacteria was extracted using the boiling lysis method as described by Meacham *et al.* (2003). This method extracts both genomic and plasmid DNA from bacteria. The boiling lysis method provides wholesome DNA for PCR amplification. However, genetic material extracted is unstable on storage for long periods due to degradation by enzymes. Thus, fresh extractions of DNA was used for each PCR reaction. Pure colonies of *P. aeruginosa* cultured on 20 mL nutrient agar were transferred into 25 µL of Tris-Ethylenediamine tetraacetic acid (TE) buffer. The

suspension was heated at 95ºC for 10 min to lyse the bacterial cells, cooled at -20ºC for 5 min to shrink the cells and release the genetic material into the buffer. The celluar debris was then pelleted by centrifuging at 13000 x g for 5 min. The supernatant was then stored at -20ºC and used as the template for PCR amplification of the genes of interest.

3.2.7.5.2 PCR amplification and gel electrophoresis

PCR was used to amplify a *Pseudomonas* specific outer membrane lipoprotein gene (oprL), screen for genes encoding aminoglycoside modifying enzymes, integrons, penicillin, cephalosporin, monobactam, and quinolone resistance. The amplified PCR products were resolved by conventional electrophoresis on 2% w/v agarose gels at 60V for 120 min. The primers and reaction conditions used are provided in Table 3.6.

3.2.7.5.3 PCR protocol for amplification of a 504 base pair *Pseudomonas aeruginosa* species specific oprL gene

Molecular identification of *P. aeruginosa* by amplification of the species specific outer membrane lipoprotein gene oprL, provides confirmation for all phenotypes of this species (De Vos *et al.*, 1997). Using the forward primer oprL-F (5'-ATG GAA ATG CTG AAA TTC GGC-3') and reverse primer oprL-R (5'-CTT CTT CAG CTC GAC GCG ACG-3'), polymerase chain reaction was carried out using a thermal cycler in a final volume of 25 µL containing 2 µL of DNA template, 12.5 µL of GoTaq master mix, 0.75 µL of a 0.5 mM magnesium chloride, 8.55µL of nuclease free water. The DNA template was initially denatured at 94ºC for 5 min, followed by 35 cycles of denaturation of 94ºC for 30 sec, annealing at 64ºC for 30 sec and extension of 72ºC for 1 min. Finally, the products were extended at 72ºC for 10 min. The PCR products were examined on a 2% w/v agarose gel at 60V and visualized using a transilluminator.

3.2.8 Antibiotic susceptibility testing of *P. aeruginosa* isolates

The susceptibility of the isolates to selected antibiotics was determined according to the approved method of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015).

The inoculum size for susceptibility test was standardized to 5 x 10^8 CFU/mL (0.5 McFarland) in a calibrated densitometer. Within 15 min after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped in the solution, rotated several times and pressed firmly on the inside wall of the tube above the liquid. The dried surface of a Mueller Hinton agar plate was inoculated by streaking the swab over the surface of the agar and rotated at approximately 60^o each time to ensure even distribution of the inoculum. The surface of the inoculated media was left for 15 min to dry. Antibiotic discs were then applied to the surface of the inoculated agar plate using a disc dispenser- (Oxoid 6-place, 90 mm) and incubated for at 37^oC for 18 h. The zones of growth inhibition for each of the antibiotics was measured in millimeters and compared with breakpoint values provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015).

3.2.9 Genotyping of *P. aeruginosa* isolates by Repetitive-element-based (ERIC) PCR assay

Non-coding intergenic repetitive sequences in the genome of *P. aeruginosa* were amplified using ERIC1 (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') primers as described by Versalovic *et al.* (1991). Using two microliter (2 µL) of extracted DNA as template, PCR was performed in a final reaction volume of 25 µL containing 12.5 µL of Green Taq master mix, 0.75 µL of 0.5 mM magnesium chloride and 8.55 µL of nuclease free water. With an initial denaturation of 94^oC for 5 min, the reaction continued with 30 cycles of denaturation at 94^oC for 1 min, annealing at 53^oC for 1 min and extended at 72^oC

for 4 min. The products were finally extended at 72°C for 10 min. Five microliters (5µL) of the amplicon was loaded into a 20 well 1.5% w/v agarose gel in 1X TAE (1 mM EDTA, 40 mM Tris-acetate) and run for 340 min at 65V.

The ability of the typing methods to assign different types to randomly sampled unrelated strains from the sampled population species (discriminatory index-D value) was calculated for each typing method using Simpson's index of diversity(Achtman, 2002) $(D) = 1 - \frac{1}{\mathrm{N(N-1)}} \sum_{j=1}^{s} x_j(x_j - 1)$

N= Total number of strains in the sample population, S= total number of types described

x_j= Number of strains belonging to the jth type

3.2.10 Determination of mechanism of antibiotic resistance

Resistance in bacteria can be determined through phenotypic and genotypic methods. Phenotypic methods rely on the morphological and biochemical characteristics of bacteria whilst genotypic methods detect molecular changes in the genetic material which encode resistance.

3.2.10.1 Phenotypic tests for detection of mechanism responsible for antibiotic resistance in *P. aeruginosa*.

3.2.10.1.1 Double disc synergy test (DDST) for extended spectrum β-lactamase (ESBL) detection

Twenty milliliters of Mueller-Hinton agar plates containing 200 µg/mL of cloxacillin was inoculated by swabbing a standardized inoculum of *P. aeruginosa* on the surface of the agar using a sterile cotton swab. Amoxicillin-clavulanic acid (20/10) µg disc was placed at the center of the inoculated media. Cefepime (30 µg), Ceftazidime (30 µg), Cefotaxime (30 µg), Ceftriaxone (30 µg), Imipenem (10 µg) and Aztreonam (30 µg) discs were placed 20 mm from the central

Amoxicillin-clavulanate disc. The plates were then incubated at 37ºC for 24 h. The presence of a "ghost inhibition zone" or a synergistic inhibition of any of the antibiotics towards the central antibiotic was recorded.

3.2.10.1.2 Imipenem-EDTA synergy test for metallo-β-lactamase (MBL) detection

EDTA, a polyamino carboxylic acid binds metal ions like zinc and inactivate metallo-β-lactamases that use zinc to break the amide bond in substrate antibiotics (Lee *et al.*, 2003). Imipenem–EDTA synergy test was performed according to the method described by Lee *et al* (2003). Mueller-Hinton agar (20 mL) plates were inoculated with 5 x 10^8 CFU/ml of the test organisms. An imipenem disc (10 µg) was placed 20 mm from a blank disk containing 10 µL of 0.5 M EDTA. Enhancement of the zone of inhibition in the area between Imipenem and EDTA disks was considered as positive for metallo-beta lactamase production.

3.2.10.1.3 Phenylboronic acid test for KPC Carbapenemase

Phenylboronic acid acts as an inhibitor of the hydrolytic activity of KPC carbapenamases and class A and C beta-lactamases (Tsakris *et al.,* 2010). *P. aeruginosa* suspension diluted to 0.5 MacFarland was swabbed on 20 mL Mueller-Hinton agar and two meropenem discs were placed 30 mm from each other. 20 µL of a 20g/L phenylboronic acid was added to the second meropenem disc and incubated at 37ºC for 20 h. A ≥ 5 mm increase in inhibition zone between the combined meropenem and phenylboronic acid disc and the meropenem disc alone indicated production of KPC carbapenemase enzyme by the *P. aeruginosa* strain (Pournaras *et al.,* 2010).

3.2.10.1.4 D test for detection of inducible AmpC beta-lactamases.

The D test which incorporates an inducer of AmpC enzyme together with a substrate antibiotic as described by Dunne *et al.* (2005) was used for the detection of AmpC beta-lactamase production.

An antibiotic disc inducing production of AmpC beta-lactamase enzyme (imipenem) was placed between two substrate antibiotic discs (ceftazidime and piperacillin-tazobactam) on an inoculated 20 mL Mueller-Hinton agar. The plate was incubated for 24 h at 37ºC. The formation of a D-shaped inhibition zone around any of the substrate discs indicates the imipenem-mediated induction of the AmpC production and the subsequent inactivation of the substrate antibiotic by the beta-lactamase.

3.2.10.1.5 Assessment of efflux pump activity in *P. aeruginosa*

3.2.10.1.5.1 Ethidium-Bromide agar cartwheel method

Ethidium bromide (EtBr)-agar cartwheel method as described by Martins *et al.* (2011) was used to determine the efflux capacity of *P. aeruginosa*. Isolates of *P. aeruginosa* were cultured in 5 mL of nutrient broth for at 37ºC for 24 h. The optical density (OD) of the cultures were adjusted to 0.5 of a McFarland. Mueller-Hinton agar (20 mL) plates containing ethidium bromide at concentrations of 0 to 2.5 mg/L were divided into sectors to form a cartwheel pattern. The OD adjusted cultures were swabbed on EtBr agar plates from the centre to the edge of the plate. Each plate was swabbed with *P. aeruginosa* ATCC 27853 that served as a comparative control. The agar plates were then incubated for 16 h at 37ºC and examined under an UV-transilluminator.

3.2.10.2 Genotypic methods for detection of antibiotic resistant genes in *P. aeruginosa*

3.2.10.2.1 Detection of β-lactamases and aminoglycoside modifying enzyme encoding genes

Antibiotic resistance genes encoding some genetic variants of ESBLS (bla_{SHV1}, bla_{TEM1}, bla_{CTMX}), MBLS (bla_{VIM}, bla_{IMP}), and aminoglycoside modifying enzymes (aac (3)-IV) were sought in the MDR isolates. Using the forward and reverse primers in Table 3.6, polymerase chain reaction was carried out using a thermal cycler in a final volume of 25 µL containing 2 µL of DNA template, 12.5 µL of GoTaq master mix, 0.75 µL of a 0.5 mM magnesium chloride, 8.55 µL of nuclease free

water. The DNA template was initially denatured at 94°C for 5 min, followed by 35 cycles of denaturation of 94°C for 30 sec and extension of 72°C for 1 min. Annealing temperatures for bla_{SHV1}, bla_{TEM1}, bla_{CTMX}, bla_{VIM}, $bla_{IMP,}$ and aac (3)-IV were 56°C for 1 min, 58°C for 1 min, 60°C for 30 sec, 51°C for 1 min, 55°C for 1 min and 50°C for 1 min, respectively. Finally, the products were extended at 72°C for 10 min. The PCR products were examined on a 2% w/v agarose gel at 60V for 120 min and visualized using a transilluminator.

3.2.10.2.2 Detection of quinolone resistance genes in *P. aeruginosa* isolates

Substitution of amino acids in the quinolone-resistance determining region (QRDR) of DNA gyrase and topoisomerase IV results in less efficient binding of quinolone antibiotics (Robicsek, 2006). Primers targeting mutations in *gyr*A, *gyr*B genes (DNA gyrase) and *par*C gene (Topoisomerase IV) were used to amplify genes encoding mutant DNA gyrase and Topoisomerase IV in the MDR isolates through conventional PCR. The primers and reaction conditions are as stated in Table 3.6. With an initial denaturation of 94°C for 5 min, the reaction continued with 35 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 57°C for 40s (*gyr*A), 60°C for 30 sec (*gyr*B) and 70°C for 40 sec (*par*C) and extension of 72°C for 4 min. The products were finally extended at 72°C for 10 min. Five microliters (5 µL) of the amplicon was loaded into a 20 well 1.5% w/v agarose gel in 1X TAE (1 mM EDTA, 40 mM Tris-acetate) and run for 340 min at 65V.

3.2.10.2.3 Detection of integrons in *P. aeruginosa* isolates

Bacteria genomic or plasmid DNA may contain antibiotic resistance markers and gene cassettes (integrons) that encode resistance to several antimicrobial agents. In order to determine the prevalence of integrons in the isolates, amplification of the 5' conserved (GGCATCCAAGC GCAAG) and 3' conserved (AAGCAGACTTGACCTGA) regions of class 1 integron and class 2

integrons (with forward and reverse primers int2-F and reverse Int2-R) were performed. The reaction conditions were an initial denaturation at 94^{o}C for 5 min, further denaturation at 94^{o}C for 1 min, annealing at 59.5^{o}C for 1 min (class I integron) and 60^{o}C for 1 min extension of 72^{o}C (class 2 integron) for 4 min. The products were finally extended at 72^{o}C for 10 min. Five microliters (5 µL) of the amplicon was loaded into a 20 well 1.5% w/v agarose gel in 1X TAE (1 mM EDTA, 40 mM Tris-acetate) and run for 340 min at 65V.

3.2.11 Plasmid characterization in MDR *P. aeruginosa* isolates

3.2.11.1 Extraction of plasmids in *P. aeruginosa* isolates (Alkaline lysis method)

Extra-chromosomal genetic material (plasmids) which may carry antibiotic resistance genes to other bacteria genera (Johnson *et al.,* 2012) were isolated from the *P. aeruginosa* isolates using alkaline lysis method as described by Kado and Liu (1981). *E. coli* control strains 39R and V517 with known plasmid sizes and *P. aeruginosa* isolates were cultured in 2 mL Luria-Bertani (LB) broth for 24 h at 37^{o}C. A 1.5 mL aliquot of both reference and *P. aeruginosa* culture grown in LB broth was pelleted by centrifugation at 13,400 x g for 3 min. The pellets were resuspended by vortexing in 20 µL of Tris- ethylene diamine tetra acetic acid (TE) buffer (50:10). Hundred microliters (100 µL) of lysis buffer (SDS, NaOH) was then added and mixed by repetitive inversions of the 1.5 mL eppendorf tube. The suspension was incubated at 56^{o}C for 30 min in a dry bath. A mixture of 100µL of phenol: chloroform:isoamylalcohol (25:24:1) was then added to the mixture and vortexed until it turned milky white. The mixture was centrifuged at 13000 x g for 30 min to remove excess protein from the mixture. Forty microlitres 40 µL of the supernatant was transferred into a new Eppendorf tube containing 15 µL of loading dye and stored at 4^{o}C until use. Twenty microliters (20 µL) of the plasmid-dye mix was loaded into a 0.8%w/v agarose gel wells and run at 60V for 4h. The gel was stained in ethidium bromide (0.0002%) and destained for 20

min in 1 L sterile distilled water. The gels were then viewed under ultraviolet light and images captured. The plasmid sizes of the reference *E. coli* strains were V517(54 kb, 7.2 kb, 5.6 kb, 5.1 kb, 4.4 kb, 3 kb, 2.7 kb and 2.0 kb) and R39 (147 kb, 63 kb, 36 kb, 7 kb).

3.2.12 Analysis of data

Statistical analysis of the prevalence of the *P. aeruginosa* in the various samples and the susceptibility of the isolates to the studied antipseudomonal agents were determined using Chi-square analysis with Graphpad Prism version 5.01. *p- value* < 0.05 were considered to be statistically significant. The ability of the typing methods to assign different types to randomly sampled unrelated strains from the sampled population species (discriminatory index-D value) was calculated for each typing method using Simpson's index of diversity. Dendrogram was generated from the ERIC-PCR patterns using Gel j software version 1.2. This established genetic relationships between the various MDR strains using Pearsons correlation as a similarity measure and unweighted pair group method with arithmetic averages (UPGMA) as a distance measure.

CHAPTER FOUR

RESULTS

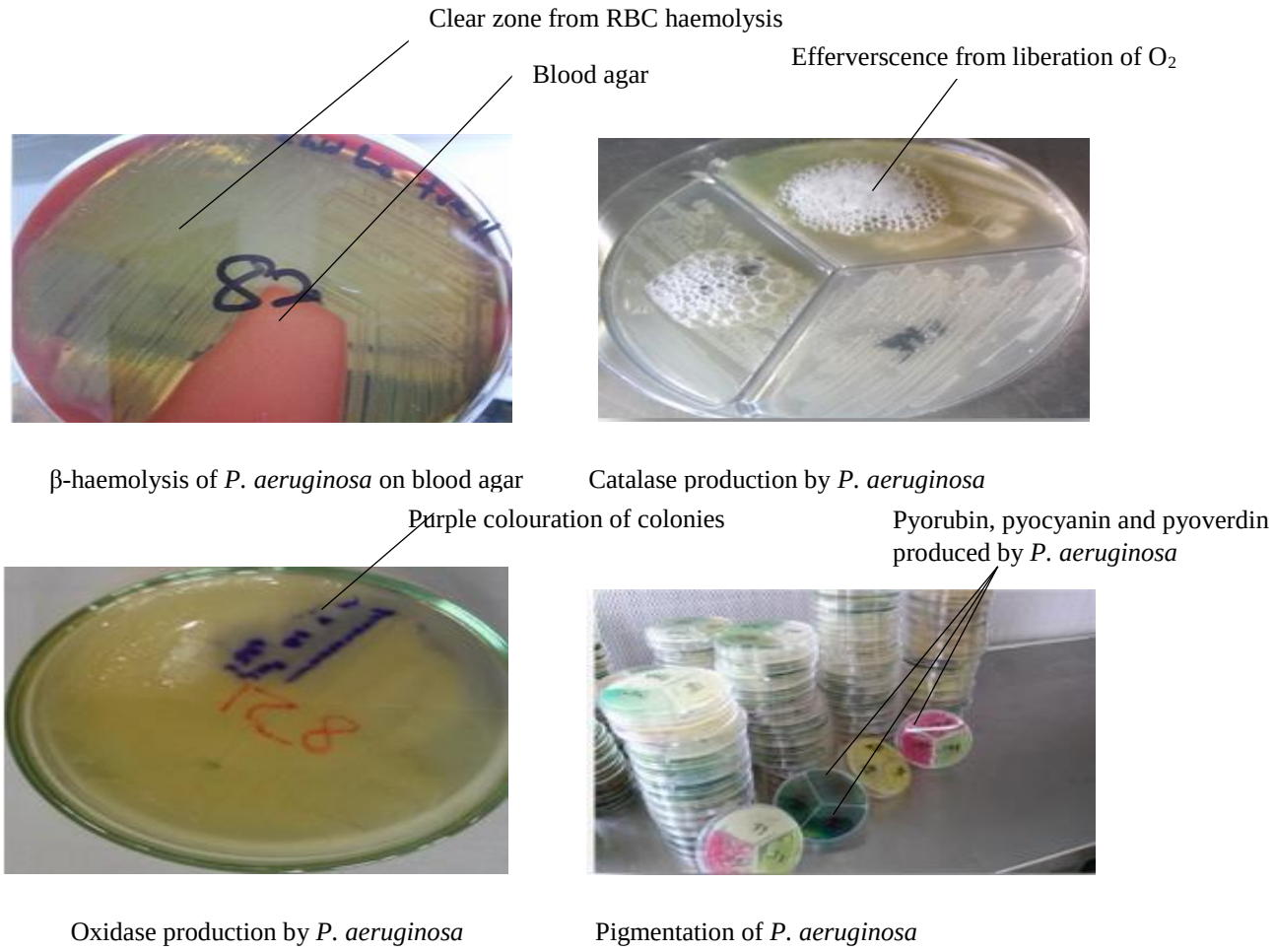
4.1 Isolation and Identification of bacterial isolates

A variety of phenotypic and genotypic identification techniques were employed to identify all the *P. aeruginosa* isolates. These included strain morphological characteristics, biochemical testing, pigment production and detection of species specific membrane lipoprotein genes (oprL) through polymerase chain reaction. Nine hundred (900) clinical, environmental and poultry litter samples were screened for the presence of *P. aeruginosa*. 273 presumptive *P. aeruginosa* isolates were obtained from the samples after growth in trypticase soy broth and subsequent culturing on *Pseudomonas* cetrimide agar.

4.1.1 Morphological and biochemical characteristics of *P. aeruginosa*

Preliminary identification tests conducted on all the isolates included Gram staining, growth at 42^{o}C, tests for catalase activity, oxidase activity and haemolysin production. Gram staining differentially stains bacteria distinguishing Gram-positive bacteria from Gram-negative bacteria. Staining of the isolates allows determination of cell morphology, size and arrangement of bacteria cells, enhancing presumptive identification of the isolates (Betty *et al.,* 2007). Bacteria that undergoes aerobic respiration may have cytochrome C oxidase enzyme which oxidises cytochrome C in the electron transport chain (Leboffe and Pierce, 2010). Catalase, an enzyme of aerobes, microaerophiles and facultative anaerobes catalyses conversion of hydrogen peroxide produced through the electron transport chain to oxygen. Oxidative bacteria such as *P. aeruginosa* thus produce catalase and oxidase enzymes. Several species of bacteria produce exotoxins called haemolysins that destroy red blood cells and haemoglobin. Complete (β) or partial (α) destruction of red blood cells and haemoglobin produce a clearing of the medium around the colonies or

greenish discolouration of medium around the colonies. *P. aeruginosa* produce β-haemolysis of red blood cells (Leboffe and Pierce, 2010). In spite of an optimum growth temperature of 37^{o}C, *P. aeruginosa* remains viable at temperatures as high as 42^{o}C but loses its viability when cultured at 4^{o}C (Cowan *et al.*, 2004). All the *P. aeruginosa* strains were identified as Gram-negative rods with mucoid and non- mucoid forms as outlined in Appendix III. Growth of the isolates on nutrient agar produced either whitish, bluish-green or yellowish-green bacteria colonies. Biochemically, the isolates were catalase and oxidase positive producing β-haemolysis on blood agar (Figure 4.1).



β-haemolysis of *P. aeruginosa* on blood agar

Catalase production by *P. aeruginosa*

Oxidase production by *P. aeruginosa*

Pigmentation of *P. aeruginosa*

Figure 4.1a: Biochemical characteristics of clinical, environmental and poultry litter *P. aeruginosa* isolates.

Also, the presumptive *P. aeruginosa* isolates grew well at both 37ºC and 42ºC in nutrient broth. Pigments produced (Table 4.1, Figure 4.1) after culturing the isolates on *Pseudomonas* isolation agar (Alpha Biosciences, USA) which was indicative of *P. aeruginosa* were yellow-green fluorescein, blue-green pyocyanin, red pyorubin and brown pyomelanin (Appendix III). One hundred and ten (110), 131, 23, and 18 of the presumptive *P. aeruginosa* isolates produced fluorescein, pyocyanin, pyorubin and pyomelanin respectively (Appendix III).

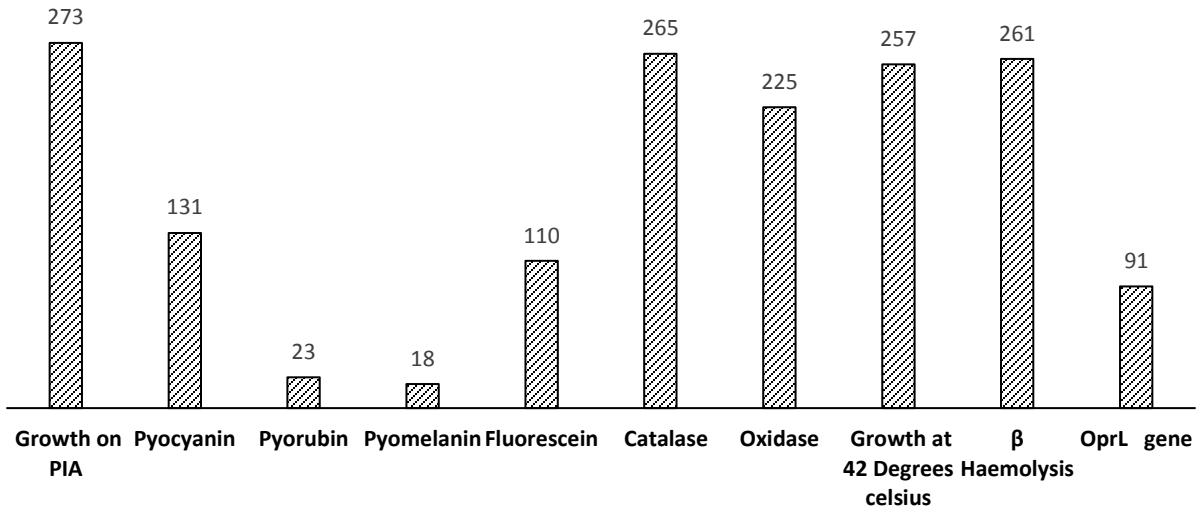


Figure 4.1b: Identification of *P. aeruginosa* isolates using biochemical characteristics and molecular properties. PIA: Pseudomonas isolation agar

4.1.2 Genotypic confirmation of *P. aeruginosa* by amplification of a 504 bp Pseudomonas aeruginosa species specific OprL gene

Phenotypic, morphological and biochemical changes that may arise in different strains of a bacteria species presents a limitation in the exclusive use of these methods for bacteria identification. Molecular methods that target species conserved regions in the genome of a bacteria species enables a more confirmatory bacteria identification (Kidd *et al.,* 2011). Extraction of bacteria

DNA, amplification of conserved gene targets and electrophoresis of the amplified products enables detection and characterization of specific target genes.

P. aeruginosa is highly plastic (Kung *et al.,* 2010) adapting to varied environmental niches (Pirnay *et al.,* 2002). This introduces different morphotypes in the species and makes conventional identification by observing morphological characteristics and biochemical reactions inconsistent and difficult. Molecular identification of *P. aeruginosa* by amplification of the species specific outer membrane lipoprotein gene oprL provides confirmation for all phenotypes of this species (De Vos *et al.,* 1997). This genes (oprL) encodes an immunogenic peptidoglycan associated lipoprotein (PAL) which is conserved in the species *aeruginosa* providing high specificity and sensitivity to *P. aeruginosa* identification (Lim *et al.,* 1997). Targeting of the gene locus with species specific primers in Table 3.6 through PCR enabled amplification of a 504 bp PAL gene (Figure 4.2) which was indicative of *P. aeruginosa* colonization of the isolates. The *P. aeruginosa* outer membrane specific oprL gene was amplified in 87 of the 273 culture identified isolates from clinical, environmental and poultry litter sources.

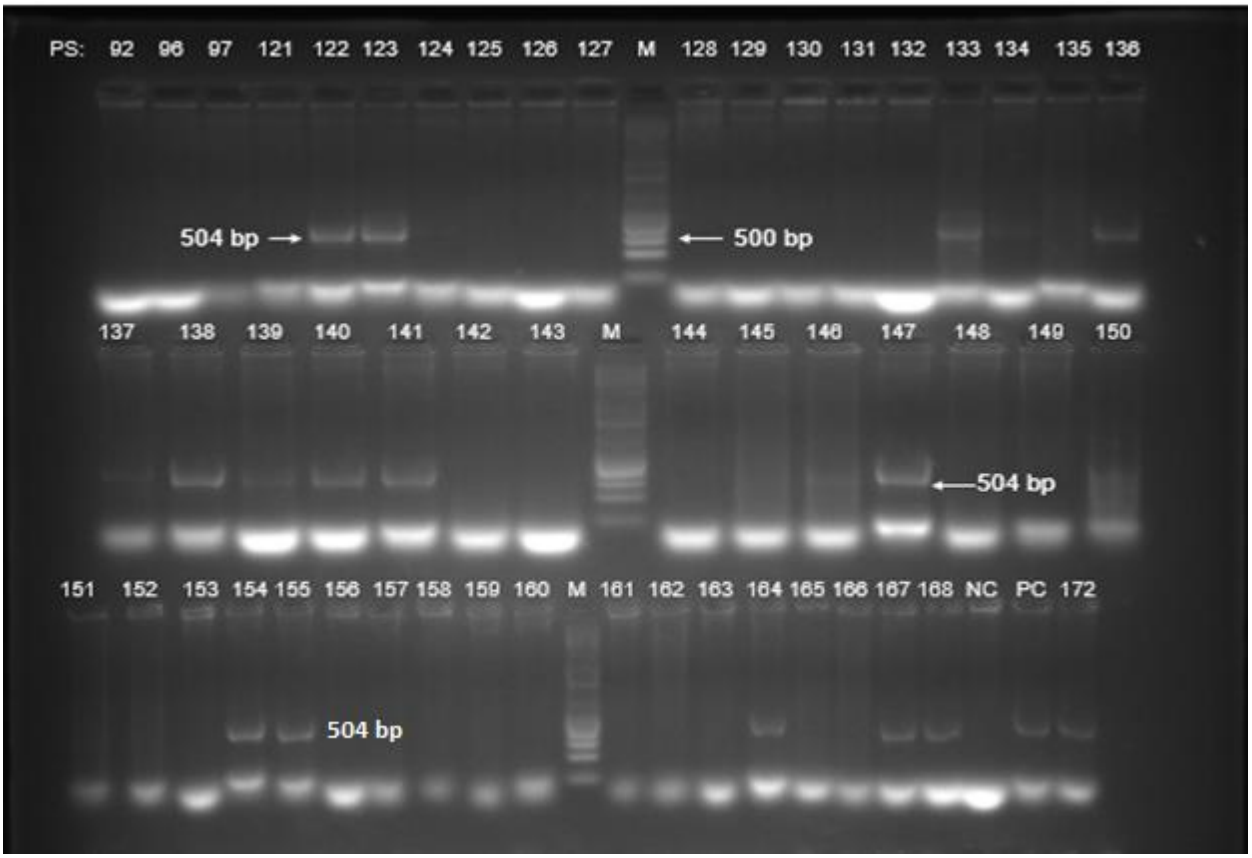


Figure 4.2: Gel image showing a 504 bp PCR amplicon of a peptidoglycan associated outer membrane lipoprotein gene (oprL) in *P. aeruginosa*. M: DNA marker; PC: positive control (*Pseudomonas aeruginosa* ATCC 27853); NC: Negative control

4.1.3 *P. aeruginosa* isolates recovered from clinical, environmental and poultry litter samples

Based on the morphological, cultural, biochemical and molecular characteristics of the isolates, a total of 87 (9.6%) *P. aeruginosa* strains were recovered from the 900 samples (Figure 4.3). Clinical samples obtained from patients included blood (100), stool (78), urine (97) and oral swabs of poultry farm workers (89). *P. aeruginosa* was more prevalent in the environmental samples 35 (13.4%) compared to the clinical (12.9%) and poultry litter (1.8%) samples (Table 4.2).

