University of Ghana

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Metallo-beta-lactamase-producing *Acinetobacter* spp. from clinical isolates at a tertiary care hospital in Accra, Ghana.

By

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This thesis /dissertation is submitted to the University of Ghana, Legon in partial fulfilment of the requirement for the award of MSc Medical Lab. Science degree.

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DECLARATION

I here-in declare that the work presented in this thesis is my own original research undertaken in the Department of Medical Laboratory Science, School of Biomedical and Allied Health Science (SBAHS); under the joint supervision of Dr Akua Obeng Forson and Dr Japheth A. Opintan and that no part of this work has been presented for another degree in the University or elsewhere. All references have been duly acknowledged.

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Supervisor

(Dr Japheth A. Opintan)

DEDICATION

This work is dedicated to my lovely and prayerful wife,

Mrs Samantha Dzifa Xetsa Olu-Taiwo,

Your understanding and prayers have been most helpful

Divine Mercy, 'Jesus, I Trust In You'

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LIST OF ABBREVIATIONS

β	Beta
µg	Microgram
µl	Microliter
AmpC	Ampicillin hydrolyzying Cephalosporinase
AFLP	Amplified fragment length polymerase
ATCC	American type culture collection
β-lactamase	Betalactamase
CDT	Combined disc test
CTX	Cefotaxime
CAZ	Ceftazidime
COT	Co-trimoxazole
CIPRO	Ciprofloxacin
CLSI	Clinical laboratory standard institute
DDST	Double disc synergy test
EDTA	Ethylene diamine tetra acetic acid
DNA	Deoxyribonuucleic acid
ESBL	Extented-spectrum-betalactamase

GEN	Gentamicin
ICU	Intensive care unit
IMP-1	Imipenemase-1
IPM	Imipenem
KPC	*Klebsiella pneumoniae* carbapenemase
LEV	Levofloxacin
KBTH	Korle-Bu Teaching Hospital
MHT	Modified Hodge test
MDR	Multidrug resistant
MBL	Metallo-betalactamase
MER	Meropenem
NCTC	National collection type culture
NDM-1	New Delhi Metallo-betalactamase-1
OXA	Oxacillinase
PCR	Polymerase chain reaction
SIM	Seoul imipenemase
TAE	Tris acetate ethylene diamine tetreacetic acid

VIM-1	Verona integron-encoded metallo-betalactamase -1

ABSTRACT

Background: One of the most pertinent issues in health-care institutions is the emergence and global spread of metallo-β-lactamase (MBL) producing bacteria of *bla*$_{VIM}$-, *bla*$_{IMP}$- and *bla*$_{NDM}$-types. Metallo-β-lactamase producing-*Acinetobacter* has become a public health concern due to therapeutic treatment challenges associated with nosocomial infections. In Ghana, limited information is available on clinical isolates of MBL-producing-*Acinetobacter*.

Aim: The aim of the study was to determine the prevalence of MBL-producing-*Acinetobacter* spp. of routinely collected clinical isolates from the Korle-Bu Teaching Hospital, Accra.

Methodology: A total of 87 clinical isolates of *Acinetobacte*r were routinely collected from cultures of aspirates, urine, ear, eye and wound swabs between August 2014 to July 2015. Susceptibility pattern was done by Kirby-Bauer disk diffusion method. Meropenem-resistant *Acinetobacter* isolates were screened for enzymes using Modified Hodge test (MHT) and carbapenem-EDTA combined disc test (CDT). Additionally, multiplex PCR was used to determined MBL genes (*bla*$_{VIM,}$ *bla*$_{IMP}$ and *bla*$_{NDM}$) in MBL screen positive *Acinetobacter* isolates.

Results: The 87 *Acinetobacter* isolates showed high levels of antibiotic resistance to cefotaxime (90.8%), ceftadizime (75.9%), co-trimoxazole (70.1%), ciprofloxacin (64.4%), gentamicin (72.4%), levofloxacin (67.8%) and meropenem (59.8%). A total of 54 (62.1%) of *Acinetobacter* isolates were multidrug-resistant. Out of 52 (59.8%) meropenem-resistant *Acinetobacter*, 3 (5.8%) were carbapenemase producers by MHT whilst, 23 (44.2%) were MBL screen positive by CDT. There was no significant difference between the resistance pattern of amikacin, ceftazidime, co-trimoxazole, ciprofloxacin and meropenem amongst MBL screen positive and

MBL screen negative isolates (p-value >0.05). A total of 7 of 87 (8.1%) MBL screen positive *Acinetobcter* isolates harboured $bla_{NDM.}$ Of these, 4 (57.1%) were from wound swabs, urine 2 (28.6%) and ear swab 1 (14.3%). However, no bla_{VIM} or bla_{IMP} was detected.

Conclusion: PCR analysis for $bla_{VIM,}$ bla_{IMP} and bla_{NDM} showed that less than 9% of 87 *Acinetobacter* spp. harboured NDM encoding genes. MBL-producing-*Acinetobacter* isolates showed high levels of resistance to multiple antibiotics. The detection of bla_{NDM} amongst MBL-producing-*Acinetobacter* is a cause for concern, therefore, strict antibiotics usage and infection control measures should be instituted to prevent the spread of these resistance genes.

