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CHARACTERIZATION OF ANTIMICROBIAL RESISTANT *PSEUDOMONAS* SPP. AND OTHER BACTERIA FROM INFECTED WOUND

BY

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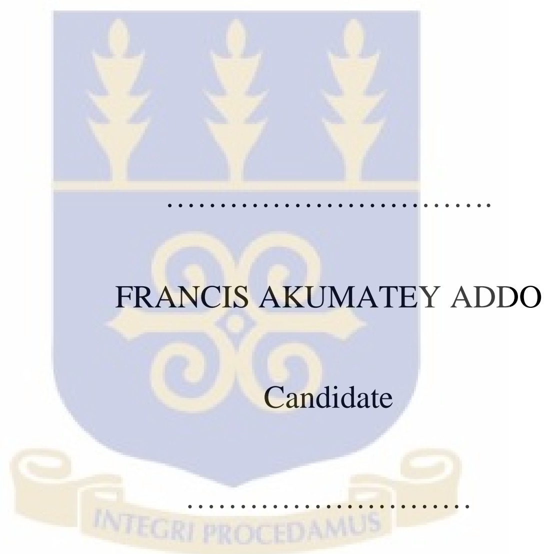
DECLARATION

I hereby declare that the work presented in this thesis is my own original research undertaken in the Department of Microbiology, University of Ghana Medical School (UGMS); under the joint supervision of Dr. Japheth A. Opintan and Professor Mercy J. Newman and that no part of this work has been presented for another degree in this University or elsewhere. All references have been duly acknowledged.



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DEDICATION

This work is affectionately dedicated to all patients whose wound specimen were used in the study, my mother Mrs. Comfort Mamle Addo and my late father, George Tetteh Addo.

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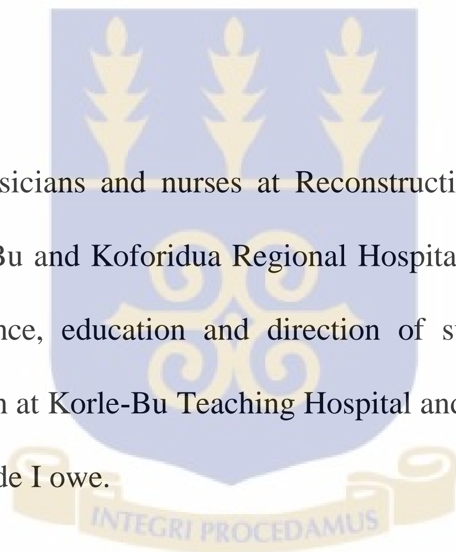

My profound thanks also go to Mr. Richard Harry Asmah, Head of Department, School of Allied Health Sciences, Korle-Bu and Ms. Marjorie Ntiwaa Quarchie for their support of direction in the molecular characterization.

Last but not least, I wish to thank Ms. Asafo Eunice Nukunya for her priceless prayer support.

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ABBREVIATIONS

µg	microgram
µl	microliter
µM	micro molar
ATCC	American Typed Culture Collection
CLSI	Clinical Laboratory Standards Institute
CRE	Carbapenem-resistant Enterobacteriaceae
CR-MDRSA	Carbapenem-resistant Multidrug Resistant *P. aeruginosa*
CRPA	Carbapenem-resistant *Pseudomonas aeruginosa*
DNA	Deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	Ethelene diamine tetra acetate
ESBL	Extended spectrum beta-lactamase
FDA	Food and Drug Administration
GIM	German imipenemase
GNB	Gram negative bacteria
GPB	Gram positive bacteria
IMP	Imipenemase
KBTH	Korle-Bu Teaching Hospital
KRH	Koforidua Regional Hospital
MBL	Metallo-beta-lactamase

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MDR	Multidrug resistant
MDRPA	Multidrug-resistant *Pseudomonas aeruginosa*
ml	milliliter
MRSA	Methicillin-resistant *Staphylococcus aureus*
NDM	New Delhi metallo-β-lactamase
OPD	Out Patient Department
OXA	Oxacillinase
PCR	Polymerase chain reaction
pH	Hydrogen-iron exponent
QS	Quorum sensing
SIM	Seoul imipenemase metallo-beta-lactamase
SPM	São Paulo metallo-beta-lactamase
Spp	Species
TAE	Tris-acetate EDTA
Tris	2-amino-2-(hydroxymethyl)-1, 3-propanediol
TSB	Trypticase Soy Broth
TSI	Triple sugar iron
U.A.E	United Arab Emirates
VIM	Verona integron metallo-beta-lactamase

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ABSTRACT

Background: Wound infections mostly caused by *Pseudomonas* are on the increase in hospitals in Ghana. Most of these infected wounds are very difficult to treat with available antibiotics. This failure of treatment has resulted in increased need for healthcare, undue financial burden on both hospitals and patients and finally death of many of these patients. This has become a source of worry for both patients and healthcare providers in Ghana.

General Aim: The main aim of this study was to characterize antimicrobial resistant bacteria from infected wounds at the Korle-Bu Teaching Hospital (KBTH) and the Koforidua Regional Hospital (KRH) in Ghana.

Methodology: Two hundred and twenty-two (222) consecutive, non-duplicated patients with infected wounds attending the KBTH and the KRH were swabbed. Bacteria were isolated, identified and characterized using standard microbiological methods. Antimicrobial susceptibility of the clinical isolates was determined according to the Clinical Laboratory Standard Institute guidelines (CLSI). Phenotypic and molecular techniques were used to detect carbapenem-resistant *Pseudomonas* strains and presence of metallo-beta-lactamase (MBL) genes, $bla_{\text{VIM-type}}$, $bla_{\text{SPM-type}}$ and $bla_{\text{IMP-type}}$. Vital clinical information of patients was obtained from folders at the time of sample collection and analyzed.

Results: A total number of 194 bacteria were isolated from patients attending KBTH and KRH. The bacteria isolates included *Pseudomonas* spp. 92(47.4%), *Proteus* spp. 27(13.9%), *Citrobacter* spp. 20(10.3%), *Escherichia coli* 15(7.7%), *Klebsiella* spp. 15(7.7%), *Staphylococcus aureus* 14(7.2%), *Enterobacter* spp.

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6(3.1%), *Acinetobacter* spp. 3(1.5%), methicillin-resistant *Staphylococcus aureus* 1(0.5%) and *Morganella morgani* 1(0.5%). At KBTH, *Pseudomonas* spp. represented 67.0% of the 103 patients compared to 25.3% of the 91 patients from KRH.

The antibiotic resistance pattern of *Pseudomonas* spp. showed high level of resistance to ciprofloxacin 24(26.1%) followed by gentamicin 20(21.3%), ceftazidime 18(19.6%), amikacin 15(16.3%), piperacillin/tazobactam 7(7.6%) and meropenem 1(1.1%). Phenotypically, one metallo-beta-lactamase (MBL) producing, carbapenem-resistant multidrug-resistant *Pseudomonas aeruginosa* (CR-MDRPA) was detected from KBTH. Up to 35.1% of 194 bacteria isolated met the criteria used to define multidrug resistance (MDR).

An average of two years duration of non-healing wound was observed among patients with infected wounds attending KBTH and KRH. Increased systemic treatment with ciprofloxacin and metronidazole (Flagyl) were observed. There was no record of any patient treated with carbapenem at the time of sample collection.

Conclusions: In the current study, high prevalence of multidrug resistance varied among isolates; *Pseudomonas* (13.0%), *Proteus* spp. (29.6%), *Acinetobacter* spp. (33.3%), *Klebsiella* spp. (40.0%), *Enterobacter* spp. (66.7%), *Staphylococcus aureus* (66.7%), *Escherichia coli* (73.3%) and *Morganella morgani* (100%). Very low levels of carbapenem-resistant *Pseudomonas* (1.1%) and methicillin-resistant *Staphylococcus aureus* (6.7%) were detected in the infected wounds.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Wound infection is one of the major health problems in the world because it poses serious and disastrous complications that result in many deaths (Mulu *et al*., 2006). Patients with chronic non-healing wounds due to carbapenem resistance multidrug resistant bacteria strains live poorer quality of life due to undue financial burden resulting from frequent hospital visits for wound dressing with high associated costs, many days off work and sometimes loss of employment. These negative effects associated with hard-to-heal wounds come at a great cost not only to the patient but to the general society (Person *et al*., 2004; Chandan *et al*., 2009; Leonard *et al.,* 2009).

Wound infections are caused by the invasion of pathogenic microorganisms from the environment and other parts of the body. Bacteria including *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella* species, *Escherichia coli, Proteus* species, *Streptococcus* species and *Enterobacter* species are the most common pathogens isolated from wound infections (Mulu *et al*., 2006; Frank *et al*., 2009; Gontcharova *et al*., 2010).

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Metallo-beta-lactamase (MBL) producing *Pseudomonas* strains have emerged worldwide and are associated with outbreaks in health-care settings causing serious infections (De *et al*., 2010). *P. aeruginosa, P. fluorescence, P. putida, P. cepacia, P. stutzeri, P. maltophila and P. putrefaciens* are associated with chronic opportunistic infections in humans (Agarwal *et al*., 2005). *Pseudomonas aeruginosa* has developed resistance to most antibiotics thereby jeopardizing the selection of appropriate treatment options (Obritsch *et al.,* 2004; Micek *et al.,* 2005).

Wound gets infected by either single or multiple microorganisms (Mulu *et al.,* 2006). Hard-to-heal wounds such as venous ulcers, pressure ulcers and diabetic foot ulcers are usually infected by multiple microorganisms (Dowd *et al.,* 2008; Gontcharova *et al.,* 2010) that may exist predominantly in the form of a biofilm resistant to antimicrobial treatments (Wolcott *et al.,* 2008; Black and Costerton, 2010). Vigorous dressing with increased debridement is the best method for reducing biofilm burden (Lipsky *et al*., 2010; Nusbaum *et al.,* 2012).

The escalating prevalence of carbapenem-resistant multidrug resistant bacteria wound infections; inter-hospital and intra-hospital spread of carbapenem-resistant multidrug resistant bacteria strains are of great challenge to medical care (Goswami *et al*., 2011). The production of highly versatile carbapenem hydrolyzing enzymes; carbapenemases became one main mechanism of carbapenem resistance across the globe (Walsh, 2010).

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Carbapenemase encoding genes are located on mobile genetic elements which contained other resistant genes and also have no respect for species and geographical boundaries (Okeke *et al.,* 2001).

No doubt, the emergence of metallo-β-lactamases (molecular class B), Oxacillinase (molecular class D) and *Klebsiella pneumoniae* carbapenemases (KPCs, molecular class A) has rapidly caused several drastic changes in approach in antibiotic therapy against bacterial infections (Wirth *et al*., 2009; Samuelson *et al.,* 2010; Cornaglia *et al*., 2011; Boucher *et al*., 2013).

1.1.1 Problem statement

The high rate of antimicrobial resistance worldwide put the future prospects of healthcare delivery in great dilemma (Cotton *et al*., 1992; Levy and Marshall, 2004; Roca *et al*., 2006; Shashikala *et al.,* 2006). Due to the pacing advent of new resistance mechanisms, microbial response to standard treatment of common infectious diseases has become a great challenge, leading to prolong illness, higher expenditure for health care and immense risk of death (Jyoti *et al.,* 2014).

Anecdotal reports in most hospitals in Ghana show that wound infections mostly caused by *Pseudomonas* are on the increase with many of these infected wounds very difficult to treat with available antibiotics. This failure of treatment has resulted in increased need for health care, undue financial burden on patients and hospitals as well as death of many of these patients. Additionally, there is limited information on the various circulating species of *Pseudomonas* and their carbapenem-resistant profiles. This limited information on circulating species of

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Pseudomonas as well as their antimicrobial resistance pattern hinders effective monitoring and treatment of these organisms.

Wound infection and its associated treatment failures are worldwide phenomenon. A study in Nigeria observed the following prevalence rates in wound infections: *Staphylococcus aureus* 44.0%, *Proteus* species 19.0%, *Klebsiella* species 14.0%, *Pseudomonas aeruginosa* 11.0% and *Escherichia coli* 11.0% (Garba *et al*., 2012). Possible explanation to antibiotic treatment failures of many infected wounds might be that these bacteria are harboring antibiotic resistant genes. Hence antimicrobial resistant bacteria including carbapenem-resistance encoding genes in *Pseudomonas* would be characterized from infected wounds.