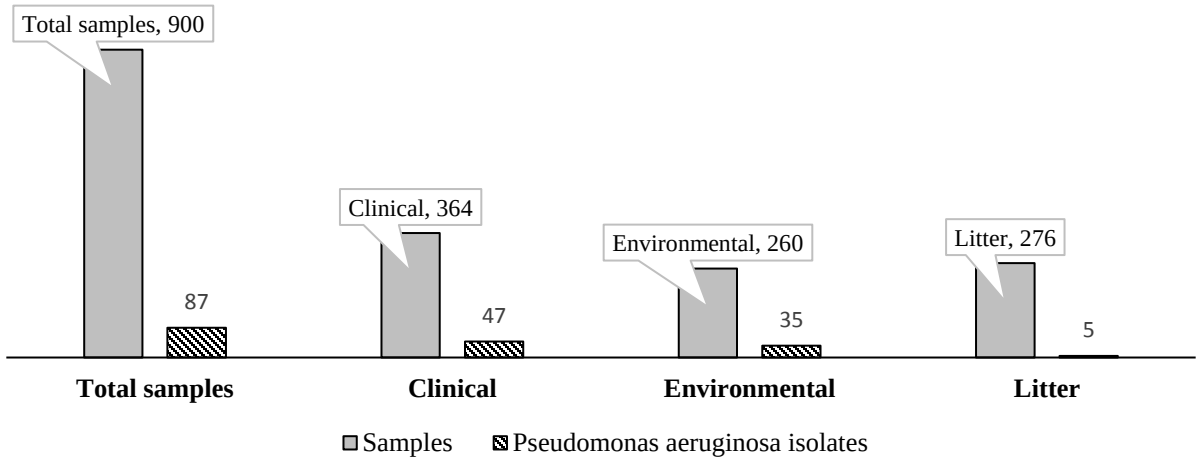


Figure 4.3: Distribution of *P. aeruginosa* isolates in the samples collected

In the clinical samples, *P. aeruginosa* had a high occurrence in the stool (39.7%) and urine (15.4%) of patients from the various hospitals. Blood sampled from patients showed no presence of the study bacteria. Thirty five (13.4%) of the environmental samples haboured *P. aeruginosa*. Low prevalence of the bacteria (1.8%) was detected in litter samples from the various poultry farms.

Table 4.1: Various samples screened and the number of *P. aeruginosa* isolates obtained

SOURCE OF SAMPLE	TOTAL SAMPLE	NUMBER OF ISOLATES	%
CLINICAL	364	47	12.9
Stool	78	31	39.7
Urine	97	15	15.4
Blood	100	0	0
Farm Hands	89	1	1.1
ENVIRONMENTAL	260	35	13.4
Sewage	96	12	12.5
Market floors	104	15	14.4
Others[a]	60	8	13.3
POULTRY LITTER	276	5	1.8

Others[a]: Environment samples collected from market tables, soil, and community based latrines and water sources.

4.2 Antibiotic resistance

Resistance to antibiotics in *P. aeruginosa* may be intrinsic or acquired. Changes that occur in the bacteria genome may confer resistance which may be expressed phenotypically. Genotypic methods such as PCR and DNA sequencing can be used to detect these genetic changes that occur in bacteria (Aarts *et al.,* 2001). Culture based methods also play useful roles in the detection of emerging or new forms of antibiotic resistance and resistance mechanisms.

P. aeruginosa has high intrinsic resistance to commonly used antibacterial agents (Poole *et al.,* 2011). Treatment of associated infections has thus been limited to a few antibacterial classes such as quinolones (ciprofloxacin and levofloxacin), aminoglycosides (amikacin, gentamicin and tobramycin), carbapenems (meropenem and imipenem), cephalosporins (ceftazidime and cefepime), monobactams (aztreonam) and semi-synthetic penicillins (carbenicillin, ticarcillin and piperacillin) (Mesaros *et al.,* 2007). These antibiotics act through different pathways to kill or inhibit bacterial growth. Fluoroquinolones bind to the enzymes DNA gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE) which are responsible for the removal of supercoils in DNA and thus play an essential role in DNA replication, transcription, recombination and repair (Hooper, 1993). β-lactam antibiotics (penicillins, cephalosporins, monobactams and carbapenems) bind to and inactivate penicillin-binding proteins (PBPs) that are transpeptidases involved in bacteria cell wall synthesis (Tipper, 1985). Aminoglycosides inhibit protein synthesis by binding to the 30S or 50S ribosomal subunit (Dozzo *et al.,* 2010).

The antibiograms of the *P. aeruginosa* isolates were carried out as described by Kirby-Bauer to determine the susceptibility or resistance profiles of the isolated strains to selected antipseudomonal agents. For an antibacterial agent to exert a bactericidal or bacteriostatic effect,

it has to be in direct contact with the bacteria. The modified Kirby-Bauer disc diffusion test provides contact between the bacteria and the antibiotic in a nutritive growth environment and was used to determine the *in vitro* sensitivity of the isolates to the selected antipseudomonal agents (Bauer *et al.,* 1966). Breakpoint values set by EUCAST (2015) (Appendix IV) were used to evaluate the sensitivity of the isolates to the various antibiotics.

4.2.1 Antibiogram profiles of all *P. aeruginosa* isolates

The isolated strains were classified as susceptible or resistant to the antipseudomonal depending on the size of inhibition zone diameters when compared to breakpoint values from EUCAST (2015). Susceptibility data were compared by using a Chi-square test and level of significance ($p < 0.05$). Susceptibility rate of the *P. aeruginosa* to all the antipseudomonal agents were in a range of zero (0) to 90%. Resistance rate of all the isolates ranged from 7% to 69% (Figure 4.3).

Table 4.2: Susceptibility profiles of *P. aeruginosa* isolates to various antipseudomonal agents

Antibiotic	Number of *P. aeruginosa* isolates									
	Total isolates (N=87)				Clinical (N=47)		Environmental (N=35)		Poultry Litter (N=5)	
	R	%	S	%	R	S	R	S	R	S
CAZ-10	9	10	78	90	5	42	3	32	1	4
PIP-30	13	15	74	85	9	38	3	32	1	4
MEM-10	6	7	71	82	3	40	2	30	1	1
TIM-85	16	18	71	82	7	40	5	30	4	1
FEP-30	11	13	71	82	5	42	5	25	1	4
IPM-10	8	9	63	72	5	28	2	31	1	4
LEV-5	17	20	61	70	12	30	5	26	0	5
TIC-75	49	56	38	44	21	26	24	11	4	1
CIP-5	54	62	27	31	30	13	22	11	2	3
CN-10	60	69	26	30	34	12	25	10	1	4
ATM-30	22	25	0	0	5	0	15	0	2	0

CIP-5: Ciprofloxacin 5µg; LEV-5: Levofloxacin 5 µg; MEM-10: Meropenem 10 µg; IPM-10: Imipinem 10 µg; CN 10: Gentamycin 10 µg; TIC-75: Ticarcillin 75 µg; TIM-85: Ticarcillin/Clavulanic acid 85 µg; FEP-30: Cefepime 30 µg; ATM-30: Aztreonam 30 µg; CAZ-10: Ceftazidime 10 µg; PIP-30: Piperacillin 30 µg; R: resistant; S: sensitive; N: Number of isolates.

All the *P. aeruginosa* isolates showed high susceptibility to ceftazidime (90%), piperacillin (85%), meropenem (81.6%), imipenem (72.4%), ticarcillin/clavulanic acid (81.6%), cefepime (81.6%) and levofloxacin (72.4%) (Table 4.3). Aztreonam was not active against any of the *P. aeruginosa* isolates. Gentamicin showed the least activity with 69% of the isolates being resistant.

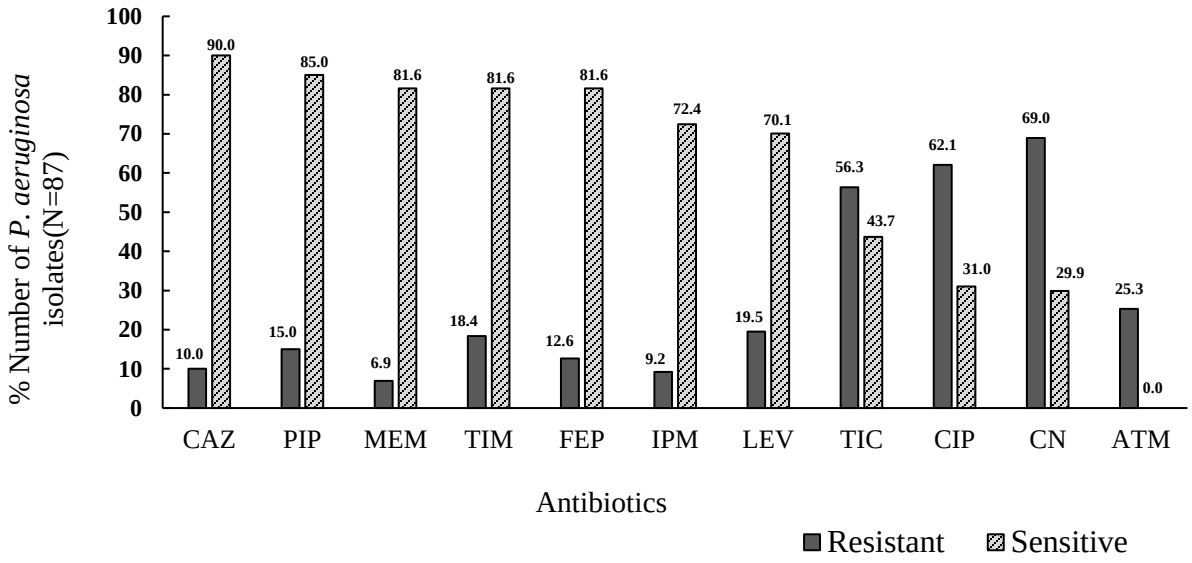


Figure 4.4: Susceptibility pattern of all isolated *P. aeruginosa.* CIP: Ciprofloxacin 5µg; LEV: Levofloxacin 5 µg; MEM: Meropenem 10 µg; IPM: Imipinem 10 µg; CN: Gentamycin 10 µg; TIC: Ticarcillin 75 µg; TIM: Ticarcillin/Clavulanic acid 85 µg; FEP: Cefepime 30 µg; ATM: Aztreonam 30 µg; CAZ: Ceftazidime 10 µg; PIP: Piperacillin 30 µg

High resistance of the isolates were also observed against ciprofloxacin (62.1%) and ticarcillin (56.3%). The *P. aeruginosa* isolates showed the least resistance to meropenem while most of the *P. aeruginosa* isolates were resistant to gentamicin (Figure 4.5).

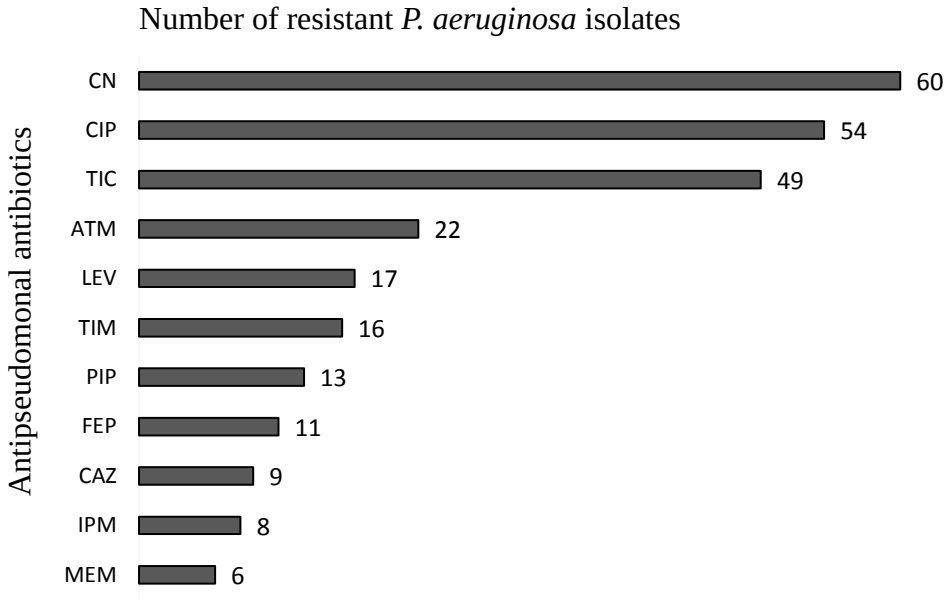


Figure 4.5: Resistance pattern of *P. aeruginosa* to selected antipseudomonal antibiotics. CIP: Ciprofloxacin 5µg; LEV: Levofloxacin 5 µg; MEM: Meropenem 10 µg; IPM: Imipinem 10 µg; CN: Gentamycin 10 µg; TIC: Ticarcillin 75 µg; TIM: Ticarcillin/Clavulanic acid 85 µg; FEP: Cefepime 30 µg; ATM: Aztreonam 30 µg; CAZ: Ceftazidime 10 µg; PIP: Piperacillin 30 µg

There was no significant difference of the *in vitro* activity within the carbapenem group (meropenem and imipenem; p=0.47) and cephalosporin group (ceftazidime and cefepime; p=0.51) of antibiotics. The difference in sensitivity within the penicillin group (piperacillin, ticarcillin, ticarcillin/ clavulanic acid) was significant (p<0.05). Piperacillin and ticarcillin/clavulanic acid were active in most strains of *P. aeruginosa* compared to ticarcillin. Among the quinolone group, levofloxacin exerted greater activity in most strains than ciprofloxacin (p<0.05).

4.2.3 Antibiotic susceptibility pattern of *P. aeruginosa* isolates

P. aeruginosa strains isolated from the environment and clinical sources showed high susceptibility rates to cefepime, ceftazidime, meropenem, piperacillin, ticarcillin/clavulanic acid, levofloxacin and imipenem (Figure 4.6). The percentage of environmental isolates that were susceptible to these antibiotics were in the range of 71% to 91% whereas clinical isolates

susceptible to these antibiotics were in the range of 60% to 89%. Ticarcillin, ciprofloxacin and gentamicin showed lower activity in the environmental isolates compared to the clinical isolates. Susceptibility of isolates from poultry litter to all the antibiotic groups were in the range of 20% to 100% with levofloxacin being active against all 5 poultry litter isolates. Most of the *P. aeruginosa* isolates (74.7%) demonstrated intermediate susceptibility to aztreonam. None of the isolates were sensitive to aztreonam but resistance ranged from 11% to 40% in the clinical, environmental and poultry litter isolates. The most frequent pattern of resistance in the isolates were CIP-CN, CN, CIP-LEV-CN-TIC and CIP-CN-TIC (Table 4.4).

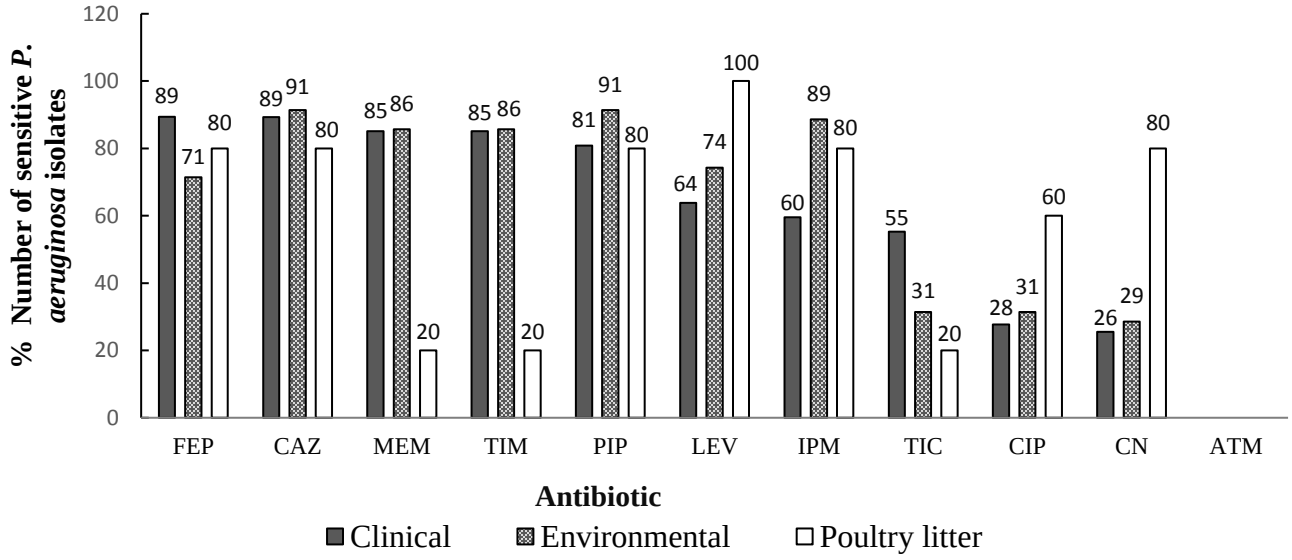


Figure 4.6: Susceptibility pattern of *P. aeruginosa* isolates from various sources CIP: Ciprofloxacin 5µg; LEV: Levofloxacin 5 µg; MEM: Meropenem 10 µg; IPM: Imipinem 10 µg; CN: Gentamycin 10 µg; TIC: Ticarcillin 75 µg; TIM: Ticarcillin/Clavulanic acid 85 µg; FEP: Cefepime 30 µg; ATM: Aztreonam 30 µg; CAZ: Ceftazidime 10 µg; PIP: Piperacillin 30 µg

Table 4.3: Antibiotic resistance pattern of *P. aeruginosa* isolates from various sources

Resistance pattern	Number of isolates	Resistance pattern	Number of isolates
CIP-MEM-IPM-CN-TIC-PIP-TIM-FEP-CAZ-ATM	2	CN-TIC-ATM	3
CIP-LEV-MEM-IPM-CN-TIC-PIP-FEP-CAZ-ATM	1	TIC-TIM-ATM	3
CIP-MEM-IPM-CN-TIC-PIP-FEP-ATM	1	CIP-TIC-CAZ	3
CIP-CN-TIC-PIP-TIM-FEP-ATM	1	CIP-TIC-TIM	3
CIP-LEV-CN-TIC-PIP-TIM-ATM	1	CN-TIC-CAZ	3
CIP-LEV-CN-TIC-PIP-TIM-CAZ	1	CN-TIC-TIM	3
CIP-LEV-CN-TIC-TIM-CAZ-ATM	1	CIP-CN	2
CIP-CN-TIC-FEP-CAZ-ATM	1	CIP-TIC	2
CIP-CN-TIC-PIP-TIM-FEP	1	CN-TIC	2
CIP-LEV-CN-TIC-PIP-FEP	1	TIC-ATM	2
MEM-IPM-TIC-PIP-TIM-ATM	1	CIP-LEV	2
CIP-CN-TIC-FEP-ATM	1	CIP-PIP	2
CIP-LEV-IPM-CN-TIC	1	CN-PIP	2
CIP-LEV-CN-TIC	6	MEM-CN	2
CIP-CN-TIC-FEP	1	TIC-PIP	2
CIP-CN-TIC-TIM	1	TIC-TIM	2
CIP-IPM-CN-TIM	1	CN	1
CIP-LEV-CN-CAZ	1	CIP	1
CIP-TIC-CAZ-ATM	1	TIC	1
CN-TIC-TIM-ATM	1	ATM	1
CIP-CN-TIC	6	IPM	1
CIP-LEV-CN	4	CIP-CN-ATM	3
Number of strains susceptible to all antibiotics	5		

CIP: Ciprofloxacin 5µg; LEV: Levofloxacin 5 µg; MEM: Meropenem 10 µg; IPM: Imipinem 10 µg; CN: Gentamycin 10 µg; TIC: Ticarcillin 75 µg; TIM: Ticarcillin/Clavulanic acid 85 µg; FEP: Cefepime 30 µg; ATM: Aztreonam 30 µg; CAZ: Ceftazidime 10 µg; PIP: Piperacillin 30 µg.

4.2.2 Susceptibility of *P. aeruginosa* to various antibiotic groups

Of the 87 *P. aeruginosa* strains isolated, 6 (7%) of the isolates were sensitive to all antipseudomonal groups studied. Resistance varied from a single antibiotic group (18%) to six

antibiotic groups (5%). Strains found to be resistant to two and three groups of antibiotics were 31% and 27% respectively. Four (4), 5, 7, 18, 23, and 61 of the *P. aeruginosa* isolates were resistant to cephalosporins, carbapenems, penicillins, quinolones, monobactam and aminoglycoside respectively

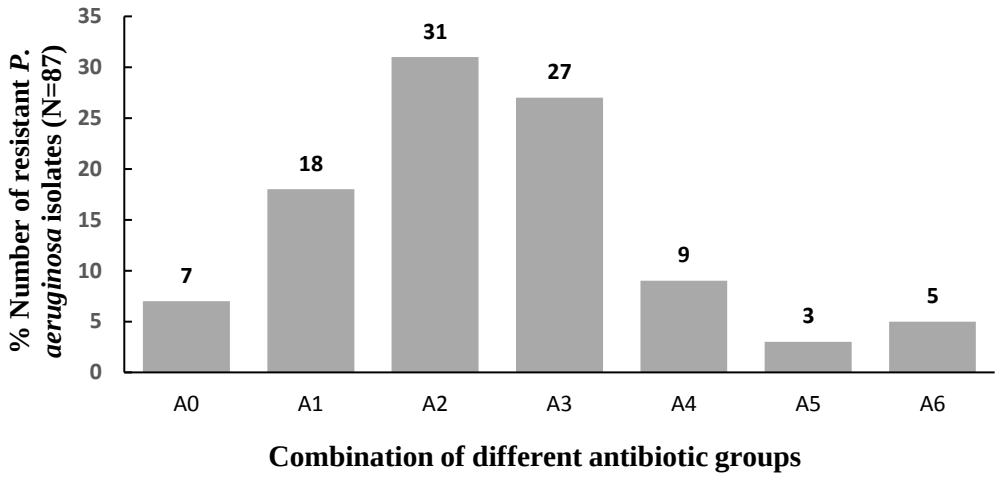


Figure 4.7: Number of resistant *P. aeruginosa* to the various antipseudomonal groups
AO: No antibiotics group; A1: One antibiotic group A2: Two antibiotic groups; A3: Three antibiotic groups; A4: Four antibiotic groups; A5: Five antibiotic groups; A6: Six antibiotic groups.

4.2.3 Cross-resistance and pattern of resistance of *P. aeruginosa* isolates

The P. aeruginosa isolates were studied for cross-resistance to related and unrelated antibiotic classes. in the isolated bacteria strains. Bacteria can become resistant to several different members of a group of chemically related or unrelated antibiotics by the same resistance mechanism (Sefton, 2002). In this study, all the isolates that were resistant to levofloxacin were also resistant to ciprofloxacin. The level of levofloxacin-gentamicin and ciprofloxacin-gentamycin cross-resistance were 94% and 81% respectively (Table 4.5). Majority of meropenem and imipenem resistant isolates (63 to 83%) demonstrated cross-resistance to ciprofloxacin, gentamicin, ticarcillin, piperacillin and aztreonam but remained susceptible to levofloxacin. β-lactam resistant

strains also exhibited cross-resistance to ciprofloxacin and gentamicin. Quinolone (levofloxacin and ciprofloxacin) resistant isolates exhibited high susceptibility (88 to 96%) to the carbapenem group of β-lactams. Carbapenems also showed high activity in strains that were resistant to gentamicin and other β-lactam antipseudomonal groups.

Table 4.4: Cross-resistance of *Pseudomonas aeruginosa* isolates to antibiotics

	N	Number of resistant isolates (%)										
		CIP	**LEV**	**MEM**	**IPM**	**CN**	**TIC**	**PIP**	**TIM**	**FEP**	**CAZ**	**ATM**
CIP	54	*	17(31)	4(7)	6(11)	44(81)	33(61)	9(17)	10(19)	10(19)	9(17)	13(24)
LEV	17	17(100)	*	1(6)	2(12)	16(94)	11(65)	3(18)	2(12)	2(12)	4(24)	2(12)
MEM	6	4(67)	1(17)	*	5(83)	5(83)	5(83)	5(83)	3(50)	4(67)	3(50)	5(83)
IPM	8	6(75)	2(25)	5(63)	*	6(75)	6(75)	5(63)	4(50)	4(50)	3(38)	5(63)
CN	60	44(73)	16(27)	5(8)	6(10)	*	34(57)	9(15)	11(18)	10(17)	8(13)	14(23)
TIC	49	33(67)	11(22)	5(10)	6(12)	6(12)	*	10(20)	15(31)	10(20)	9(18)	18(37)
PIP	13	9(69)	3(23)	5(38)	5(38)	9(69)	10(77)	*	6(46)	7(54)	4(31)	6(46)
TIM	16	10(63)	2(13)	3(19)	4(25)	11(69)	15(94)	6(38)	*	4(25)	4(25)	9(56)
FEP	11	10(91)	2(19)	4(36)	4(36)	10(91)	10(91)	7(64)	4(36)	*	4(36)	7(64)
CAZ	9	9(100)	4(44)	3(33)	3(33)	8(89)	9(100)	4(44)	4(44)	4(44)	*	6(67)
ATM	22	13(59)	2(9)	5(23)	5(23)	14(64)	18(82)	6(27)	9(41)	7(32)	6(27)	*

CIP: Ciprofloxacin 5µg; LEV: Levofloxacin 5 µg; MEM: Meropenem 10 µg; IPM: Imipinem 10 µg; CN: Gentamycin 10 µg; TIC: Ticarcillin 75 µg; TIM: Ticarcillin/Clavulanic acid 85 µg; FEP: Cefepime 30 µg; ATM: Aztreonam 30 µg; CAZ: Ceftazidime 10 µg; PIP: Piperacillin 30 µg**;** N: Number of resistant isolates; * Not applicable.

4.3 Multi-drug resistance of *P. aeruginosa* isolates

Multiple antibiotic resistance may be regulated by multiple resistance mechanisms in both related and unrelated strains (Markowska *et al.*, 2014) . Isolates that were resistant to three or more antibiotic groups were identified and their phenotypic and genotypic resistance profiles characterized.

4.3.1 Characterization of multi-drug resistant (MDR) *P. aeruginosa* isolates

A total of 38 multi-drug resistant *P. aeruginosa* isolates were identified having multiple antimicrobial resistance indexes (MAR) (ratio of number of resistant antibiotics to the total number

of antibiotics studied) ranging from 0.3 to 0.9. One poultry litter isolate (PS_{231}), 16 clinical strains and 21 environmental strains were multi-drug resistant. Among the clinical isolates, 75% (12/16) were isolated from stool while 25% (4/16) were isolated from urine. Of the multi-drug resistant strains, 24 (63.1%) and 6 (15.7%) were resistant to three and four antipseudomonal groups respectively. 4(10.5%) showed resistance to five and six antibiotic groups each.

4.3.1.1 Relatedness of MDR *P. aeruginosa*

Clonal relatedness of the different multidrug resistant *P. aeruginosa* strains from the various sources were determined by their antibiogram patterns and genomic fingerprint profiles using Gelj version 1.2 software (Heras *et al.*, 2015). This was to help identify particular multidrug-resistant strains that had been disseminated between poultry farms, hospitals and the environment. Similar bacteria strains may exhibit the same genomic fingerprint with or without specific phenotypic and biochemical properties (Tenover *et al.*, 1997).

4.3.1.1.1 Phenotyping of multi-drug resistant *P. aeruginosa* isolates

Phenotyping enable bacteria to be grouped based on similar observable characteristics resulting from their genotypic expression. Phenotyping of bacteria may involve colony morphology, colour, odour and other macroscopic features that may characterize a particular bacteria strain (Achtman, 2002). The ability of bacterial isolates to grow in the presence of specific chemical substances such as antibiotics (antibiogram typing) may be used to assess relatedness of specific bacteria strains (Belkum *et al.*, 2007). Using the antibiogram patterns, the MDR isolate from poultry (PS_{231}) shared similar antipseudomonal resistance pattern with a urine isolate (PS_{185}). At a similarity level of 98%, the dendrogram obtained from the antibiogram (Figure 4.8) generated 24 antibiotypes of *P.*

aeruginosa with 5 clusters and 19 distinct strains. The diversity index (*D*=0.9502) indicated good discriminatory ability of the antibogram typing method employed.