CHAPTER ONE

INTRODUCTION

1.1 Background

Acinetobacter spp. are Gram-negative cocco-bacilli bacteria, commonly found in soil, water and hospital environments (Peleg *et al*.,2008). They are aerobic in nature, non-fermentative, non-motile, oxidase-negative and catalase-positive bacteria (Peleg *et al*.,2008; Fournier *et al*., 2006). In the last few years, *Acinetobacter* spp, has been implicated in nosocomial infections of clinical importance mostly in the elderly, infants as well as in immunocompromised patients (Peleg *et al*., 2008). Some of these infections include urinary tract infections, lung infections, conjunctivitis, endocarditis, meningitis, wound infections, septicemia, skin and soft tissues infections (Peleg *et al*., 2008; Souli *et al.*, 2008). *Acinetobacter* infections from community acquisition are on the increase, the most prevalent being community-acquired pneumonia, followed by meningitis and cellulitis (Clark et al., 2016; Souli *et al.*, 2008). Presently, there are over 40 species of *Acinetobacter*, however, *A. baunmannii* accounts for over 80 % of isolates causing human diseases (Al Atrouni *et al*., 2016; Nemec *et al.,* 2011; Allen & Hartman, 2000). Infections with *Acinetobacter* spp. can lead to high mortality and morbidity, prolonged hospital stays with increased treatment costs (Higgins *et al*., 2010; Souli *et al.*, 2008).

Acinetobacter spp. associated with nosocomial infections were previously susceptible to 3rd generation broad-spectrum cephalosporins (ceftriaxone, cefotaxime, ceftazidime) (Livermore, 2011). However, with the emergence of extended spectrum-β-lactamases (*ESBLs*) Gram-negative bacilli, carbapenems (meropenem, imipenem, ertapenem) became the primary antibiotics of choice in the treatment of serious bacterial infections (Livermore, 2012; Papp-

Wallace *et al*., 2011). Furthermore, *Acinetobacter* spp. that are non susceptible to carbapenem drugs have been increasing (Doi *et al*., 2015; Livermore 2012; Mezzatesta *et al*., 2008; Poirel & Nordamnn 2006). Resistance to carbapenem can evolve by the following mechanisms; development of efflux pump, decreased cell permeability and the production of intrinsic or acquired carbapenemases resistance belonging to either the class B metallo-β-lactamase or class D oxacillinase (OXA) (Nordmann *et al*., 2011). Some of the main oxacillinases genes include $bla_{\text{OXA-48,}}$ $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-51}}$ (Queenan & Bush 2007). Several metallo-β-lactamase encoding genes have been found in *A. baunmanii* such as Imipenemase (IMP), Verona integron-encoded metallo-β-lactamase (VIM), New Delhi metallo-β-lactamase (NDM) and Seoul Imipenemase (SIM) (Nordmann *et al*., 2011; Poirel & Nordmann, 2006). The most prevalent MBLs include IMP-type, VIM-type and NDM-type (Nordmann *et al*., 2011; Poirel *et al.*, 2011). Metallo-β-lactamsase are known to be associated with genes residing on intergrons and plasmids which can enable the dispersion of resistant genotypes (Nordmann *et al*., 2011). Metallo-β-lactamase-producing *Acinetobacter* spp. is mostly resistant to most available commercial antibiotics (β-lactams, aminoglycoside, cephalosporins, quinolones and carbapenems). In order to screen these resistance phenotypes, several phenotypic carbapenemase detection methods have been employed such as the combined disc test (CDT) with EDTA, the double disc synergy test (DDST) using EDTA or 2-mercaptoacetic acid, and imipenem/imipenem-EDTA E-test strips (Franklin *et al.*, 2006; Walsh *et al*., 2005: Lee *et al.*, 2003; Yong *et al*., 2002). All these screening methods use carbapenem as indicator β-lactam and a metallic ion chelator like ethylene diamine tetra acetic acid (EDTA) or thiol based compound (mercaptopropionic acid) and dipicolinic acid (Lee *et al*., 2003). Molecular techniques can also be employed to confirm carbapeemase encoding genes (Poirel *et al.*, 2011; Ellington *et al*., 2007)

1.2 PROBLEM STATEMENT

In humans, β-lactams are often prescribed because of their comparatively high effectiveness, broad spectrum activity, low toxicity and minimal side effect (Wilke *et al*., 2005). The over-dependence on β-lactams class of antibiotics has conrtibuted to the development of β-lactamases-producing strains of bacteria. The emergence of extended spectrum-β-lactamases (*ESBLs*) led to the dependence on carbapenems as last options for treating serious bacterial infections (Livermore 2012). Not long after the introduction of carbapenems, the emergence of carbapenemase-producing bacteria also evolved (Livermore 2012). The emergence of MBL-producing strains resistance to β-lactams and non-β-lactams drugs has become an alarming issue. Furthermore, the resistance problem is escalated by the fact that some of these MBLs encoding genes are located on integrons and plasmids which have a higher propensity to dissemination among *Enterobacteriaceae* (Walsh *et al*., 2005). The appearance and worldwide dispersion of metallo-β-lactamase-producing-*Acinetobacter* spp. have become an importance public health issue globally (Papp-Wallace *et al*., 2011). However, despite these global issue on resistance, limited investigations have been performed to evaluate the occurrence of metallo-β-lactamase in Western Africa (Codjoe, 2016; Manenzhe *et al*., 2015; Olaitan *et al*.,2012). An investigation by Codjoe, (2016) with clinical isolates of Gram-negative bacilli revealed carbapenemase prevalence of 7.2% amongst *Acinobacter baunmanii*. Codjoe, (2016) also determined that the overall prevalence of carbapenemase-production among Gram-negative bacilli to be 13.5%.

Currently, in Ghana, routine laboratory screening of carbapenemases-production in clinical isolates is absent. To monitor the depth of this issue, this study examined routinely collected clinical isolates of *Acinetobacter* spp. from KBTH, Accra, Ghana, for the prevalence of metallo-β-lactamase production.