1.1.2 Justification/Relevance

In Ghana there is limited information on antibiotic resistant organisms from wound infections, particularly carbapenem encoding resistance genes. Such genes can be transferred to other Gram negative bacteria, further worsening available treatment options.

Knowledge about antimicrobial resistance profile of a particular pathogen helps make prompt decision on the best choice of antibiotic for effective treatment. Understanding the mechanisms of antimicrobial resistance for a particular human pathogen and antimicrobial agent, helps devise strategies to contain further transmission (Simonsen *et al*., 2004; Hindler and Stelling, 2007; Morrissey and Northwood, 2007).

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This research will provide vital information on antimicrobial susceptibility profiles of bacteria isolated from infected wounds with valuable insight into carbapenem-resistance trends of *Pseudomonas*.

This vital information will help clinicians make prompt decision on the best choice of antibiotics for effective treatment and control of wound infections which will go a long way to prevent the spread of antibiotic resistant bacteria strains among patients and hospitals.

1.1.3 Main objective

The main objective of this study is to characterize antimicrobial resistant bacteria from infected wounds at the Korle-Bu Teaching Hospital and the Koforidua Regional Hospital in Ghana.

1.1.3.1 Specific objectives

i) To determine the various bacteria from the infected wound.

ii) To characterize antimicrobial resistant bacteria from the infected wound.

iii) To phenotypically speciate and molecularly characterize MBLs producing *Pseudomonas.*

iv) To determine duration of hospital stay and antimicrobial therapy during admission from patients′ folders.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Wound infections

Although it is possible to measure the direct health costs of wound care, its implications for individual patients and their family are beyond measure. Treatment of chronic non-healing wound is time and energy demanding on both the patient and care-givers (Leonard and Vudo, 2009; Green *et al.,* 2010).

The high frequencies of *Staphylococcus aureus* and *P. aeruginosa* in hard-to-heal wounds is due to their characteristic ubiquitous nature, active role in nosocomial infections (Nagoba *et al*., 2009), synergistic effect that impairs wound healing and high resistance to antimicrobial agents (Gjodsbol *et al*., 2006; Dowd *et al*., 2008; Fazli *et al*., 2009; Pastar *et al.,* 2013).

Staphylococcus aureus, in its methicillin sensitive and methicillin-resistant form (*MRSA*), is a common opportunistic pathogen, responsible for majority of acute and chronic superficial skin infections, resulting in increased morbidity, mortality, and undue health-care costs (Roghmann *et al.,* 2001; Talan *et al.,* 2011). Methicillin-resistant *Staphylococcus aureus* (*MRSA*) is a dangerous human pathogen (Schiavo *et al.,* 2001; Cosgrove *et al.,* 2003).

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Wound with mixed-species infection shows significant wound healing impairment compared to wound with single-species infection. Mixed-species infection delays epithelialization through suppression of keratinocyte growth factor 1 (KGF 1) expression. This results in delayed wound healing (Pastar *et al.,* 2013).

In developing countries, high prevalence of bacteria wound infections is largely due to poor hygienic conditions, mass production and use of low quality antiseptic and medicinal solutions for wound treatment (Hsueh *et al*., 1998; Henwood *et al*., 2001; Goswami *et al*., 2011; Chitnis *et al*., 2012).

Studies elsewhere observed the following prevalence rates for *Pseudomonas* wound infections: 35.6% in United Arab Emirates (Al-Akayleh, 1999), 9.9% in Uganda (Anguzu *et al.,* 2007), 32.9%, 11.0% in Nigeria, respectively (Nwachukwu *et al.,* 2009) and (Garba *et al.,* 2012), 27.8% in Saudi Arabia (Masaadeh and Jaran, 2009), 59.0% in India (Agnihotri *et al.,* 2004), 32.0% in India (Anupurba *et al.,* 2010), 29.6% in Haryana, India (Prabhat *et al.,* 2010) and 33.3% in Pakistan (Oguntibeju *et al.,* 2004).

2.1.1 Significance of pus cells in wound culture

The presence of pus cells in wound is an indication of inflammation and proves that the microorganisms isolated in the culture are clinically significant (Mahon and Manuselis, 2000; Bailey and Scott`s, 2002).

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2.1.2 Emergence of antimicrobial resistance and its impact on healthcare delivery

One of the greatest medical achievements of the 20th century was the discovery and use of antimicrobial agents to treat human infections because antimicrobial agents have saved many lives from serious bacterial infections (Levy and Marshall, 2004). Few years after the introduction of antimicrobial agents, populations of antimicrobial resistant pathogenic bacteria emerged resulting from use, misuse, and abuse of antimicrobials (Wilke, 2010; Korczak and Schoffmann, 2010). The threat of antibiotic resistance is growing rapidly, more especially in developing countries (Amabile-Cuevas, 2003).

In Ghana, antibiotics may be sold without prescription; usage is largely without guidance from healthcare professionals thereby resulting in the emergence of antibiotic resistant bacteria strains (Wolf-Gould *et al*., 1991). Additionally, many patients fail to finish the full treatment once they start to feel better. These practices result in selection pressure leading to antibiotic resistance (Roberts *et al*., 2008). Unfortunately, no one will be spared when antimicrobials finally become ineffective (Tablan *et al.,* 2004; Moellering, 2010).

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2.1.3 Metallo-β-lactamases (MBLs)

Metallo-β-lactamases (MBLs) are metalloenzymes of Ambler class B and are clavulanic acid-resistant enzymes that require divalent cations of zinc as co-factors for enzymatic activity but of which are universally inhibited by ethylenediamine tetra-acetic acid (EDTA), as well as other chelating agents of divalent cations (Anuradha *et al*., 2010).

Metallo-β-lactamases hydrolyse all beta lactams including carbapenems except monobactams such as aztreonam (Walsh *et al*., 2005; Cornaglia *et al.,* 2011). Acquired MBL is encoded by integron borne mobile gene cassettes which contained other resistant genes; hence MBL producing strains are often resistant to different classes of antimicrobial agents with transferable properties to various types of bacteria (Okeke *et al.,* 2001; Walsh *et al*., 2005; Valenza *et al.,* 2010).

The most common MBLs include the Verona integron-encoded metallo-β-lactamase (VIM-type), Imipenemase Metallo-β-lactamase (IMP-type), German imipenemase (GIM-type), São Paulo metallo-β-lactamase (SPM), Seoul imipenemase (SIM-type) and New Delhi metallo-β-lactamase (NDM-1) (Syuntaro *et al.,* 2009; Samuelson *et al.,* 2010).

In particular, VIM, IMP and SPM have emerged as dominant MBL variants worldwide (Samuelson *et al.,* 2010; Cornaglia *et al*., 2011).

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2.1.4 Carbapenem-resistance through Metallo-β-lactamases (MBLs)

Carbapenem-resistance populations of bacteria due to metallo-β-lactamases (MBLs) production emerged first in Japan, few years after the introduction of the first carbapenem in 1985 (Birnbaum *et al*., 1985; Sader *et al*., 2005; Clatworthy *et al.,* 2007). Since then, MBLs have become increasingly distributed worldwide (Poirel *et al*., 2007; Kaleem *et al*., 2010). Brazil and Korea were the first two developing nations to identify, respectively São Paulo metallo-β-lactamase (SPM) and Seoul imipenemase metallo-β-lactamases (SIM) among *P. aeruginosa* (Sader *et al*., 2005; Castanheira *et al*., 2007; Poirel *et al*., 2007).

Currently, MBL producing *Pseudomonas* strains have emerged worldwide and are associated with outbreaks in healthcare settings causing serious infections and panic (Levy and Marshall, 2004; De *et al*., 2010).

2.1.5 Prevalence of carbapenem-resistant *P. aeruginosa* in infected wounds

Reported prevalence of carbapenem resistant *P. aeruginosa* from wound infections ranged between 42.0% and 45.0% in South Africa (Brink *et al*., 2007), 6.8% to imipenem and to meropenem, 87.5% to imipenem, 78.1% to meropenem in France during 2007 (Jose-Manuel *et al.,* 2009), 5.2% to imipenem in Canada (Alan *et al*., 2002), 39.0% to imipenem, 35.0% to meropenem and 33.0% doripenem from 16 Spanish hospitals during 2008-09 (Riera *et al*., 2011). Manoharan *et al.,* (2010) reported 42.6% to imipenem and meropenem in India.

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From Latin America, Casellas *et al.,* (2006) reported 28.0% to imipenem, 24.0% to meropenem in Argentina, 24.0% to imipenem, 22.0% to meropenem in Ecuador, 35.0% to imipenem, 35.0% to meropenem in Paraguay and 17.0% to imipenem, 10.0% to meropenem in Venezuela.

2.1.6 Prevalence of MBL-positive carbapenem-resistant *P. aeruginosa*

Prevalence of MBL-positive among carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) ranged from 17.0 to 36.0% and was as high as 76.9% among multidrug-resistant *P. aeruginosa* (MDRPA) in China (Yan *et al*., 2001), 44.8% to 54.1% among MDRPA in Brazil (Paula *et al*., 2012) and 13.7% in Kenya, the first report of VIM-2 producing *Pseudomonas aeruginosa* from the African continent (Pitout *et al*., 2008). Mariana *et al*., (2014) reported 20.0% from fourteen European and Mediterranean countries during 2009-11.

Many regions have reported significant increase in MBL-positive among MDRPA in recent years. For example, in Hiroshima, Japan, the prevalence of MBL-positive among MDRPA rose from 42.3% to 81.4% between 2004 and 2006 (Syuntaro *et al.,* 2009). The high carbapenem-resistance rates found in many institutions were due to the dissemination of MBL-producing clones (Kimura *et al*., 2005; Sader *et al*., 2005; Yu *et al*., 2006).

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2.2 Pathogenesis of bacteria wound infections

When skin intergument is damaged, bacterial microorganisms from the environment and other parts of the skin get entry. Coagulation and acute inflammatory response is triggered (Yah *et al*, 2010; Pastar *et al.,* 2013). This creates a favourable subcutaneous tissue environment for bacteria colonization and proliferation (Eming *et al.,* 2007; Pastar *et al*., 2013).

Pathogenic microorganisms upon entry, expressed different virulent factors including enzymes and toxins to cause tissue degradation and acute wound infections (Pastar *et al*., 2013).

In chronic wound infections, bacteria pathogens employ additional strategies including biofilm formation, quorum sensing (Bodey *et al.,* 1983; Yah *et al*., 2010), acquisition of numerous multidrug resistance genes (Vasil *et al.,* 1999) and mutations to express more potent virulent factors to pursue their prolong disastrous cause (Cornelis *et al.,* 2008).

Antimicrobial resistance is a major predisposing factor in antibiotic treatment failures. The biofilm mode of growth provides a key protection against host defenses and antibiotics (Engleberg *et al.,* 2007).

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CHAPTER THREE

MATERIALS AND METHODS

3.1 DESCRIPTION OF HOSPITALS

i) Korle-Bu Teaching hospital (KBTH)

The Korle-Bu Teaching hospital (KBTH) is in the Greater Accra region of Ghana. Currently, KBTH is the third largest hospital in Africa and a leading national referral centre in Ghana. The Reconstructive Plastic Surgery and Burns Centre of KBTH where this research was carried out provides services in plastic surgery, skin and soft tissue grafting, burns, road traffic accident/laceration cases, leg ulcers, buruli ulcers, keloids, male circumcision, cleft lip and palates, scrotum among others. The centre has 67 beds distributed as follows; ulcer wards (19 beds), burns wards (18 beds), Reconstructive Plastic Surgery (RPS) wards (27 beds) and three executive wards (1 bed per ward). Sixty-five In-patients and forty-six Out-patients were investigated from the Korle-Bu Teaching hospital.

ii) Koforidua Regional hospital (KRH)

The Koforidua Regional hospital (KRH) is in the Eastern region of Ghana and is a referral hospital. The surgical block of the hospital provides services in general surgery (mostly abdominal surgery), leg ulcers, wounds, amputations, road traffic accidents, intestinal obstructions, appendicitis, hernia, benign prostatic hyperplasia (BPH), eyes, ears and throat impacted coin among others. The hospital has 77 beds distributed as follows; surgical block (male ward=46 beds, female ward=18 beds).