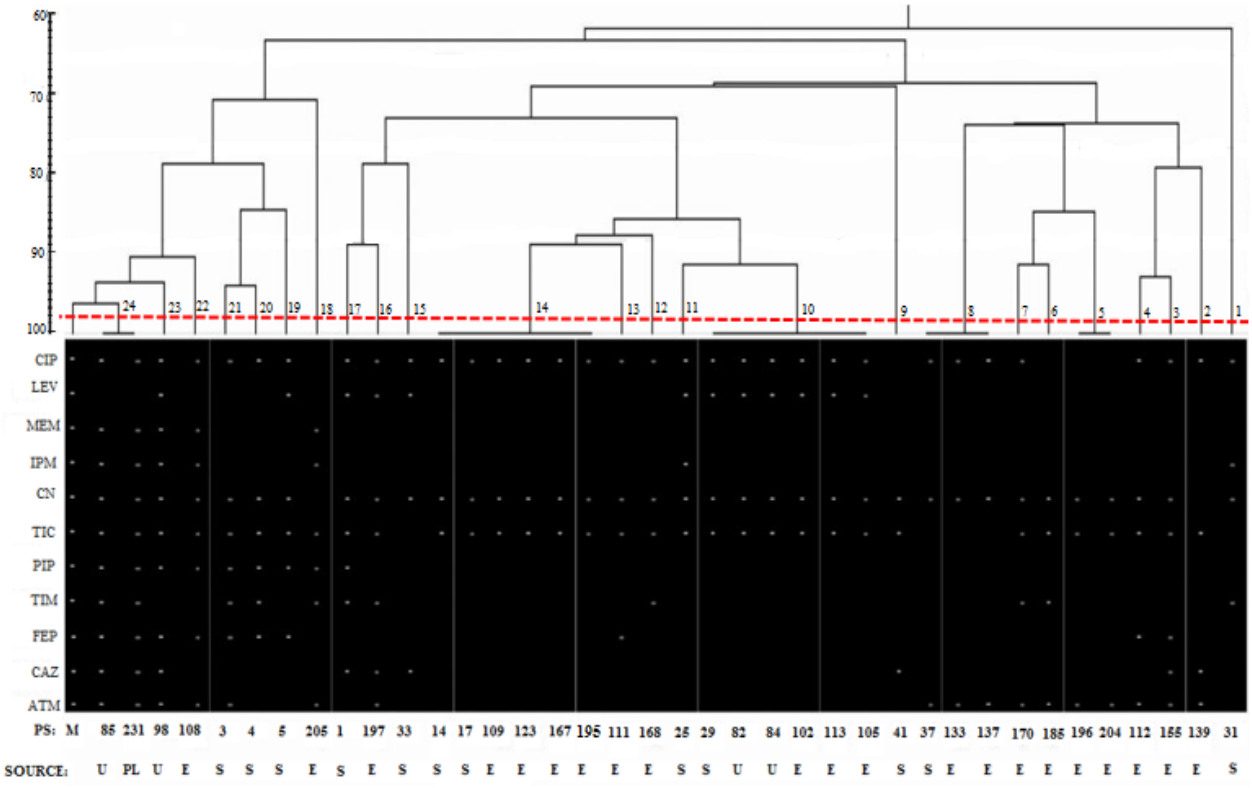


Figure 4.8: Dendrogram derived from antibiogram using Gelj ver.1.2 with Dice coefficient and UPGMA (unweighted pair group method with arithmetic averages). PS 1-231: *Pseudomonas aeruginosa* isolate, U: Urine; S: Stool; E: Environment; PL: Poultry litter; M: resistance marker

The probability that the multi-drug resistant (MDR) isolate was resistant to carbapenem, cephalosporin, monobactam, quinolone, penicillin and aminoglycoside were respectively 0.15, 0.31, 0.44, 0.86, 0.89 and 0.95. The most dominant resistance patterns among the MDR isolates were CIP/LEV/CN/TIC and CIP/CN/TIC which were each observed in six MDR *P. aeruginosa* isolates.

4.3.1.1.2 Molecular typing of MDR *P. aeruginosa*

Single bacteria clones cause most infectious disease outbreaks (Tenover *et al.*, 1997). Bacteria, through time evolve into varied clonal groups during reproduction. The genetic changes (mutations, recombination, insertion, deletion, rearrangement and loss or gain of plasmids) that occur favours the rise of strains with varied virulence and morphological characteristics (Achtman, 2002). To establish clonality in the *P. aeruginosa* strains, an electrophoretic fingerprint pattern of the genome of the various isolates were determined by amplification of repeat intergenic sequences spread across the bacteria genome. PCR-based fingerprinting of the genomic regions in bacteria species through amplification of the conserved repeat regions (ERIC-PCR) and subsequent gel electrophoresis enable similarity to be drawn between isolated bacteria strains in diverse environmental niches (Bruijn, 1992). This may help establish if the occurrence of particular organism in the various environments is due to the spread of a single strain. Similar strains may likely have similar mutations, resistance determining markers and resistance mechanisms (Tenover *et al.*, 1997). Dendrograms depicting the strain relatedness were generated using Pearson coefficient as a similarity measure and UPGMA (unweighted pair group method with arithmetic averages) cluster analysis as a distance measure. Strains with a threshold linkage value of $\geq 98\%$ were assigned the same subtype (designated A1-A27). Two or more strains were assigned the same type if they showed identical banding patterns. ERIC fingerprint patterns generated 2 to 9 bands with molecular weight ranging from 161 base pairs to 850 base pairs (Figure 4.9).

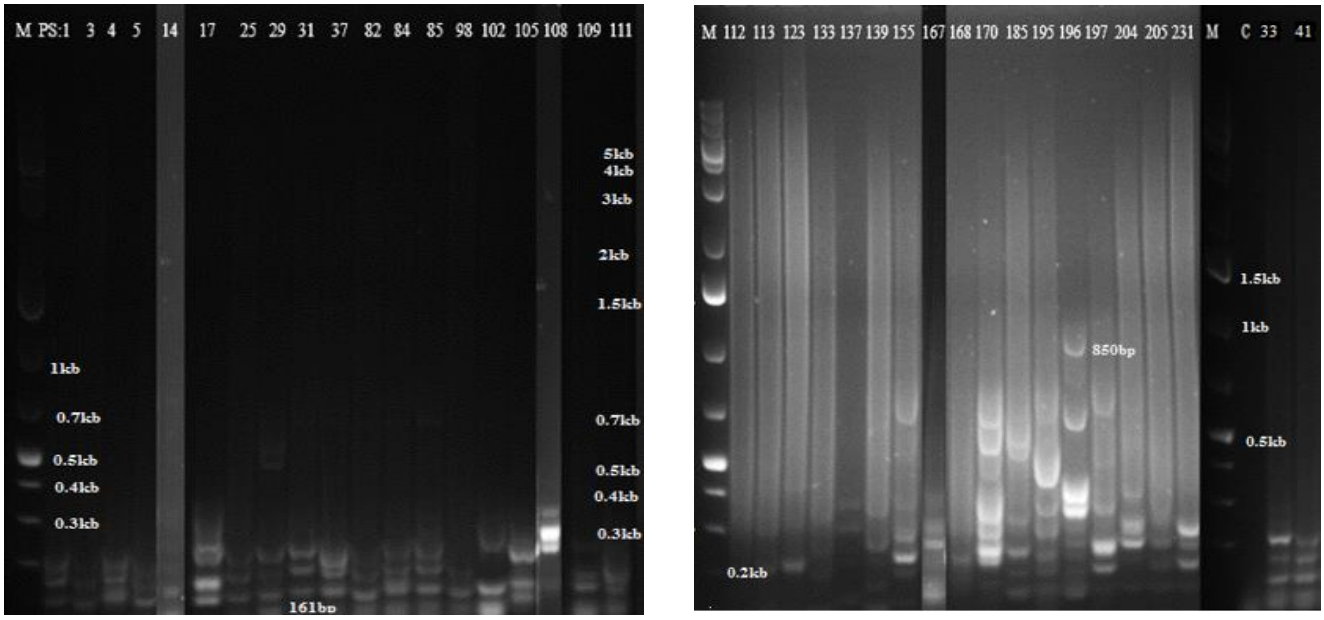


Figure 4.9: Gel image showing amplified ERIC sequences in MDR P. aeruginosa. M: DNA marker, PS1-231: *Pseudomonas aeruginosa* isolate

Genotypically, all the *P. aeruginosa* strains belonged to three groups according to the dendrogram generated from the UPGMA analysis (Figure 4.10). The lowest similarity threshold between the *P. aeruginosa* strains from all the sources was 62% and this showed two major clusters designated M (cluster I) and N (cluster II) (Figure 4.10). Cluster I further differentiated into two sub-clusters M1 and M2. The environmental MDR *P. aeruginosa* isolates were distributed among clusters M and N. The poultry litter isolate also shared similarity with environmental strains from cluster I. Cluster II comprised all the clinical MDR isolates (16) and 28.5% (6/28) of the environmental isolates. Some strains isolated from either sources were phenotypically similar with some strains showing related resistance patterns and plasmid profiles. All the clinical *P. aeruginosa* isolates did not differ by more than two bands and hence could be possibly closely related.

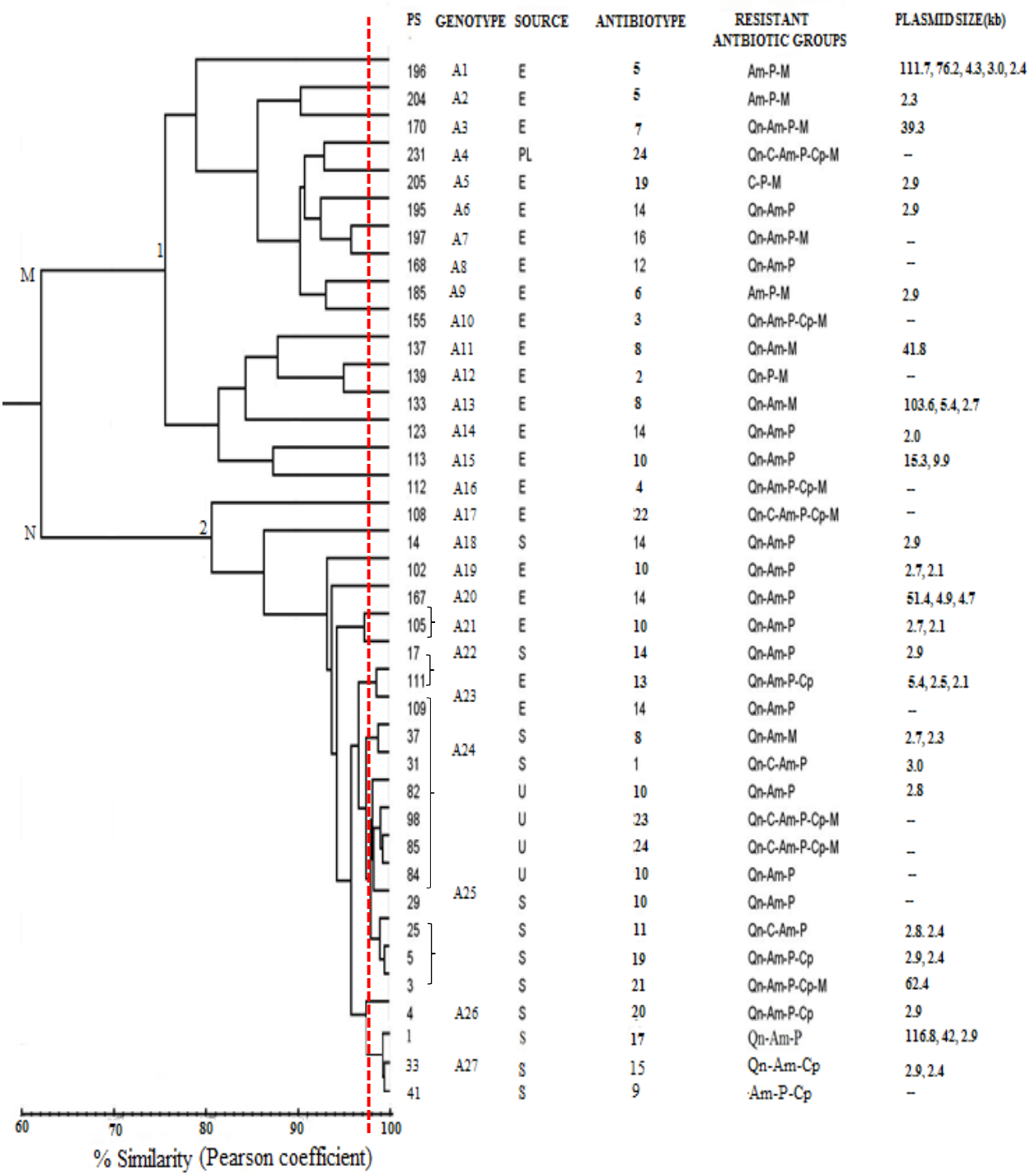


Figure 4.10: Cluster analysis by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) fingerprinting of 38 multidrug resistant *P. aeruginosa* isolates generated by Gelj v.1.2 software. S: Stool; U: urine; E: Environment; Qn:quinolone; Am: aminoglycoside; Cp:cephalosporin; C:carbapenem; P:penicillin; M: Cluster I; N : Cluster II.

None of the isolates showed 100% similarity. At a similarity level of 98%, a total of 27 subtypes (A1-A27) were generated with a discriminatory index (*D*) of 0.9559. 23 distinct subtypes and 4 subtype groups were identified. The MDR poultry litter isolate showed 93% similarity to environmental *P. aeruginosa* strain PS_{205} of subtype A5. Subtypes A23 (PS_{111}, PS_{109}), A24 (PS_{37}, PS_{31}), A25 (PS_{82}, PS_{98}, PS_{85}, PS_{84}, PS_{29}, PS_{25}, PS_{5}, PS_{3}) and A27 (PS_{1}, PS_{33}, PS_{41}) demonstrated >98% similarity indicating possible clonality (Figure 4.10). These strains were prevalent in stool and urine samples from patients as well as the environment indicating possible transfer of related strains between patients and the environment.

All the urine *P. aeruginosa* isolates clustered at subtype A25 indicating possible relatedness of renal inhabited *P. aeruginosa* strains (Figure 4.10). Only three (3) strains of the same genotype group (A25), PS_{82} and PS_{84} from urine and PS_{29} exhibited similar antibiotic resistance pattern. These strains were resistant to the quinolones (ciprofloxacin and levofloxacin) and the penicillin, ticarcillin/ clavulanic acid. Other genotypes however showed varied resistance patterns to the various antibiotics but similarity could be drawn from their resistance to the various antibiotic groups.

P. aeruginosa strains that inhabited the study environments were genetically diverse. The clinical strains however were more closely related with either the same or similar resistance patterns. Strains isolated from stool and urine of infectious patients may be similar to some environmental strains.

4.3.1.2 Mechanism of resistance in MDR *P. aeruginosa*

The routine determination of antibiotic resistance mechanisms in indicator pathogenic bacteria can impact the selection and evaluation of both empiric and definitive antibiotic therapy. Multiple

resistance in a strain of bacteria may be due to a multiplicity of resistance factors which may be inherent in the bacteria or acquired through evolution or the environment. Strains of the same species may exhibit different resistance mechanisms due to gain or loss of specific resistance determining factors and this may select for resistance to particular antibiotics.

4.3.1.2.1 Phenotypic resistance in multi-drug resistant *P. aeruginosa*

4.3.1.2.1.1 Detection of β-lactamase antibiotic degrading enzymes.

Enzymes that hydrolyse the β-lactam ring of antibiotics may be extended spectrum β-lactamases (ESBLS), metallo- β-lactamase (MBLS), inducible cephalosporinases (AmpC) or carbapenamases (KPC) (Strateva and Yordanov, 2009). β-lactamase inhibitors such as clavulanic acid inhibit β-lactamases produced in bacteria and thus may augment the activity of β-lactamase substrate antibiotics (Figure 4.11) (Jiang *et al.,* 2006). It can therefore be used for the detection of enzyme induction in a bacteria species using the double-disc synergy test (DDST). Addition of cloxacillin inhibits the activity of AmpC enzyme (Georgios *et al.,* 2014).

In this study, ESBLS were detected in 84.2% (32/38) of the MDR *P. aeruginosa* isolates. Almost 88% (14/16) of the clinical and 80.9% (17/21) of the environmental MDR P. aeruginosa isolates produced ESBLS indicating a high prevalence of these enzymes in both clinical and environmental *P. aeruginosa.* The MDR *P. aeruginosa* isolate from poultry litter (PS_{231}) was also found to produce ESBLs.

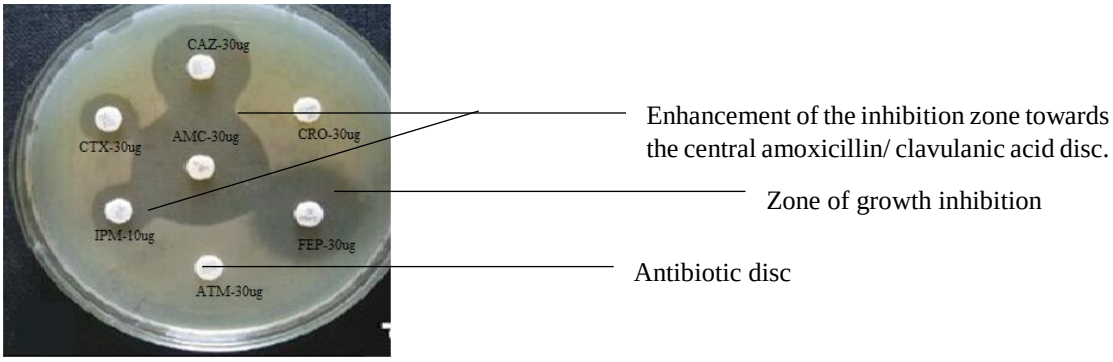


Figure 4.11: Double disk synergy test (DDST) showing production of extended spectrum β-lactamase (ESBL) in MDR *P. aeruginosa*. AMC-30 µg: Amoxicillin/clavulanic acid 30 µg; CAZ-30 µg: Ceftazidime 30 µg; CRO-30 µg: Ceftriaxone 30 µg; FEP-30 µg: Cefepime 30 µg; ATM-30 µg: Aztreonam 30 µg; IPM-10 µg: Imipenem 10 µg; CTX-30 µg: Cefotaxime.

Carbapenemases constitute a group of enzymes that inactivate carbapenems and other groups of β-lactams (Marsik and Nambiar, 2011). Majority of genes that encode carbapenemase production are transferrable by plasmids. These enzymes may act through a zinc residue (metallo-β-lactamases) or a serine residue at the active site. In view of the zinc dependency of metallo-β-lactamases (MBLS), chelators such as EDTA (ethylene-diamine-tetraacetic acid) inhibit MBL activity. The imipenem-EDTA synergy test was used to detect metallo-β-lactamase production in the MDR isolates. KPC, the most common form of serine carbapenemase was determined using the boronic acid test. Genes that control the production of the serine carbapenemases can also be located on plasmids or in the bacteria chromosome (Marsik and Nambiar, 2011).

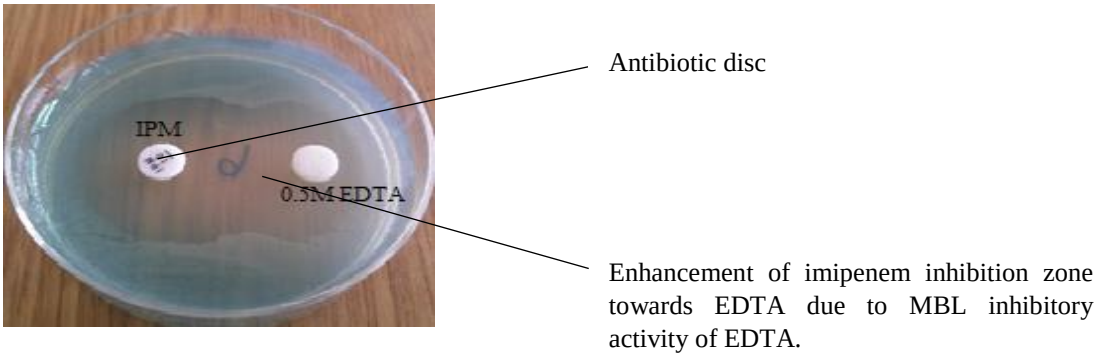


Figure 4.12: Imipenem-EDTA synergy test for detection of metallo- β-lactamases production. IPM: Imipenem EDTA: ethylene-diamine-tetraacetic acid.

Almost 35% (13/38) of the MDR *P. aeruginosa* isolates produced metallo-β-lactamase enzymes. Thirty-one percent (5/16) of the MBL producing strains were isolated from stool and urine samples while 38.1% (8/21) were from environmental isolates. None of the MDR isolate produced KPC-type carbapenemase enzyme.

AmpC-β-lactamase encoding genes may exist in the chromosome or plasmids of *P. aeruginosa.* Chromosomal genes produce low levels of β-lactamases but may be induced by antibiotics which de-repress the gene. Plasmid encoded genes constitutively produce AmpC - β-lactamases. These enzymes have limited activity against carbapenems and monobactam.

Among the MDR isolates assessed for AmpC production (Figure 4.13), 55% produced AmpC - β-lactamases. Nearly 67% of the environmental MDR *P. aeruginos*a strains produced AmpC- β-lactamases compared to 37.5% of the hospital strains. ESBL with AmpC production was prevalent in 44.7% of the MDR strains, followed by production of only ESBLS (34.2%) and ESBL with MBL (28.9%). Two (2) MDR isolates produced only MBL and AmpC .Two isolates produced no β-lactamase (Table 4.6).

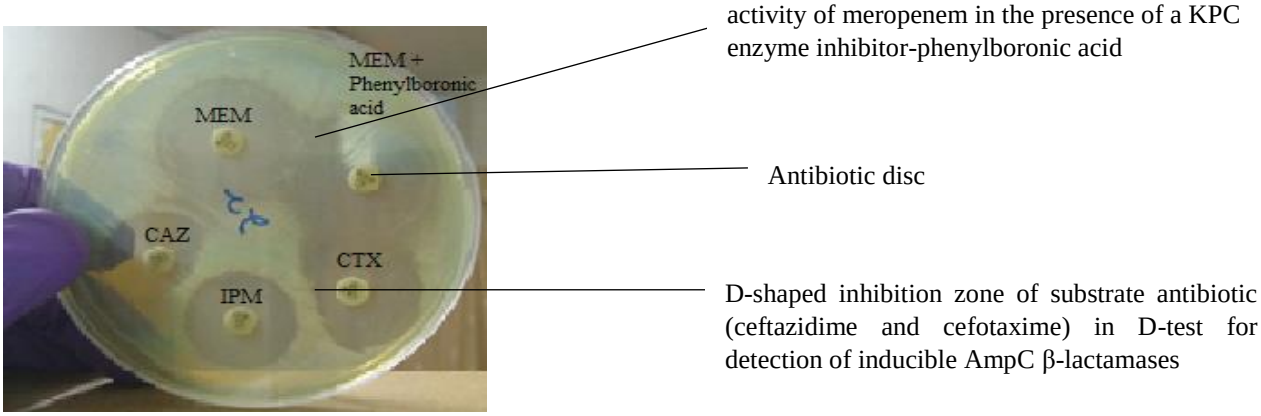


Figure 4.13: Boronic acid test and D-test for detection of KPC-type carbapenemases and inducible AmpC β lactamases. IPM: Imipenem; MEM: Meropenem; CAZ: Ceftazidime; CTX: Cefotaxime

Table 4.5: Prevalence of β-lactamase enzymes in MDR *P. aeruginosa* and the number of resistant isolates

Type of β-lactamase	N (%)	Number of resistant multi-drug *P. aeruginosa* resistant isolates										
		CIP	LEV	MEM	IPM	CN	TIC	PIP	TIM	FEP	CAZ	ATM
No β-lactamase	2(5.3%)	2		1	1	1	2	1	1	1	2	2
Only ESBL,	13(34.2%)	13	7	1	1	13	10	3	5	5	3	4
Only MBL,	2(5.3%)	1				2	1					2
Only AmpC	2(5.3%)	2		1		1	1	1		1		2
ESBL+MBL,	11(28.9%)	8	5	1	2	8	10	2	3		1	2
ESBL+AmpC,	17(44.7%)	15	6	2	4	16	14	3	4	3	3	4
MBL+AmpC,	8(21.1%)	8	4	1	1	8	9	2	2		1	1
ESBL+MBL+AmpC,	9(23.7%)	8	5	1	2	8	9	2	2		1	1

CIP: Ciprofloxacin; LEV: Levofloxacin; MEM: Meropenem; IPM: Imipinem; CN: Gentamycin; TIC: Ticarcillin; TIM: Ticarcillin/Clavulanic acid; FEP: Cefepime; ATM: Aztreonam; CAZ: Ceftazidime; PIP: Piperacillin**;** N: Number of resistant isolates; ESBL: extended spectrum β-lactamase; MBL: metallo-β-lactamase; AmpC: Cephalosporinase.

4.3.2.1.2 Assessment of efflux pump activity in MDR *P. aeruginosa*

Ethidium-bromide (EtBr) intercalates between DNA and produces fluorescence under ultra-violet radiation. It also acts as a substrate for most bacteria efflux pumps. It is thus rapidly pumped out by an overexpressed efflux pump resulting in lack of fluorescence of the bacteria mass (Martins *et al.,* 2011). The minimum concentration of EtBr (MC_{EtBr}) that produced fluorescence of the bacteria mass after incubation for 24 h was used in determining the efflux capacity of the various isolates (Figure 4.14). The capacity of each bacteria strain to efflux EtBr was ranked relative to the reference strain (*P. aeruginosa* ATCC 27853) by calculating the efflux capacity index (σ).

$$\sigma = \frac{MC_{EtBr(MDR)} - MC_{EtBr(Ref)}}{MC_{EtBr(Ref)}}$$

$MC_{EtBr(MDR)}$ = Minimum concentration of EtBr that produced fluorescence in MDR *P. aeruginosa.* MC_{EtBr}(Ref)=Minimum concentration of EtBr that produced fluorescence in *P. aeruginosa* ATCC 27853

Efflux activity was ranked as being very high (σ^{****}=7-9), high (σ^{****}=4-6), moderate (σ^{****}=1-3) and low (σ^{****}=0). A total of 57.8% of the MDR isolates demonstrated moderate to very high efflux pump activity. Forty-two percent showed low efflux capacity. Strains PS_{155} and PS_{111} had the highest efflux capacity (Table 4.7).

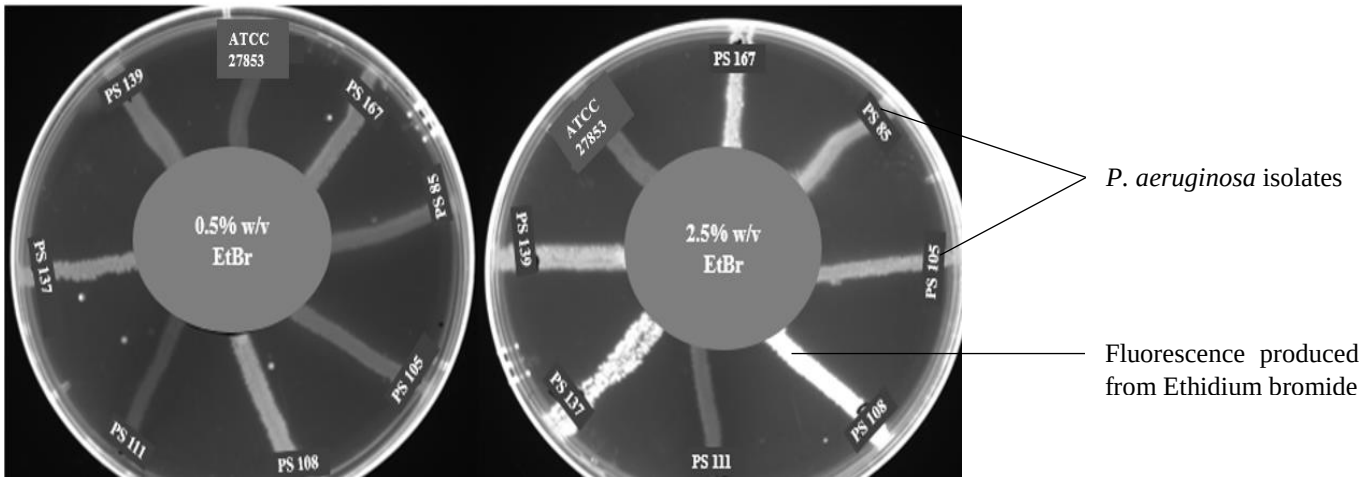


Figure 4.14: Fluorescence of MDR *P. aeruginosa* on Ethedium bromide incorporated Mueller-Hinton agar plates. EtBr: Ethedium bromide

Table 4.6: Efflux capacity of MDR *P. aeruginosa*

Efflux pump activity	Very high(σ^{****})	High(σ^{***})	Moderate (σ^{**})	Low (σ^{*})
Number of MDR isolates	4	4	14	16
%	10.50%	10.50%	36.80%	42.10%

4.3.1.2.2 Molecular detection of some antibiotic resistance genes and resistance determining factors

4.3.1.2.2.1 Detection of bla_{SHV}, bla_{TEM}, bla_{CTMX} bla_{IMP}, bla_{VIM} β-lactamase resistance genes and class 1 integrons in MDR *P. aeruginosa*

Genetic variants of common ESBL encoding antibiotic resistance genes (bla_{SHV}, bla_{TEM}, bla_{CTMX}) were not detected in any of the MDR isolates. The most common MBL encoding genes (bla_{IMP}, bla_{VIM}) were also not detected in any of the isolates. Class 1 integrons, which may habour different antibiotic resistance genes were detected in 89.4% (34/38) of the MDR isolates.