1.3 SIGNIFICANCE OF STUDY

While report of the spread of bacteria producing carbapenemase have been in wide circulation in Europe, United States, Canada and Asia (Codjoe, 2016; Canton *et al*., 2012), limited information is available on the importance and spread of carbapenemase in West Africa (Codjoe, 2016 Manenzhe *et al*., 2015). It is imperative to understand the prevalence and type of metallo-β-lactamase, as early detection is critical for the effective formulation of antibiotic treatment guidelines for metallo-β-lactamase-producing *Acinetobacter* spp. Presently, only few information is available on the occurrence of metallo-β-lactamase producing *Acinetobacte*r spp. in Ghana (Codjoe, (2016). This study is designed to provide baseline data on the prevalence of metallo-β-lactamase-producing *Acinetobacter* spp. The information generated in this study will provide data needed to inform laboratory screening and surveillance of metallo-β-lactamase in Ghana. This will eventually help in successful prevention and control of metallo-β-lacatamase (MBLs) in *Enterobacteriaceae* and *Acinetobacter* strains in Ghana.

1.4 Aim

The main aim of this study is to determine the prevalence of metallo-β-lactamse (MBL) in routinely collected clinical isolates of *Acinetobacter* spp.

1.5 Specific objectives

The specific objectives of this study

1 To determine the antibiogram of routinely collected clinical isolates of *Acinetobacter* spp.

2 To determine phenotypically, the presence of carbapenemase (MBL) production among carbapenem-resistant *Acinetobacter* spp.

3 To determine the prevalence of VIM, IMP and NDM carbapenemase encoding genes among phenotypically confirmed metallo-β-lactamase screen positive *Acinetobacter* spp.

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical background of the genus *Acinetobacter*

In 1911, Beijerinck described the genus *Acinetobacter* with the name *Micrococcus calcoaceticus* that he obtained from soil samples (Henriksen, 1973). *Acinetobacter* group was at various times confusedly classified into different genera such as *Diplococcus mucosus*, *Micrococcus calcoaceticus, Alcaligenes haemolyticus, Minapolymorpha, Moraxella iwoffii, Herellea vaginicola, Bacterium anitratum, Moraxella iwoffii var. glucidolytica, Neisseria winogradskyi, Achromobacter anitratus and Achromobacter mucosus* (Bergogne-Bérézin & Towner 1996; Peleg *et al*., 2008). However, in 1954 Brisou and Prevot suggested the present designation of *Acinetobacter* (derived from the Greek word 'Akinetos' i.e. non-motile) to distinguish the organism's motility potential in the group *Achromotobacter* (Henriksen, 1973). By 1968, the name *Acinetobacter* began to receive acceptability by the research community (Baunmann, *et al*., 1968). In 1971, due to the research findings on the organism's nutritional requirements, Baunmann *et al*., (1968) demonstrated that oxidase-positive strains are distinct from oxidase-negative strains hence, the subcommittee on Moraxella and Allied bacteria decided that the genus *Acinetobacter* consist of a single oxidase-negative strain ((Lessel, 1971). Thereafter, the 1974 book edition of *Bergeys manual of systematic bacteriology* listed the genus *Acinetobacter* in addition to its only species *Acinetobacter calcoaceticus*. Subsequently, the genus *Acinetobacter* was added to the family *Neisseriaceae* (Lautrop, 1974; Juni, 1984). Presently, the genus *Acinetobacter* belongs to family *Moraxellaceae* along with other genus such as *Moraxella, Psychrobacter* and related ogranisms (Rossau, 1991).

2.2 Taxonomy of the genus *Acinetobacter*

In 1986, Bouvet and Grimont through genetic analysis of DNA-DNA hybridization were able to identified 12 genospecies including *Acinetobacter baunmannii, Acinetobacter calcoaceticus, Acinetobacter johnsonii*, *Acinetobacter haemolyticus,* and *Acinetobacter iwoffi* (Bouvet & Grimont, 1987). Further studies carried out by Bouvet & Jeanjean (1989) and Tjernberg & Ursing (1989), on the genus *Acinetobacter* resulted in the identification and addition of more genospecies.

Acinetobacter calcoaceticus-A. baumannii complex consists of 4 closely related genospecies (Peleg *et al*., 2008). They are difficult to differentiate phenotypically with routine commercial systems, except with the help of genotypic techniques such amplified 16S rRNA gene restriction analysis (ARDRA), sequencing *rpoB* gene (RNA polymerase β-subunits) and Amplified fragment length polymerase (AFLP) (Peleg *et al*., 2008). These methods are tedious to be applied in routine laboratory diagnosis and are performed mostly in reference laboratories (*Peleg et al*., 2008). Species of the *Acinetobacter baunmannii* complex include: *A. calcoaceticus,* an environment resident bacteria usually inhabitants of water and soil, *Acinetobacter* genomic species 3 (now known as *A. pitti*), *Acinetobacter* genomic specie 13TU (now known as *A. nosocomialis*) and *A. baunmannii* that comprise more than 80% of the complex and has been found to be the most clinicaly relevant and is severally implicated in nosocomial infection (Lee *et al*., 2013; Nemec *et al*., 2011; Allen & Hartman, 2000). *Acinetobacter pitti*, *A. nosocomialis* and *A. baunmannii* are mostly termed *A. baunmannii* group (Peleg *et al*., 2008). Currently, there are at least 41 recognized species of the genus *Acinetobacter* (Al Atrouni *et al*., 2016; Nemec *et al.,*2011; Peleg *et al*. 2008) (Table 2.0).