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The main block (male ward=9 beds, female ward=10 beds, staff room=2 beds, special room=1 bed). Forty-three In-patients and sixty-eight Out-patients were investigated from the Koforidua Regional hospital.

3.2 SPECIMEN COLLECTION AND STUDY SITES

From January to May 2014, consecutive, non-duplicated clinical wound swabs were collected from two hundred and twenty-two (222) patients with infected wounds attending the Korle-Bu Teaching Hospital and the Koforidua Regional Hospital. These patients included 121 males and 101 females, aged between two (2) weeks to ninety-seven (97) years.

Infected wounds of patients not on active systemic antibiotic treatment were swabbed. The wounds were swabbed using sterile swab sticks from the Outpatient department (OPD), the wards and various dressing rooms. Surfaces of infected wounds were cleaned with normal saline before swabbing to reduce contamination with skin flora. The samples were Gram stained and cultured. Surfaces of objects including dressing trolleys, beds and sinks in patients' rooms at the KBTH and the KRH were also swabbed. All swabs were immediately transported to the laboratory in sterile containers for microbiological investigations.

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3.2.1 Culture and identification of bacteria isolates

Swabs were inoculated onto Blood, MacConkey and Chocolate agar plates and incubated overnight (approximately 18 hours) at 37°C. Blood and MacConkey plates were incubated aerobically. The Chocolate plates were incubated in jar for an increased carbon dioxide concentration (facultative). The Blood, MacConkey and Chocolate plates were read after the overnight incubation and bacteria identified using a combination of colonial morphology, Gram stain characteristics and biochemical tests (Gencer *et al*., 2002; Cheesbrough, 2006). Biochemical tests used included oxidase, indole, urease, citrate, triple sugar iron (TSI), catalase and coagulase. *Pseudomonas* strains were identified by oxidase positive, non-lactose fermentation and indole negative biochemical tests.

3.2.2 Pus cells enumeration

Wound swabs were Gram stained and pus cells quantified as number of cells/High power field (Hpf). Results interpreted as none (0/Hpf), few (1-4/Hpf), moderate (5-9/Hpf) and many (>10/Hpf) (Anguzu *et al.,* 2007).

3.2.3 Antimicrobial susceptibility testing

Commercially available and commonly used antimicrobial discs (Abtek Biological Ltd UK) were used to determine the drug susceptibility patterns of the isolates. The antimicrobial susceptibility testing was carried out on Mueller Hinton agar as described by the Kirby–Bauer disc diffusion method (Bauer *et al*.,

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1996) and interpreted using the Clinical Laboratory Standards Institute guidelines (CLSI, 2012).

Antibiotic discs tested included ceftazidime (30µg), gentamicin (10µg), amikacin (30µg), ciprofloxacin (5µg), meropenem (10µg), ampicillin (10µg), cefuroxime (30µg), erythromycin (15µg), penicillin (10µg), tetracycline (30µg), piperacillin/tazobactam (100/10µg), cloxacillin (15µg) and cefoxitin (30µg). Diameters of zones of inhibitions were measured with a caliper after 16 to 18 hours of incubation. Measured zones of inhibitions were compared with zone diameter interpretative chart (CLSI, 2012).

3.2.4 Characterization of antimicrobial resistant bacteria

Antimicrobial resistant bacteria isolates were characterized according to (Gencer *et al*., 2002; Cheesbrough, 2006; CLSI, 2012). The non-lactose fermenting, Gram negative and oxidase positive *Pseudomonas* strains were sub-cultured onto Nutrient Agar (Difco) plates. The plates were incubated aerobically at 37°C overnight (approximately 18 hours) for the characteristic blue-green pyocyanin pigmentation (Aysel *et al*., 2012). *Pseudomonas* strains including the carbapenem-resistant *Pseudomonas aeruginosa* were stored in cryo-tubes containing Mueller Hinton agar slants for further investigation.

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3.2.5 Multidrug-resistant (MDR) bacteria strains

Multidrug-resistant (MDR) was determined by methods described elsewhere (Falagas *et al.,* 2006; CLSI, 2012). The isolates were tested against more than three different classes of antimicrobials including fluoroquinolones, aminoglycosides, cephalosporins, penicillins, phenicols, carbapenems and tetracyclines on Mueller Hinton agar according to Kirby–Bauer disc diffusion method (Bauer *et al*., 1996) and Clinical Laboratory Standards Institute guidelines (CLSI, 2012).

Isolates that showed resistance to ≥ 1 antimicrobial agent in ≥ 3 different antimicrobial classes were described as multidrug-resistant (MDR) strains (Falagas *et al*., 2006).

Pseudomonas isolates that show resistance to three or more of the following antimicrobial agents: anti-pseudomonal penicillins (e.g., piperacillin/tazobactam), anti-pseudomonal cephalosporins (e.g., ceftazidime), fluoroquinolones (e.g., ciprofloxacin), carbapenems (e.g., meropenem) and the aminoglycosides (e.g., gentamicin or amikacin) were considered MDR (Defez *et al.,* 2004).

3.2.6 Methicillin-resistant *Staphylococcus aureus* (*MRSA*) screening

Multidrug-resistant *Staphylococcus aureus* (*MRSA*) were further screened for methicillin resistance. The methicillin resistance screening was carried out using cefoxitin (FOX) disc diffusion test on Mueller Hinton agar according to Kirby–Bauer disc diffusion method (Bauer *et al*., 1996) and Clinical Laboratory

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Standards Institute guidelines (CLSI, 2012). The plates were incubated aerobically at 33-35°C (maximum of 35°C) overnight (approximately 18 hours). Diameters of the zones of inhibitions were measured with a caliper after the overnight incubation. Measured zones of inhibitions were compared with zone diameter interpretative chart for methicillin-resistant *Staphylococcus aureus* (CLSI, 2012).

3.2.7 Phenotypic screening for metallo-beta lactamase production

Phenotypic screening for metallo-beta-lactamase production in *Pseudomonas* isolates was carried out using CAZ-EDTA/MEM-EDTA combined disc test (Hemalatha *et al.,* 2005; Franklin *et al.,* 2006; Arunagiri *et al.,* 2012; CLSI 2012).
A 0.5M EDTA solution was prepared by adding 1.86g of disodium EDTA in 10mL of distilled water. The pH of the solution was adjusted to 8.0 using sodium hydroxide and sterilized by autoclaving. Inoculums of *Pseudomonas* isolates were prepared. Opacity was adjusted to 0.5 McFarland standard and plated on Muller Hinton agar.

Two ceftazidime (30µg) and meropenem (10µg) discs were placed on the plates and 5µL of EDTA solution was added to one of each ceftazidime and meropenem disc. The zone of inhibition around individual ceftazidime and meropenem discs and those with EDTA was recorded and compared after 16 to 18 hours of incubation at 37°C. An increase in zone size of at least 7mm around ceftazidime-EDTA disc and meropenem-EDTA disc was considered a positive result for metallo-beta-lactamase production in *Pseudomonas*.

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3.2.8 Quantification of bacteria isolates

Quantification of bacteria isolates was done using the "3-phase streaking techniques" according to (Mahon and Manuselis, 2000; Bailey and Scott`s 2002). Blood and MacConkey plates were divided into four sections. Sterile loops dipped into 0.5 ml prepared inoculums were used to streak the quadrants, starting from the first to the forth without dipping it back into the inoculums. The plates were incubated aerobically at 37°C overnight (approximately 18 hours). Bacteria were described as "few", "moderate" and "many" with respect to growth in the first, second and third or fourth quadrants respectively.

Quality control test for both media and antibiotic discs used were carried out using *Escherichia coli* control strain (ATCC 25022) and *Pseudomonas aeruginosa* control strain (ATCC 27053).

3.3 Medical records review of patients

Sixty (60) folders of patients with infected wounds were randomly sampled to estimate length of hospital stay at the time of specimen collection. Information regarding duration of wounds at the time of sample collection was gathered from the sampled folders. Average duration of non-healing wounds calculated in weeks and subsequently converted into years. Information regarding patient's age, sex, diagnosis, history of disease condition and wound management procedures were also obtained from patients' folders. Some of the infected wounds were photographed after verbal consent.

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3.4 Molecular detection of metallo-beta lactamase (MBL) encoding genes in carbapenem-resistant *Pseudomonas* strain.

The carbapenem-resistant multidrug resistant *P. aeruginosa* (CR-MDRPA) isolate was molecularly screened for MBL encoding genes.

3.4.1 DNA isolation

Total DNA of the carbapenem-resistant *Pseudomonas aeruginosa* isolate was extracted from overnight growth broth culture using QIAMP Mini kit for Gram negative bacteria (LGC Biotecnologia) according to manufacturer's instructions (Appendix IV). The extracted DNA was stored at -20°C for further analysis.

3.4.2 Polymerase chain reaction (PCR)

PCR was performed to detect the presence of MBL encoding genes *bla* $_{VIM}$, *bla* $_{IMP}$ and *bla* $_{SPM}$ using the following primers:

bla$_{SPM}$-1 F (5'-CCTACAATCTAACGGCGACC-3'),

bla$_{SPM-}$1 R (5'TCGCCGTGTCCAGGTATAAC3'),

bla$_{IMP}$ F (5'GGAATAGAGTGGCTTAATTCTC-3'),

bla$_{IMP}$ R (5'-CGTGTGATGCYCCAAYTTCACT-3'),

bla$_{VIM}$ F (5'-CAGATTGCCGATGGTGTTTGG-3'),

bla$_{VIM}$ R (5'-AGGTGGGCCATTCAGCCAGA-3').

Amplification reactions were prepared in a total volume of 25µL per tube, comprising: 5µL of genomic DNA, 0.5 µL of 10µM primers, 5x buffer, 0.5µL each deoxyribonucleotide triphosphate (Ludwig Biotechnology), 16.875 nuclease

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free H_2O and 0.125µL Taq DNA polymerase (Promega Inc. Madison, USA). In each round of amplification, positive and negative controls were included.

PCR cycle parameters were initial 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1minute, annealing for 1min at 50.6ºC, 55.3°C and 62ºC, for bla_{IMP}, bla_{SPM} and bla_{VIM}, respectively; extension at 68°C for 1minute, and final 10 minutes at 68°C. PCR products were stained and subjected to electrophoresis on 1% agarose gel in TAE buffer (Tris-borate 0.089 M EDTA and 0.002M) under constant voltage of 100V. Bands were visualized under UV light as described by Paula *et al.,* (2012).

3.5 Data analysis

Data was stored in Microsoft Access files and later analyzed using Minitab 16 version. Percentages were calculated for categorical variables and results presented in tabular and graphical forms. Statistical comparisons of total bacteria isolated, antibiotic resistance patterns of *Pseudomonas* strains and *Staphylococcus aureus* from the KBTH and the KRH were performed using Student`s t-test. Statistically significant differences were defined as $p<0.05$.

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CHAPTER FOUR

RESULTS

4.1 PATIENT CHARACTERISTICS

Patients investigated at the Korle-Bu Teaching hospital (KBTH) aged between 2 weeks to 97 years included 63 males and 48 females. Injuries and infections of patients investigated included surgical site infections (5), diabetic foot infections (5), wounds and chronic wound infections (26), burns and post burnt ulcer infections (15), leg ulcers, cellulitis and post cellulitic ulcer infections (47) and others (13) (Figure 4.1).

Patients investigated at the Koforidua Regional hospital (KRH) aged between 3 months to 88 years included 58 males and 53 females. Injuries and infections of patients investigated included; surgical site infections (7), diabetic foot infections (5), wounds and chronic wound infections (65), burns and post burnt ulcer infections (4), leg ulcers, cellulitis and post cellulitic ulcer infections (19) and others (11) (Figure 4.1).

Patients with chronic wound infections, ulcers and post cellulitic ulcer infections were high at the KBTH ranging from 23.4% to 55.9%, respectively. At the KRH, high numbers of patients with ulcers and chronic wound infections were detected (Figure 4.1) and ranged from 20.7% to 58.6%, respectively.