4.3.1.2.2.2 Detection of aminoglycoside acetyltransferase (AAC (3)-IV) enzyme encoding gene

P. aeruginosa may gain resistance to aminoglycosides through a series of resistance mechanisms including enzymatic modification (Meletis and Bagkeri, 2013). The presence of aminoglycoside resistance gene AAC (3)-IV (resistance to gentamycin, tobramycin and netilmicin) was determined in both gentamycin resistant and sensitive MDR isolates of *P.* aeruginosa. AAC (3)-IV gene was not detected in any of the MDR *P. aeruginosa* isolates.

4.3.1.2.2.3 Detection of *gyr*A, *gyr*B and *par*C quinolone resistance genes in MDR *P. aeruginosa*

Quinolones over a long period have remained effective antibacterial agents against *P. aeruginosa.* Structural changes in target enzymes and active efflux are two major mechanisms that lead to resistance in quinolones (Hooper, 2001). Structural enzymatic changes which are engineered by point mutations in genes encoding these enzymes; gyrA and gyrB of DNA gyrase and parC and parE of topoisomerase IV limit the binding of quinolones to the target enzyme rendering them ineffective (Jalal and Wretlind, 1998).

In the current study, high ciprofloxacin resistance (62.1%) was detected in the *P. aeruginosa* isolates. The probability of a multi-drug resistant *P. aeruginosa* isolate being resistant to a quinolone antibiotic was 0.86 indicating high quinolone resistance. *gyrA* antibiotic resistance genes were found in 15.8% (6/38) of the isolates. Two strains isolated from stool (PS_{14}, PS_{37}) and 4 environmental strains (PS_{108}, PS_{109}, PS_{137} and PS_{195}) produced a 360bp amplicon after amplification with *gyr*A specific primers (Figure 4.15). *par*C genes that encode a structurally modified topoisomerase IV enzyme were detected in 5 of the multi-drug resistant isolates (Figure 4.16). Strains PS_{17}, PS_{25} and PS_{41} from stool, PS_{105} and PS_{108} from the environment produced a 209 bp PCR product.

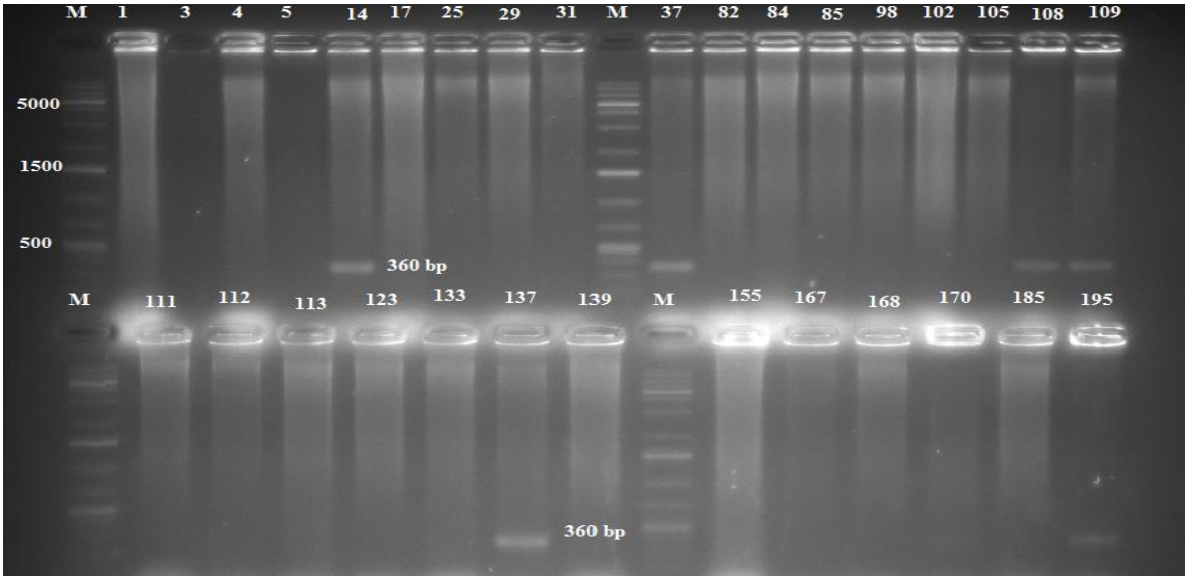


Figure 4.15: Gel image showing amplification of a 360 bp gyrA gene in MDR *P. aeruginosa*. M: molecular; 1- 195: *P. aeruginosa* isolates.

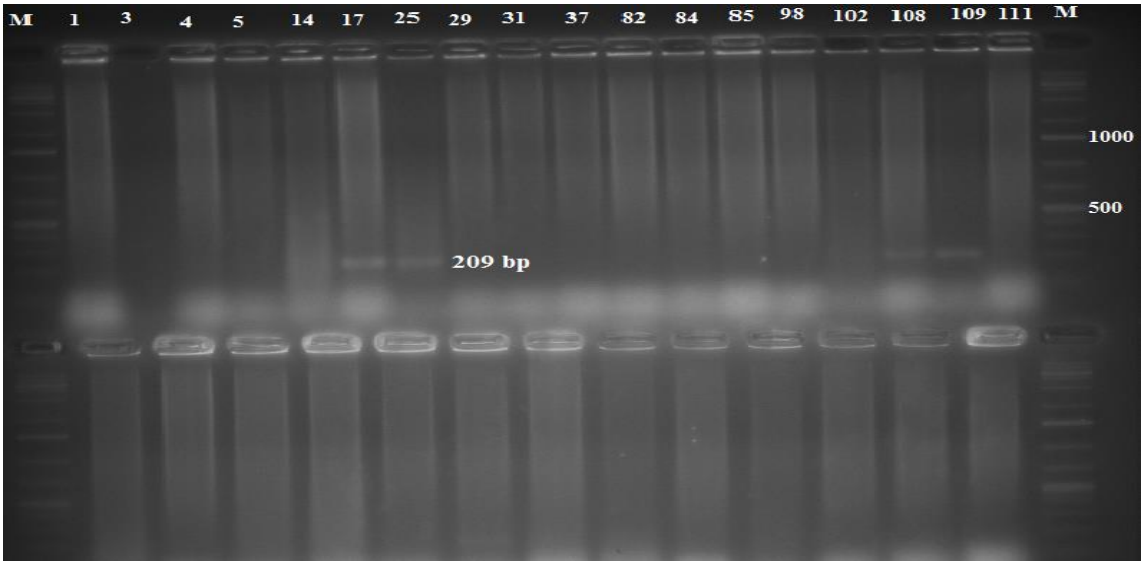


Figure 4.16: Gel image showing 209 bp amplified parC gene in *P. aeruginosa*.
M: molecular marker PS_1- PS_{195}: *P. aeruginosa* isolates.

Twenty six percent of the isolates haboured quinolone resistance genes with *gyr*A gene detected in 13.1%; *par*C gene detected in 10.5% and *gyr*A and *par*C detected in 2.6% of the MDR isolates.

4.3.1.3 Plasmid characterization in multi-drug resistant *P. aeruginosa*

The presence of plasmids in many Gram-negative bacteria impact antibiotic resistance (Bennett, 2008). These extrachromosomal genetic elements carry antibiotic resistance genes and are involved in dissemination of antibiotic resistance genes from one bacteria to another. The plasmid copy number and plasmid size of all the 38 multi-drug resistant strains were determined. Selection pressure and fitness cost may influence the number and size of plasmids in the cell. Large plasmids can harbour cassettes of antibiotic resistance genes to several antibiotics. Twenty five *P. aeruginosa isolates* (65.7%) of all the multidrug resistant isolates harboured between one to five plasmids. Thirteen (34.2%) of the isolates had no plasmids. Nearly 35% had one plasmid, 18.4% had two plasmids and 10.5% had three plasmids and only one isolate haboured five plasmids (Table 4.8, Figure 4.17)

The plasmid sizes of the isolates were determined from calibration curves constructed from plasmids sizes of control *E. coli* V517 and *E. coli* R39 strains and their migration distances (Figure 4.18)

Table 4.7: Distribution of plasmids in multidrug resistant *P. aeruginosa*

No. of plasmids	None	1	2	3	5
No. of MDR isolates	13	13	7	4	1

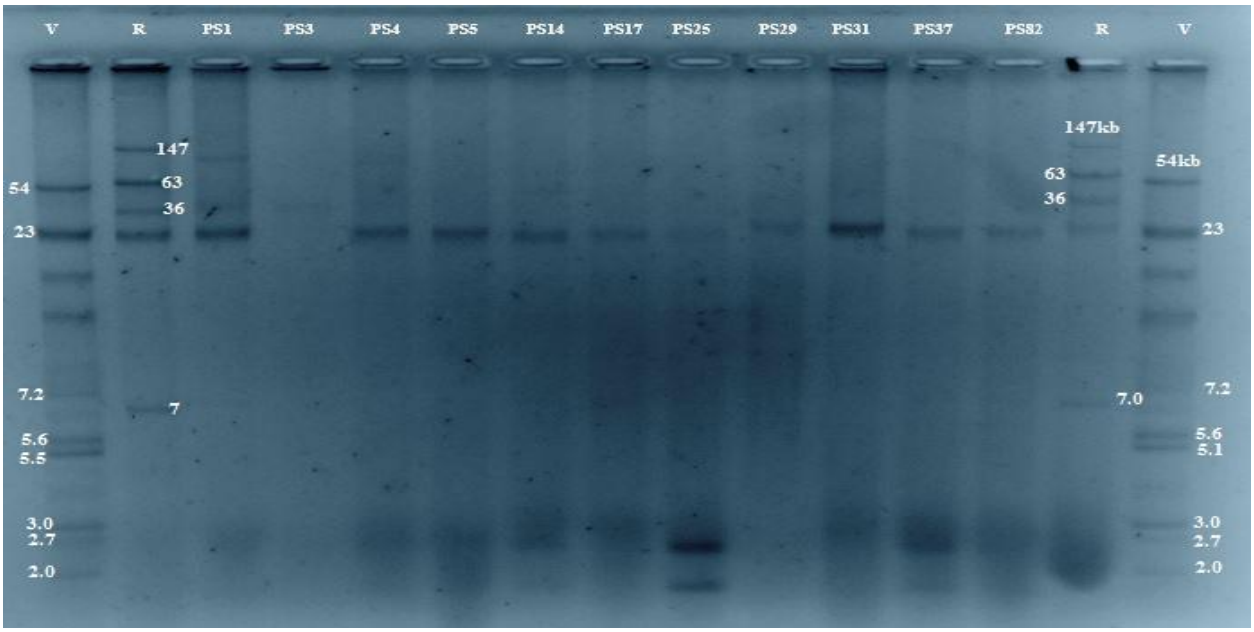


Figure 4.17: Image of a gel showing number and sizes of plasmids in MDR *P. aeruginosa* isolates. V 517= *E. coli* control strain V517; R 39 = *E. coli* control strain R39; C-DNA-Chromosomal DNA

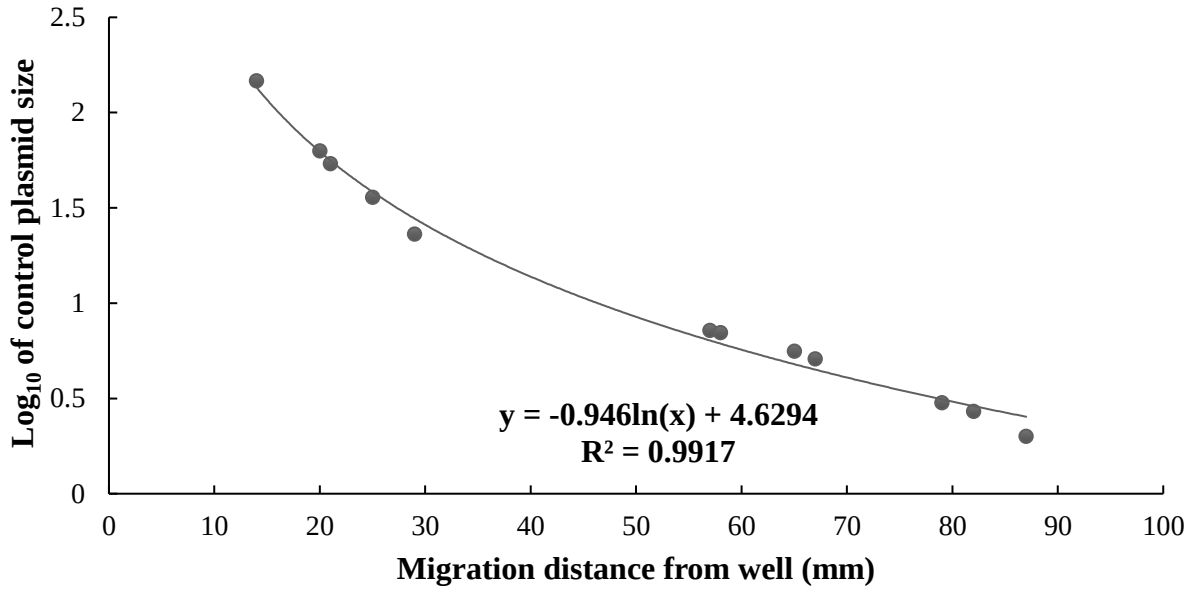


Figure 4.18: Calibration curve for determination of plasmid sizes of *P. aeruginosa*

Table 4.8: Plasmid size distribution among the multidrug resistant *P. aeruginosa* isolates

Plasmid size (kb)	2.0-9.9	10-20	30-50	51-70	71-99	100-110	111-120
Number of strains	24	1	3	2	1	2	2

Table 4.9: Phenotypic and genotypic characteristics of *P. aeruginosa* isolates

					PHENOTYPIC RESISTANCE						ANTIBIOTIC REISISTANCE GENES			
MDR Strain	ERIC type	Source	Antibiogram type	MAI	Plasmid (a+)	ESBL	KPC	MBLS	Inducible AmpC	Efflux capacity index	Class 1 integron	gyrA	gyrB	ParC
PS_{196}	A1	E	5	0.3	5+	+	-	-	+	0	+	-	-	-
PS_{204}	A2	E	5	0.3	+	-	-	+	-	0	+	-	-	-
PS_{170}	A3	E	7	0.5	+	+	-	-	-	0	+	-	-	-
PS_{205}	A5	E	18	0.5	+	+	-	+	+	0	+	-	-	-
PS_{195}	A6	E	14	0.3	+	+	-	-	-	1	+	+	-	-
PS_{197}	A7	E	16	0.6	-	+	-	-	-	7	+	-	-	-
PS_{168}	A8	E	12	0.4	-	+	-	-	+	1	+	-	-	-
PS_{185}	A9	E	6	0.4	+	+	-	+	-	0	+	-	-	-
PS_{155}	A10	E	3	0.5	-	+	-	-	+	9	+	-	-	-
PS_{137}	A11	E	8	0.3	+	-	-	-	+	0	+	+	-	-
PS_{139}	A12	E	2	0.4	-	-	-	-	-	0	-	-	-	-
PS_{133}	A13	E	8	0.3	3+	+	-	-	-	1	+	-	-	-
PS_{123}	A14	E	14	0.3	+	+	-	+	+	0	+	-	-	-
PS_{113}	A15	E	10	0.4	+	+	-	+	+	1	+	-	-	-
PS_{112}	A16	E	4	0.5	-	+	-	-	-	5	+	-	-	-
PS_{108}	A17	E	22	0.7	-	-	-	-	+	0	-	+	-	+
PS_{102}	A19	E	10	0.4	2+	+	-	+	+	1	+	-	-	-
PS_{167}	A20	E	14	0.3	3+	+	-	+	+	0	-	-	-	-
PS_{105}	A21	E	10	0.4	2+	+	-	+	+	5	+	-	-	+
PS_{109}	A23	E	14	0.3	-	+	-	-	+	1	+	+	-	-
PS_{111}	A23	E	13	0.4	+	+	-	-	+	9	+	-	-	-
PS_{231}	A4	PL	24	0.9	-	+	-	-	-	1	+	-	-	-
PS_{14}	A18	S	14	0.3	+	+	-	-	+	0	-	+	-	-

						PHENOTYPIC RESISTANCE					GENOTYPIC RESISTANCE			
MDR strain	ERIC type	Source	Antibiogram type	MAI	Plasmid (a+)	ESBL	KPC	MBLS	Inducible AmpC	Efflux capacity index	Class 1 integron	*gyr*A	*gyr*B	*Par*C
PS_{17}	A22	S	14	0.3	+	+	-	+	+	4	+	-	-	+
PS_{31}	A24	S	1	0.4	+	+	-	-	+	1	+	-	-	-
PS_{37}	A24	S	8	0.3	2+	-	-	+	-	0	+	+	-	-
PS_{5}	A25	S	5	0.5	2+	+	-	-	-	0	+	-	-	-
PS_{25}	A25	S	11	0.5	2+	+	-	+	+	5	+	-	-	+
PS_{29}	A25	S	10	0.4	-	+	-	-	-	0	+	-	-	-
PS_{4}	A26	S	20	0.5	+	+	-	-	-	0	+	-	-	-
PS_{1}	A27	S	17	0.6	3+	+	-	+	+	7	+	-	-	-
PS_{33}	A27	S	15	0.4	+	+	-	-	-	1	+	-	-	-
PS_{41}	A27	S	9	0.3	-	+	-	+	-	0	+	-	-	+
PS_{82}	A25	U	10	0.4	+	+	-	-	-	1	+	-	-	-
PS_{84}	A25	U	10	0.4	-	+	-	-	-	0	+	-	-	-
PS_{85}	A25	U	24	0.9	-	-	-	-	-	1	+	-	-	-
PS_{98}	A25	U	23	0.9	-	+	-	-	+	3	+	-	-	-

+ (present); 2+ (two plasmids present) a: Plasmid copy number); A1-A27: ERIC genotype; MAI: multiple antibiotic resistance index; ESBL: Extended spectrum beta-lactamase; MBL: Metallo beta-lactamase; *gyr*A, *gyr*B, *par*C: Quinolone resistance genes; S: Stool; U: Urine; Environment; PL: Poultry litter.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

Microorganisms including bacteria, fungi and viruses have generated much attention due to their positive and negative impacts on human health. Some of these microorganisms have an established symbiotic relationship with humans providing some essential growth factors and preventing colonization of pathogenic ones (Reichenberger *et al.*, 2013).

Pathogenic bacteria over time have become increasingly virulent and problematically resistant to most common antibiotics. The surge in the prevalence and spread of these resistant species of bacteria has been a major public health concern, raising the need for increased local, regional, national and global surveillance. The undertaken study is an effort to establish the distribution and characteristics of these virulent and resistant organisms as well as assess the impact of anthropogenic factors on the emergence and spread of these bacteria. The World Health Organisation and a host of other research groups have made efforts to paint a global picture of the problem of antibiotic resistance. These studies have however been largely limited to high income countries. Data on the distribution and characteristics of common indicator bacteria from many countries in the African region including Ghana is limited due to lack of adequate resources. Resistance characteristics that may be developed by a bacteria from the wild type strain may be geographically dependent, hence localized surveillance of resistance and resistance determining factors is key to combating the problem of antibiotic resistance.

Newman *et al.* (2015) in a recent study sought to generate baseline data on antibiotic resistance and to assess the readiness of Ghana in laboratory-based surveillance. The study reported

predominant occurrence of *Escherichia coli*, *Pseudomonas spp*, *Staphylococcus aureus*, *Streptococcus spp* and *Salmonella enterica serovar typhi* with most of the isolates being multi-drug resistant and over 80% producing ESBLs. However, a lot of the samples used for this surveillance were from clinical sources with blood, urine, stool, ear, wounds and aspirate habouring most of the identified bacteria, leaving a gap in the AMR state of strains from other environmental sources.

Infections caused by *P. aeruginosa*, an ubiquitous member of the genus *Pseudomonas* are very difficult to treat (Krieg *et al.,* 2005). It mostly causes nosocomial infections especially in immune-compromised patients, damaging multiple organs (Carmeli *et al.,* 1999). Treatment of these infections have therefore been limited to a few class of antibacterials usually referred to as antipseudomonal antibiotics. Selection of multi-drug resistant, extensively drug resistant and pandrug resistant strains through continuous use of subinhibitory antibiotic concentrations in varied environments such as human medicine, aquaculture and animal husbandry may gradually alter the susceptibility of *P. aeruginosa* and other bacteria species to established antipseudomonal agents as well as common antibiotics. Resistant bacteria selected from these environments may be disseminated between humans, animals and the environment through waste products such as human excreta and manure from these animals.

Boamah (2015) and Boamah *et al.* (2016) reported that most poultry farmers in Ghana including the Ashanti region employ antibiotic containing agents for prophylactic, metaphylactic and treatment purposes in poultry production. These may leave residual concentrations of these antibiotics on the farms and hence select for resistance strains in resident bacteria. It has also been reported that 93.6% of most fish farmers in Ashanti region use poultry manure for fertilizing ponds (Agoba, 2015). Manure from poultry are heavily employed to fertilize soil for growth of cabbage,

carrots and a range of agricultural produce. Thus resistant bacteria strains in poultry litter may easily be disseminated in the environment.

Routine surveillance of the distribution, resistance pattern and resistance mechanisms of common pathogenic bacteria including *P. aeruginosa* strains from various sources thus provide local data on the effectiveness of these antibiotics as well as their resistance pathways. This goes to inform antipseudomonal selection for both empiric and definitive therapy.

In this study, blood, urine and stool samples from patients together with environmental samples from market tables and floors, soil, community based latrines, domestic water sources, sewages and poultry litter from various poultry farms were screened for the presence of *P. aeruginosa*. Pathogenic bacteria from these environments have a greater chance of coming in direct contact with resident human population.

The study of any bacteria starts with thorough identification of the species of interest. Differentiating strains based on morphological and biochemical characteristics has been traditionally utilized as the primary means of identifying bacteria (Woo *et al.,* 2000). Over time, analytical techniques have evolved due to the advancements in molecular diagnostics enabling rapid and definitive identification of bacteria (Wiedmann *et al.*, 2000). Morphologically, all the *P. aeruginosa* isolates were Gram-negative unicellular rods appearing as mucoid or non-mucoid colonies. The mucoid form is mainly due to alginate slime formation which is presumed to play a role in colonisation and virulence (Todar, 2004). All strains were catalase and oxidase positive. Definitive identification of the species, *Pseudomonas aeruginosa,* includes identifying the production of the soluble pigments, pyocyanin (blue-green) (Kanner *et al.,* 1978), fluorescein

(greenish-yellow), pyorubin (red) (Kandela *et al.,* 1997) or pyomelanin (reddish-brown) (Yabuuchi and Ohyama, 1972), growth at 42ºC and β-haemolysis on blood agar. Pyocyanin enhances the virulence of *P. aeruginosa* while pyorubin and pyomelanin function in protecting against oxidative stress (Ferguson, 2007). Pyoverdine acts as a siderophore binding and transporting soluble iron (Fe^{3+}) from the environment under iron deficient conditions. The morphological and biochemical characteristics of the isolates were consistent with the description of typical *Pseudomonas aeruginosa* according to Cowan and Steel (1993) and Bergey's Manual for Systematic Bacteriology (2005).

Complementary confirmation of *P. aeruginosa* by molecular amplification of the conserved outer membrane lipoprotein L gene (*opr*L) was found to be highly sensitive in both pigmenting and non-pigmenting strains. Thirty eight *P. aeruginosa* strains from both clinical, environmental and poultry litter sources which failed to produce characteristic pigments were identified though *opr*L gene amplification. This is consistent with reports by De Vos *et al.* (1997) on the sensitivity of *opr*L outer membrane gene amplification in the identification of *P. aeruginosa* from both clinical and environmental sources.

There was low prevalence of *P. aeruginosa* (9.6%) in all the collected samples from the various sources. Among the clinical samples, *P. aeruginosa* was significantly present in stool samples (39.7%) than in urine samples (15.4%) of patients (p=0.00077). This may be so because aside the resident *P. aeruginosa* colonisation of the gastrointestinal tract, most ingested food, especially uncooked foods like salads (Mensah *et al.*, 2002) and slightly cooked foods like macroni (Feglo and Sakyi, 2012) may be contaminated by *P. aeruginosa* and other pathogenic bacteria which may increase its colonization of the gastrointestinal tract and hence its high prevalence in stool. All

blood samples from the patients did not contain *P. aeruginosa*. The absence of *P. aeruginosa* in the blood samples however contradicts a previous report by Opoku (2013) from Komfo Anokye Teaching Hospital in which 11.83% of 187 *P. aeruginosa* isolates were obtained from blood sampled from patients. This suggests that, patients who presented to the hospital (study site) during the study period had no sepsis caused by *P. aeruginosa.*

Susceptibility of all the *P. aeruginosa* isolates to six antipseudomonal classes which include quinolones (ciprofloxacin and levofloxacin), aminoglycosides (gentamicin), penicillins (ticarcillin, piperacillin, ticarcillin/clavulanic acid), monobactams (aztreonam), cephalosporins (cefepime and ceftazidime) and carbapenems (meropenem and imipenem) were determined.

Among the antibiotics, ceftazidime was active against 90% of all the *P. aeruginosa* isolates. Also, susceptibility rates of the isolates to piperacillin, meropenem, cefepime, levofloxacin and imipenem ranged between 70% and 85%. The high activity of these antibiotics in *P. aeruginosa* may be due to the infrequent use of these antibiotics both in community and clinical settings in the country. Seventy five percent of the *P. aeruginosa* isolates were resistant to more than a single antipseudomonal agent with resistance to gentamicin (69%), ciprofloxacin (62%), ticarcillin (56%) and aztreonam (25%) being the most frequent. All the *P. aeruginosa* isolates showed intermediate susceptibility to aztreonam. According to Pitondo-Silva *et al.* (2014), high resistance rate of *P. aeruginosa* to ticarcillin and aztreonam have also been observed in countries such as Brazil.

From the study, nearly half (43.6%) of the isolates were resistant to at least three antipseudomonal groups. Comparing these findings to a study by Addo (2015) who reported 13.04% MDR *in P. aeruginosa* isolates from wounds of patients shows an increment in the number of MDR strains in the environment. Sixty percent (60%) to 100% of the *P. aeruginosa* isolates from the various

sources were susceptible to cefepime, ceftazidime, piperacillin, levofloxacin, meropenem and imipenem. This suggests that these antipseudomonals may still remain effective in management of *P. aeruginosa* infections. Equally high susceptibility of clinical *P. aeruginosa* isolates to meropenem and ceftazidime confirms the reports of Feglo and Opoku (2014). Isolates from the environment exhibited low level of susceptibility to ciprofloxacin and gentamicin. This however is inconsistent with reports by Addo (2015) and Feglo and Opoku (2014) on the antibiogram studies of *P. aeruginosa* isolates from other hospitals in the Ashanti and Greater Accra regions of Ghana. Similarly, high ciprofloxacin and gentamicin resistance has been reported in Gram-negative isolates from the southern sector of Ghana. This may suggest the ineffectiveness of these antibiotics in treatment of infections in which *P. aeruginosa* is implicated. High quinolone-aminoglycoside (ciprofloxacin-gentamicin) cross-resistance was also observed in the current study. This finding is similar to those from a week nationwide study of *P. aeruginosa* isolates from 136 hospitals in Spain and burn patients from Iran (Japoni *et al.,* 2006; Bouza *et al.,* 1999). The changes in the susceptibility patterns of the isolates may be accounted for by the different antibiotic use practices in environments where samples were collected.

Carbapenem resistant isolates of *P. aeruginosa* were found to show cross-resistance to antibiotics from multiple classes including ciprofloxacin, gentamicin, ticarcillin, piperacillin and aztreonam. This high cross-resistance of the isolates suggests a combination of multiple unrelated resistance mechanisms among the isolates. Also, significant differences in the antipseudomonal activity of antibiotics from the same class (quinolone and penicillin groups) implies regulation of resistance by different mechanisms (Sefton, 2002).

The multiplicity of mechanisms and resistance determining factors that may have regulated the resistance of *P. aeruginosa* to quinolones, aminoglycosides and the β-lactam antibiotics were evident in the selected multi-drug resistant strains. β-lactamase antibiotic resistance genes including bla_{SHV}, bla_{TEM}, bla_{CTX-M}, bla_{VIM} and bla_{IMP} were not detected in any of the isolates. A similar study by Addo (2015) in the Korle Bu Teaching Hospital and Koforidua regional hospitals also found no bla_{VIM} and bla_{IMP} carbapenemase encoding genes in *P. aeruginosa* isolates fom diabetic, burn and cellulitic wounds of patients suggesting their low prevalence in *P. aeruginosa* in Ghana.