Table 2.1 currently recognized *Acinetobacter* species

	Acinetobacter species	*Genomic species*	*Source*
1	*A. calcoaticecus*	1	Soil & Human
2	*Acinetobacter baunmannii*	2	Human
3	*Acinetobacter pittii*	3	Human & Soil
4	*A. larvae*	4	Moth larval gut
5	*A. junii*	5	Human
6	*A johnsonii*	7	Human & animal
7	*A. iwoffii*	9	Soil & Human
8	*A. radioresistens*	12	Soil & Human
9	*A. bereziniae*	10	Soil & Human
10	*A. guillouiae*	11	Human & animal
11	*A. nosocomialis*	13TU	Human
12	*A. seiferitii*	Close to 13tu	Human & animal
13	*A. ursingii*	Phenon 1	Human
14	*A. schindleri*	Phenon 2	Human & animal
15	*A. parvus*	Phenon 4	Human& animal
16	*Acinetobacter beijerincki*	Phenon 7	Human& animal
17	*Acinetobacter courvalini*	14BJ	Human& animal
18	*Acinetobacter variabilis*	15TU	Human
19	*Acinetobacter dispersus*	17	Soil & Human
20	*Acinetobacterdijkshoorniae*	BN14	Human & animal
21	*Acinetobacter modestus*	Taxon 18	Human
22	*Acinetobacter vivianii*	Taxon 20	Human & water
23	*Acinetobacter bouveti*		Activated sludge
24	*Acinetobacter brisouli*		Peat
25	*Acinetobacter equi*		Horse
26	*Acinetobacter gerneri*		Activated sludge
27	*Acinetobacter grimontii*		Activated sludge

28	*A.guagdongensis*	Lead-zinc ore
29	*A.harbinensis*	River water
30	*A.indicus*	Soil
31	*A.kookii*	Soil & water
32	*A.nectaris*	Floral necter
34	*A.pakistancensis*	Wastewater
35	*A.populi*	Populus bulk
37	*A.puyangensis*	Populus bulk
38	*A.qingfengensis*	Populus bulk
39	*A.rudis*	Rawmilk
40	*A.soli*	Hunam & soil
41	*A.tandoii*	Hunam & soil

Source: Al Atrouni *et al*., (2016); Nemec *et al.,* (2011); Peleg *et al*., (2008)

2.3 Morphological and Physioloical characteristics of *Acinetobacter* species

Members of the genus *Acinetobacter* are known to be obligate aerobes, non-motile, glucose-non-fermentative, catalase-positive, citrate-positive, indole-negative, oxidase-negative (Peleg *et al*., 2008). They are Gram-negative and cocco-bacilli that usually occurring in diploid formation with G+C content of 39%+47% (Peleg *et al*., 2008). *Acinetobacter* spp. are short, plump, mostly 1.5-3 mm in size, with a form varying from coccoid to coccobacillary, in consonance with the state of growth phase (Peleg *et al*., 2008; Henriksen, 1973). *Acinetobacter* spp. sometimes, appear as smooth and mucoid as well as varying from pale yellow to grayish-white colonies on routine agar plate. The colony morphology of *Acinetobacter* spp. is on some occasions comparable in size to members of the *Enterobacteriaceace* family (Bergogne-Berezin & Towner, 1996).

Acinetobacter spp. are non-fastidious in nature and normally grow easily on regular culture media such as MacConkey agar, Cysteine lactose electrolyte deficiency agar (CLED) and nutrient agar over a diverse range of temperature (Doughari *et al*., 2011). Although, most clinical isolates grow at 33°C - 37°C, some of the known *Acinetobacter* isolates from the environment prefer incubation temperature between 20°C-30°C. However, few species such as *A. baunmannii* have been reported to withstand growth at higher temperature of 41°C - 44°C, growth at such temperature have been used to distinguish *A. baunmannii* from *A. calcoaceticus* (Bouvet and Grimont, 1987).

2.4 Natural habitat of *Acinetobacter* spp.

Acinetobacter are heterogeneous group and mostly ubiquitous in nature (Peleg *et al*., 2008). The isolation of a variety of known species from different sources that include soil, water, sewage, human, food and animals are well documented (Doughari *et al*, 2011; Kurcik-Trajkovska, 2009;

Peleg *et al.*, 2008). Studies have shown that most *Acinetobacter* spp. that were recovered from human specimens are pathogens of clinical relevance (Peleg *et al*., 2008). The mucus membrane of the pharynx and human respiratory secretions have been associated with local and systemic *Acinetobacter* infections that include pneumonia, septicemia and wound infection (Munoz-Price and Weinstein, 2008; Beggs *et al*., 2006). However, the natural habitat of *A. baunmannii* and *A. nosocomialis* is yet to be defined (Peleg *et al*., 2008).

There has been the recovery of *Acinetobacter* spp. from the skin, oropharynx and digestive tracts of in-patients (Junk and Park, 2015). Likewise, *Acinetobacter* spp. have also been isolated from food (Kanafani & Kanj, 2014). Reports of *Acinetobacte*r spp. from the skin of health care workers, mattresses and pillows is well documented (Beggs *et al*., 2006; Griffith *et al*., 2006).

2.5 Dessication resistance of the genus *Acinetobacter*

Acinetobacter baunmannii ability to survive on dry surfaces with limited nutrient enables their persistence and transmission in medical settings (Kanafani & Kanj, 2014). Furthermore, during prolonged hospital outbreaks, colonized medical devices and equipments could serve as reservoirs (Kanafani & Kanj, 2014). It has been found that majority of *A. baunmannii* strains could persist longer when compared to *Escherichia coli* on dry and nutrient deficient surfaces, Certain strains of *Acinetobacter* spp could remain viable as long as four months (Lee *et al.*, 2011). This potential enables the organism to survive in hospital and spread infections. In addition, military personnel's infections by *Acinetobacte*r were found to have been acquired during admissions in medical facilities and not from the environment (Kanafani & Kanj, 2014; Lee *et al.*, 2011).