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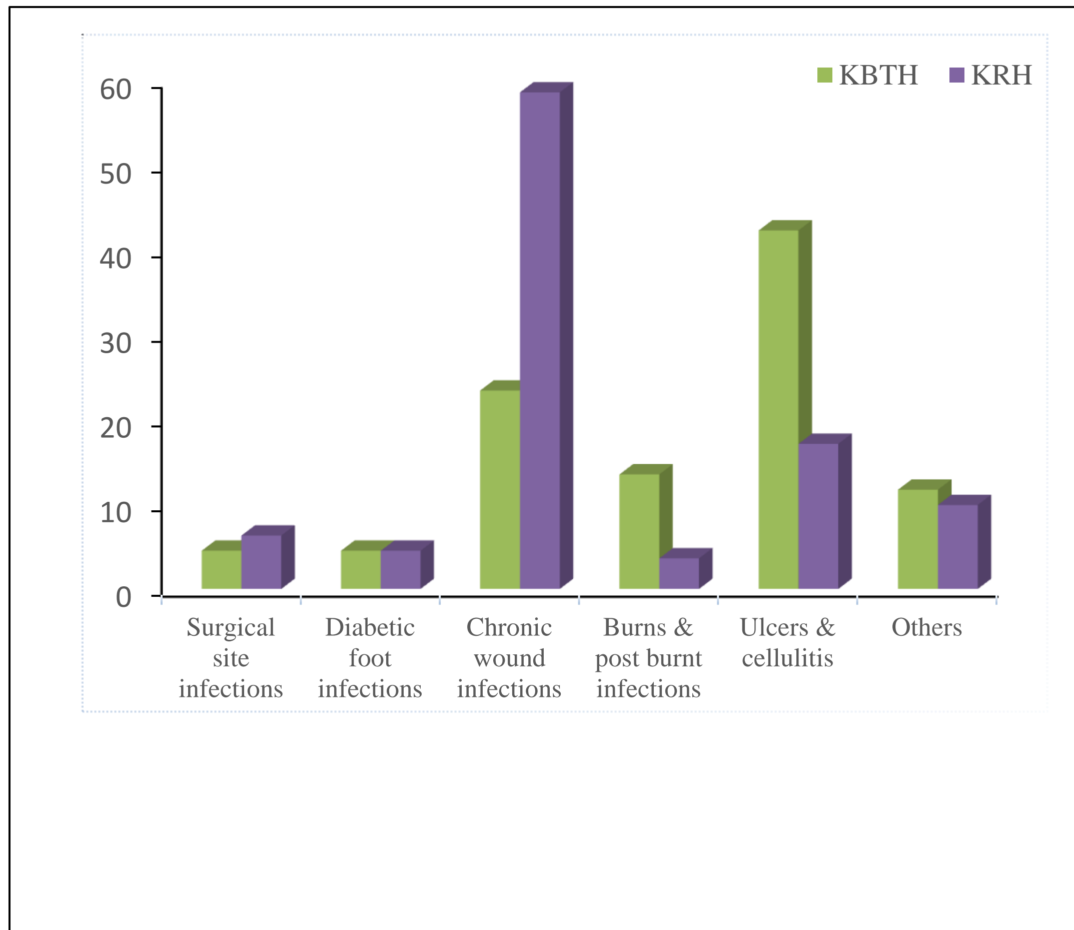

FIGURE 4.1 Percentage comparisons of types of wounds and infections of patients attending KBTH and KRH.

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4.2 Wound Duration and Antibiotic therapy

Duration of wounds at the time of sample collection ranged from a few weeks to nineteen (19) years of non-healing wound (Figure 4.2). On average, patients with infected wounds spend two years accessing healthcare at the KBTH and the KRH.

Different types of infected wounds were investigated and some photographed; surgical site infection (Figure 4.3a), post burnt ulcer (Figure 4.3b), diabetic foot (Figure 4.3c), venous ulcer (Figure 4.3d), septic wound (Figure 4.3e) and post cellulitic ulcer (Figure 4.3f).

Topical treatment of infected wounds with one or two combinations of nadoxin, 1% silver sulfadiazine, bactroban, lexporin, dermacin, ionsil, 1% hydrocortisone and herbamin creams was observed. Due to many treatment failures with available antibiotics, systemic antibiotic treatment for many patients with infected wounds attending the KBTH and the KRH was at its barest minimum.

Increased usage of ciprofloxacin and metronidazole (Flagyl) were detected. There was no record of any patient treated with carbapenem at the time of sample collection.

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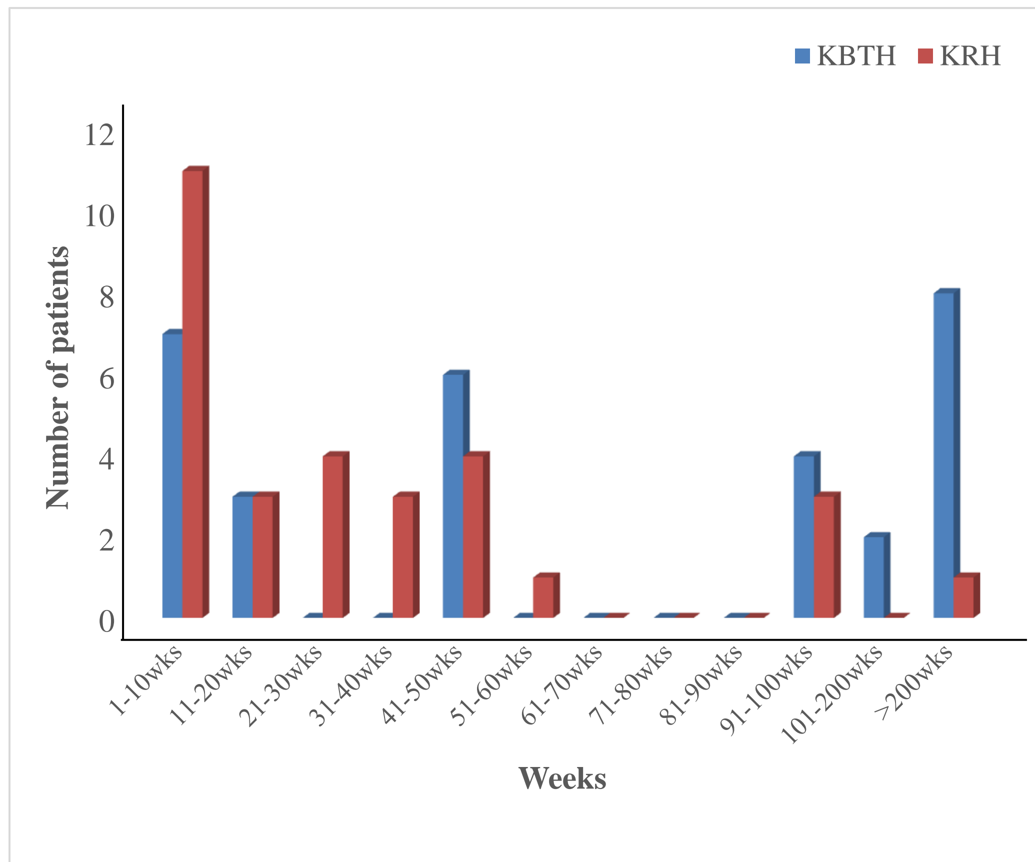

FIGURE 4.2 Durations of infected wounds of patients attending KBTH and KRH

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FIGURE 4.3a Surgical site infection of a 15 year old female student diagnosed of Pelvic abscess and Peritonitis secondary to typhoid perforation investigated at the KRH.

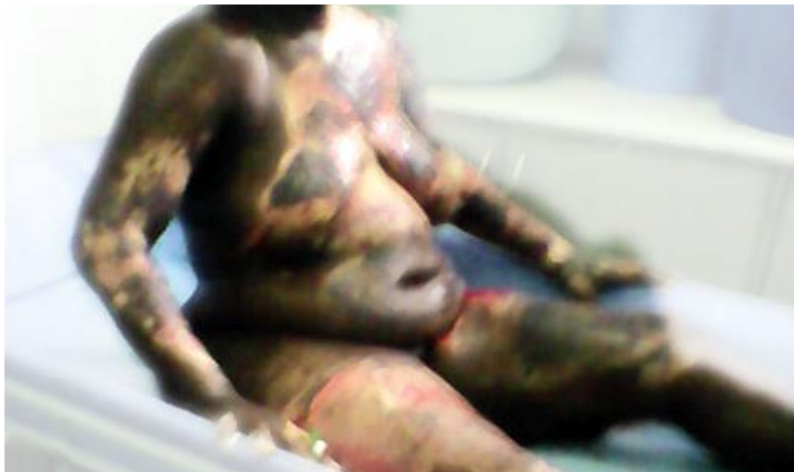

FIGURE 4.3b Post burnt ulcer from hot palmnut soup of a 42 year old nursing mother investigated at the KBTH.

Patient was shopping on a market when a "kayayo" carrying hot soup fell and poured the hot soup on the patient.

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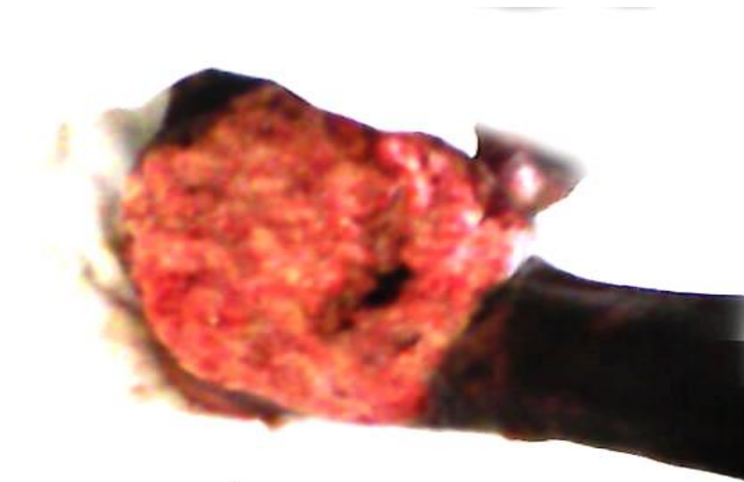

FIGURE 4.3c Diabetic foot of a 60 year old male patient investigated at the KRH.

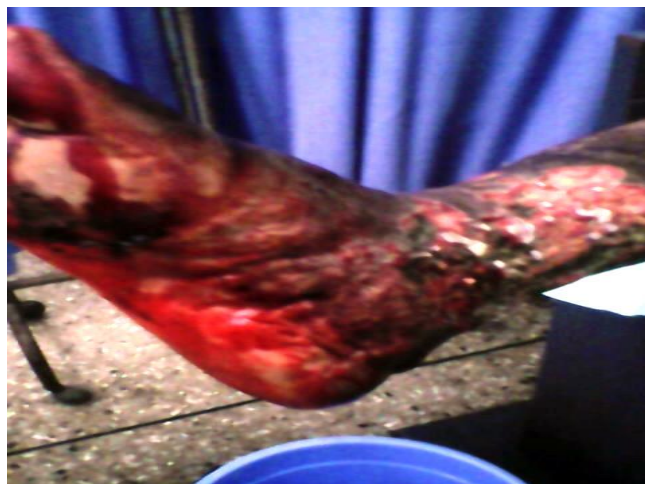

FIGURE 4.3d Venous ulcer on right leg of a 42 year old male patient investigated at the KBTH

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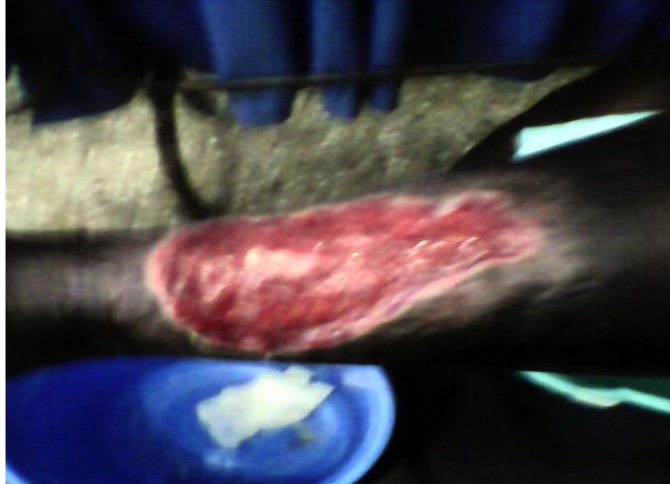

FIGURE 4.3e Septic wound on left leg of a 76 year old male patient investigated at the KBTH

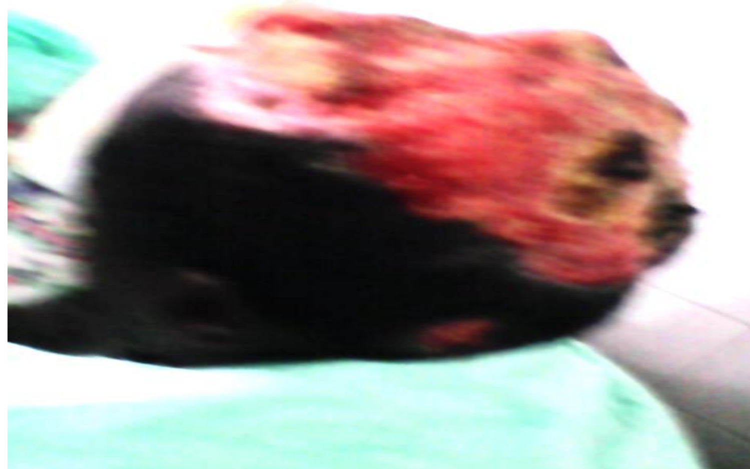

FIGURE 4.3f Post cellulitic ulcer of the head of a 46 year old male patient referred from Holy Trinity hospital, Tema to KBTH.