Mobile genetic elements (integrons and plasmids) which are known to habour varied antibiotic resistant genes were prevalent in the *P. aeruginosa* isolates. Class I integron resistant gene cassettes were identified in 89.3% of the MDR isolates but no Class II or III integrons were identified. Also, extrachromosomal genetic material (plasmids) were prevalent in 65% of the MDR strains with sizes ranging from 2.0 kb to 116.8 kb.

Characterisation of *P. aeruginosa* class I integron gene cassettes through sequencing and gene cloning in other related studies have revealed the prevalence of quaternary ammonium compound resistance gene (qacEΔ1), carbapenem resistance gene (blaVIM-2), aminoglycoside modifying enzyme encoding genes (aacA4, aac(6)-Ib, aac(6')-IIa, aac(6')-Ib, aadA1), broad spectrum β-lactam resistance genes (blaOXA-1, blaOXA-10) and folate-pathway inhibitor resistance genes (dfrA17) in most strains of *P. aeruginosa* (Hwan *et al.,* 2014). These resistance gene cassettes are inserted at the attI site downstream of the class I integron using an integron-mediated circular gene cassette capturing system with the help of an integrase gene. Expression of integron cassette genes depends on the gene copy number, promoter strength and relative distance of the gene cassette

from the promoter (Martinez-Freijo *et al.,* 1998). The regulation of these gene cassettes under the control of a single promoter (Pc) strongly expresses these antibiotic resistance genes and may cause cross-resistance to varied antipseudomonal agents (Levesque *et al.,* 1994). The high prevalence of plasmids and integrons may suggest an association enabling wide dissemination of other antibiotic resistance genes leading to the observed antibiotic resistance pattern (Leverstein *et al.,* 2002).

Even though some common β-lactamase antibiotic resistant genes were not detected, enzymes that are products of these genes were phenotypically detected in the isolates. About 5% of the study isolates produced no β-lactamase enzymes. Even though extended spectrum β-lactamase (ESBLS) enzymes were prevalent in 84.2% of the MDR isolates only 34.2% produced only ESBLs. This is similar to the findings of Newman *et al.* (2015) who detected high ESBL production (90 to 98%) in Gram-negative isolates from the southern, middle and northern sectors of Ghana. Metallo-enzymes (MBLs) and inducible AmpC β-lactamases were detected in 34.2% and 50% of the MDR isolates, respectively. This indicates that other β-lactamase encoding genes such *bla*$_{PER}$, *bla*$_{VEB}$, *bla*$_{GES,}$ *bla*$_{PSE}$, *bla*$_{SPM}$, *bla*$_{GIM}$, *bla*$_{AIM}$, *bla*$_{NDM}$, AmpC and *bla*$_{OXA}$ which have been found in *P. aeruginosa* from other geographical regions (Strateva and Yordanov, 2009) may be responsible for the regulation of β-lactamase enzyme production.

None of the *P. aeruginosa* isolates produced KPC type carbapenemase enzymes. Co-production of ESBL, MBL and AmpC were predominant in the isolates with occurrence rates ranging from 21.1 to 44.7%. ESBL and AmpC co-production were the most prevalent (44.7%) in the MDR isolate but the carbapenems (meropenem and imipenem) remained effective against these enzyme

producers. ESBL producers were also susceptible to carbapenems as reported by (Feglo and Opoku, 2014) and this confirms their role in the definitive treatment of ESBL producing bacteria strain infections. In a related report by Feglo and Opoku (2014) on 187 *P. aeruginosa* isolates from Kumasi, 44.9% were AmpC producers and 21.9% were ESBL producers. From the above, these findings indicate an increase in both ESBLs and MBLs prevalence.

The prevalence of AmpC was relatively high compared to other study reports in Delhi, India (20.7%) (Manchanda and Singh, 2003). MBL prevalence was similar to findings from Brazil which reported 36.4% ± 14.1 MBL occurrence in *P. aeruginosa* (Zavascki *et al.*, 2007). These findings could indicate geographical variations in the prevalence of AmpC producing *P. aeruginosa* and may be due to low usage of cephalosporinases that induce production of this enzyme.

From the study, resistance of *P. aeruginosa* to quinolones was partly regulated by both mutations in gyrA and parC subunits of DNA gyrase and topoisomerase IV (Table 4.10). Sixty three percent (63.15%) of the selected MDR strains haboured no quinolone resistance genes but showed resistance to either ciprofloxacin, levofloxacin or both (Table 4.10). This indicates that, aside mutations in resistance genes other resistance mechanisms such as over-expression of efflux pumps could also be largely responsible for mediation of quinolone resistance among the *P. aeruginosa* isolates (Strateva and Yordanov, 2009). This is evident in the pronounced efflux capacity (57.8%) of the MDR strains showing moderate to very high efflux capacity of most of the strains compared to the reference *P. aeruginosa* ATCC 27853 strain.

P. aeruginosa isolates from the clinical, environmental and poultry litter sources showed both phenotypic and genotypic relatedness with only a few strains being diverse. Genotyping of bacteria has been widely accepted as ideal in establishing strain relatedness compared to phenotypic methods like antibiotyping, ribotyping and serotyping due to their high discriminatory power. Phenotypic methods have only modest reproducibility mainly because most bacteria can unpredictably alter the expression of many cellular products (Tenover *et al.,* 1997). In this study, antibiogram typing was employed as a phenotypic method and enterobacterial repetitive intergenic consensus sequence typing was used as a genotypic method to establish the relatedness among the bacteria strains from the various sources.

Antibiogram typing of the isolates was found to be highly discriminatory among the *P. aeruginosa* isolates (D=0.9502), differentiating the isolates into 24 antibiotypes with 5 clusters and 19 distinct antibiotypes. Therefore it can be said that, appropriate selection of antibiotics would enable good discrimination of bacteria based on their susceptibility profiles. Hence, the choice of antibiotics selected were appropriate for typing of the *P. aeruginosa* isolates from Kumasi, Ghana.

Pulsed field gel electrophoresis (PFGE), described as the gold standard for genotypic typing of *P. aeruginosa* (Tenover *et al.,* 1997) is costly, technically demanding and time consuming. Another method which has gained wide usage and even said to be more suitable than PFGE is multi-locus strain typing (MLST) (Ranjbar *et al.,* 2014). This method relies on determining allelic variations in seven housekeeping genes as a means of monitoring changes in a small portion of the genome. The method employed in this study (Enterobacterial Repetitive Intergenic Consensus Sequence Typing (ERIC-PCR), which creates a genomic fingerprint map of the bacteria through amplification of conserved intergenic repetitive sequences has also been described to demonstrate

high level concordance, discrimination (Syrmis *et al.,* 2004, Bruijn, 1992) and typeability of *P. aeruginosa* comparable to PFGE and MLST (Kidd *et al.,* 2011). The method was found to have a good discriminatory power (D=0.9559) but not significantly different from the antibiogram typing method.

P. aeruginosa isolated from the three sampling sites belonged to 3 groups with 62% similarity. This indicates high genetic similarity between *P. aeruginosa* strains from stool, urine, and environmental samples. All the clinical isolates belonged to the same cluster indicating a close genetic relationship between them. Clinical strains however were more closely related than environmental isolates (Figure 4.10). Some environmental strains showed some similarity with clinical strains (PS105 from environmental sources and PS17 from stool) suggesting possible exchange of resistant bacteria between humans and the environment. Among the clinical isolates sampled from different patients, some strains were genotypically similar. Two strains of *P. aeruginosa* (PS_{84} from urine and PS_{29} from stool) which showed >98% genetic similarity, had the same antibiogram pattern, plasmid profile and resistance mechanisms (Table 4.10), suggesting clonality and possible transfer between the two patients who had been visiting the same hospital. Cross-infection of the patients could have resulted through acquisition of nosocomial MDR *P. aeruginosa* strains in the hospital. The genotypic relatedness of the clinical strains may also indicate nosocomial transmission of related bacteria strains to patients visiting the study hospital.

The findings of this study have implications for both empirical and definitive treatment of *P. aeruginosa* infections and should be considered when making decisions regarding selection of antibiotics for management of nosocomial and community acquired *P. aeruginosa* infections. The

study revealed high prevalence of *P. aeruginosa* in stool samples of patients suggesting that hygienic management of human waste is very crucial to preventing cross-infection. Also, the prevalence of quinolone resistant genes and a surge in the occurrence of ESBL, MBL and AmpC enzyme producing *P. aeruginosa* was demonstrated, calling for the routine examination of the phenotypic and genotypic resistance mechanisms of *P. aeruginosa* isolated in the various hospitals so as to guide antibiotic selection during therapy.

5.2 CONCLUSION

A total of 900 samples from blood, urine, stool, market tables and floors, soil, community based latrines, domestic water sources, sewages and poultry litter from various poultry farms were screened for the presence of *P. aeruginosa*. Of the 900 samples, 87 *P. aeruginosa* isolates were confirmed. There was low prevalence (1.8%) of *P. aeruginosa* on the various poultry farms. Among stool, urine and blood samples of patients, *P. aeruginosa* colonisation was more prevalent in stool (39.7%). Gentamicin and ciprofloxacin were not active against most of the *P. aeruginosa* isolates. The carbapenems (meropenem and imipenem), piperacillin and cefepime, ceftazidime were highly active against the *P. aeruginosa* isolates. Carbapenem resistant strains of *P. aeruginosa* were found to show cross-resistance to antibiotics from multiple classes including ciprofloxacin, gentamicin, ticarcillin, piperacillin and aztreonam. Also, there was high occurrence (45.1%) of multi-drug resistant *P. aeruginosa* in clinical and environmental samples having moderate to very high efflux capacity. There was high prevalence of extended spectrum β-lactamase (ESBLs), metallo β-lactamase (MBLs) and inducible cephalosporinase (AmpC) enzymes in the *P. aeruginosa* isolates but no KPC type carbapenemases were detected in any of the strains. ESBL and AmpC producing *P. aeruginosa* were susceptible to meropenem. Quinolone resistant genes (*gyr*A and *par*C) showing mutations in gyrA subunits of DNA gyrase and parC

subunits topoisomerase IV were detected in 26.3% of the isolates. Mobile genetic elements (plasmids and class I integrons) were highly prevalent in *P. aeruginosa* with plasmid sizes ranging from 2.0 kb to 116.8 kb. Isolates from all the three sources were genotypically related (62%) with some strains having similar resistance patterns. Clinical strains were more closely related than environmental isolates. The above findings suggest possible transfer of multi-drug resistant *P. aeruginosa* strains between humans and the environment.

5.3 RECOMMENDATIONS

- The use of ciprofloxacin and gentamicin in the treatment of *P. aeruginosa* should be further re-evaluated and further decision taken on the use of these agents in the treatment of *P. aeruginosa* infections.
- Meropenem should be maintained as the last line of defense against multi-drug resistant *P. aeruginosa.*
- Routine localized surveillance and characterization of *P. aeruginosa* as well as other indicator pathogenic bacteria should be conducted on regional and national bases.
- Phenotypic and genotypic determination of antibiotic resistance mechanisms should be routinely done to complement susceptibility studies so as to inform selection of antibiotics and to identify developing areas of high antibiotic resistance selection.
- Mobile genetic elements (plasmids and integrons) which enable transfer of resistant genes among bacteria should be adequately characterized to determine the resistance gene cassettes.
- Health workers should be adequately informed about the emergence of high ESBL, MBL and AmpC β-lactamase enzyme producing bacteria and their negative implications on antibiotic therapy.

- Typing techniques should be routinely employed in the various district, regional and teaching hospitals to track the source and dissemination of MDR bacteria among patients.

REFERENCES.

Aarts, H. J. M., Boumedine, S., K., Nesme, X. and Cloeckaert, A. (2001). Molecular tools for the characterisation of antibiotic-resistant bacteria. *Veterinary Research*, 32:363–380.

Abavelim, D. A. (2014). Determination of antibiotic residues in beef and mutton from selected markets in Kumasi, Ghana. Master of Philosophy (Mphil) thesis submitted to the *Kwame Nkrumah University of Science and Technology*, Kumasi, Ghana, 1-72.

Abdullahi, R., Lihan, S., Carlos, B. S., Bilung, M. L., Mikal, M. K. and Collick, F. (2013). Detection of oprL gene and antibiotic resistance of *Pseudomonas aeruginosa* from aquaculture environment. *European Journal of Experimental Biology*, 3(6):148–152.

Abraham, E. P., and E. Chain. (1940). An enzyme from bacteria able to destroy penicillin. *Reviews of Infectious Disease,* 10:677–678.

Achtman, M. (2002). A Phylogenetic Perspective on Molecular Epidemiology, in: *Max Sussman Molecular Medical Microbiology*, Medical School, Newcastle upon Tyne, UK, 486–502.

Addo, F. A. (2015). Characterization of antimicrobial resistant *Pseudomonas spp*. And other bacteria from infected wound. Master of Philosophy thesis submitted to the *University of Ghana*, Legon, 1-72.

Agoba, E. E. (2015). Antibiotic use in selected fish farms in the Ashanti region, Ghana. Master of Philosophy thesis submitted to the Department of Pharmaceutics, *Kwame Nkrumah University of Science and Technology*, 1-45.

Ambler, R. P. (1980). The structure of β-lactamases. *Philosophical Transactions of the Royal Society,* 289:321-331.

Ampofo, J. A., Clerk, G. C. (2010). Diversity of bacteria contaminants in tissues of fish cultured in organic waste-fertilized ponds: health implications. *Open Fish Science Journal*, 3: 142-146.

Barr, V., K. Barr, M.R. Millar, and R.W. Lacey. (1986). β -Lactam antibiotics increase the frequency of plasmid transfer in *Staphylococcus aureus*. *Journal Antimicrobial. Chemotherapy*, 17:409–413.

Bauer, A.W., Kirby, W. M. M., Sherris, J.C. and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, 45(4): 493.

Belkum, A. V., Tassios, P. T., Dijkshoorn, L., Haeggman, S., Cookson, B., Fry, N. K. and Fussing, V. (2007). Guidelines for the validation and application of typing methods for use in bacterial epidemiology. *Clinical Microbiology and Infectious Diseases*, 13:1–46.

Bennett, P. M. (2008). Plasmid encoded antibiotic resistance : acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology*, 347–357.

Betty, F., Daniel, S., Alice, W., (2007). *Bailey & Scott's Diagnostic Microbiology.* Elsevier Science Publishing Company, New York, 12th edition: 322-325.

Bieranye, S. B. M. S. (2011). Antibiotic resistance patterns of strains of *Salmonella typhi* and staphylococcus aureus isolated from patients in three hospitals in Kumasi. Master of Philosophy thesis submitted to Department of Pharmaceutics, *Kwame Nkrumah University of Science and Technology* Ghana, 109.

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O. and Piddock, L. J. V. (2014). Molecular mechanisms of antibiotic resistance. *Nature Reviews,* 13(1):42–51.

Boamah, V. E. (2015). Susceptibility patterns and prevalence of antibiotic resistant genes of *Escherichia coli* and *Staphylococcus* species isolated from poultry farms in the Ashanti, Brong Ahafo and Greater Accra regions of Ghana. Doctor of philosophy (Phd) thesis submitted to the *Kwame Nkrumah University of Science and Technology*, 1-202

Boamah, V. E., Agyare C., Odoi H., Dalsgaard A. (2016). Practices and factors influencing the use of antibiotics in selected poultry farms in Ghana. *Journal of Antimicrobial Agents, 1-6*

Bockstael, K. and Aerschot, A. (2009). Antimicrobial resistance in bacteria. *Central European Journal of Medicine*, 4(2):141–155.

Boeckel, T. P. Van, Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., Teillant, A. and Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences*, 112(18):5649 – 5654

Boeckel, T. P. Van, Gandra, S., Ashok, A., Caudron, Q., Grenfell, B. T., Levin, S. A. and Laxminarayan, R. (2014). Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. *The Lancet Infectious Diseases,* 14(8):742–750.

Bogaard, A. E. and Stobberingh, E. E. (2000). Epidemiology of resistance to antibiotics. Links between animals and humans. *International Journal of Antimicrobial Agents,* 14(4):327–35.

Bouza, E., Garcia-Garrote, F., Cercenado, E., Marin, M. and Diaz, M. S. (1999). *Pseudomonas aeruginosa:* A survey of resistance in 136 hospitals in Spain. *Antimicrobial Agents and Chemotherapy*, 43(4):981–982.

Brian, G. S., Feil, E. J. and Noel, H. S. (2002). Population Genetics of Bacterial Pathogens, in: *Max Sussman Molecular Medical Microbiology*, 535–545.

Bruijn, F. J. D. E. (1992). Use of Repetitive Extragenic Palindromic and Enterobacterial Repetitive Intergeneric Consensus) Sequences and the Polymerase Chain Reaction to Fingerprint the

Genomes of *Rhizobium meliloti* Isolates and Other Soil Bacteria *Applied and Environmental Microbiology,* 58(7), 2180-2187.

Brüssow, H., C. Canchaya, and Hardt, W.D. (2004). Phages and the evolution of bacterial pathogens: From genomic rearragements to lysogenic conversion. *Microbiology and Molecular Biology Review*, 68:560–602.

Bush, K, Jacoby GA, Medeiros, A.A. (1995). A functional classification scheme for β-lactamases and its correlation with molecular structure. *Antimicrobial Agents of Chemotherapy*, 39:1211-1233.

Cambray, G., Guerout, A.-M., Mazel, D. (2010). Integrons. *Annual Review of Genetics,* 44(1):141–166.

Carmeli, Y; Troillet, N; Eliopoulos, GM; Samore, MH (1999). "Emergence of Antibiotic-Resistant *Pseudomonas aeruginosa:* Comparison of Risks Associated with Different Antipseudomonal Agents". *Antimicrobial Agents and Chemotherapy,* 43 (6): 1379–82.

Center for Disease Control and Prevention, CDC. (2013). Antibiotic resistance threats in the United States.1-94

Centre for Disease Dynamics, Economics and Policy, CDDEP. (2015). State of the world's antibiotics, Washington, D. C. 1-66

Cérèmonie, H., F. Buret, P. Simonet, and Vogel, T.M. (2004). Isolation of lightning-competent soil bacteria. *Applied and Environmental Microbiology*, 70:6342–6346.

Cogliani, C., Goossens, H. and Greko, C. (2011). Restricting Antimicrobial Use in Food Animals: Lessons from Europe. *Microbe*, 6(6):274–279.

Cowan, S.T., Barrow, G.I., Steel, K.J. and Feltham, R.K.A., (2004). *Cowan and Steel's manual for the identification of medical bacteria*. Cambridge university press.

Cristina, N. and Eusébio, L. (2013). Identification and Genotyping of *Pseudomonas aeruginosa* in Bronchiectasis. Thesis submitted to the *Institute of Molecular Pathology and Immunology of University of Porto.1-83*

Dantas, G. and Sommer, M. O. A. (2014). How to Fight Back Against *Antibiotic Resistance*, 42–51.

Davies, J. and Davies, D. (2010). Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews*, 74(3):417–433.

Davies, J., G. B. Spiegelman, and Yim. G. (2006). The world of sub-inhibitory antibiotic concentrations. *Current Opinion in Microbiology*, 9:1–9.

De Vos, D., Lim, A., Pirnay, J. P., Struelens, M., Vandenvelde, C., Duinslaeger, L., Vanderkelen, A. and Cornelis, P. (1997). Direct detection and identification of *Pseudomonas aeruginosa* in clinical samples such as skin biopsy specimens and expectorations by multiplex PCR based on two outer membrane lipoprotein genes, oprI and oprL. *Journal of Clinical Microbiology*, 35(6):1295–1299.

Demanèche, S., E. Kay, F. Gourbière, and P. Simonet. (2001). Natural transformation of Pseudomonas fluorescens and Agrobacterium tumefaciens in soil. *Applied and Environmental Microbiology*. 67:2617–2621.

Denyer, S. P., Hodges, N. A. and Gorman, S. P. (2004). *Hugo and Russell's Pharmaceutical Microbiology*.1-153

Donkor, E. S., Newman, M. J. and Yeboah-Manu, D. (2012). Epidemiological aspects of non-human antibiotic usage and resistance: implications for the control of antibiotic resistance in Ghana. *Tropical Medicine & International Health*, 17(4):462–8.

Donkor, E. S., Newman, M. J., Tay, S. C. K., Dayie, N. T. K. D., Bannerman, E. and Olutaiwo, M. (2011). Investigation into the risk of exposure to antibiotic residues contaminating meat and egg in Ghana. *Food Control*, 22(6):869–873.

Dozzo, P., Moser, H.E. (2010). New aminoglycoside antibiotics. *Expert Opinion on Therapeutic Patents,* 20:1321-1341

Drenkard, E., Ausubel, F. M. (2002). *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature,* 416, 740-743.

Dunne, W.M. Jr, Hardin D. J. (2005). Use of several inducer and substrate antibiotic combinations in a disk approximation assay format to screen for AmpC induction in patient isolates of *Pseudomonas aeruginosa*, *Enterobacter spp.*, *Citrobacter spp*., and *Serratia spp*. *Journal Clinical Microbiology*, 43: 5945-5949.

Dworzanski, J.P., Snyder, A.P. (2005) Classification and identification of bacteria using mass spectrometry-based proteomics. *Expert Reviews Proteomics*, 2: 863–878

Dzidic, S., Suskovic, J. and Kos, B. (2008). Antibiotic Resistance Mechanisms in Bacteria: Biochemical and Genetic Aspects. *Food Technology and Biotechnology*, 46(1):11–21.

European Antibiotic Resistance Surveillance Network, EARS-Net. (2012). Antimicrobial resistance surveillance in Europe 2012.

Euzéby, J.P. (1997). "List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet". *International Journal of Systematic and Evolutionary Microbiology,* 47 (2): 590–2.

FAO. (2014). Poultry Sector Ghana. FAO Animal Production and Health Livestock Country Reviews. No. 6. Rome.

FAOSTATS (2013). Food and Agricultural Organisation Statistics. Faostat.fao.org. Accessed November 2015.

Feglo, P. and Opoku, S. (2014). AmpC beta-lactamase production among *Pseudomonas aeruginosa* and *Proteus mirabilis* isolates at the Komfo Anokye Teaching Hospital, Kumasi, Ghana. *Journal of Microbiology and Antimicrobials*, 6(January):13–20.

Feglo, P. and Sakyi, K. (2012). Bacterial contamination of street vending food in Kumasi, Ghana. *Journal of Medical and Biomedical Sciences*, 1(1):1–8.

Ferguson, D. B. (2007). A study of clinical strains of *Pseudomonas aeruginosa* and the investigation of antibiotic resistance mechanisms in the multidrug resistant strain PA13 by the National Institute for Cellular Biotechnology, Dublin City University, 1-229

Fiedler, S. and Wirth, R. (1988). Transformation of bacteria with plasmid DNA by electroporation. *Analytical Biochemistry*, *170*(1), 38-44.

Fluit A.C., Schmitz F.J. (2004). Resistance integrons and super-integrons. *Clinical Microbiology and Infection*, 10:272–88

Fluit, A. D. C., Visser, M. R. and Schmitz, F. (2001). Molecular Detection of Antimicrobial Resistance. *Clinical Microbiology Reviews*, 14(4):836–871.

Forkner, C.E. (1960). *Pseudomonas aeruginosa* infections. In *Modern medical monographs*, I. S. Wright. Grune and Stratton, New York and London, N0. 22

Fox, G. E., Stackebrandt, E., Hespel, R. B., Gibson, J., Maniloff, J., Dyer, T.A., Wolfe, R. S., Balch, W. E., Tanner, R. S., Magrum, L. J., Zablen, L. B., Blakemore, R., Gupta, R., Bonen, L., Lewis, B. J., Stahl, D. A., Leuhrsen, K. R., Chen, K. N. and Woese, C. R. (1980). The phylogeny of prokaryotes. *Science*, 209, 457-463.

Garrity, George M., Julia A. Bell, and Timothy Lilburn. (2005) "Class I. Alphaproteobacteria class.", *Bergey's Manual of Systematic Bacteriology*.

Gilbert, D. N., Moellering, R. C. Jr., and Sande, M. A. (2003). *The Sanford Guide to Antimicrobial Therapy.*

Gilson, E., Clément, J.M., Brutlag, D. and Hofnung, M., (1984). A family of dispersed repetitive extragenic palindromic DNA sequences in E. coli. *The EMBO journal*, *3*(6), p.1417.

Grinsted J, Bennett PM (1988) Plasmid Technolooy, 2nd edn. London: Academic Press

Hancock, R. E. W. and Speert, D. P. (2000). Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. *Drug Resistance Updates*, 3(4):247–255.

Harbottle, H., Thakur, S., Zhao, S., White, D. G. and Taylor, P. (2007). Genetics of Antimicrobial Resistance. *Animal Biotechnology*, 37–41.

Harel, J., H. Lapointe, A. Fallara, L. A. Lortie, M. Bigras-Poulin, S.Lariviere, and J. M. Fairbrother. (1991). Detection of genes for fimbrial antigens and enterotoxins associated with *Escherichia coli* serogroups isolated from pigs with diarrhea. *Journal of Clinical Microbiology.* 29:745–752.

Hassan, K. A., Elbourne, L. D. H., Li, L., Gamage, H. K. A. H., Liu, Q., Jackson, S. M., Sharples, D., Kolst A. B., Henderson, P. J. F. and Paulsen, I. T. (2015). An ace up their sleeve: A transcriptomic approach exposes the AceI efflux protein of *Acinetobacter baumannii* and reveals the drug efflux potential hidden in many microbial pathogens. *Frontiers in Microbiology*, 6:1–6.

Hausner, M., and S. Wuertz. (1999). High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. *Applied Environmental Microbiology*. 65:3710–3713.

Heras, J., Domínguez, C., Mata, E., Pascual, V., Lozano, C., Torres, C. and Zarazaga, M. (2015). GelJ--a tool for analyzing DNA fingerprint gel images. *BMC bioinformatics*, 16(1):270. Retrieved from http://www.biomedcentral.com/1471-2105/16/270

Hocquet, D., Bertrand, X., Kohler, T., Talon, D., Plesiat, P. (2003) Genetic and phenotypic variations of a resistant *Pseudomonas aeruginosa* epidemic clone. *Antimicrobial Agents Chemotherapy*. 47: 1887-1894.

Hooper, D. C. (2001). Emerging mechanisms of fluoroquinolone resistance. *Emerging Infectious Diseases*, 7:337–341.

Hooper, D.C. (1993). Quinolone mode of action-new aspects. *Drugs*, 45. 8-14.

Hulton, C.S.J., Higgins, C.F., and Sharp, P.M. (1991). ERIC sequences: a novel family of repetitive elements in the genomes of Escherichia coli, Salmonella typhimu-rium and other enteric bacteria. *Molecular Microbiology* 5:825-834.

Hwan, M., Jangwon, O., Seung, L., Choi, K. and Son, S. (2014). Analysis of class I integrons responsible for antibiotics resistance in *Pseudomonas aeruginosa. Genes & Genomics, 37(3)241-246.*

Ibrahim, A.I., Junaidu, A.U. and Garba, M.K. (2010). Multiple antibiotic residues in meat from slaughtered cattle in Nigeria. *Internet Journal of Veterinary Medicine*, *8*(1).

Jacoby, G. (2006). β- lactamase nomenclature. *Antimicrobial Agents and Chemotherapy*, 50, 1123-1129.

Jacoby, G. A. (2005). Mechanisms of resistance to quinolones. *Clinical Infectious Diseases*, 41, 120–126.

Jalal, S. and Wretlind, B. (1998). Mechanisms of quinolone resistance in clinical strains of Pseudomonas aeruginosa. *Microbial Drug Resistance*, 4:257–261

Jalal, S. and Wretlind, B. (1998). Mechanisms of quinolone resistance in clinical strains of Pseudomonas aeruginosa. Microb.Drug Resist., 4(1076-6294 (Print)):257–261.

Japoni, A., Alborzi, A., Kalani, M., Nasiri, J., Hayati, M. and Farshad, S. (2006). Susceptibility patterns and cross-resistance of antibiotics against *Pseudomonas aeruginosa* isolated from burn patients in the South of Iran. *Burns* 32 (2006) 343–347.

Jiang, S.C., and J.H. Paul. (1998). Gene transfer by transduction in the marine environment. *Applied and Environmental Microbiology*. 64:2780–2787.

Jiang, X, Zhang, Z, Li M. (2006). Detection of extended-spectrum beta-lactamases in clinical isolates of *Pseudomonas aeruginosa*. Antimicrobial Agents Chemotherapy, 50. 2990-2995.

Joanne, C. C.-S., Mackie, R. I., Koike, S., Krapac, I. G., Lin, Y.-F., Yannarell, A. C., Maxwell, S. and Aminov, R. I. (2009). Fate and Transport of Antibiotic Residues and Antibiotic Resistance Genes. *Journal of Environmental Quality*, 38(3)1086-1108.