2.6 Virulence factors of the genus *Acinetobacter*

Acinetobacter spp. are often classified as organisms of low virulence (Kurcik-Trajkovska, 2009; Peleg *et al*., 2008). However, irrespective of the growing clinical significance of *Acinetobacter* infections, very few data is available in respect of the conditions that contribute to the pathogenesis of *Acinetobacter* spp. (McConnell *et al.*, 2013). Some preliminary virulence factors identified include: cell surface hydrophobicity, outer membrane proteins (OMPs), toxic slime polysaccharide and verotoxins. *Acinetobacter* spp. have been found to exhibit cell surface hydrophobicity involve in bacterial adhesion as well as help to evade phagocytosis (Doughari *et al*., 2011; Kurcik-Trajkovska, 2009). Some *Acinetobacter* strains have been found to possess various OMPs of the OmpA family (Kim *et al*., 2009). Likewise, some Gram-negative bacilli have been found to exhibit outer membrane proteins (OMPs), that are involve in pathogenesis and adaptation in host cells, they are also implicated in antimicrobial resistance. (Kanafani & Kanj, 2014; Doughari *et al*., 2011). *Acinetobacter baunmannii* lipopolysaccharides (endotoxin) have been found to be a potent stimulator of circulating white blood cells that release proinflammatory substances They are detrimental to neutrophils and inhibit their migration as well as their phagocytosis (Kanafani & Kanj, 2014; Doughari *et al*., 2011).

2.7 *Acinetobacter* nosocomial infections

*Acinetobacte*r spp. are major nosocomial pathogens **(**Dijkshoorn *et al.*, 2007). *Acinetobacter baunmannii* has been found as the most clinically relevant *Acinetobacter* spp. however, *A. nosocomialis* and *A. pittii* are severally implicated in hospital-acquired and health- associated infections (Chuang *et al*., 2011). Hospital-acquired and health-associated infections linked with *A. baunmannii* complex include; pneumonia, urinary tract infections, bloodstream infection,

wound infections, surgical site infections, meningitis, soft and skin infections (Kempf & Rolain, 2012; Peleg *et al*., 2008). The risk factors that predisposes patients to colonization or infection with multidrug-resistant *Acinetobacter* spp. Include prolonged stay in hospital, exposure in intensive care units, receipt of mechanical ventilation, colonization pressure, recent surgery, exposure to antimicrobial pressure, invasive pressure and immunocompromised, (Dijkshoorn *et al.* 2007; Fournier *et al.*, 2006). *Acinetobacter* nosocomial infection is estimated to be the cause of 2-10% of reported Gram-negative infections in the (ICU) in the United States and Europe (Eveilard *et al*., 2010). *Acinetobacte*r spp. have been found to be the second most prevalent non-fermentative Gram-negative pathogens recovered from clinical specimens after *Pseudomonas aeroginosa* (Gautam *et al*., 2011). The distribution by site of *Acinetobacter* infections have been found to be similar to those of other *Enterionacteriaceae* (Livermore, 2012).

In Iran, an investigation by Noori *et al*., (2014) have found the tracheal tube (52.8%) to be the site with the highest *Acinetobacter* infection, followed by urine (26.9%). In Pakistan, Anwar *et al*., (2016) found the highest infection by site to be the urinary tract (21.2%), followed by bloodstream (18.2%) and the respiratory tract (13.6%). Likewise, in Morocco, Uwingabiye *et al*., (2016) revealed that the broncho-pulmonary (44.67%) to have the maximum number of *Acinetobacter* isolates, followed by blood culture (14.51%), deep pus (12.47%), urine (12%), superficial pus (9%), catheters (3.85), tissues (1.81%) and puncture liquid (1.59%). Uwingabiye *et al*., (2016) also found *Acinetobacter* isolates to be (87.11%) and (1.0%) for *A. baunmannii* and *A. iwoffii* respectively.

In Ghana, a study by Enweronu-Laryea and Newman, (2007) has found *Acinetobacter* spp., *Enterobacter species.* and *Klebsiella pneumoniae* to be the major cause of neonatal infections.

2.8 Community-acquired *Acinetobacter* infections

Occasionally, *Acinetobacter* spp. have been implicated in community-acquired infections which comprise pneumonia, bacteremia, wound infections, urinary tract infection, eye infections and otitis media (Davis *et al*., 2014). A study in a Kenyan hospital have found *Acinetobacter* spp. to account for 10% of community-acquired bacteremia (Aiken *et al*., 2011). Furthermore, community-acquired pneumonia is associated with a mortality rate of between 40-60% (McConnell *et al.*, 2013).

2.9 Treatment of *Acinetobacter* infections

Acinetobacter. baunmanii has been listed as one of the 3 bacteria placed in the critical priority group (WHO, 2017). Due to their escalating prevalence of non susceptibility to β-lactams and non-β-lactams (Souli *et al.,* 2008). Only a few antimicrobial can be effectively used as therapeutic measure for MDR *Acinetobacter* infection. Furthermore, since only limited antimicrobial remain reliable as therapeutic means for *Acinetobacter* infections, the research for the discovery of new drugs and the reassessment of older drugs have been a necessity (Jain & Danziger, 2004).

2.9.1 Antibiotic treatment options for *Acinetobcter* infections

2.9.2 Sulbactam

Sulbactam one of the known inhibitors of β-lactamase with capacity to bind penicillin binding protein (PBP) of *Acinetobacter* spp. (Bassetti *et al*., 2008). Whilst its mode of action relies on hindering the degradative effect of β-lactams by β-lactamase, They are known to also possess an *in vitro* bactericidal activity against *Acinetobacter* spp. when used as monotherapy (Dinc *et al*.,

2015; Higgins *et al*., 2004). However, the clinical usage of sulbactam is mostly as combination therapy with other antibiotics (Kempf *et al*., 2013). Furthermore, a combination therapy like ampicillin-sulbactam has been a reliable drug of choice in the empirical treatment of multidrug-resitant *Acinetobacter* spp. (Levin *et al*., 2003). However, non susceptibility of an ampicillin-sulbactam combination have been reported in a Taiwan study where (70%) of *Acinetobacter* from clinical source were found to be non susceptible to ampicillin-sulbactam (Yang *et al*., 2010).