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4.3 Culture Results from Korle-Bu Teaching Hospital (KBTH) and Koforidua Regional Hospital (KRH).

Of 222 patients swabbed, 74.8% (166) produced pathogenic bacteria growth. A total number of 194 bacteria were isolated from patients attending the KBTH and the KRH. *Pseudomonas* strains were found to be the most prevalent isolates 92(47.4%), followed by *Proteus* spp. (13.9%), *Citrobacter* spp. (10.3%), *Escherichia coli* (7.7%), *Klebsiella* spp. (7.7%), *Staphylococcus aureus* (7.2%), *Enterobacter* spp. (3.1%), *Acinetobacter* spp. (1.5%), methicillin-resistant *Staphylococcus aureus* (*MRSA*) (0.5%) and *Morganella morgani* (0.5%). All these isolates yielded heavy growth.

From the Korle-Bu Teaching hospital (KBTH), *Pseudomonas* strains represented 67.0% (69/103) as compared to 25.3% (23/91) from the Koforidua Regional hospital (KRH). Eighty-one percent (69/85) of patients from the KBTH had wounds infected with *Pseudomonas* strains as compared to 26.0% (21/81) from the KRH.

Observed percentage ratios of male to female patients with *Pseudomonas* wound infection were 58.0% (40/69) males to 42.0% (29/69) females from KBTH, and 52.2% (12/23) males to 39.1% (9/23) females from KRH. High level of *Pseudomonas* detection was observed among patients aged 40-59 years from both KBTH and KRH. *Pseudomonas* age distribution between KBTH and KRH is shown in (Table 4.1).

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TABLE 4.1 *Pseudomonas* wound infection and age of patient

	***Pseudomonas* strains (N=92)**		
Age cat./ Years	**Total no.**	**KBTH (N=69) n, (%)**	**KRH (N=23) n, (%)**
0-19	11	6 (8.7)	5 (21.7)
20-39	31	24 (34.8)	7 (30.4)
40-59	33	25(36.2)	8 (34.8)
60-79	13	10 (14.5)	3 (13.0)
>79	4	4 (5.8)	0 (0)
Total	**92**	**69**	**23**

N-represents total number of *Pseudomonas* isolated, n-number of isolates per age category

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Bacteria isolated from the KBTH and the KRH is shown in (Table 4.2). Differences of *Pseudomonas* strains, *Enterobacter* spp., *Klebsiella* spp., and *Citrobacter* spp. observed between KBTH and KRH were statistically significant (Table 4.2).

TABLE 4.2 Bacteria isolated from infected wounds among patients attending the KBTH and the KRH.

Bacteria isolated	**KBTH (N=103)** **n, (%)**	**KRH (N=91)** **n, (%)**	**95% CI**	**p-value**
Pseudomonas spp.	69 (67.0)	23 (25.3)	28.98-54.45	**0.000**
Acinetobacter spp.	3 (2.9)	0 (0)	-0.92-2.86	1.000
Enterobacter spp.	6 (5.8)	0 (0)	1.30-10.35	**0.031**
Proteus spp.	10 (9.7)	17 (18.7)	-18.81-0.87	0.096
Klebsiella spp.	2 (1.9)	13 (14.7)	-20.0-4.68	**0.002**
Escherichia coli	5 (4.9)	10 (11.0)	-13.78-1.51	0.177
Staphylococcus aureus	5(4.9)	9 (10.0)	-12.44-2.37	0.266
Citrobacter spp.	2 (1.9)	18 (19.8)	-26.45-9.23	**0.000**
MRSA	1(1.0)	0 (0)	-3.24-1.04	0.469
Morganella morgani	0 (0)	1 (1.1)	-3.24-1.04	0.469
Total	**103**	**91**		

N-represents total bacteria isolated, n-number of each species isolated, *MRSA*: Methicillin-Resistant *Staphylococcus aureus*.

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4.4 Mixed-species infections

Mixed infections were detected in cultures of 24 patients with ulcers, diabetic foot and chronic wounds. At both the KBTH and the KRH, more mixed infections were detected in the males as compared to the females. *Pseudomonas* and *Proteus* species were the predominant isolates detected in the mixed infections (Table 4.3).

TABLE 4.3 Bacteria isolates in mixed infections

ID	Age (yrs)	Sex	Isolate(s)
KBTH (n=17)			
KB-31	39	M	*P.aeruginosa*, *Proteus* spp, *Klebsiella* spp.
KB-2	59	M	*P. aeruginosa*, *Acinetobacter* spp.
KB-5	41	F	*P. aeruginosa*, *Proteus* spp.
KB-6	30	F	*P. aeruginosa*, *Proteus* spp
KB-12	52	F	*P. aeruginosa*, *Klebsiella* spp.
KB-57	42	F	*P. aeruginosa*, *MRSA*
KB-14	46	M	*P. aeruginosa*, *E. coli*
KB-87	64	M	*P. aeruginosa*, *Citrobacter* spp.
KB-75	2/52	M	*P. aeruginosa*, *E. coli*,
KB-64	49	M	*P. aeruginosa*, *Staphylococcus aureus*
KB-7	50	M	*Pseudomonas* spp. *Proteus* spp.
KB-15	76	M	*Pseudomonas* spp. *Proteus* spp.
KB-34	81	M	*Pseudomonas* spp., *Enterobacter* spp.
KB-86	45	M	*Pseudomonas* spp., *Proteus* spp.
KB-97	80	F	*Pseudomonas* spp., *Proteus* spp.
KB-88	19	M	*Citrobacter* spp., *Proteus* spp.
KB-71	34	M	*Staphylococcus aureus, Proteus* spp
KRH (n=7)			
P-65	48	F	*P.aeruginosa*, *Proteus* spp.
P-66	8	F	*P.aeruginosa*, *Proteus* spp.
P-67	53	F	*P. aeruginosa*, *Proteus* spp.
P-1	22	M	*Pseudomonas* spp., *Proteus* spp.
P-27	64	M	*E.coli*, *Klebsiella* spp.
P-44	45	M	*Citrobacter* spp., *Proteus* spp.
P-70	6	M	*Klebsiella* spp., *Proteus* spp.

KB-Korle-Bu Teaching hospital, P-Koforidua Regional hospital.

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4.5 Pus cells enumeration

Pus cells were detected from the 166 patients whose wound cultures produced 194 clinically significant bacteria. Many pus cells were detected in wound swabs from patients with mixed infections, multidrug resistant isolates and *Pseudomonas* wound infections. At the KBTH, many wound swabs yielded moderate pus cells whilst at the KRH many wound swabs yielded many pus cells.

One metallo-beta lactamase producing, carbapenem-resistant multidrug-resistant *Pseudomonas aeruginosa* (CR-MDRPA) was detected from the KBTH. The three most widely spread metallo-beta-lactamase (MBL) encoding genes; *bla* $_{\text{VIM-type}}$ (Figure 4.4), *bla* $_{\text{SPM-type}}$ (Figure 4.5) and *bla* $_{\text{IMP-type}}$ (Figure 4.6) out of the six dominant MBL variants investigated were not detected in the carbapenem-resistant *Pseudomonas aeruginosa* strain. The antibiotic resistance of *Pseudomonas* strains from the KBTH and the KRH is shown in (Table 4.4). Although *Pseudomonas* strains isolated from the KBTH showed higher percentage resistance to each anti-pseudomonal agent as compared to those isolated from the KRH, they were not statistically significant (Table 4.4).

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TABLE 4.4 Resistance profile of *Pseudomonas* isolated from infected wounds of patients attending KBTH and KRH.

Antibiotic	KBTH (N=69) n, (%)	KRH (N=23) n, (%)	95% CI	p-value
Meropenem	1 (1.1)	0 (0)	-1.37-4.27	1.000
Piperacillin/Tazobactam	4 (4.3)	3 (3.3)	-22.7-7.58	0.361
Ceftazidime	15 (16.3)	5 (5.4)	-19.46-19.46	1.000
Gentamicin	15 (16.3)	5 (5.4)	-19.46-19.46	1.000
Amikacin	10 (10.9)	5 (5.4)	-19.46-19.	1.000
Ciprofloxacin	20 (21.7)	4 (4.3)	-7.24-30.42	0.411

N-represents number isolated and tested, n-number resistant.

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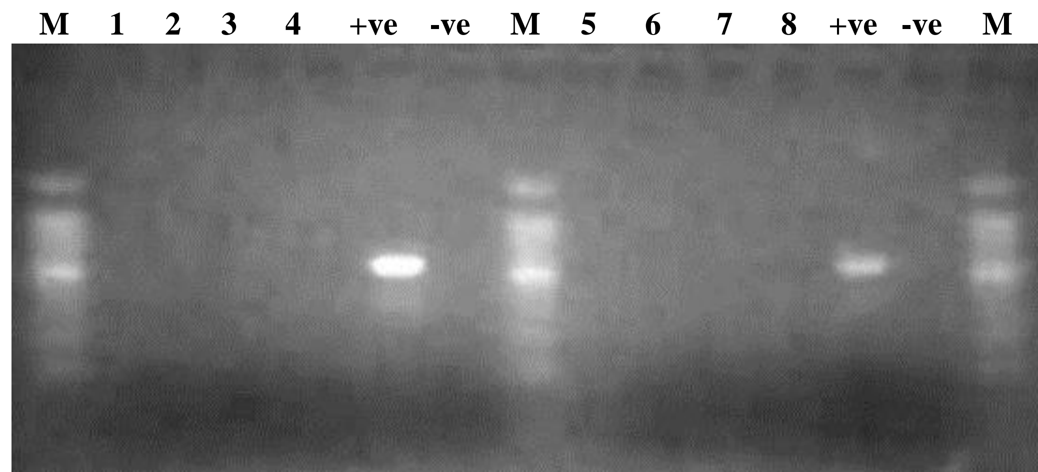

FIGURE 4.4 PCR result of MBL gene *bla* $_{\text{VIM-type}}$ detection in CRPA

VIM gene amplification gel showing molecular marker (100bp DNA ladder) lanes (M), Negative control lanes (-ve), Positive control lanes (+ve) and *bla* $_{\text{VIM}}$ negatively amplified test lanes (1, 2, 3, 4,5,6,7 and 8).