Johnson, T. J., Logue, C. M., Johnson, J. R., Kuskowski, M. A., Sherwood, J. S., Barnes, H. J., DebRoy, C., Wannemuehler, Y. M., Obata-Yasuoka, M., Spanjaard, L. and Nolan, L. K. (2012). Associations between multidrug resistance, plasmid content, and virulence potential among extraintestinal pathogenic and commensal *Escherichia coli* from humans and poultry. *Foodborne Pathogens and Disease*, 9(1):37–46.

Jukes, T. H., Stokstad, E. L. R., Taylor, R. R., Cunha, T. J., Edwards, H. M. and Meadows, G. B. 1950. Growth-Promoting Effect of Aureomycin on Pigs. *Archives of Biochemistry*, 26:324–25.

Kado, C. I. and Liu, S. T. (1981). Rapid Procedure for Detection and Isolation of Large and Small Plasmids. *Journal of Bacteriology*, 146(3):1174.

Kandela, S. A., al-Shibib, A. S. and al-Khayat, B. H. (1997). A study of purified pyorubin produced by local *Pseudomonas aeruginosa*. *Acta microbiologica Polonic*a, 46: 37- 43.

Kanner, D., Gerber, N. N. and Bartha, R. (1978). Pattern of phenazine pigment production by a strain of *Pseudomonas aeruginosa*. *Journal of Bacteriology*, 134, 690,692.

Katayama, Y., Ito, T. & Hiramatsu, K. A new class of genetic element, staphylococcus cassette chromosome mec, encodes methicillin resistance in Staphylococcus aureus. *Antimicrobial Agents Chemotherapy*. 44, 1549–1555 (2000).

Kenneth, T., (2008) Todar's Online Textbook of Bacteriology, http://www.textbookofbacteriology .net/ken_todar.htm, Accessed January 2016.

Kidd, J. T., Whiley M.D., Bell C. S., Grimwood K. (2011). Pseudomonas in: Molecular detection of human bacterial pathogens, 86:1009-1020

Kidd, T. J., Grimwood, K., Ramsay, K. A., Rainey, P. B. and Bell, S. C. (2011). Comparison of three molecular techniques for typing *Pseudomonas aeruginosa* isolates in sputum samples from patients with cystic fibrosis. *Journal of Clinical Microbiology*, 49(1):263–268.

Koeuth, T., Versalovic, J. and Lupski, J.R. (1995). Differential subsequence conservation of interspersed repetitive *Streptococcus pneumoniae* BOX elements in diverse bacteria. *Genome Research*, *5*(4), pp.408-418.

Kohler, T. Van Delden, C. Curty, LK. Overexpression of the MexEF-OprN multidrug efflux system affects cell-to-cell signaling in *Pseudomonas aeruginosa*. *Journal of Bacteriology*. 183(18), 5213–5222 (2001).

Kotwani, A., and Holloway, K. (2011). Trends in Antibiotic Use among Outpatients in New Delhi, India. *BMC Infectious Diseases*, 11(1):99.

Krieg, N.R., Holt, J.G. (2005). *Bergey's Manual of Systematic Bacteriology* Williams and Wilkins Publishers, Baltimore Vol. 2, 25-250

Krieg, Noel (1984). *Bergey's Manual of Systematic Bacteriology*, Volume 1. Baltimore: Williams & Wilkins. ISBN 0-683-04108-8.

Krieg, R., N., Brenner, D. J. and Staley, J. T. (2005). Bergey's Manual of Systematic Bacteriology, 2nd edition. 20-250.

Kung, V. L., Ozer, E. A. and Hauser, A. R. (2010). The Accessory Genome of *Pseudomonas aeruginosa*, 74(4):621–641.

Kunin, C.M., (1993). Resistance to antimicrobial drugs – a worldwide calamity. *Annals of Internal Medicine*. 1(18): 557 – 561.

Kureishi, A., J. M. Diver, B. Beckthold, T. Schollaardt, and L. E. Bryan. (1994). Cloning and nucleotide sequence of *Pseudomonas aeruginosa* DNA gyrase gyrA gene from strain PAO1 and quinolone-resistant clinical isolates. *Antimicrobial Agents and Chemotherapy*. 38:1944–1952.

Lalitha, M.K., (2004). Manual on antimicrobial susceptibility testing. 7th edition. *Indian Association of Medical Microbiologists.* Delhi. 10-4.

Lambert P.A. (2005) Bacterial resistance to antibiotics: modified target sites, *Advanced Drug Delivery Reviews*, 57:1471-1485.

Lambert, P.A. (2002). Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. *Journal of the Royal Society of Medicine*, 95:22–6.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K. M., Wertheim, H. F. L., Sumpradit, N., Vlieghe, E., Hara, G. L., Gould, I. M., Goossens, H., Greko, C., So, A. D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A. Q., Qamar, F. N., Mir, F.(2013). Antibiotic resistance-the need for global solutions. The *Lancet Infectious Diseases*, 13(12):1057–1098.

Leboffe, M. J., and Pierce, B. E. (2010). Differential tests, in: Microbiology Laboratory Theory and applications, 166–168.

Lee K, Lim YS, Yong D, et al. Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-beta-lactamase-producing isolates of Pseudomonas spp. and Acinetobacter spp. *Journal of Clinical Microbiology*, 2003;41. 4623-4629.

Leverstein-van Hall M.A., Box, A.T.A., Blok H.E.M., Paauw A, Fluit A.C, Verhoef J. (2002). Evidence of extensive interspecies transfer of integron-mediated antimicrobial resistance genes among multidrug-resistant Enterobacteriaceae in a clinical setting. *Journal of Infectious Disease*, 186: 49-56.

Levesque, C., Brassard, S., Lapointe, J., Roy, P.H. (1994). Diversity and relative strength of tandem promoters for the antibiotic resistance genes of several integrons. *Gene*; 142: 49-54.

Levison, M. (1977). Factors influencing colonization of the gastro intestinal tract with *Pseudomonas aeruginosa.* IN: Young VM, ed. *Pseudomonas aeruginosa*: 97–109

Levy S. B., FitzGerald G. B., Macone A. B. (1976). Changes in intestinal flora of farm personnel after introduction of a tetracycline-supplemented feed on a farm. *New England Journal of Medicine*, 295, 583–588.

Levy, S. B., Marshall, B. (2004). Antibacterial resistance worldwide : causes, challenges and responses. *Nature Medicine*, 10(12):122–129.

Levy, S. B. (1998). The challenge of antibiotic resistance. *Scientific American.* 278, 46–53.

Levy, S.B., and R.V. Miller. (1989). Gene transfer in the environment. *Environmental Biotechnology*, McGraw-Hill, New York

Lewis K. (2013) Antibiotic resistance and tolerance. *Nature Reviews Drug Discovery*, 12, 371–387

Lim, Jr. A., De Vos, D., Brauns, M., Mossialos, D., Gaballa, A., Qing, D. and Cornells, P.(1997). Molecular and immunological characterization of OprL, the 18 kDa outer-membrane peptidoglycan-associated lipoprotein (PAL) of *Pseudomonas aeruginosa*. *Microbiology*, *143*(5), 1709-1716.

Lister, P.D., Wolter, D.J., Hanson N.D. (2009). Antibacterial-resistant Pseudomonas aeruginosa: Clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clinical Microbiology Reviews*; 22. 582-610.

Liu, L., Oza, S., Hogan, D., Perin, J., Rudan, I., Lawn, J. E. (2015). Global, Regional, and National Causes of Child Mortality in 2000–13, with Projections to Inform Post- 2015 Priorities: An Updated Systematic Analysis. *The Lancet*, 385(9966):430-440.

Livermore, D. M. (1987). Clinical significance of beta-lactamase induction and stable derepression in gram-negative rods. *European Journal of Clinical Microbiology*, 6:439-445.

Lodge, J. M., Minchin, S. D., Piddock, L. J., and Busby, J. W. (1990). Cloning, sequencing and analysis of the structural gene and regulatory region of the *Pseudomonas aeruginosa* chromosomal ampC β-lactamase. *Biochemical Journal*, 272, 627–631.

Lorenz, M.G., and W. Wackernagel. (1994). Bacterial gene transfer by natural genetic transformation in the environment. *Microbiology Reviews*. 58:563–602.

Lupski J.R. (1987) Molecular mechanisms for transposition of drug- resistance genes and other movable genetic elements. *Reviews of Infectious Diseases*. 9:357-368.

Madhavan, H. N., Murali, S. (2011). Mechanisms of Development of Antibiotic Resistance in. Bacteria among Clinical Specimens. *Journal of Clinical and Biomedical Sciences*, 42–48.

Magiorakos, a, Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S. and Hindler, J. F. (2011). Bacteria : an International Expert Proposal for Interim Standard Definitions for Acquired Resistance. *Microbiology*, 18(3):268–281.

Mahillon J, Chandler M (1998). Insertion sequences. *Microbiology and Molecular Biology Reviews*. 62: 725-774.

Manchanda V, Singh NP (2003). Occurrence and detection of AmpC beta-lactamases among Gram negative clinical isolates using a modified three-dimensional test at Guru Tegh Bahadur Hospital, Delhi. Indian Journal of Antimicrobial Chemotherapy. 51:415-418.

Mann, S. 1969. Melanin-forming strains of Pseudomonas aeruginosa. *Archives of Microbiology*. 65: 359–379.

Markowska, K., Grudniak, A. M., Krawczyk, K., and Wolska, K. I. (2014). Modulation of antibiotic resistance and induction of a stress response in Pseudomonas aeruginosa by silver nanoparticles. Journal of Medical Microbiology, 63:849–854.

Maron, D. F., Smith, T. J. S. and Nachman, K. E. (2013). Restrictions on antimicrobial use in food animal production: an international regulatory and economic survey. *Globalization and Health*, 9(1):48.

Marsik, F.J. and Nambiar, S., (2011). Review of carbapenemases and AmpC-beta lactamases. *The Pediatric Infectious Disease Journal*, *30*(12).1094-1095.

Martinez-Freijo, P., Fluit, F. J., Schmitz, V. S., Grek, V.S.C., Verhoef and Jones, M. E. (1998). Class 1 integrons in Gram-negative isolated and association with decreased susceptibility to multiple antibiotic compounds. Journal of Antimicrobial Chemotherapy, 42, 689-696.

Martins, M., Viveiros, M., Couto, I., Costa, S.S., Pacheco, T., Fanning, S., Pages, J.M. and Amaral, L., (2011). Identification of efflux pump-mediated multidrug-resistant bacteria by the ethidium bromide-agar cartwheel method. *in vivo*, *25*(2), 171-178.

Masterton, R. (2003). A quick guide to antibiotic resistance-antibiotic resistance in noscomial infections. Available online at http://www.infectionacademy.org (March 2016)

Mazel, D. (2006). Integrons: agents of bacterial evolution. *Nature Reviews Microbiology*. 4:608–20

Mazel, D., Dychinco, B., Webb, V.A. and Davies, J. (2000) Antibiotic resistance in the ECOR collection: integrons and identification of a novel and gene. *Antimicrobial Agents Chemotherapy*, 44, 1568–1574.

Mazodier, P., and J. Davies. (1991). Gene transfer between distantly related bacteria. *Annual Review of Genetics*, 25:147–171

Meacham, K. J., Zhang, L., Foxman, B., Bauer, R. J. and Marrs, C. F. (2003). Evaluation of Genotyping Large Numbers of Escherichia coli Isolates by Enterobacterial Repetitive Intergenic Consensus-PCR. *Journal of Clinical Microbiology*, 41(11):5224–5226.

Meletis, G. and Bagkeri, M. (2013) *Pseudomonas aeruginosa* : Multi-Drug-Resistance Development and Treatment Options.

Mena, K.D. and Gerba, C.P., (2009). Risk assessment of Pseudomonas aeruginosa in water. In *Reviews of Environmental Contamination and Toxicology Vol 201*:71-115.

Mensah, P., Yeboah-Manu, D., Owusu-Darko, K. and Ablordey, A. (2002). Street foods in Accra, Ghana: How safe are they? *Bulletin of the World Health Organization*, 80(7):546–554.

Mesaros, N., Nordmann, P., Plésiat, P., Roussel-Delvallez, M., Van Eldere, J., Glupczynski, Y., Van Laethem, Y., Jacobs, F., Lebecque, P., Malfroot, A. and Tulkens, P.M. (2007). *Pseudomonas aeruginosa*: Resistance and therapeutic options at the turn of the new millennium. *Clinical Microbiology and Infection*, 13(6):560–578.

Meyer, J.M. and J.M. Hornsperger. (1978). Role of pyoverdine, iron-binding fluorescent pigment of Pseudomonas fluorescens, in iron transport. *Journal of General Microbiology*. 107: 329–331.

Meyer, J.M. and M.A. Abdallah. 1978. Fluorescent pigment of Pseudomonas fluorescens-biosynthesis, purification and physicochemical properties. *Journal of General Microbiology,* 107: 319–328.

Millen, D. D., Lauritano Pacheco, R. D., Meyer, P. M., Mazza Rodrigues, P. H., and De Beni Arrigoni, M. (2011). Current Outlook and Future Perspectives of Beef Production in Brazil. *Animal Frontiers*, 1(2):46–52.

Miller, R.V. 2001. Environmental bacteriophage-host interactions: Factors contributing to natural transduction. *Antonie van Leeuwenhoek* 79:141–147.

Moats, A. W. (1986). Agricultural Uses of Antibiotics. ACS Symposium Series; *American Chemical Society*: 1–193.

Monstein, H.J., Stholm-Balkhed, A., Nilsson, M.V., Nilsson, M., Dornbusch, K & Nilsson, L.E (2007) Multiplex PCR amplification assay for the detection of blaSHV, blaTEM and blaCTX-M genes in Enterobacteriaceae. *Acta Pathologica Microbiologica et Immunologica Scandinavica,* 115: 1400–1408.

Newman, M. J., Arhin, R. E., Donkor, E. S., Gyansa-lutterodt, M. and Mills-pappoe, W. (2015). Laboratory-based nationwide surveillance of antimicrobial resistance in Ghana. : 379–389.

Newman, M.J., Frimpong, E., Asamoah-Adu, A. and Sampene-Donkor, E. (2006). Resistance to antimicrobial drugs in Ghana. The Ghanaian-Dutch Collaboration for Health research and Development. *Technical report series*.8-26.

Nikaido H, Vaara M. (1985) Molecular basis of bacterial outer membrane permeability. *Microbiology Reviews*, 49:1-32.107.

Nikaido H. (1985) Role of permeability barriers in resistance to beta-lactam antibiotics. *Pharmacology & Therapeutics*; 27:197-231.

O'Neill, J. (2014). Antimicrobial Resistance : Tackling a crisis for the health and wealth of nations. (December).

Ogle (2013) "They've Doubled Gains with New Drugs". Successful Farming. Antibiotics Now Proved in Hog and Poultry Ratios, They're the Biggest Feeding News in 40 Years! Successful Farming 49 (3): 33. March 1951.

Ohlsen, K., T. Ternes, G. Werner, U. Wallner, D. Löffler, W. Ziebuhr, W. Witte, and J. Hacker. 2003. Impact of antibiotics on conjugational resistance gene transfer in Staphylococcus aureus in sewage. *Environmental Microbiology*. 5:711–716.

Opal, S. M. and Pop-Vicas, A. (2004). Basic Principles in the Diagnosis and Management of Infectious Diseases: Molecular Mechanisms of Antibiotic Resistance in Bacteria, in: *Principles and Practice of Infectious Diseases,* 19:279–295.

Opoku, S. (2013). AmpC beta-lactamase production among Pseudomonas aeruginosa and *Proteus mirabilis* clinical isolates at Komfo Anokye Teaching Hospital in Kumasi, Ghana. Masters thesis submitted to the Kwame Nkrumah University of Science and Technology, 1-84

Pagel, S. W. and Gautier, P. 2012. Use of Antimicrobial Agents in Livestock. *Revue Scientifique et Technique International Office of Epizootics*, 31(1):145–88

Pattison M, McMullin PF, Bradbury JM, Alexander DJ (eds.) Poultry Diseases Philadephia, Saunders Elsevier,6th Edition. 160-163.

Pechère JC (September 2001). "Patients' interviews and misuse of antibiotics". *Clinical Infectious Diseases*, 33, 3:170–3.

Petrosino, J., Cantu III, D., Palzkill, T. (1998). Beta-lactamases: protein evolution in real time. *Trends Microbiology*, 6:323-327.

Piddock, L. J. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clinical Microbiology Reviews*. 19, 382–402 (2006).

Pirnay, J.-P., De Vos, D., Cochez, C., Bilocq, F., Vanderkelen, A., Zizi, M., Ghysels, B. and Cornelis, P. (2002). Pseudomonas aeruginosa displays an epidemic population structure. *Environmental Microbiology*, 4(12):898–911.

Pitondo-Silva, A., Martins, V. V., Fernandes, A. F. T. and Stehling, E. G. (2014). High level of resistance to Aztreonam and Ticarcillin in *Pseudomonas aeruginosa* isolated from soil of different crops in Brazil. *Science of the Total Environment*, 473-474:155–158.

Poole K. (2005). Efflux-mediated antimicrobial resistance. *Journal Antimicrobial Chemotherapy* 56(1), 20–51 (2005).

Poole, K. (2004). Resistance to -lactam antibiotics. *Cellular and Molecular Life Sciences,* 61, 2200–2223.

Poole, K. (2007). Efflux pumps as antimicrobial resistance mechanisms. *Annals of Medicine*. 39, 162–176.

Poole, K., Hazlett, L. D., State, W. and Greenberg, E. P. (2011). *Pseudomonas aeruginosa* : resistance to the max., *Frontiers in Microbiology* 2:1–13.

Pournaras S, Poulou A, Tsakris A.(2010) Inhibitor-based methods for the detection of KPC carbapenemase-producing Enterobacteriaceae in clinical practice by using boronic acid compounds. *Journal Antimicrobial Chemotherapy*, 65. 1319-1321.

Qinghu, R., Chen, K. and Paulsen, I. T. (2006). Transport DB: a comprehensive database resource for cytoplasmic membrane transport systems and outer membrane channels. *Nucleic Acids Research*, 35, 274-279.

Ramirez MS, Tolmasky ME. (2010) Aminoglycoside modifying enzymes. *Drug Resistance*, 13. 151–171.

Ranjbar, R., Karami, A., Farshad, S., Giammanco, G. M. and Mammina, C. (2014). Typing methods used in the molecular epidemiology of microbial pathogens: a how-to guide. *The New Microbiological*, 37(1):1–15.

Ratkai, C. (2011). Institute of Clinical Microbiology Faculty of Medicine University of Szeged Characterization of medically important Pseudomonas aeruginosa isolates. Doctor of Philosophy (Phd) thesis submitted to the Institute of Clinical Microbiology, Faculty of Medicine, University of Szeged, 1-62

Rayner, C. F. J, Cole, P. J. and Wilson R. (1994).The management of chronic bronchial sepsis due to bronchiectasis. *Clinical Pulmonary Medicine*, 1:348-355.

Recchia, G. D., and R. M. Hall. (1997). Origins of the mobile gene cassettes found in integrons. *Trends in Microbiology*. 5: 389–394.

Reichenberger, E.R., Alexander, G.M., Perreault, M.J., Russell, J.A., Schwartzman, R.J., Hershberg, U. and Rosen, G. (2013). Establishing a relationship between bacteria in the human gut and complex regional pain syndrome. *Brain, Behavior, and Immunity*, 29, 62-69.

Robicsek A, Jacoby GA, Hooper D.C. (2006). The worldwide emergence of plasmid-mediated quinolone resistance. *The Lancet infectious diseases*, 6:629-40.

Rodriguez-Martinez, J. M., Poirel, L., and Nordmann, P. (2009). Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa*. *Antimicrobial Agents Chemotherapy*, 53:4783–4788.

Roe M. T., Pillai S. D. (2003). Monitoring and identifying antibiotic resistance mechanisms in bacteria. *Poultry Science*. 82:622–626.

Sabat, A. J., Budimir, A., Nashev, D., Sá-Leão, R., van Dijl, J., Laurent, F., Grundmann, H., Friedrich, A. W. and ESCMID Study Group of Epidemiological Markers (ESGEM). (2013). Overview of molecular typing methods for outbreak detection and epidemiological surveillance. Euro surveillance : bulletin européen sur les maladies transmissibles *European Communicable Disease Bulletin*, 18(4):20380.

Salyers, A.A., N.B. Shoemaker, A.M. Stevens, and L.Y. Li. 1995. Conjugative transposons: An unusual and diverse set of integrated gene transfer elements. *Microbiology Reviews*. 59:579–590.

Schobert, Max; Jahn, Dieter (2010). "Anaerobic physiology of Pseudomonas aeruginosa in the cystic fibrosis lung". *International Journal of Medical Microbiology* 300 (8): 549–556.

Schwarz, S. and Nobel, W. C. (1999). Aspects of bacterial resistance to antimicrobials used in veterinary dermatological practice. *Veterinary Dermatology*, 10, 163-176.264

Sefton, A. M. (2002). Mechanisms of Antimicrobial Resistance Their Clinical Relevance in the New Millennium. *Drugs*, 62(4):557–566.

Seol, B., Naglic, T., Madic, J. and Bedekovic, M. (2002). In vitro antimicrobial susceptibility of 183 Pseudomonas aeruginosa strains isolated from dogs to selected antipseudomonal agents. *Journal of Veterinary Medicine*, 49, 188-192.

Severino, P. and Magalhaes, V.D. (2002). The role of integrons in the dissemination of antibiotic resistance among clinical isolates of Pseudomonas aeruginosa from an intensive care unit in Brazil. *Research in Microbiology,* 153, 221–226.

Shaw, K. J., Rather, P. N., Hare, R.S and Miller, G. H. (1993). Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside modifying enzymes. *Microbiology and Molecular Biology Reviews*, 57, 138-163.

Sigmund, C. D., Ettayebi, M., Morgan, E. A. (1984). Antibiotic resistance mutations in 16S and 23S ribosomal RNA genes of Escherichia coli. *Nucleic Acids Research*, 12, 4653-4663.

Skerman, McGowan; Sneath (1980). "Approved Lists of Bacterial Names". *International Journal of Systematic and Evolutionary Microbiology*, 30: 225–420.

Soonthornchaikul N., Garelick H. (2009). Antimicrobial resistance of *Campylobacter* species isolated from edible bivalve molluscs purchased from Bangkok markets, Thailand. *Foodborne Pathogens and Disease*. 6, 947–951.

Strateva, T. and Yordanov, D. (2009). *Pseudomonas aeruginosa* – a phenomenon of bacterial resistance, *Journal of Medical Microbiology*, 58, 1133–1148

Su XZ, Chen J, Mizushima T, Kuroda T, Tsuchiya T. (2005) AbeM, an H+coupled Acinetobacter baumannii multidrug efflux pump belonging to the MATE family of transporters. *Antimicrobial Agents of Chemotherapy*. 49(10), 4362–4364.

Syrmis, M. W., Carroll, M. R. O., Sloots, T. P., Coulter, C., Wainwright, C. E., Bell, S. C. and Nissen, M. D. (2004). Rapid genotyping of Pseudomonas aeruginosa isolates harboured by adult and paediatric patients with cystic fibrosis using repetitive-element-based PCR assays. *Journal of Medical Microbiology*: 1089–1096.

Tenover, F.C., Arbeit, R.D. and Goering, R.V., (1997). How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections a review for healthcare epidemiologists. *Infection Control & Hospital Epidemiology*, 18(06), 426-439.

Thomas, C.M. and Summers, D. (2008) Bacterial Plasmids. In: eLS. John Wiley & Sons Ltd, Chichester. http://www.els.net. Accessed January 2016.

Tipper, D.J. (1985) Mode of action of beta-lactam antibiotics. *Clinical Pharmacology & Therapeutics*, 27.1-35

Todar, K. (2004). Pseudomonas and related bacteria. *Todar's Online Textbook of Bacteriology*. Available online http://textbookofbacteriology.net/pseudomonas.html. Accessed 14th July 2016.

Trias J, Nikaido H. Outer membrane protein D2 catalyzes facilitated diffusion of carbapenems and penems through the outer membrane of Pseudomonas aeruginosa. *Antimicrobial Agents Chemotherapy*, 34: 52-57

Tsakris, A., Kristo, I., Poulou, A., Themeli-Digalaki, K., Ikonomidis, A., Petropoulou, D., Pournaras, S. and Sofianou, D., (2009). Evaluation of boronic acid disk tests for differentiating KPC-possessing Klebsiella pneumoniae isolates in the clinical laboratory. *Journal of Clinical Microbiology*, *47*(2), 362-367.

Turkson, P. K. (2008). Use of drugs and antibiotics in poultry production in Ghana. Ghana *Journal of Agricultural Science*, 41(1):23–32.

Van Bambeke, R., E. Balzi, and P. M. Tulkens. (2000). Antibiotic efflux pumps. *Biochemical Pharmacology*, 60:457–470

Versaslovic, J., Koeuth, T., and Lupski, J.R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research*, 19:6,823-6,831.

Vetting, M. W. et al. Structure of QnrB1, a plasmid- mediated fluoroquinolone resistance factor. *Journal of Biological Chemistry*, 286, 25265–25273 (2011).

Villegas MV, Lolans K, Correa A, (2007). First identification of Pseudomonas aeruginosa isolates producing a KPC-type carbapenem-hydrolyzing β-lactamase. *Antimicrobial Agents Chemotherapy*, 51. 1553–1555.

Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance. *Nature*, 406, 775-781.

Walsh, C. and Cole, S.T., (2003). Books and arts-Antibiotics: Actions, Origins, Resistance. *Nature*, 424(6948), 491.

Walton, J.R., (1983). Antibiotics, animals, meat and milk. *Zentralblatt für Veterinärmedizin Reihe* A, 30(2), 81-92.

Wang, J., Zhou, J. Y., Qu, T. T., Shen, P., Wei, Z. Q., Yu, Y. S., and Li, L. J. (2010). Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Chinese hospitals. International Journal of Antimicrobial Agents 35, 486–491

Wang, Z., Xiang, L., Shao, J., Węgrzyn, A. and Węgrzyn, G., 2006. Effects of the presence of ColE1 plasmid DNA in *Escherichia coli* on the host cell metabolism. *Microbial Cell Factories*, *5*(1), 1.

Weinbauer, M.G., and F. Rassoulzadegan. 2004. Are viruses driving microbial diversify cation and diversity? *Environmental Microbiology*, 6:1–11.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991). "16S ribosomal DNA amplification for phylogenetic study". *Journal of Bacteriology.* 173 (2): 697–703.

Wellinghausen, N., Köthe, J., Wirths, B., Sigge, A. and Poppert, S. (2005). Superiority of molecular techniques for identification of gram-negative, oxidase-positive rods, including morphologically nontypical *Pseudomonas aeruginosa*, from patients with cystic fibrosis. *Journal of Clinical Microbiology*, 43(8):4070–4075.

Westbrock-Wadman, S. Sherman, D.R. Hickey, M.J. (2004). Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. *Antimicrobial Agents Chemotherapy*. 43(12), 2975–2983 (2004)

WHO. (2000). Antimicrobial resistance: a global threat, *Essential Drugs Monitor* 29.

WHO. (2014). Antimicrobial resistance: global report on surveillance: 3.

WHO. (2015). Influenza Update Number 244. *World Health Organization*, No. 244:1–9.

Wiedmann, M., Weilmeirer, D., Dineen, S. S., Ralyea, R. and Boor, K. J. (2000). Molecular and phenotypic characterization of *Pseudomonas spp*. isolated from milk. *Environmental Microbiology*, 66, 2085-2095.

Woo, P. C. Y., Leung, K. W. and Yuen, K.Y. (2000). Identification by 16S ribosomal RNA gene sequencing of an Enterobacteriaceae species from a bone marrow transplant recipient. *Journal of Clinical Pathology: Molecular Pathology*, 53, 211- 215.

Yabuuchi, E. and Ohyama, A. (1972). Characterisation of 'pyomelanin'-producing strains of Pseudomonas aeruginosa. *Bacteriology*, 22, 53-64.

Yildirim, Ibrahim. (2011). Molecular methods for bacterial genotyping and analyzed gene regions. *Journal of Microbiology and Infectious Diseases,* 1(1):42–46.

Yoneyama, H. and Katsumata, R., (2006). Antibiotic resistance in bacteria and its future for novel antibiotic development. *Bioscience, Biotechnology, and Biochemistry*, 70(5), 1060-1075.

Zavascki, A.P., Goldani, L.Z., Goncalves, A.L.S., Martins, A.F. and Barth, A.L., (2007). High prevalence of metallo-β-lactamase-mediated resistance challenging antimicrobial therapy against Pseudomonas aeruginosa in a Brazilian teaching hospital. *Epidemiology and infection*, *135*(02), 343-345.