2.9.3 Polymyxin

Polymyxin is polycationic lipopeptide antimicrobial that have bactericidal activity towards nonfermenter Gram-negative bacilli such as *Acinetobacter* spp. They consist of polymyxin B and polymyxin E (colistin). These agents were adminstered in the 1960 and 1970s, but their usuage have been restricted due to concern over neurotoxicity and nephrotoxicity (Cisneros & Rodriguez-Bano 2002). However, with the high prevalence of multidrug-resistant *Acinetobacte*r, the utility of polymyxin has escalated and at times prescribed as an empirical therapy for carbapenem non susceptible *Acinetobacter* spp. (Kim *et al.*, 2009).

2.9.4 Tigecycline

Tigecycline is a semi-synthetic modified minocycline and a member of the new glycyclines**.** It possesses a potent invitro activity toward clinical sources of *Acinetobacter* spp. (Deny *et al*., 2013). Poulakou *et al*., 2009) have reported microbiological and clinical response of multidrug-resitant *Acinetobacter* spp. to tigecycline. In another study, a succuss rate of 82.4% documented during the administration of tigecyclcine in the treatment of *Acinetobacter baunmanii* infection

(Vasilev *et al.*, 2008) found a success level of 82.4% in the treatment of *A. baunmanii* resistant infection.

2.9.5 Aminoglycoside

Tobramycin and amikacin are some of the aminoglycoside agents that have retain susceptibility, used as therapeutic choice for treatment of multidrug-resistant *A. baunmanii* infections (Bassetti *et al.*, 2008). These treatment options are normally adminstered as combination therapy with other active antimicrobial agents. Many MDR *A. baunmanii* isolates have been found to maintain an intermediate susceptibility to amikacin and tobramycin ((Bassetti *et al.*, 2008). However, *A. baunmanii* resistant to Amikacin and Tobramycin have been identified to be associated with Aminogycoside modifying enzyme or efflux pump mechanism (Bassetti *et al.*, 2008).

2.9.6 Carbapenem

Carbapenem was developed in the 1980s; they belong to a potent class of β-lactams antimicrobial with vast spectrum of inhibitory effective. (Papp-Wallace *et al.*, 2011). It possesses a dimension that renders them virtually stable and less selective to many β-lactamase (Papp-Wallace *et al.*, 2011) (Figure 2.0). They are known to be antibiotic of last choice for the treatment of many grievious Gram-negative bacilli infections (Nordmann *et al.*, 2012; Papp-Wallace *et al.*, 2011). The US Food and drug adminstration (FDA) approved carbapenem and some commonly used carbapenems include; Imipene (FDA approved 1985), Meropenem (FDA approved 1996), Ertapenem (FDA approved 2000), Doripenem (FDA approved 2007), Panipenem/betamipron (Japanese approval 1993) and Biapenem (Japanese approval 2001). Reinert *et al.*, (2007) study on the susceptibility patterns of *Acinetobacter* isolates collected from

different continents have reported Carbapenem (Imipenem) susceptibility of 60.6%, 86.6%, 85.9%, and 69.2% in South America, North America, Europe and Asia respectively.

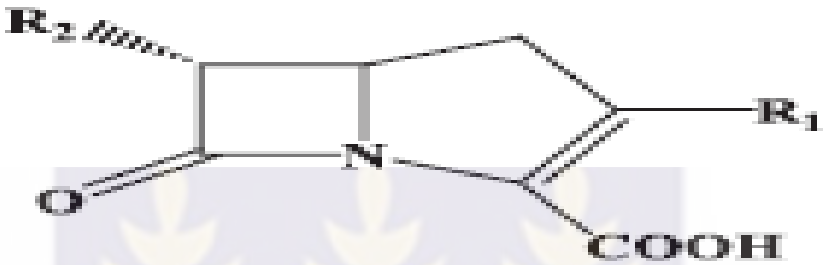

Figure: 2.1 Structure of Carbapenem

Source: Papp-Wallace *et al*., (2011)

However, the situation has changed with emerging carbapenem resistance to *Acinetobacter* spp. , *Pseudomonas* spp. as well as *Enterobacteriaceae* in the last few years (Poirel *et al*., 2011; Poirel & Nordamnn, 2006). Likewise, Bonnin *et al*., (2013) have identified a systematically carbapenems resistance increase among Gram-negative bacilli in recent years. Different investigations have emphasized the existence of carbapenem-resistant isolates in health institutions (Kempf & Rolain 2012; Lee *et al*., 2011). Studies in India and Morocco have reported *Acinetobacter* resistance of 89.6% and 87.7% to imipenem respectively (Uwingabiye *et al*., 2016; Jaggi *et al*., 2012;). In a worldwide study of the collection of 5127 *Acinetobacter* spp. between 2005 & 2009, from 140 hospitals in 32 countries that included North America (17.1%), Europe (22.9%), Latin America (25.2%) and Asia-Pacific (34.8%) revealed the overall resistant prevalence of imipenem and meropenem to be 45.9% and 48.2% respectively (Mendes *et al*.,

2010). Furthermore, the prevalence rate of carbapenem resistance in *A. baunmanii* have been found to vary from country to country with rates of 40-60% in Southern Europe, Middle East, Turkey, Southern America and Asia (Mendes *et al*., 2010).

2.9.6.1 Mechanism of carbapenem resistance

Resistance to carbapenem have been found to evolve by diverse mechanisms that include: development of efflux pump, decreased cell permeability due to loss of OMP and the production of intrinsic as well as acquired carbapenemase belonging to either metallo-β-lactamase (MBL) of class B and OXA carbapenemase of class D (Nordmann *et al*., 2010).