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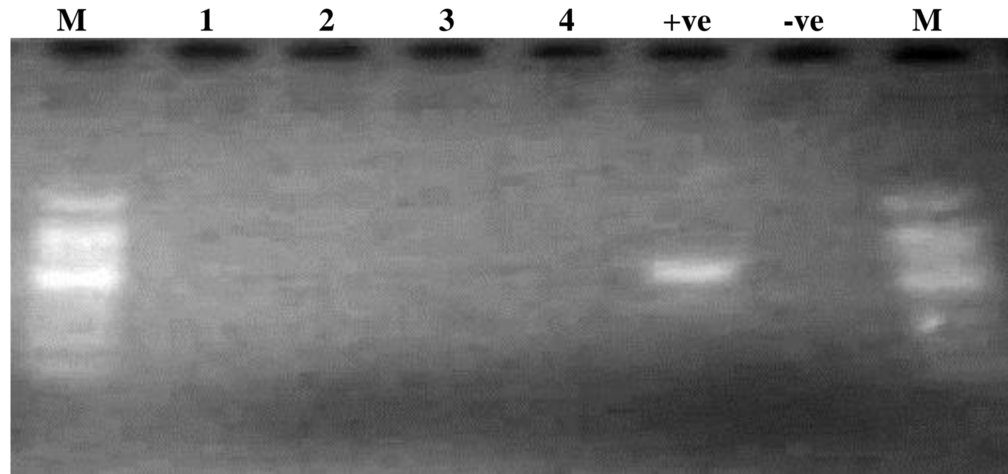

FIGURE 4.5 PCR result of MBL gene *bla* $_{\text{SPM-type}}$ detection in CRPA

SPM gene amplification gel showing molecular marker (100bp DNA ladder) lanes (M), Negative control lane (-ve), Positive control lane (+ve) and *bla* $_{\text{SPM-type}}$ negatively amplified test lanes (1, 2, 3 and 4).

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FIGURE 4.6 PCR result of MBL gene *bla* $_{\text{IMP-type}}$ detection in CR

IMP gene amplification gel showing molecular marker (100bp DNA ladder) lanes (M), Negative control lanes (-ve), Positive control lanes (+ve) and *bla* $_{\text{IMP-type}}$ negatively amplified test lanes (1, 2,3,4,6 and 9).

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Sixty-eight multidrug-resistant (MDR) bacteria strains were detected from the KBTH (23) and the KRH (45). The MDR strains were recovered from 30 males and 35 females and included *Citrobacter* spp. (n=15), *Pseudomonas* strains (n=12), *Escherichia coli* (n=11), *Staphylococcus aureus* (n=9), *Proteus* spp. (n=8), *Klebsiella* spp. (n=6), *Enterobacter* spp. (n=4), *Morganella morgani* (n=1), methicillin-resistant *Staphylococcus aureus* (n=1) and *Acinetobacter* spp. (n=1). Multidrug-resistant patterns of *Pseudomonas* strains shown in (Table 4.5).

TABLE 4.5 Multidrug-resistant pattern of *Pseudomonas* isolated from KBTH and KRH

ID	Age(yrs)	Sex	MDR Isolates	Resistant Pattern
KBTH (n=9)				
KB-16	18	F	*Pseudomonas* spp.	CIP,CAZ,LEV,TZP
KB-41	11/52	M	*Pseudomonas* spp.	AMK,CIP,CAZ,GEN
KB-42	36	F	*Pseudomonas* spp.	AMK,CIP,CAZ,GEN,LEV
KB-69	18	M	*Pseudomonas* spp.	AMK,CIP,CAZ,GEN
KB-91	64	F	*Pseudomonas* spp.	AMK,CIP,CAZ,GEN,TZP
KB-55	31	F	*P. aeruginosa*	AMK,CIP,CAZ,GEN,TZP,MEM
KB-64	49	M	*P. aeruginosa*	AMK,CIP,CAZ,GEN,LEV
KB-73	18	M	*P. aeruginosa*	AMK,CIP,CAZ,GEN,TZP
KB-98	46	M	*P. aeruginosa*	AMK,CIP,CAZ,GEN
KRH (n=3)				
P-13	42	F	*P. aeruginosa*	AMK,CIP,CAZ,GEN,LEV
P-14	60	M	*P. aeruginosa*	AMK,CIP,CAZ,GEN,LEV
P-20	35	M	*P. aeruginosa*	AMK,CAZ,GEN,LEV

KB-Patients unique identification at Korle-Bu Teaching Hospital, P-Patients unique identification at Koforidua Regional Hospital, n-number of MDR isolates.

Escherichia coli, *Citrobacter* spp., *Proteus* spp., *Acinetobacter* spp., *Enterobacter* spp., *Klebsiella* spp., and *Morganella morgani* isolated from the KBTH and the KRH showed varied resistances to the antibiotics tested (Figure 4.7).

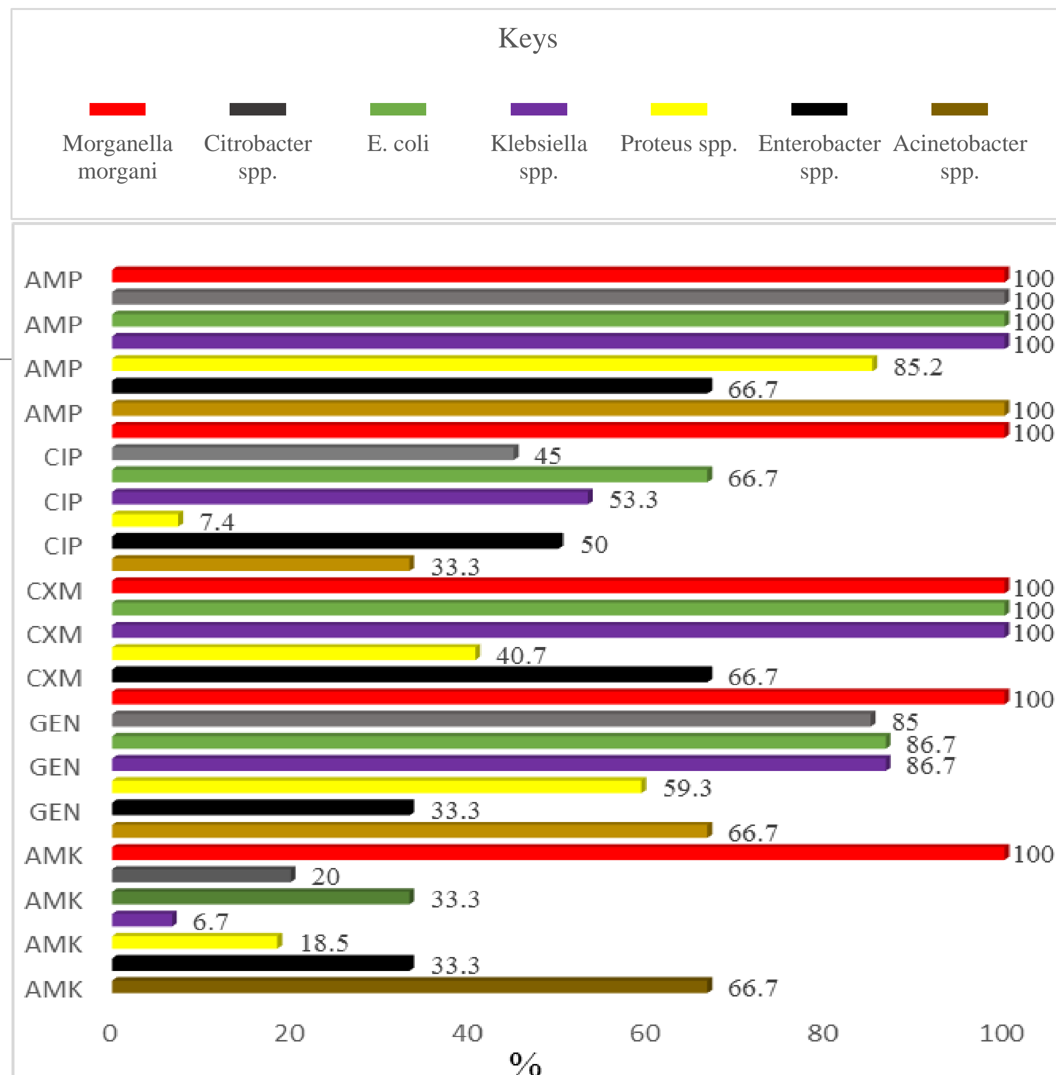

FIGURE 4.7 Percentages of Antibiotic Resistance of total GNB Isolates except *Pseudomonas*.

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One methicillin-resistant *Staphylococcus aureus* (*MRSA*) was detected from the KBTH. All other *Staphylococcus aureus* isolated from the KBTH and the KRH were 100% resistant to ampicillin, penicillin and tetracycline (Table 4.6). Co-trimoxazole resistance was 88.9% (8/9) from KRH against 0% (0/6) from KBTH. Co-trimoxazole percentage resistant difference between KBTH and KRH was statistically significant (Table 4.6).

Pseudomonas strains and *Citrobacter* spp. were isolated from surfaces in dressing rooms at both the KBTH and the KRH.

TABLE 4.6 Antibiotic resistance of *Staphylococcus aureus* in wounds

Antibiotics	KBTH (N=6) n, (%)	KRH (N=9) n, (%)	95% CI	p-value
Cefuroxime	1 (16.7)	5 (55.6)	-82.97-5.19	0 .287
Erythromycin	2 (33.3)	3 (33.3)	-48.70-48.70	1.000
Gentamicin	2 (33.3)	3 (33.3)	-48.70-48.70	1.000
Ampicillin	6 (100)	9 (100)	-	
Penicillin	6 (100)	9 (100)	-	
Tetracycline	6 (100)	9 (100)	-	
Co-trimoxazole	0 (0)	8 (88.9)	-100.0-68.36	**0.001**
Cloxacillin (*OXA)	1 (16.7)	0 (0)	-13.15-46.49	0.400
Cefoxitin (*FOX)	1 (16.7)	0 (0)	-13.15-46.49	0.400

N-represents number isolated and tested, n-number resistant, *FOX (indicator for methicillin resistance), *OXA (indicator for oxacillin resistance).

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CHAPTER FIVE

DISCUSSIONS

5.1 ANTIBIOGRAM

In this study, high numbers of multidrug-resistant bacteria strains were detected from both KBTH and KRH. Phenotypically, one metallo-beta lactamase producing, carbapenem-resistant multidrug-resistant *Pseudomonas aeruginosa* (CR-MDRPA) was detected from the Korle-Bu Teaching Hospital (KBTH). A high level of antibiotic resistance to anti-pseudomonal drugs were detected; including ciprofloxacin, gentamicin and ceftazidime.

In a study on prevalence of MDR *P. aeruginosa* in surgical units in Nigeria, Olayinka *et al.,* (2004) found that 19.6% of isolates were resistant to three or more of the antibiotics tested, with the most prevalent resistance pattern being ceftazidime, gentamicin, perfloxacin and ofloxacin. In this study, 13.0% of *Pseudomonas* isolates showed resistance to three or more of the antibiotics tested, with the most prevalent resistance pattern being ciprofloxacin, ceftazidime and gentamicin.

Results of this study detected 1.1% carbapenem resistance among MDR *Pseudomonas* isolates. Brink *et al*., (2007) observed a range of between 42.0% and 45.0% carbapenem resistance among MDR *P. aeruginosa* in South Africa.

In Kenya, Pitout *et al.,* (2008) detected 13.7% carbapenem resistance among MDR *P. aeruginosa* during 2006 and 2007.

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Pseudomonas resistance to third generation cephalosporins, ciprofloxacin, carbapenems (used as last resort) is a real threat (Goswami *et al.,* 2011; Lautenbach *et al*., 2006). Carbapenem resistance posed a major challenge in the management of infected wounds because such bacteria strains cause invasive disease and are associated with a higher hospital case-fatality rate compare with other strains (Hirakata *et al.,* 2003).

High usage of anti-pseudomonal drugs like ciprofloxacin has been reported to be an independent risk factor for the development of fluoroquinolone and carbapenem resistance (Lautenbach *et al*., 2006). Exposure to ciprofloxacin mediates development of resistance to both fluoroquinolones and carbapenems, by mechanism of selecting mutation that unregulate MexEF-OprD efflux system and decrease levels of outer membrane porin protein OprD (Ochs *et al*., 1999; Livermore *et al*., 2002; Lewis *et al.,* 2012).

Meropenem was the most active compound against *Pseudomonas* isolates followed by piperacillin/tazobactam, amikacin, ceftazidime, gentamicin and ciprofloxacin. From the results of this study, piperacillin/tazobactam, amikacin, ceftazidime, gentamicin and ciprofloxacin may be considered as empirical therapy of first choice for *Pseudomonas* wound infections in KBTH and KRH. At hospitals in Mandeville, Kingston and St. Andrew in United Kingdom, ceftazidime, gentamicin and ciprofloxacin were second line antibiotics administered for wound infections (Gilbert *et al.,* 2003).