APPENDICES

APPENDIX I

PREPARATION OF CULTURE MEDIA

Pseudomonas isolation agar (Alpha Biosciences, Maryland)

45g of the powder was mixed in 980ml of distilled water. 20 mL of glycerol was added until evenly dispersed. The mixture was heated with repeated stirring to dissolve. It was sterilized by autoclaving at 121^{o}C for 15 minutes

Composition (/L): Pancreatic digest of gelatin(20.0g), potassium sulphate (10.0g), magnesium chloride (1.4g), irgasan (0.025g), agar (13.6g)

Mueller-Hinton agar (Oxoid, Basingstoke, UK)

To prepare the medium, 35 grams of the powder was dissolved in 1 litre of distilled water. It was then boiled shortly to dissolve the medium completely. It was sterilized by autoclaving at 121^{o}C for 15 minutes

Composition (/L): Beef infusion (300g), casein hydrolysate (17.5g), starch (1.5g), agar (17.0g), pH(7.2-7.4)

Nutrient agar (Oxoid, Basingstoke, UK)

To prepare nutrient agar, 28g was suspended in 1 litre of distilled water. It was then boiled to dissolve completely and 20ml each was distributed into test tubes. The tubes were sterilised by autoclaving at 121°C for 15 minutes.

Composition(/L): Lab-Lemco powder(1.0g), yeast extract (2.0g), peptone (5.0g), sodium chloride (5.0g), agar (15.0g), pH (7.4 ± 0.2 at 25°C)

Columbia Blood Agar Base (Oxoid, Basingstoke, UK)

To prepare this medium 39g of the dehydrated powder was added to 1 litre of distilled water. It was then boiled to dissolve the medium completely and distributed in 20ml quantities in test tubes. They were then sterilised by autoclaving at 121°C for 15 minutes, cooled to 50°C and 5% sterile defibrinated Horse blood added.

Composition(/L): Pancreatic Digest of Casein (10.0g), peptone (5.0g), yeast extract (5.0g), beef Heart Digest (3.0g), starch (1.0g), sodium chloride (5.0g), agar (15.0g)

Nutrient broth (Oxoid, Basingstoke, UK)

25g was added to 1 litre of distilled water. It was the mixed well, distributed into final containers and sterilised by autoclaving at 121°C for 15 minutes.

Composition(/L): Lab-Lemco powder(10.0g), peptone (10.0g), sodium chloride (5.0g), pH (7.5 ± 0.2)

Tryptone soya broth (Casein soya bean digest medium) (Oxoid, Basingstoke, UK)

30g of Tryptone Soya Broth powder was added to 1 litre of distilled water, mixed well and distributed into final tubes. It was sterilized by autoclaving at 121°C for 15 minutes

Composition(/L): Pancreatic digest of casein17.0, enzymatic digest of soya bean(3.0), sodium chloride(5.0), dipotassium hydrogen phosphate (2.5), glucose (2.5 pH 7.3 ± 0.2 @ 25°C)

Cetrimide agar (Oxoid, Basingstoke, UK)

45.3 g of Cetrimide agar was suspended in 1 litre of sterile distilled water and brought to boil completely. It was sterilized by autoclaving at 121°C for 15 minutes, cooled to approximately 50°C and poured into sterile Petri dishes.

Composition(/L): Gelatin peptone(20.0g), magnesium chloride(1.4g), potassium sulphate(10.0g), Cetrimide, agar(0.3g), pH 7.2 ± 0.2 @ 25°C

MacConkey agar (Oxoid, Basingstoke, UK)

This was prepared by suspending 51.5 grams of powder in 1 litre of distilled water. It was then boiled shortly to dissolve completely. Sterilization was done by autoclaving at 121oC for 15 minutes.

Composition(/L): Peptone Lactose(20.0g), bile salts No. 3 (1.5g), sodium chloride (5.0g), neutral red (0.03g), crystal violet agar(0.001g), pH (6.9-7.3)

APPENDIX II

SAMPLING SITES AND NUMBER OF SAMPLES COLLECTED

A: Images of poultry farms and environments where samples collected

Drains at Ayigya market where samples where some environmental samples were collected (Author, July 2015)

Some poultry farms in the Ashanti region where litter samples were collected (Author, July 2015)

Some areas of Kumasi central market where environental samples were collected(Author, July 2015)

B: Location of studied farms and samples collected

Location	Latitude N	Longitude W	Number of farms visited	Number of samples
Besease	6.71389	1.4348	10	20
Atin	6.71262	1.42168	2	4
Kubease	6.67141	1.36729	2	4
Jachie	6.57454	1.52946	9	18
Fumesua	6.71490	1.5187	1	2
Boadi junction	6.69508	1.54884	1	2
Maaban	6.82460	1.72772	3	6
Amanfrom	6.76626	1.67541	4	8
Adankwame-Kokoben	6.78821	1.70195	1	2
Adankwame	6.82599	1.7294	2	4
Kokoben	6.82599	1.7294	5	10
Mankrano	6.81254	1.84548	2	4
Mfensi	6.77700	1.79271	7	14
Atwima-Koforidua	6.73219	1.73058	1	2
Nyarboo	6.65304	1.19292	4	8
Fomena	6.28015	1.51351	1	2
Akwanserem	6.18613	1.47953	1	2
Asokwa-Adansi	6.24271	1.51063	1	2
Apemso-Juabeng	6.76963	1.78343	1	2
Tepa	6.64136	1.3463	2	4
Bekwai	6.45462	1.59801	2	4

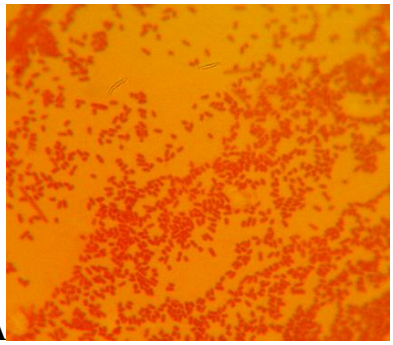
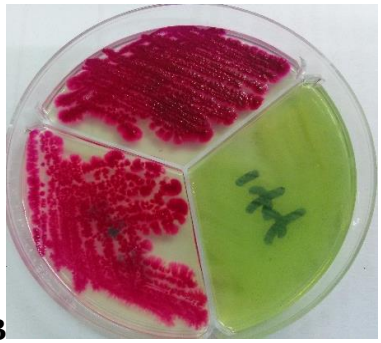
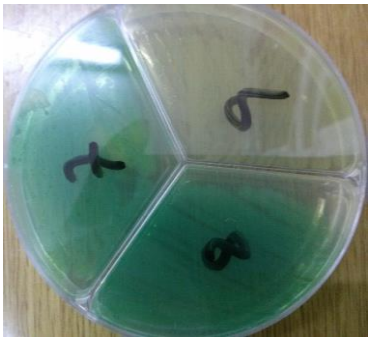
Pekyi No. 2	6.21238	1.59103	4	8
Obuasi	6.20864	1.5967	4	8
Ejura	7.37054	1.36987	1	2
Mampong	7.06391	1.38501	1	2
Offinso	6.94064	1.66043	2	4
Akomadan	7.39505	1.96671	1	2
Kuntenase-Toafom	7.05381	1.39715	1	2
Kuntenase	6.54426	1.53211	1	2
Onwe	6.68188	1.44695	1	2
Tetrefu	6.61899	1.51974	1	2
Sawia	6.62287	1.53323	2	4
Dominase	6.51968	1.65475	2	4
Adjemesu	6.52283	1.65715	1	2
Ampabame No.2	6.60129	1.68767	2	4
Bebu	6.61464	1.68773	4	8
Nyinahin	6.59280	2.11976	1	2
Akontonsu-Nkwanta	6.60885	2.08353	1	2
Yaw Barimakrom	6.60702	1.94231	1	2
Humabenase	6.62781	1.49629	1	2
Nkrumah-Nkwanta	6.61917	1.98664	1	2
Nkawie	6.66796	1.80398	1	2
Akrofrom	6.70351	1.7909	1	2
Afari	6.69831	1.78577	1	2
Sepaase	6.70071	1.75259	1	2
Akrofrom	6.70455	1.79101	2	4
Abuakwa-Makro	6.70198	1.73776	2	4
Nsuta	6.01497	1.38248	1	2
Beposo	6.98579	1.36136	3	6
Boko	6.66866	1.70037	6	12
Atwima-Takyiman	6.68455	1.69433	2	4
Akropong	6.73748	1.71989	1	2
Esaase	6.73687	1.70892	3	6
Daabaa	6.75416	1.72626	4	8
Jamasi	6.97271	1.48325	8	16
Afari	6.69661	1.78615	3	6
Tikrom	6.74399	1.51574	2	4
Antoa	6.74779	1.52033	1	2
Ayigya Market	6.690583	1.572826	NA	156
Kumasi Central Market	6.696599	1.618429	NA	104
Suntreso Government Hospital	6.702628	1.642265	NA	275
KNUST Hospital	6.686255	1.574414	NA	100

NA-Not applicable

APPENDIX III

MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR CHARACTERISTICS OF *PSEUDOMONAS AERUGINOSA*

A: Some results obtained during biochemical screening of isolates

A  **B**  **C** 

A: Gram-negative unicellular rods of *Pseudomonas aeruginosa*
B: Pyorubin (red) and pyoverdine (yellowish- green) pigments produced on Pseudomonas isolation agar
C: Pyocyanin (greenish) producing and non-pigmenting *P. aeruginosa* on Pseudomonas isolation agar

B: Colony characteristics of multi-drug resistant isolates

Isolate	Cetrimide agar	Texture	Gram +/-	Shape
PS_1	Blue-green	Non-mucoid	-	Rod
PS_3	Blue-green	Non-mucoid	-	Rod
PS_4	Blue-green	Non-mucoid	-	Rod
PS_5	Blue-green	Non-mucoid	-	Rod
PS_{14}	Blue-green	Non-mucoid	-	Rod
PS_{17}	Blue-green	Non-mucoid	-	Rod
PS_{25}	Blue-green	Non-mucoid	-	Rod
PS_{29}	Blue-green	Non-mucoid	-	Rod
PS_{31}	Blue-green	Non-mucoid	-	Rod
PS_{37}	Blue-green	Non-mucoid	-	Rod
PS_{82}	Blue-green	Mucoid	-	Rod
PS_{84}	Blue-green	Mucoid	-	Rod
PS_{85}	Blue-green	Non-mucoid	-	Rod
PS_{98}	Blue-green	Non-mucoid	-	Rod
PS_{102}	Blue-green	Mucoid	-	Rod
PS_{105}	Blue-green	Non-mucoid	-	Rod
PS_{108}	Blue-green	Non-mucoid	-	Rod
PS_{109}	Blue-green	Non-mucoid	-	Rod
PS_{111}	Blue-green	Mucoid	-	Rod
PS_{112}	Blue-green	Mucoid	-	Rod
PS_{113}	Blue-green	Non-mucoid	-	Rod
PS_{123}	Blue-green	Non-mucoid	-	Rod
PS_{133}	Blue-green	Non-mucoid	-	Rod
PS_{137}	Blue-green	Non-mucoid	-	Rod
PS_{139}	Blue-green	Mucoid	-	Rod
PS_{155}	Blue-green	Mucoid	-	Rod
PS_{167}	Blue-green	Mucoid	-	Rod
PS_{168}	Blue-green	Mucoid	-	Rod
PS_{170}	Blue-green	Non-mucoid	-	Rod
PS_{185}	Blue-green	Non-mucoid	-	Rod
PS_{195}	Blue-green	Non-mucoid	-	Rod
PS_{196}	Blue-green	Mucoid	-	Rod
PS_{197}	Blue-green	Mucoid	-	Rod
PS_{204}	Blue-green	Mucoid	-	Rod
PS_{205}	Blue-green	Mucoid	-	Rod
PS_{231}	Blue-green	Mucoid	-	Rod

PS1-PS231: *P. aeruginosa* isolates

C: Pigmentation, biochemical and growth properties of *P. aeruginosa* isolates

N#	Strain ID	cetrimide agar	Blue-green (pyocyanin)	Red (pyorubin)	Yellow (fluorescein)	Brown (pyomelanin)	Catal ase	Oxid ase	Growth at 42^{O}C	*Opr*L gene	β haemoly sis
					STOOL ISOLATES						
1	**PS_1**	+	+	+	−	−	+	+	+	+	+
2	**PS_2**	+	+	+	−	−	+	+	+	+	+
3	**PS_3**	+	+	−	−	−	+	+	+	+	+
4	**PS_4**	+	+	+	−	−	+	+	+	+	+
5	**PS_5**	+	+	+	+	−	+	+	+	+	+
6	**PS_6**	+	+	+	+	−	+	+	+	+	+
7	**PS_7**	+	+	−	−	−	+	+	+	+	+
8	**PS_8**	+	+	−	−	−	+	+	+	+	+
9	**PS_9**	+	−	−	+	−	+	+	+	+	+
10	**PS_{10}**	+	+	−	−	−	+	+	+	+	+
11	**PS_{11}**	+	+	+	+	−	+	+	+	+	+
12	**PS_{12}**	+	+	+	+	−	+	+	+	+	+
13	**PS_{13}**	+	+	−	+	−	+	+	+	+	+
14	**PS_{14}**	+	+	+	+	−	+	+	+	+	+
15	**PS_{15}**	+	+	+	+	−	+	+	+	+	+
16	**PS_{16}**	+	+	−	−	−	+	+	+	+	+
17	**PS_{17}**	+	−	−	+	−	+	+	+	+	+
18	**PS_{18}**	+	+	+	+	−	+	+	+	+	+
19		+	+	−	+	−	+	+	+	−	+
20		+	+	+	−	−	+	+	+	−	+
21		+	−	−	−	−	−	+	+	−	+
22	**PS_{19}**	+	−	−	−	−	+	+	+	+	+
23		+	−	−	−	−	+	+	+	−	+
24	**PS_{20}**	+	−	−	−	−	+	+	+	+	+
25	**PS_{21}**	+	+	−	−	−	+	+	+	+	+

N#	Strain ID	Growth on cetrimide agar	Blue-green (pyocyanin)	Red (pyorubin)	Yellow (fluorescein)	Brown (pyomelanin)	Catal ase	Oxid ase	Growth at 42^{O}C	*Opr*L gene	β haemoly sis
26		+	+	–	–	–	+	+	+	–	+
27		+	+	–	–	–	+	+	+	–	+
28		+	–	–	–	–	–	+	+	–	+
29	**PS$_{22}$**	+	+	–	–	–	+	+	+	+	+
30		+	+	–	+	–	+	+	+	–	+
31	**PS$_{23}$**	+	–	–	–	–	–	–	+	+	+
32		+	–	–	–	+	+	+	+	–	+
33	**PS$_{24}$**	+	+	–	+	–	+	+	+	+	+
34		+	+	–	–	–	+	–	+	–	+
35	**PS$_{25}$**	+	+		+	–	+	+	+	+	+
36		+	+	–	–	–	+	+	+	–	+
37	**PS$_{26}$**	+	+	–	–	–	+	+	+	+	+
38		+	–	–	–	–	–	–	+	–	+
39	**PS$_{27}$**	+	–	–	–	–	+	+	+	+	+
40		+	–	–	–	–	–	–	+	–	+
41	**PS$_{28}$**	+	+	+	–	–	+	+	+	+	+
42	**PS$_{29}$**	+	–	–	+	–	+	+	+	+	+
43	**PS$_{30}$**	+	+	–	–	–	+	+	+	+	+
44		+	–	–	–	–	–	–	+	–	+
45	**PS$_{31}$**	+	+	–	–	–	+	+	+	+	+
46		+	–	–	–	–	–	–	+	-	+
47	**PS$_{32}$**	+	+	–	–	–	+	+	+	+	+
					URINE ISOLATES						
48	**PS$_{33}$**	+	+	–	–	–	+	+	+	+	+
49		+	+	–	–	–	+	+	+	–	+
50	**PS$_{34}$**	+	+	–	–	–	+	+	+	+	+
51		+	–	–	–	–	+	+	+	–	+
52		+	–	–	–	–	+	+	+	–	+

N#	Strain ID	Growth on cetrimide agar	Blue-green (pyocyanin)	Red (pyorubin)	Yellow (fluorescein)	Brown (pyomelanin)	Catal ase	Oxid ase	Growth at 42^{O}C	*Opr*L gene	β haemoly sis
53		+	–	–	–	–	+	+	+	–	+
54		+	–	–	–	–	+	+	+	–	+
55		+	–	–	–	–	+	+	–	–	+
56		+	–	–	–	–	+	–	–	–	+
57		+	–	–	–	–	+	+	–	–	+
58		+	–	–	–	–	+	+	+	–	+
59		+	+	+	+	–	+	+	+	–	+
60		+	–	+	–	–	+	+	+	–	+
61		+	–	–	–	–	+	+	+	–	+
62	**PS$_{35}$**	+	+	–	–	–	+	+	+	+	+
63	**PSP$_{36}$**	+	+	–	–	–	+	+	+	+	+
64		+	–	+	–	–	+	+	+	–	+
65		+	+	–	+	–	+	+	+	–	+
66		+	–	–	–	–	+	–	+	–	+
67		+	–	–	–	–	+	+	+	–	+
68		+	–	–	–	–	+	+	+	–	+
69		+	–	–	–	–	+	+	+	–	+
70		+	–	–	–	–	–	+	–	-	+
71		+	+	–	+	–	+	+	+	–	+
72		+	+	–	+	–	+	+	+	–	+
73		+	–	–	–	–	+	–	+	–	+
74		+	–	–	–	–	+	–	+	–	+
75		+	–	–	–	–	+	+	+	–	+
76	**PS$_{37}$**	+	+	–	+	–	+	+	+	+	+
77		+	–	–	–	–	+	+	+	–	+
78		+	–	–	–	–	+	+	+	–	+
79		+	+	–	+	–	+	+	+	–	+
80	**PS$_{38}$**	+	+	–	+	–	+	+	+	+	+

N#	Strain ID	Growth on cetrimide agar	Blue-green (pyocyanin)	Red (pyorubin)	Yellow (fluorescein)	Brown (pyomelanin)	Catal ase	Oxid ase	Growth at 42^{O}C	*Opr*L gene	β haemoly sis
81	**PS$_{39}$**	+	+	–	–	–	+	+	+	+	+
82	**PS$_{40}$**	+	+	–	+	–	+	+	+	+	+
83	**PS$_{41}$**	+	–	–	–	–	+	+	+	+	+
84	**PS$_{42}$**	+	–	–	–	–	+	+	+	+	+
85	**PS$_{43}$**	+	–	–	–	–	+	+	+	+	+
86		+	–	–	–	–	+	+	+	-	+
87		+	–	–	–	–	+	–	+	-	+
88		+	–	–	–	–	+	–	+	-	+
89	**PS$_{44}$**	+	+	–	+	–	+	+	+	+	+
90	**PS$_{45}$**	+	+	–	+	–	+	+	+	+	+
91		+	–	–	–	–	+	–	+	-	+
92		+	–	–	–	–	+	–	+	–	+
93		+	–	–	–	–	–	–	+	-	+
94		+	–	–	–	–	+	–	–	-	+
95		+	–	–	–	–	+	–	+	-	+
96		+	–	–	–	–	+	–	–	–	+
97		+	–	–	+	–	+	+	+	–	+
98	**PS$_{46}$**	+	–	–	–	–	–	+	+	+	+
					BLOOD ISOLATES						
99		+	–	–	–	–	+	–	–	–	+
100		+	–	–	–	–	+	–	–	–	+
					ENVIRONMENTAL ISOLATES						
101		+	–	–	–	–	+	–	+	-	+
102	**PS$_{47}$**	+	+	–	+	+	+	+	+	+	+
103		+	+	–	–	+	+	–	+	-	+
104		+	+	–	+	–	+	–	+	-	+
105	**PS$_{48}$**	+	+	–	+	–	+	+	+	+	+

N#	Strain ID	Growth on cetrimide agar	Blue-green (pyocyanin)	Red (pyorubin)	Yellow (fluorescein)	Brown (pyomelanin)	Catal ase	Oxid ase	Growth at 42^{O}C	*Opr*L gene	β haemoly sis
106	**PS$_{49}$**	+	+	−	−	+	+	+	+	+	+
107		+	+	−	+	−	+	+	+	−	+
108	**PS$_{50}$**	+	−	−	−	−	+	−	+	+	+
109	**PS$_{51}$**	+	+	−	−	−	+	+	+	+	+
110	**PS$_{52}$**	+	+	−	+	−	+	+	+	+	+
111	**PS$_{53}$**	+	−	−	−	−	+	−	+	+	+
112	**PS$_{54}$**	+	+	−	+	−	+	+	+	+	+
113	**PS$_{55}$**	+	+	−	+	−	+	+	+	+	+
114		+	+	−	+	−	+	+	+	−	+
115		+	+	−	+	−	+	+	+	−	+
116		+	+	−	+	+	+	+	+	−	+
117	**PS$_{56}$**	+	−	−	−	−	+	+	+	+	+
118		+	−	−	−	−	+	−	+	+	+
119		+	−	−	−	−	−	+	+	−	+
120		+	−	−	−	−	−	−	+	+	+
121		+	+	−	+	−	+	+	+	−	−
122		+	+	−	+	−	+	+	+	+	+
123	**PS$_{57}$**	+	−	−	+	−	+	+	+	+	+
124		+	+	−	+	−	+	+	+	−	+
125		+	+	−	+	−	+	+	+	−	+
126		+	+	−	+	−	+	+	+	−	+
127		+	+	−	+	−	+	+	+	−	+
128		+	+	−	+	+	+	+	+	−	+
129		+	+	−	+	+	+	+	+	−	+
130		+	+	−	+	+	+	+	+	−	+
131		+	+	−	+	−	+	+	+	−	+
132		+	+	−	+	−	+	+	+	−	+
133	**PS$_{58}$**	+	+	−	+	−	+	+	+	+	+

N#	Strain ID	Growth on cetrimide agar	Blue-green (pyocyanin)	Red (pyorubin)	Yellow (fluorescein)	Brown (pyomelanin)	Catal ase	Oxid ase	Growth at 42^{O}C	*Opr*L gene	β haemoly sis
134		+	–	–	–	–	+	+	+	–	+
135		+	–	+	–	–	+	+	+	–	+
136	**PS_{59}**	+	–	–	–	–	–	+	+	+	–
137	**PS_{60}**	+	–	–	–	–	–	+	+	+	+
138	**PS_{61}**	+	+	–	–	–	+	+	+	+	+
139	**PS_{62}**	+	+	–	+	–	+	+	+	+	–
140	**PS_{63}**	+	+	–	+	–	+	+	+	+	+
141	**PS_{64}**	+	+	–	+	–	+	+	+	+	+
142		+	–	+	–	–	+	+	+	–	+
143		+	–	+	–	–	+	+	+	–	+
144		+	+		+	–	+	+	+	–	+
145		+	+	–	–	–	+	+	+	–	+
146		+	–	+	–	–	+	+	+	–	+
147	**PS_{65}**	+	–	–	–	–	+	+	+	+	+
148		+	+	–	+	–	+	+	+	–	+
149		+	+	–	+	–	+	+	+	–	+
150		+	–	–	+	–	+	+	+	–	+
151		+	–	–	–	–	+	+	+	–	+
152		+	–	+	–	–	+	–	+	–	+
153		+	+	–	–	–	+	+	+	–	+
154	**PS_{66}**	+	+	–	+	–	+	+	+	+	+
155	**PS_{67}**	+	+	–	+	–	+	+	+	+	+
156		+	–	–	–	–	+	–	+	–	+
157		+	+	–	+	–	+	+	+	–	+
158		+	–	–	–	–	+	+	+	–	+
159		+	–	–	–	–	–	+	+	–	+
160		+	+	–	+	–	+	+	+	–	+
161		+	–	–	–	–	+	+	+	–	–

N#	Strain ID	Growth on cetrimide agar	Blue-green (pyocyanin)	Red (pyorubin)	Yellow (fluorescein)	Brown (pyomelanin)	Catal ase	Oxid ase	Growth at 42^{O}C	*Opr*L gene	β haemoly sis
162		+	+	−	+	−	+	+	+	−	+
163		+	+	−	+	+	+	+	+	−	+
164	**PS$_{68}$**	+	+	−	+	−	+	+	+	+	+
165		+	+	−	+	−	+	+	+	−	+
166		+	−	+	−	−	+	+	+	−	+
167	**PS$_{69}$**	+	+	−	+	−	+	+	+	+	+
168	**PS$_{70}$**	+	+	−	+	−	+	+	+	+	+
169		+	−	−	−	−	+	+	+	−	+
170	**PS$_{71}$**	+	+	+	+	+	+	+	+	+	+
171	**PS$_{73}$**	+	+	−	+	−	+	+	+	+	+
172		+	+	−	+	−	+	+	+	−	+
173		+	−	−	−	−	+	+	+	−	+
174	**PS$_{72}$**	+	−	−	+	−	+	+	+	+	+
175		+	+	−	+	−	+	+	+	−	+
176		+	−	−	+	−	+	+	+	−	+
177		+	−	−	−	−	+	+	+	−	+
178		+	+	−	+	−	+	+	+	−	+
179		+	+	−	+	−	+	+	+	−	+
180		+	−	−	−	−	+	+	+	−	+
181		+	+	−	+	−	+	+	+	−	+
182		+	+	−	+	−	+	+	+	−	+
183	**PS$_{74}$**	+	+	−	+	−	+	+	+	+	+
184		+	+	−	+	−	+	+	+	−	+
185	**PS$_{75}$**	+	−	−	−	−	+	+	+	+	+
186		+	−	−	−	−	+	+	+	−	+
187		+	+	−	+	−	+	+	+	−	+
188		+	−	−	−	−	+	+	+	−	+
189		+	−	−	−	−	+	+	+	−	+

N#	Strain ID	Growth on cetrimide agar	Blue-green (pyocyanin)	Red (pyorubin)	Yellow (fluorescein)	Brown (pyomelanin)	Catal ase	Oxid ase	Growth at 42^{O}C	*Opr*L gene	β haemoly sis
190		+	–	–	–	–	+	+	+	–	+
191		+	+	–	+	–	+	+	+	–	+
192	**PS$_{76}$**	+	+	–	+	–	+	+	+	+	+
193		+	–	–	–	–	+	–	+	–	+
194	**PS$_{77}$**	+	+	–	+	–	+	+	+	+	+
195	**PS$_{78}$**	+	+	–	+	–	+	+	+	+	+
196	**PS$_{79}$**	+	–	–	–	–	+	+	+	+	+
197	**PS$_{80}$**	+	–	–	–	–	+	+	+	+	+
198		+	+	–	+	–	+	+	+	–	+
199		+	–	–	–	–	+	+	+	–	+
200		+	+	–	–	–	+	+	+	–	+
201		+	–	–	–	–	+	+	+	–	+
202		+	–	–	–	–	+	–	+	–	+
203		+	+	–	+	–	+	+	+	–	+
204	**PS$_{81}$**	+	+	–	+	–	+	+	+	+	+
205	**PS$_{82}$**	+	+	–	+	–	+	+	+	+	+
206		+	–	–	–	–	+	+	+	–	–
207		+	–	–	–	–	+	+	+	–	–
208		+	+	–	+	–	+	+	+	–	+
209		+	+	–	+	–	+	+	+	–	+
210		+	–	–	–	–	+	–	+	-	–
211		+	–	–	–	–	–	–	+	–	+
212		+	–	–	–	–	+	–	+	–	+
213		+	+	–	+	+	+	+	+	–	+
214		+	+	–	+	–	+	+	+	–	+
215		+	–	–	–	+	+	+	+	–	+
216		+	+	–	+	–	+	+	+	–	+
217		+	–	–	+	–	+	+	+	–	+

N#	Strain ID	Growth on cetrimide agar	Blue-green (pyocyanin)	Red (pyorubin)	Yellow (fluorescein)	Brown (pyomelanin)	Catal ase	Oxid ase	Growth at 42^{O}C	*Opr*L gene	β haemoly sis
218		+	+	+	–	–	+	+	+	–	+
219		+	+	–	+	–	+	+	+	–	+
220		+	–	+	–	–	+	+	+	–	–
221		+	+	–	+	–	+	+	+	–	+
222		+	+	–	+	–	+	+	+	–	+
223		+	–	–	–	–	+	–	+	–	+
224		+	+	–	+	+	+	+	+	–	+
225		+	–	–	+	–	+	+	+	–	+
226		+	+	–	+	–	+	+	+	–	+
227		+	+	–	+	+	+	+	+	–	+
228		+	+	–	+	–	+	+	+	–	+
					POULTRY LITTER						
229		+	–	–	–	–	+	–	+	–	+
230		+	–	–	–	–	+	+	+	–	+
231	**PS$_{83}$**	+	–	–	+	–	+	+	+	+	+
232		+	–	–	–	–	+	–	–	–	–
233		+	–	–	+	–	+	+	+	–	+
234		+	–	–	–	+	+	+	+	–	+
235		+	+	–	–	–	+	+	+	–	+
236		+	–	–	+	–	+	+	+	–	–
237		+	+	–	+	–	+	+	+	–	+
238		+	–	–	–	–	+	–	–	–	+
239		+	–	–	–	–	+	–	–	–	+
240		+	–	–	+	–	+	+	+	–	+
241		+	+	–	–	+	+	+	+	–	+
242		+	–	–	–	+	+	–	–	–	+
243		+	–	–	+	–	+	+	+	–	+
244		+	–	–	–	–	+	–	+	–	–