Antibiotic drug resistance mediated through overexpression of efflux pump development is a common factor affecting antimicrobial susceptibility pattern in Gram-negative bacteria and various types of efflux pumps have been identified in *Acinetobacter baunmanii* (Magnet *et al*., 2001). The AdeABC (*Acinetobacter* drug efflux) pump is a member of the Resistance Nodulation cell Division (RND) family. Intrinsic AdeABC efflux over expression in association with oxacillinases may confer a high level resistance to carbapenem (Heritier *et al.,* 2005). Furthermore, a second RND efflux pump (AdeIJK) has been found to contribute to β-Lactam resistance in *A. baunmanii*, however, its effect on carbapenem resistance has not been observed (Coyne *et al*., 2011).

Reports of the loss of outer membrane protein (OMPs) in *Acinetobacter* spp. has been associated with resistance to carbapenem (Perez *et al.,* 2007). Porin of the OMPA family have been identified in various species of *Acinetobacte*r such as *A. radioresistens*. *A.junii* and *A. baunmanii*. Furthermore, altered expressions of OMPs in response to antibiotic challenge have

been found to further reduce permeability to antimicrobial and subsequently lead to multidrug resistance (Perez et al., 2007).

2.9.7 Carbapenemase

Carbapenemases are reputed to be the most potent and diverse of all known beta-lactamases . They possess the avidity to recognize virtually all hydrolysable beta-lactam (Livermore & Woodford, 2006; Poirel & Nordmann, 2006). The first carbapenemase-producing *Enteriobacteria*ceae was reported by Naas and Nordmann, (1994). Since this discovery, different carbapenemase enzymes have been identified in *Acinetobacter* spp. and classified to belong to three major classes: Ambler classes A, B and D carbapenemases. These three classes mode of action is to split the amide bond of the beta lactams ring. however, their ability to bind and hydrolyze beta lactams varies considerably. (Queenam and Bush, 2007; Walsh *et al.*, 2005)

Based on molecular studies, two active sites have been described, class A and D with serine as its active site and class B with a zinc atom as its act site (Queenam and Bush, 2007). The appearance of carbapenemases has become one of a major concern worldwide (Cornaglia *et al*., 2011). The threat to global healthcare is on the increase due to their non suscptibility to β-lactams antibiotics as well as to other non-β-lactams like Aminoglycosides, Fluoroquinolones and Co-trimoxazole (Cornaglia *et al*., 2011; Souli *et al.*, 2008).

2.9.7.1 Class A carbapenemase

Klebsiella pneumoniae carbapenemases (KPCs) is the most clinically significant enzyme in class A carbapanemase (Nordamnn *et al.*, 2009). Their mode of action is inhibited by β-lactamase inhibitors such as clavulanic acid and tazobactam. The first KPC producer (KPC-2 positive

Klebsiella pneumonia) was detected in North Carolina State of the United States (Queenan and Bush 2007). Since this discovery, KPC producers have been identified in almost every state in the United States and have spread to different parts of the world and now of public health concern (Nordamnn *et al*., 2009, Yigit *et al*., 2001). These KPC enzymes have been identified in several genera of *Enterobacteriaceae and Pseudomonas* spp. as well as *Acinetobacter spp*. in particular *A.baunmanii* (Nordamnn *et al*., 2009). Mortality from KPC producers now ranged between 25% - 60% (Nordamnn *et al*., 2011).

2.8.7.2 Class D carbapenemase

Oxacillinases (OXA-type) β-lactamases constitute class D and possess the capacity to hydrolyse carbapenems. But are not inhibited by EDTA or clavulanic acid (Nordmann *et al*., 2011). In 1985, the first incidence of oxacillinase type of gene was isolated from *A. baunmanii* in Scotland (Paton *et al*., 1993). Subsequently, OXA-23 producers have been identified worldwide in *A. baunmanni*, while OXA-4O and OXA-58 are less distributed (Bonnin *et al*., 2013). Furthermore, OXA-48 producers were first identified in a clinical isolate of *Klebsiella pneumonia* from Turkey (Poirel *et al.,* 2004). Since then, OXA-48 producers have been reported in North African countries and lately in Middle East and Asia (Girlich *et al*., 2014).

2.8.7.3 Class B Carbapenemase: **Metallo-β-lactamases (MBLs)**

Metallo-β-lactamase (MBLs) is representative of class B carbapenemase (Walsh *et al*., 2005). They require zinc as a divalent cation for their activity and possess capacity to hydrolyse almost all β-lactams except monobactams such as astreonam as well as confer resistance to aminoglycoside and flouroquinolones (Walsh *et al*., 2005). Metallo-beta-lactamases are inhibited

by ethylene diamine tetra acetic acid (EDTA) or other chelators but not clavulanic acid. They are carried either on plasmids or on chromosomes (Queenan & Bush 2007).

Nineteen-ninetys (1990s) witnessed a vast acceleration of cases of horizontal gene transfers in *Pseudomonas* spp, *Enterobacteriaceae* and lately in *Acinetobacter* spp. in particular *A.baunmanii* (Nordmann *et al*., 2012). The five main metallo-β-lactamase (MBLs) that have been identified in *A.baunmanii* include the Verona integron-coded metallo-β-lactamase (VIM-type), Imipenemase (IMP-type), Seoul Imipenemase (SIM-type), German Imipenemase (GIM-type) and the recently New Delhi metallo-β-lactamase (NDM-type) (Corniglia *et al*., 2011; Brown & Amyes, 2006). As illustrated in (Figure 2.2) there has been a global dispersion of MBLs. Since, the discovery of bla_{IMP} in the late 1980s in Asia from clinical isolates, several MBLs types have spread to different parts of the globe. Subsequently, the death rate associated with MBLs production ranges from 18% to 67% (Cornaglia *et al.*,2011; Nordamann *et al*., 2011).