Shashikala *et al.,* (2006) in a study on carbapenem resistant *Pseudomonas* in hospital acquired infection concluded that unless conscious efforts are made to

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contain the menace of drug resistance, multi-drug resistant organisms, untreatable by every known antibiotic, may emerge, reversing the medical progress made by mankind and throwing us back to the pre-antibiotic era where many people die of common illnesses.

Research has shown that the lowest concentration required to kill or eliminate bacteria biofilm for many antibiotics actually exceeds the maximum prescription levels for the antibiotics (Brooun *et al.,* 2000). Evidence to date suggests that physical debridement or vigorous physical cleansing of wounds are the best methods for reducing biofilm burden (Flemming *et al.,* 2007; Wolcott *et al.,* 2008; Wolcott *et al.,* 2009; Hall-Stoodley and Stoodley, 2009).

5.1.2 *Pseudomonas* wound infection and age of patient

This study shows high prevalence of *Pseudomonas* wound infection among patients aged 40-59 years at both the KBTH and the KRH as compared to Prabhat *et al.,* (2010) who reported 21-40 years and Siguan and colleagues who reported 21-30 years (Siguan *et al.,* 1990).

This observed age distribution of *Pseudomonas* in infected wounds implies that a high percentage of Ghanaians working age group may be out of work or spending many working hours accessing healthcare. Persoon *et al.,* (2004) observed that patients with chronic leg ulcers and diabetic foot have poorer quality of life due to many time off work or loss of employment, frequent hospital visits for dressing appointments with high associated costs.

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The observed high rate of MDR bacteria strains including carbapenem-resistant multidrug-resistant *Pseudomonas aeruginosa* in this study may be the cause of many antibiotic treatment failures among patients with infected wounds in KBTH and KRH.

Based on the high prevalence of multidrug resistant, antibiotic resistant screening should be carried out before systemic antibiotic treatment commences. Physical and surgical debridement should be part of key wound management procedures for patients with infected wounds. Stringent preventive measures are urgently needed at the Korle-Bu Teaching hospital and the Koforidua Regional hospital to prevent the spread of multi-drug resistant bacteria strains among patients.

CONCLUSIONS

The aim of this study was to characterize antimicrobial resistant bacteria from infected wounds at the Korle-Bu Teaching hospital and the Koforidua Regional hospital. There was a high prevalence (35.1%) of multidrug resistant among *Pseudomonas, Escherichia coli*, *Proteus* spp, *Klebsiella* spp., and *Staphylococcus aureus.* Low prevalence of carbapenem resistant *Pseudomonas* (1.1%) and methicillin-resistant *Staphylococcus aureus* (6.7%) was detected at the Korle-Bu Teaching hospital. Phenotypically, one metallo-beta-lactamase producing, carbapenem resistant *Pseudomonas* strain was detected at the Korle-Bu Teaching hospital.

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The three widely spread metallo-beta-lactamase (MBL) encoding genes; *bla* $_{\text{VIM-type}}$, *bla* $_{\text{SPM-type}}$ and *bla* $_{\text{IMP-type}}$ investigated were not detected in the carbapenem-resistant *Pseudomonas aeruginosa* strain.

An average of two years non-healing wound among patients with infected wounds was detected.

5.1.3 Recommendations

There should be further studies to investigate other MBLs encoding genes and other carbapenem resistant mechanisms in carbapenem resistant *Pseudomonas* strains.

Genotyping should be carried out to identify novel genes and also to monitor inter-and intra-hospital spread of antimicrobial resistant bacteria strains.

Other mechanisms of antibiotic resistance in other multi-drug resistant bacteria isolated from infected wounds should be investigated.

Studies into quality of antiseptic and medicinal solutions for wound treatment are highly recommended.

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5.1.4 Limitations of study

Due to limited anaerobic facilities at both the Korle-Bu Teaching hospital and the Koforidua Regional hospital, anaerobes were not cultured.

API 20 NE and API ZYM biochemical tests could not be used to fully identify other *Pseudomonas* species due to non-availability of Analytical Profile Index kits at both the Korle-Bu Teaching hospital and the Koforidua Regional hospital.

Due to the short period of the study, only three out of the six emerged dominant MBL variants were molecularly investigated in the carbapenem-resistant *Pseudomonas* strain detected.

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APPENDIX I

Media and Standard solutions

Preparation of agar media and standard solutions for culture, identification and antimicrobial susceptibility testing of bacteria isolates

The following media and standard solutions were aseptically prepared according to manufacturer`s instructions using sterile distilled water. Where necessary the media and solutions were autoclaved at 121°C and 15 psi pressure for 15 min.

With the agar plates, dehydrated powders were dissolved in appropriate volumes of distilled water according to manufacturer`s instructions. Substances were mixed thoroughly and gently heated to completely dissolved and autoclaved. When cooled to about 50-55°C, approximately 25ml volumes were dispensed into 90 cm sterile Petri dishes, left to set and agar surfaces dried.

For the agar slopes, dehydrated powders were dissolved in appropriate volumes of distilled water according to manufacturer`s instructions. Substances were mixed thoroughly and gently heated to completely dissolve. Appropriate volumes were then dispensed into appropriate tubes before autoclaving. Autoclaved tubes were slanted at appropriate gradients during setting for the agar slopes.

Quality and sterility of prepared media were ascertained by inoculating randomly selected media with *Pseudomonas* and *Escherichia coli* positive control strains.

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a) Mueller Hinton Agar

Composition	*gm/Ltr*
Beef infusion solids	2.0
Acid Hydrolysed Casein	17.5
Starch	1.5
Agar No.1	17.0

pH 7.3 ± 0.1

Preparation

Prepared according to the manufacturer`s (BIOTEC) instructions. When cooled to about 55 ^{o}C, approximately 25 ml volumes were dispensed into 90 cm sterile Petri dishes, allowed to set and agar surfaces dried.

b) MacConkey Agar

Composition	*gm/Ltr*
Peptic digest animal tissue	20.0
Lactose	10.0
Bile salts	5.0
NaCl	5.0
Neutral red	0.075
Agar	12.0

pH 7.4 ± 0.

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Preparation

Prepared according to the manufacturer`s (OXOID) instructions. When cooled to about 55 °C, approximately 25 ml volumes were dispensed into 90 cm sterile Petri dishes, allowed to set and agar surfaces dried.

c) Blood Agar

Composition

i)	Blood Agar Base	***gm/Ltr***
	Beef Extract	10.0
	Tryptose	10.0
	NaCl	5.0
	Agar	15.5
ii)	5-10 % Sheep Blood	50 ml/Ltr

pH 7.3± 0.2

Preparation

Prepared according to the manufacturer`s instructions. When cooled to about 55 °C, approximately 25 ml volumes were dispensed into 90 cm sterile Petri dishes, allowed to set and agar surfaces dried.

***d)* Chocolate Agar**

Composition

i)	Blood Agar Base	*gm/Ltr*
	Beef Extract	10.0
	Tryptose	10.0
	NaCl	5.0
	Agar	15.5
ii)	5-10 % Sheep Blood	50 ml/Ltr
	pH 7.3± 0.2	

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Preparation

Prepared according to the manufacturer`s instructions. When cooled to about 55 ^{o}C, approximately 25 ml volumes were dispensed into 90 cm sterile Petri dishes, allowed to set and agar surfaces dried.

e) Nutrient Agar

Composition	***gm/Ltr***
Peptone	5.0
Beef/Yeast Extract	3.0
NaCl	5.0
Agar	15.0

pH 7.0 ± 0.2

Preparation

Prepared according to the manufacturer`s instructions. When cooled to about 55 ^{o}C, approximately 25 ml volumes were dispensed into 90 cm sterile Petri dishes, allowed to set and agar surfaces dried.

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f) Triple Sugar Iron (TSI)

Composition	*gm/Ltr*
Peptone	20.0
Yeast extract	3.0
`Lab-Lemco powder`	3.0
NaCl	5.0
Lactose	10.0
Glucose	1.0
Ferric citrate	0.03
Sodium thiosulphate	0.3
Phenol red	*q.s*
Agar	12.0

pH 7.4± 0.2

Preparation

Prepared according to the manufacturer`s (OXOID) instructions. Completely dissolved mixtures were dispensed into appropriate tubes before they were autoclaved. The tubes were slanted at appropriate gradient before setting.

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g) Urea slope

Composition	*gm/100ml*
Glucose	1.0
Peptone	1.0
NaCl	5.0
Di-sodium phosphate	1.2
Potassium dihydrogen phosphate	0.8
Phenol red	0.012
Agar	15.0

pH 6.8 ± 0.2

Preparation

Prepared according to the manufacturer`s (OXOID) instructions. The completely dissolved mixture was autoclaved and allowed to cool. 5ml of filtered sterilized 40% urea solution was then added aseptically to the autoclaved mixture before dispensed into sterile tubes and sloped.

h) Citrate Agar Slant

Composition	*gm/Ltr*
Magnesium sulfate (heptahydrate)	0.2
Ammonium di-hydrogen phosphate	1.0
Di-potassium phosphate	1.0
Sodium citrate (dehydrate)	2.0
NaCl	5.0
Bromothymol blue	0.08
Agar	15.0

pH 6.9

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Preparation

Prepared according to the manufacturer`s (OXOID) instructions. Completely dissolved mixtures were dispensed into appropriate tubes before they were autoclaved. The tubes were slanted at appropriate gradient before setting.

i) Peptone water

Composition	***gm/Ltr***
Peptone	10.0
NaCl	5.0
pH 7.1± 0.2	

Preparation

Prepared according to the manufacturer`s (BIOTEC) instructions. Completely dissolved mixtures were dispensed into appropriate tubes before they were autoclaved.

j) Kovac`s indole reagent

Composition	
p-dimethylaminobenzaldehyde	5.0g
Amyl alcohol	75ml
Conc. HCl	25ml

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Preparation

The aldehyde was first dissolved in the alcohol by warming the mixture gently in a water bath. The mixture was allowed to cool and the acid carefully added and then kept in a brown bottle to protect it from sun light.

k) McFarland 0.5 Turbidity Standard (per 100ml)

Composition

1ml Conc. H_2SO_4

0.5g Dihydrate barium chloride ($BaCl_2.2H_2O$)

Preparation

1ml of Conc. H_2SO_4 was added to 99 ml of distilled water and thoroughly mixed for 1% v/v solution of H_2SO_4 . 0.5g of Dihydrate barium chloride ($BaCl_2.2H_2O$) was dissolved in 50ml distilled water for 1% v/v solution of barium chloride. 0.6ml of the prepared 1% v/v barium chloride solution was then added to 99.4ml of the prepared 1% v/v H_2SO_4 solution. Solutions thoroughly mixed and dispensed into capped tubes.

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APPENDIX II

Positive Biochemical Tests

Indole positive (pink ring), urease positive (pink color change of medium) and citrate positives (growth on agar slant) respectively.

APPENDIX III

Antibiotic Sensitivity Plate

CR-MDRPA Plate

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APPENDIX IV

DNA EXTRACTION USING QIAMP MINI KIT FOR GRAM NEGATIVE BACTERIA

PROTOCOL

1. Pour 1 mL of suspension of overnight growth of bacteria into 1.5 mL microcentrifuge "Eppendorf" tube (loopful of bacteria growing on plate with 1 ml of phosphate saline).
2. Centrifuge at 7500 rpm for 5 minutes
3. Decant the supernatant and add 180 mL of buffer ATL.
4. 20 µL of proteinase K is added and content mixed by vortexing to dissolve all the deposit and incubate at 56°C for 1 hour.
   Make sure tubes are mixed 2 to 3x per hour during incubation period to ensure efficient lysis.
5. Give a brief centrifugation
6. Add 200 µL buffer AL, mix by pulse-vortexing for 15 sec and incubate at 70°C for 10 minutes.
7. Give a brief centrifugation
8. 200 µL Ethanol (95-100%) is added and content mixed by pulse-vortexing for 15 sec.
9. Give a brief centrifugation
10. The mixture is carefully applied (include the precipitate) to the QIAMP spin column.
11. Centrifuge at 8000 rpm for 1 minute
12. The QIAMP spin column is placed in a clean 2 mL collection tube.
13. The QIAMP spin column is carefully opened and 500 µL buffer AW1 is added.
14. Centrifuge at 8000 rpm for 1 minute
15. The QIAMP spin column is placed in a clean 2 mL collection tube.