N#	Strain ID	Growth on cetrimide agar	Blue-green (pyocyanin)	Red (pyorubin)	Yellow (fluorescein)	Brown (pyomelanin)	Catalase	Oxidase	Growth at 42^{O}C	*Opr*L gene	β haemolysis
245		+	–	–	–	–	+	–	+	–	+
246		+	–	–	–	–	+	–	+	–	+
247		+	–	–	–	–	+	–	+	–	+
248		+	–	–	–	–	+	+	–	-	+
249		+	+	–	–	–	+	+	+	–	+
250		+	–	–	+	–	+	+	+	–	+
251		+	–	–	–	–	+	+	+	–	+
252	**PS$_{84}$**	+	–	–	+	–	+	+	+	+	+
253		+	–	–	+	–	+	+	+	–	+
254		+	–	–	–	–	+	–	–	–	+
255	**PS$_{85}$**	+	–	–	–	–	+	+	+	+	+
256		+	+	–	–	–	+	+	+	–	+
257		+	–	–	–	–	+	+	+	–	+
258		+	+	–	–	–	+	+	+	–	+
259		+	+	–	–	–	+	+	+	–	+
260		+	–	–	–	–	+	+	+	–	+
261		+	+	–	–	–	+	+	+	–	+
262	**PS$_{86}$**	+	–	–	–	–	+	+	+	+	+
263	**PS$_{87}$**	+	–	–	–	–	+	+	+	+	+
264		+	–	–	–	–	+	+	+	+	–
					ORAL SWABS OF FARM HANDS						
265		+	–	–	–	–	+	–	–	–	+
268		+	–	–	–	–	–	+	+	–	+
269		+	–	–	–	–	–	–	–	–	+
270		+	–	–	–	–	+	+	+	–	+
271		+	–	–	–	–	+	+	–	–	+
272		+	–	–	–	–	+	+	–	–	+
273		+	–	–	–	–	+	+	+	-	+

APPENDIX IV

MEAN ZONES OF GROWTH INHIBITION OF ANTIPSEUDOMONAL AGENTS

A: Breakpoint values for interpretation of zone diameters as stated by European Committee on Antimicrobial Susceptibility Testing EUCAST (2015)

INFERENCE	CIP	LEV	IPM	MEM	CN	TIC	TIM	PIP	FEP	CAZ	ATM
	Mean diameter zone of growth inhibition										
S ≥	25	20	20	24	15	18	18	18	19	16	50
R <	22	17	17	18	15	18	18	18	19	16	16

CIP: Ciprofloxacin; LEV: Levofloxacin; MEM: Meropenem; IPM: Imipenem; CN: Gentamycin; TIC: Ticarcillin; TIM: Ticarcillin/ Clavulanic acid; FEP: Cefepime; ATM: Aztreonam; CAZ: Ceftazidime; PIP: Piperacillin

B: Susceptibility of *P. aeruginosa* isolates against the selected antibiotics

STRAIN ID	QUINOLONES						CARBEPENEMS						AMINOGLYCOSIDES			PENICILLIN		
	CIPROFLOXACIN CIP			LEVOFLOXACIN LEV			MEROPENEM MEM			IMIPENEM IPM			GENTAMYCIN CN			TICARCILLIN TIC		
	MEAN	SD	I	MEAN	SD	I	MEAN	SD	I	MEAN	SD	I	MEAN	SD	I	MEAN	SD	I
PS1	0.0	0.0	R	0.0	0.0	R	27.0	0.0	S	19.3	0.5		9.0	0.0	R	0.0	0.0	R
PS2	0.0	0.0	R	20.7	0.5	S	24.7	0.5	S	19.0	0.0		16.0	0.0	S	18.7	0.5	S
PS3	12.0	0.0	R	19.0	0.0		27.3	0.5	S	20.3	0.5	S	0.0	0.0	R	10.0	0.0	R
PS4	15.0	0.0	R	20.0	0.0	S	25.0	0.0	S	22.0	0.0	S	0.0	0.0	R	0.0	0.0	R
PS5	10.3	0.5	R	15.0	0.0	R	28.0	0.0	S	18.0	0.0		0.0	0.0	R	0.0	0.0	R
PS6	24.3	0.5		24.7	0.5	S	25.0	0.0	S	20.0	0.0		10.0	0.0	R	0.0	0.0	R
PS7	24.3	0.5		25.0	0.0	S	30.0	0.0	S	18.0	0.0		0.0	0.0	R	21.3	0.5	S

STRAIN ID	CIP MEAN	SD	I	LEV MEAN	SD	I	MEM MEAN	SD	I	IPM MEAN	SD	I	CN MEAN	SD	I	TIC MEAN	SD	I
PS8	34.0	0.0	R	25.0	0.0		24.7	0.0		20.0	0.0		16.0	0.0		0.0	0.0	R
PS9	15.7	0.5	R	18.3	0.5		31.7	0.5	S	24.0	0.0	S	0.0	0.0	R	24.7	0.5	S
PS10	34.7	0.5	S	24.7	0.5	S	24.0	0.0	S	25.0	0.0	S	19.0	0.0	S	22.0	0.0	S
PS11	31.3	0.5	S	30.3	0.5	S	25.3	0.5	S	17.0	0.0		16.0	0.0	S	17.0	0.0	R
PS12	32.7	0.9	S	30.3	0.5	S	29.7	0.5	S	19.0	0.0		0.0	0.0	R	30.0	0.0	S
PS13	28.3	0.5	S	28.0	0.0	S	28.0	0.0	S	24.3	0.5	S	16.0	0.0	S	18.7	0.5	S
PS14	20.7	0.5	R	22.0	0.0	S	25.7	0.5	S	19.0	0.0		0.0	0.0	R	16.7	0.5	R
PS15	14.7	0.5	R	20.0	0.8	S	30.0	0.0	S	20.0	0.0	S	15.0	0.0	S	0.0	0.0	R
PS16	25.0	0.0	S	20.0	0.0	S	25.0	0.0	S	26.0	0.0	S	15.0	0.0	S	0.0	0.0	R
PS17	21.0	0.0	R	20.0	0.0	S	25.0	0.0	S	20.0	0.0	S	10.0	0.0	R	0.0	0.0	R
PS18	21.0	0.0	R	22.0	0.0	S	27.0	0.0	S	19.0	0.0		22.0	0.0	S	0.0	0.0	R
PS22	0.0	0.0	R	20.3	7.8	S	31.3	0.5	S	23.3	0.5	S	10.0	0.0	R	20.0	0.0	S
PS24	21.0	0.0	R	20.0	0.0	S	25.0	0.0	S	23.0	0.0	S	0.0	0.0	R	24.0	0.0	S
PS25	0.0	0.0	R	14.0	0.0	R	22.0	0.0		16.0	0.0	R	10.3	0.5	R	14.7	0.5	R
PS29	0.0	0.0	R	0.0	0.0	R	28.3	0.5	S	24.0	0.0	S	11.0	0.0	R	0.0	0.0	R
PS31	0.0	0.0	R	20.0	0.0	S	30.0	0.0	S	0.0	0.0	R	10.0	0.0	R	26.0	0.0	S
PS33	0.0	0.0	R	14.7	0.5	R	26.0	0.0	S	19.0	0.0		11.3	0.5	R	25.3	0.5	S
PS35	0.0	0.0	R	11.0	0.0	R	26.0	0.0	S	20.0	0.0	S	11.0	0.0	R	29.7	0.5	S
PS37	13.0	0.0	R	19.0	0.0		25.0	0.0	S	19.0	0.0		0.0	0.0	R	22.0	0.0	S
PS39	40.0	0.0	S	32.0	0.0	S	30.0	0.0	S	29.0	0.0	S	18.3	0.5	S	28.3	0.5	S
PS41	30.0	0.0	S	30.0	0.0	S	27.0	0.0	S	21.0	0.0	S	11.0	0.0	R	10.0	0.0	R
PS42	0.0	0.0	R	15.0	0.0	R	25.0	0.0	S	21.0	0.0	S	16.0	0.0	S	26.0	0.0	S
PS43	25.0	0.0	S	23.0	0.0	S	25.0	0.0	S	20.0	0.0	S	11.0	0.0	R	22.3	0.5	S
PS45	0.0	0.0	R	30.0	0.0	S	27.0	0.0	S	20.0	0.0	S	10.3	0.5	R	28.0	0.0	S
PS47	21.0	0.0	R	21.0	0.0	S	22.0	0.0		20.0	0.0	S	20.0	0.0	S	25.0	0.0	S
PS48	33.7	0.5	S	30.0	0.0	S	0.0	0.0	R	19.0	0.0		11.0	0.0	R	26.0	0.0	S
PS50	26.0	0.0	S	26.3	0.5	S	28.0	0.0	S	21.0	0.0	S	11.0	0.0	R	23.0	0.0	S
PS62	0.0	0.0	R	0.0	0.0	R	28.0	0.0	S	21.0	0.0	S	14.0	0.0	R	28.0	0.0	S

STRAIN ID	CIP MEAN	SD	I	LEV MEAN	SD	I	MEM MEAN	SD	I	IPM MEAN	SD	I	CN MEAN	SD	I	TIC MEAN	SD	I
PS63	0.0	0.0	R	0.0	0.0	R	28.0	0.0	S	19.0	0.0		0.0	0.0	R	28.0	0.0	S
PS76	35.7	0.5	S	30.0	0.0	S	29.0	0.0	S	15.7	0.5	R	16.0	0.0	S	26.0	0.0	S
PS80	23.0	0.0		24.0	0.0	S	26.0	0.0	S	20.0	0.0	S	10.0	0.0	R	29.0	0.0	S
PS81	22.0	0.0		19.0	0.0		23.0	0.0		20.0	0.0	S	9.0	0.0	R	0.0	0.0	R
PS82	0.0	0.0	R	0.0	0.0	R	25.0	0.0	S	23.0	0.0	S	10.0	0.0	R	0.0	0.0	R
PS83	0.0	0.0	R	24.0	0.0	S	24.0	0.0	S	22.0	0.0	S	11.0	0.0	R	25.3	0.5	S
PS84	11.3	0.5	R	16.3	0.5	R	25.7	0.5	S	23.0	0.0	S	10.0	0.0	R	0.0	0.0	R
PS85	20.0	0.0	R	20.0	0.0	S	0.0	0.0	R	0.0	0.0	R	0.0	0.0	R	0.0	0.0	R
PS89	18.0	0.0	R	25.0	0.0	S	26.0	0.0	S	22.0	0.0	S	0.0	0.0	R	25.0	0.0	S
PS90	30.0	0.0	S	30.0	0.0	S	30.0	0.0	S	22.0	0.0	S	10.0	0.0	R	26.0	0.0	S
PS98	10.0	0.0	R	14.0	0.0	R	0.0	0.0	R	0.0	0.0	R	0.0	0.0	R	0.0	0.0	R
PS102	10.0	0.0	R	10.0	0.0	R	30.0	0.0	S	25.0	0.0	S	12.0	0.0	R	0.0	0.0	R
PS105	0.0	0.0	R	15.0	0.0	R	28.0	0.0	S	22.0	0.0	S	12.0	0.0	R	0.0	0.0	R
PS106	9.0	0.0	R	17.7	4.5		26.0	0.0	S	21.0	0.0	S	0.0	0.0	R	21.0	0.0	S
PS108	0.0	0.0	R	21.0	0.0	S	0.0	0.0	R	0.0	0.0	R	7.3	5.2	R	0.0	0.0	R
PS109	17.3	1.9	R	20.7	0.5	S	21.0	0.8		19.0	0.0		11.0	0.0	R	0.0	0.0	R
PS110	20.0	0.8	R	21.0	0.8	S	25.0	0.0	S	25.0	0.0	S	14.0	0.0	R	18.0	0.0	S
PS111	15.0	0.0	R	21.0	0.0	S	28.0	0.0	S	22.0	0.0	S	0.0	0.0	R	0.0	0.0	R
PS112	18.0	0.0	R	20.0	0.0	S	32.3	0.5	S	26.0	0.0	S	0.0	0.0	R	0.0	0.0	R
PS113	0.0	0.0	R	0.0	0.0	R	29.3	0.9	S	23.0	0.0	S	11.0	0.0	R	0.0	0.0	R
PS117	30.0	0.0	S	30.0	0.0	S	24.0	0.0	S	22.0	0.0	S	12.0	0.0	R	29.0	0.0	S
PS123	0.0	0.0	R	21.3	0.5	S	25.0	0.0	S	23.0	0.0	S	12.0	0.0	R	0.0	0.0	R
PS133	10.0	0.0	R	19.7	1.2		30.0	0.0	S	25.0	0.0	S	12.0	0.0	R	20.0	0.0	S
PS136	24.3	0.5		25.7	0.5	S	29.7	0.5	S	25.0	0.0	S	13.0	0.0	R	21.0	0.0	S
PS137	18.0	0.0	R	20.0	0.0	S	29.7	0.5	S	25.0	0.0	S	0.0	0.0	R	25.0	0.0	S
PS138	0.0	0.0	R	25.7	0.5	S	26.0	0.0	S	26.3	0.5	S	15.3	0.5	S	13.0	0.0	R
PS139	21.0	0.8	R	23.0	0.0	S	30.0	0.0	S	24.0	0.0	S	15.0	0.0	S	0.0	0.0	R
PS140	27.0	0.8	S	25.0	0.0	S	30.0	0.0	S	25.0	0.0	S	15.0	0.0	S	19.0	0.0	S

STRAIN ID	CIP MEAN	SD	I	LEV MEAN	SD	I	MEM MEAN	SD	I	IPM MEAN	SD	I	CN MEAN	SD	I	TIC MEAN	SD	I
PS141	30.3	0.5	S	30.3	0.5	S	36.0	0.0	S	30.0	0.0	S	16.0	0.0	S	12.0	0.0	R
PS147	25.0	0.0	S	24.0	0.0	S	21.7	1.2		26.0	0.0	S	11.0	0.0	R	10.3	0.5	R
PS154	30.7	0.5	S	30.0	0.0	S	25.3	0.5	S	25.7	0.5	S	25.0	0.0	S	15.0	0.0	R
PS155	0.0	0.0	R	29.0	0.8	S	26.3	0.5	S	23.0	0.0	S	11.0	0.0	R	17.7	11.1	R
PS164	0.0	0.0	R	0.0	0.0	R	26.3	0.5	S	26.3	0.5	S	0.0	0.0	R	29.3	0.5	S
PS167	0.0	0.0	R	24.7	0.5	S	30.0	0.0	S	24.3	0.5	S	11.0	0.0	R	0.0	0.0	R
PS168	0.0	0.0	R	19.7	1.7		26.0	0.0	S	27.0	0.0	S	0.0	0.0	R	0.0	0.0	R
PS170	0.0	0.0	R	16.7	0.5	R	25.0	0.0	S	25.0	0.0	S	11.0	0.0	R	0.0	0.0	R
PS171	30.0	0.0	S	25.0	0.0	S	26.0	0.0	S	26.0	0.0	S	26.0	0.0	S	14.3	0.5	R
PS183	34.0	1.4	S	30.0	0.0	S	38.0	1.6	S	26.0	0.0	S	15.0	0.0	S	21.0	0.0	S
PS185	25.0	0.0	S	22.3	0.5	S	20.7	0.5		22.7	0.5	S	0.0	0.0	R	0.0	0.0	R
PS192	30.0	0.0	S	28.3	0.5	S	35.7	0.5	S	27.0	0.0	S	15.0	0.0	S	29.0	0.0	S
PS194	20.0	0.0	R	24.7	0.5	S	26.7	2.6	S	19.0	0.0		20.0	0.0	S	21.3	0.5	S
PS195	0.0	0.0	R	19.0	0.8		24.0	0.0	S	25.0	0.0	S	11.0	0.0	R	0.0	0.0	R
PS196	25.7	0.5	S	20.0	0.0	S	30.0	0.0	S	24.7	0.5	S	0.0	0.0	R	0.0	0.0	R
PS197	0.0	0.0	R	0.0	0.0	R	34.0	0.0	S	27.0	0.0	S	0.0	0.0	R	0.0	0.0	R
PS204	22.7	0.5		21.7	0.5	S	24.0	0.0	S	22.0	0.0	S	0.0	0.0	R	10.0	0.0	R
PS205	26.3	0.5	S	26.0	0.0	S	0.0	0.0	R	0.0	0.0	R	16.0	0.0	S	12.0	0.0	R
PS231	16.0	0.0	R	20.0	0.0	S	0.0	0.0	R	0.0	0.0	R	0.0	0.0	R	0.0	0.0	R
PS252	21.3	0.5	R	22.3	0.5	S	21.3	0.9		27.0	0.0	S	16.0	0.0	S	10.0	0.0	R
PS255	30.0	0.0	S	29.0	0.8	S	30.7	0.9	S	25.0	0.0	S	26.0	0.0	S	25.0	0.0	S
PS262	27.7	2.1	S	22.0	0.0	S	20.0	0.0		28.0	0.0	S	25.0	0.0	S	0.0	0.0	R
PS263	32.3	0.5	S	23.0	0.8	S	22.3	0.9		30.0	0.0	S	16.7	0.5	S	0.0	0.0	R

STRAIN ID	PENICILLIN						CEPHALOSPORINS						MONOBACTAMS		
	PIPERACILLIN PIP			TICARCILLIN/CLAVULANATE TIM			CEFIPIME FEP			CEFTAZIDIME CAZ			AZTREONAM ATM		
	MEAN	SD	I	MEAN	SD	I	MEAN	SD	I	MEAN	SD	I	MEAN	SD	I
PS1	13.3	0.5	R	16.3	0.5	R	24.0	0.0	S	0.0	0.0	R	21.7	0.5	
PS2	15.3	0.5	R	19.3	0.5	S	23.0	0.0	S	20.0	0.0	S	18.0	0.0	
PS3	16.0	0.0	R	16.0	0.0	R	14.3	0.5	R	20.0	0.0	S	11.0	0.0	R
PS4	12.7	0.5	R	0.0	0.0	R	16.0	0.0	R	23.3	0.5	S	22.0	2.4	
PS5	17.3	0.5	R	21.7	0.5	S	17.7	0.5	R	22.3	0.5	S	20.7	0.5	
PS6	20.3	0.5	S	20.0	0.0	R	23.0	0.0	S	23.0	0.0	S	19.3	0.5	
PS7	23.7	0.5	S	21.3	0.5	S	26.0	0.0	S	23.0	0.0	S	22.0	0.0	
PS8	23.0	0.0	S	21.0	0.0	S	24.0	0.0	S	0.0	0.0	R	20.0	0.0	
PS9	26.7	0.5	S	25.0	0.0	S	23.0	0.0	S	26.0	0.0	S	21.0	0.0	
PS10	25.0	0.0	S	25.0	0.0	S	25.0	0.0	S	26.0	0.0	S	22.7	0.5	
PS11	21.0	0.0	S	21.0	0.0	S	26.0	0.0	S	21.0	0.0	S	24.0	0.0	
PS12	0.0	0.0	R	30.0	0.0	S	32.0	0.8	S	27.0	0.0	S	30.0	0.0	
PS13	25.0	0.0	S	22.0	0.0	S	25.0	0.0	S	25.0	0.0	S	23.0	0.0	
PS14	24.7	0.5	S	20.0	0.0	S	26.7	0.5	S	26.0	0.0	S	24.0	0.0	
PS15	22.0	0.0	S	28.7	0.9	S	37.7	0.5	S	31.0	0.8	S	32.0	0.0	
PS16	15.0	0.0	R	25.0	0.0	S	28.0	0.0	S	25.0	0.0	S	26.0	0.0	
PS17	22.0	0.0	S	21.0	0.0	S	24.3	0.5	S	23.0	0.0	S	20.3	0.5	
PS18	25.0	0.0	S	28.0	0.0	S	32.0	0.0	S	31.3	0.5	S	30.0	0.0	
PS22	29.3	0.9	S	30.0	0.0	S	31.7	0.5	S	29.7	0.5	S	28.0	0.0	
PS24	28.0	0.0	S	25.0	0.0	S	28.3	0.5	S	27.0	0.0	S	27.7	1.2	
PS25	26.0	0.0	S	22.0	0.0	S	25.3	0.5	S	28.0	0.0	S	26.0	0.0	
PS29	25.0	0.0	S	24.3	0.5	S	28.0	0.0	S	26.0	0.0	S	26.0	0.0	
PS31	26.3	0.5	S	10.5	10.5	R	29.0	0.0	S	26.0	0.0	S	26.0	0.0	
PS33	26.0	0.0	S	25.0	0.0	S	29.0	0.0	S	0.0	0.0	R	27.0	0.0	

STRAIN ID	PIP MEAN	SD	I	TIM MEAN	SD	I	FEP MEAN	SD	I	CAZ MEAN	SD	I	ATM MEAN	SD	I
PS35	28.0	0.0	S	24.3	0.5	S	27.0	0.0	S	26.0	0.0	S	24.3	0.5	
PS37	30.7	0.5	S	22.3	0.5	S	25.0	0.8	S	32.0	0.0	S	10.3	0.5	R
PS39	27.0	0.0	S	25.0	0.0	S	27.0	0.0	S	30.0	0.0	S	28.0	0.0	
PS41	25.0	0.0	S	25.0	0.0	S	29.3	0.5	S	12.0	0.0	R	26.0	0.0	
PS42	30.0	0.0	S	27.0	0.0	S	30.0	0.0	S	30.0	0.0	S	30.0	0.0	
PS43	29.0	0.8	S	30.0	0.0	S	28.0	0.0	S	25.0	0.0	S	24.0	0.0	
PS45	27.0	0.0	S	27.0	0.0	S	30.0	0.0	S	29.0	0.0	S	28.0	0.0	
PS47	27.0	0.0	S	26.0	0.0	S	28.0	0.0	S	27.0	0.0	S	24.0	0.0	
PS48	27.7	0.5	S	25.0	0.0	S	25.0	0.0	S	28.0	0.0	S	28.0	0.0	
PS50	25.0	0.0	S	26.0	0.0	S	30.0	0.0	S	30.0	0.0	S	26.0	0.0	
PS62	27.0	0.0	S	26.3	0.5	S	28.0	0.0	S	28.0	0.0	S	30.0	0.0	
PS63	27.0	0.0	S	29.7	0.5	S	30.7	0.5	S	30.0	0.0	S	28.0	0.0	
PS76	31.0	0.0	S	25.0	0.0	S	32.0	0.0	S	30.0	0.0	S	27.0	0.8	
PS80	32.0	0.0	S	25.0	0.0	S	31.0	0.0	S	29.0	0.0	S	22.0	0.0	
PS81	25.0	0.0	S	24.7	0.5	S	27.0	0.0	S	27.3	0.5	S	25.0	0.0	
PS82	26.0	0.0	S	22.0	0.0	S	28.0	0.0	S	27.3	0.5	S	25.0	0.0	
PS83	25.7	0.5	S	22.0	0.0	S	25.0	0.0	S	27.0	0.0	S	26.0	0.0	
PS84	25.0	0.0	S	24.0	0.0	S	28.0	0.0	S	25.0	0.0	S	25.0	0.0	
PS85	0.0	0.0	R	15.0	0.0	R	0.0	0.0	R	0.0	0.0	R	0.0	0.0	R
PS89	25.0	0.0	S	25.0	0.0	S	27.0	0.0	S	26.0	0.0	S	25.0	0.0	
PS90	26.0	0.0	S	21.0	0.0	S	29.0	0.0	S	28.0	0.0	S	27.0	0.0	
PS98	0.0	0.0	R	27.0	0.0	S	0.0	0.0	R	0.0	0.0	R	0.0	0.0	R
PS102	24.0	0.0	S	25.0	0.0	S	28.0	0.0		29.0	0.0	S	26.0	0.0	
PS105	25.0	0.0	S	27.0	0.0	S	29.0	0.0		30.0	0.0	S	26.0	0.0	
PS106	28.0	0.0	S	28.0	0.0	S	29.0	0.0		30.0	0.0	S	28.0	0.0	
PS108	0.0	0.0	R	26.0	0.0	S	17.0	4.2	R	25.0	0.0	S	0.0	0.0	R
PS109	20.0	0.0	S	29.7	0.5	S	31.7	0.5		30.0	0.0	S	30.0	0.0	
PS110	25.0	0.0	S	29.3	0.5	S	26.3	0.5		29.7	0.5	S	22.0	0.0	

STRAIN ID	PIP MEAN	SD	I	TIM MEAN	SD	I	FEP MEAN	SD	I	CAZ MEAN	SD	I	ATM MEAN	SD	I
PS111	24.3	0.5	S	21.0	0.0	S	18.0	0.0	R	31.0	0.0	S	16.3	0.5	
PS112	26.0	0.0	S	26.0	0.0	S	17.0	0.0	R	31.7	0.5	S	11.3	0.5	R
PS113	27.3	0.5	S	28.3	0.9	S	28.0	0.0	S	29.0	0.0	S	27.7	0.5	
PS117	25.0	0.0	S	23.3	0.5	S	29.7	0.5	S	28.7	0.5	S	27.7	0.5	
PS123	25.0	0.0	S	30.0	0.0	S	30.0	0.8	S	30.0	0.0	S	18.0	0.0	
PS133	29.0	0.8	S	30.0	0.0	S	33.3	0.5	S	35.7	0.5	S	13.0	0.0	R
PS136	22.0	0.0	S	27.0	0.0	S	30.0	0.0	S	29.3	0.5	S	26.0	0.0	
PS137	25.0	0.0	S	29.0	0.0	S	27.0	0.0	S	24.7	0.5	S	12.0	0.0	R
PS138	25.7	0.5	S	25.0	0.0	S	31.3	0.5	S	25.3	0.5	S	25.7	0.5	
PS139	26.7	0.5	S	20.3	0.5	S	24.0	0.0	S	0.0	0.0	R	0.0	0.0	R
PS140	29.0	0.0	S	26.3	0.5	S	29.0	0.0	S	24.0	0.0	S	13.7	0.5	R
PS141	30.0	0.0	S	26.0	0.0	S	36.0	0.8	S	32.3	0.5	S	12.0	0.0	R
PS147	25.0	0.0	S	19.0	0.0	S	30.3	0.5	S	28.0	0.8		24.3	0.5	
PS154	27.0	0.0	S	22.0	0.0	S	30.3	0.5	S	30.0	0.0	S	12.0	0.0	R
PS155	30.0	0.0	S	31.3	0.5	S	12.0	0.0	R	0.0	0.0	R	0.0	0.0	R
PS164	30.0	0.8	S	26.0	0.0	S	28.0	4.2	S	32.0	0.0	S	31.7	0.5	
PS167	20.3	0.5	S	22.0	0.0	S	31.0	0.0	S	30.0	0.0	S	29.3	0.5	
PS168	25.0	0.0	S	10.0	0.0	R	27.0	0.0	S	29.3	0.5	S	19.0	0.0	
PS170	18.0	0.0	R	0.0	0.0	R	26.0	0.8	S	27.0	0.0	S	14.0	0.0	R
PS171	29.0	0.0	S	26.0	0.0	S	33.0	0.0	S	30.0	0.0	S	30.0	0.0	
PS183	27.0	0.0	S	21.0	0.0	S	26.0	0.0	S	26.3	0.5	S	24.0	0.0	
PS185	22.7	0.5	S	0.0	0.0	R	28.0	0.0	S	30.3	0.5	S	13.0	0.0	R
PS192	31.3	0.5	S	25.0	0.0	S	32.0	0.0	S	30.3	0.5	S	29.7	0.5	
PS194	0.0	0.0		24.0	0.0	S	30.3	0.5	S	31.0	0.0	S	29.0	0.0	
PS195	29.0	0.0	S	29.0	0.0	S	31.0	0.0	S	29.0	0.0	S	30.0	0.0	
PS196	24.0	0.0	S	20.3	0.5	S	32.0	0.0	S	27.0	0.0	S	13.0	0.0	R
PS197	20.7	0.5	S	13.7	0.5	R	19.3	0.5	S	0.0	0.0	R	0.0	0.0	R
PS204	30.0	0.0	S	25.0	0.0	S	30.0	0.0	S	30.0	0.0	S	10.0	0.0	R

STRAIN ID	PIP MEAN	SD	I	TIM MEAN	SD	I	FEP MEAN	SD	I	CAZ MEAN	SD	I	ATM MEAN	SD	I
PS205	10.0	0.0		15.3	0.5	R	24.0	0.0	S	30.3	0.5	S	0.0	0.0	R
PS231	0.0	0.0		0.0	0.0	R	0.0	0.0	R	0.0	0.0	R	0.0	0.0	R
PS252	28.0	0.0	S	10.7	0.5	R	30.0	0.0	S	31.0	0.8	S	27.3	0.5	
PS255	30.0	0.0	S	22.0	0.0	S	30.0	0.0	S	27.0	0.0	S	24.0	0.0	
PS262	21.0	0.0	S	0.0	0.0	R	25.0	0.0	S	22.0	0.0	S	15.3	0.5	R
PS263	25.7	0.5	S	0.0	0.0	R	30.0	0.0	S	30.3	0.5	S	16.0	0.0	

R: Resistant ; S: Sensitive; SD: Standard deviation; CIP: Ciprofloxacin; LEV: Levofloxacin; MEM: Meropenem; IPM: Imipenem; CN: Gentamycin; TIC: Ticarcillin; TIM: Ticarcillin/ Clavulanic acid; FEP: Cefepime; ATM: Aztreonam; CAZ: Ceftazidime; PIP: Piperacillin