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16. The QIAMP spin column is carefully opened and 500 µL buffer AW2 is added.
17. Centrifuge at 14000 rpm for 3 minutes (full speed).
18. *The QIAMP spin column is placed in a clean 1.5 mL Eppendorf tube.
19. *The QIAMP spin column is carefully opened and (200µL) 150µL buffer AE is added and incubated at room temperature for 1 minute.
20. Centrifuge at 8000 rpm for 1 minute.
21. Steps 18 and 19 are repeated with incubation 5 minutes at room temperature before centrifugation at 8000 rpm for 1 minute.

N.B.

To assess the quality of the DNA yield, a portion of the 10 µL is run on a 0.8% agarose with 2.5 µL ethidium bromide as follows:

STEPS:

1. A 1% agarose is prepared by dissolving 1g into 100 mL TAE buffer (concentration 1x).
2. Pour molten agarose in electrophoresis chamber and mix with 2 µL of ethidium bromide together and then put to make combs.
3. After agarose has solidified, remove the comb, put agarose in the electrophoretic instrument covered with buffer solution TAE (1x).
4. Load DNA extract in the comb after mixing with 2µL of DNA loading dye. DNA ladder in one comb as a control
5. Turn on the electrophoretic instrument. Voltage 95-100, time 45 minutes for DNA.
6. Turn off the instrument and put the block sample on UV light to see DNA band.

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APPENDIX V

i) Antimicrobial Susceptibility Pattern (zone sizes in mm) of some Bacteria Isolated from the Korle-Bu Teaching hospital (KB).

KB-16 AGE: 18 YRS SEX: F

Pseudomonas spp.

MEM+28 mm

AMK +30 mm

GEN +26mm

TZP -0 mm

CIP -0 mm

CAZ -0 mm

LEV-0 mm

KB-41 AGE: $1^1/_2$ YRS SEX: M

Pseudomonas spp.

MEM+22 mm

AMK -0 mm

GEN -0 mm

TZP +24 mm

CIP -8 mm

CAZ -0 mm

LEV+19 mm

KB-42 AGE: 36 YRS SEX: F

Pseudomonas spp.

MEM +26 mm

AMK -0 mm

GEN -0 mm

TZP +25 mm

CIP -10 mm

CAZ -8 mm

LEV -0 mm

KB-55 AGE: 31 YRS SEX: F

Pseudomonas aeruginosa

MEM -13 mm

AMK -12 mm

GEN -10 mm

TZP -13 mm

CIP -8 mm

CAZ -10 mm

LEV -7 mm

KB-64 AGE: 49 YRS SEX: M

Pseudomonas aeruginosa

MEM +30 mm

AMK -0 mm

GEN -0 mm

TZP +25 mm

CIP -13 mm

CAZ-10 mm

LEV -0 mm

KB-69 AGE: 18 YRS SEX: M

Pseudomonas spp.

MEM+20 mm

AMK -15 mm

GEN -0 mm

TZP +21 mm

CIP -10 mm

CAZ -0 mm

LEV+18 mm

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KB-73 AGE: 34 YRS SEX: M

Pseudomonas aeruginosa

MEM +20 mm

AMK -14 mm

GEN -10 mm

TZP -20 mm

CIP -10 mm

CAZ -10 mm

LEV +19 mm

KB-91 AGE: 64 YRS SEX: F

Pseudomonas spp.

MEM+20 mm

AMK -13 mm

GEN -0 mm

TZP -19 mm

CIP -14 mm

CAZ -8 mm

LEV +18 mm

KB-98 AGE: 46 YRS SEX: M

Pseudomonas aeruginosa

MEM+30 mm

AMK -16 mm

GEN -0 mm

TZP +22 mm

CIP -18 mm

CAZ-10 mm

LEV+17 mm

KB-57 AGE: 42 YRS SEX: F

MRSA

CXC -0 mm

PEN -0 mm

AMP -8 mm

ERY-19 mm

CXM-0 mm

COT+28 mm

GEN -8 mm

TET -0 mm

FOX-0 mm

KB-40 AGE: 49 YRS SEX: M

E. coli

AMK-16 mm

GEN -0 mm

CXM -0 mm

AMP -0 mm

CIP -0 mm

CAZ-10 mm

COT -0 mm

KB-75 AGE: $^{2}/_{52}$ SEX: M

E.coli

MEM +>30 mm

CIP -0 mm

AMK -14 mm

GEN -0 mm

CAZ -14 mm

COT -0 mm

CXM -0 mm

AMP-0 mm

KB-94 AGE: 63 YRS SEX: F

E. coli

CIP -10 mm

AMK+20 mm

GEN -12 mm

CAZ -19 mm

COT -0 mm

CXM -0 mm

AMP -0 mm

KB-86 AGE: 45 YRS SEX: M

Proteus spp.

CIP +28 mm

AMK +20 mm

GEN- 7 mm

CAZ +30 mm

COT -0 mm

CXM +30 mm

AMP -0 mm

KB-71 AGE: 34 YRS SEX: M

Proteus spp.

CIP -19 mm

AMK +26 mm

GEN +30 mm

CAZ +26 mm

COT -0 mm

CXM -8 mm

AMP -0 mm

KB-88 AGE: 19 YRS SEX: M

Proteus spp.

CIP -19 mm

AMK + 20 mm

GEN -12 mm

CAZ +30 mm

COT -0 mm

CXM +28 mm

AMP -9 mm

KB-4 AGE: 18 YRS SEX: M

Enterobacter spp.

CIP -0 mm

AMK-18 mm

GEN -0 mm

CAZ -10 mm

COT -0 mm

CXM -0 mm

AMP -0 mm

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KB-11 AGE: $^{2}/_{52}$ SEX: F

Enterobacter spp.

CIP +>30 mm

AMK-14 mm

GEN -0 mm

CAZ-10 mm

COT-0 mm

CXM -0 mm

AMP -0 mm

TZP -15 mm

MEM +30 mm

KB-17 AGE: 19 YRS SEX: M

Enterobacter spp.

CIP-15 mm

AMK+21 mm

GEN+21 mm

CAZ -15 mm

COT -0 mm

CXM -0 mm

AMP -0 mm

KB-22 AGE: 52 YRS SEX: M

Enterobacter spp.

CIP -0 mm

AMK-16 mm

GEN -0 mm

CAZ-0 mm

COT-0 mm

CXM-0 mm

AMP-0 mm

KB-12 AGE: 52 YRS SEX: F

Klebsiella spp.

CIP+25 mm

AMK+20 mm

GEN +18 mm

CAZ +30 mm

COT -0 mm

CXM -0 mm

AMP -0 mm

KB-87 AGE: 64 YRS SEX: M

Citrobacter spp.

CIP+22 mm

AMK+18 mm

GEN-12 mm

CAZ+30 mm

COT-0 mm

AMP -0 mm

KB-53 AGE: 9 YRS SEX: M

Acinetobacter spp.

CIP +28 mm

AMK-15 mm

GEN -0 mm

CAZ+24 mm

COT-17 mm

AMP -0 mm

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ii) Antimicrobial Susceptibility Pattern (zone sizes in mm) of some Bacteria Isolated from the Koforidua Regional hospital.

P-13 AGE: 42 YRS SEX: F

Pseudomonas spp.

MEM+29 mm

AMK -0 mm

GEN -0 mm

TZP+30 mm

CIP -0 mm

CAZ -0 mm

LEV-0 mm

P-14 AGE: 60 YRS SEX: M

Pseudomonas spp.

MEM+30 mm

AMK -0 mm

GEN -0 mm

TZP +28 mm

CIP -0 mm

CAZ -0 mm

LEV+28 mm

P-20 AGE: 35 YRS SEX: M

Pseudomonas spp.

MEM+>30 mm

AMK -0 mm

GEN -0 mm

TZP+28 mm

CIP +21 mm

CAZ -0 mm

LEV-10 mm

P-46 AGE: 45 YRS SEX: M

Morganella morgani

AMK-15 mm

GEN-10 mm

CIP-0 mm

LEV-0 mm

CAZ-9 mm

CXM-0 mm

AMP-0 mm

COT-0 mm

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P-42 AGE: 77 YRS SEX: F	**P-43 AG E: 15 YRS SEX: F**
Klebsiella spp.	*Klebsiella* spp.
AMK+27mm	AMK+20 mm
GEN-15 mm	GEN-0 mm
CIP-10 mm	CIP-0 mm
LEV-8 mm	LEV-8 mm
CAZ-15mm	CAZ-10mm
CXM-13mm	CXM-0 mm
AMP-0 mm	AMP-0 mm
COT-0 mm	COT-0 mm
P-92 AGE: 23 YRS SEX: F	**P-60 AGE: 57 YRS SEX: F**
Klebsiella spp.	*E. coli*
AMK+29 mm	AMK-0 mm
GEN-11 mm	GEN-0 mm
CIP+25 mm	CIP-0 mm
LEV+30 mm	LEV-0 mm
CAZ+>30 mm	CAZ-11 mm
CXM -10 mm	CXM-0 mm
AMP-0 mm	AMP-0 mm
COT-0 mm	COT-0 mm
P-86 AGE: 18 YRS SEX: F	**P-94 AGE: 29 YRS SEX: F**
E. coli	*E. coli*
AMK+27mm	AMK+28 mm
GEN-0 mm	GEN-0 mm
CIP+29 mm	CIP-0 mm
LEV+29mm	LEV-9 mm
CAZ+30mm	CAZ30 mm
CXM-0 mm	CXM-0 mm
AMP-0 mm	AMP-0 mm
COT-0 mm	COT-0 mm

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P-67 AGE: 53 YRS SEX: F

Proteus spp.

AMK+30 mm

GEN-0 mm

CIP +26 mm

LEV+30 mm

CAZ+30 mm

CXM-0 mm

AMP-0 mm

COT-0 mm

P-44 AGE: 77 YRS SEX: F

Citrobacter spp.

AMK-13 mm

GEN-0 mm

CIP-0 mm

LEV-0 mm

CAZ-7mm

AMP-0 mm

COT-0 mm

P-61 AGE: 26 YRS SEX: F

Citrobacter spp

AMK-0 mm

GEN-0 mm

CIP-0 mm

LEV-0 mm

CAZ-0 mm

AMP-0 mm

COT-0 mm

P-83 AGE: 26 YRS SEX: F

Citrobacter spp.

AMK+25 mm

GEN-0 mm

CIP-0 mm

LEV-8 mm

CAZ-9 mm

AMP-0 mm

COT-0 mm

P-88 AGE: 37 YRS SEX: F

Citrobacter spp.

AMK+26 mm

GEN-0 mm

CIP+25 mm

LEV+>30 mm

CAZ+30 mm

AMP-0 mm

COT-0 mm

P-93 AGE: 36 YRS SEX: M

Citrobacter spp.

AMK+28 mm

GEN-0 mm

CIP-8 mm

LEV-9 mm

CAZ+30 mm

AMP-0 mm

COT-0 mm

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P-89 AGE: 29 YRS SEX: F	**P-91 AGE: 12 YRS SEX: M**
Staphylococcus aureus	*Staphylococcus aureus*
CXC+30 mm	CXC+30 mm
PEN-0 mm	PEN-0 mm
AMP-0 mm	AMP-0 mm
ERY+30 mm	ERY-0 mm
CXM-0 mm	CXM-0 mm
COT-0 mm	COT-0 mm
GEN-0 mm	GEN-0 mm
TET-0 mm	TET-0 mm
FOX+30 mm	FOX+30 mm

P-99 AGE: 12 YRS SEX: F	**P-111 AGE: 48YRS SEX: M**
Staphylococcus aureus	*Staphylococcus aureus*
CXC+29 mm	CXC+30 mm
PEN-0 mm	PEN-0 mm
AMP-0 mm	AMP-0 mm
ERY+>30 mm	ERY-0 mm
CXM-0mm	CXM-0 mm
COT-0 mm	COT-0 mm
GEN+18 mm	GEN-0 mm
TET-0 mm	TET-0 mm
FOX+300 mm	FOX+30 mm