2.9.7.3.1 $Bla_{IMP\text{-}type}$

In 1988, bla_{IMP} gene was identified in clinical isolates of *P. aeruginosa* in Japan for the first time. (Walsh, 2005; Osano *et al*., 1994). After a period of three years, a similar type of gene was identified in *Serratia marcescens*. This bacterium was isolated from urine sample at Aichi Hospital in Okazaki, Japan (Osano *et al*., 1994).

The Asia pacific country of Japan was found to be primary source for the bla_{IMP} variants of IMP-1, IMP-2, IMP-3, IMP-6, IMP-10 and IMP-11 (Walsh, 2005). These bla_{IMP} genes have been reported in *Pseudomonas* spp., *Acinetobacter* spp. and *Enterobacteriaceae.* Presently, there are over 38 identified bla_{IMP} genes (Lahey, 2017).

In 2002, *bla*$_{IMP}$-1 was identified in clinical isolate of *A. baunmanii* from a female patient in Britain, who had earlier been hospitalized in Spain, while on holiday, furthermore *bla*$_{IMP-1}$ had equally been detected in *A. junii* in Britain (Tysall *et al*., 2002).

Allelic variants of *bla*$_{IMP}$-1, in addition to IMP-2, IMP-4, IMP-5, IMP-6 and IMP-11 have been documented from *A. baumannii* isolates of clinical sources from different countries such as Italy, Japan, Hong Kong, Portugal and Brazil (Queenan & Bush 2007; Walsh *et al*., 2005).

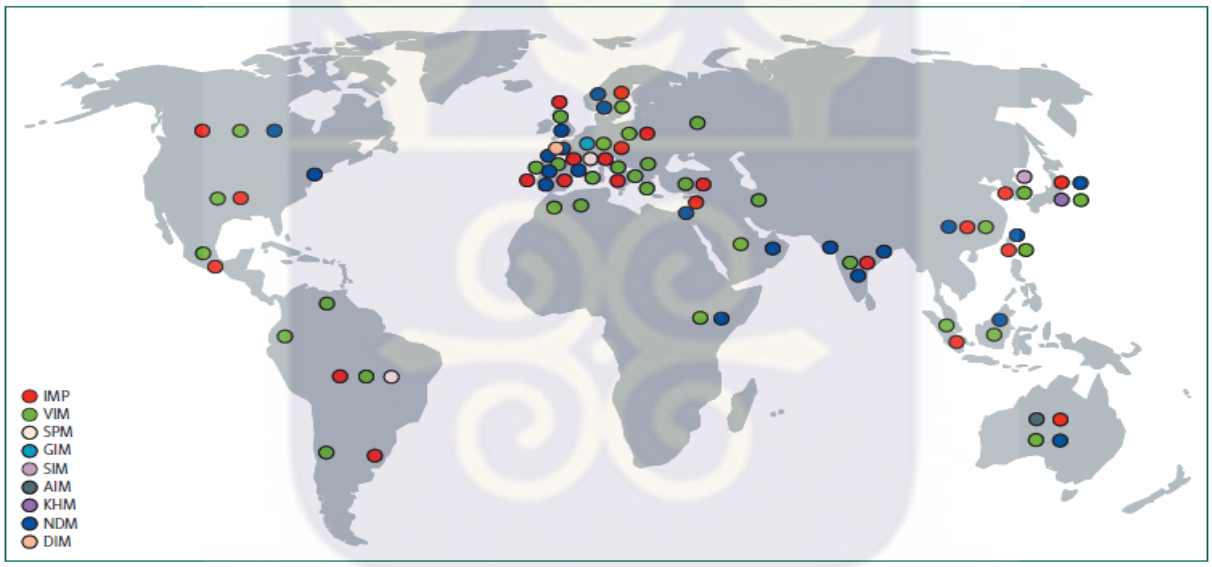

Figure 2.1 Worldwide dispersion of metallo-β-lactamases

Source : Cornaglia *et al*., (2011)

2.9.7.3.2 *Bla*$_{VIM}$-type

Bla$_{VIM}$-type, another major type of MBLs with at least 34 known variants characterized (lahey, 2017). The first *bla*$_{VIM}$-type, Vim-1 was detected in a hospital in Verona, Italy in 1997 from a clinical isolate of *Pseudomonas aeroginosa* (Lauretti *et al*., 1999). This clinical isolate was

found to be non susceptibile to different range of β-lactam antibiotics such as Ceftazidime, Imipenem, astreoman and Piperacillin (Lauretti *et al.,* 1999).

Since this discovery, VIM-1 have been identified in *Escherichia coli* as well as in *Klebsiella pneumoniae* in Greece (Giakkoupi et al., 2003). At present, five VIM-types variants including VIM-1, VIM-2, VIM-3, VIM-4 and VIM-11 have been identified in *A.baunmanii* from Greece, Taiwan and Korea (Cornaglia *et al*., 2011; Lee *et al.*, 2008).

2.9.7.3.3 *Bla*$_{\text{NDM-type}}$

In 2009, the first *bla*$_{\text{NDM}}$-1 was identified in *Klebsiella pneumonia*e of clinical source from a Swedish patient who had earlier been on hospital admission in India (Yong *et al.*, 2009). Regions such as India and Pakistan have been found to be a primary reservoir of NDM-1 gene (Nordmann *et al*., 2011). Many documentation on *bla*$_{\text{NDM}}$-1 producing *Enterobacteriaceae* isolates have been identified in the Balkan states as well as Middle east (Nordmann *et al*.,2011), probable as secondary reservoir.

Since 2010, four *bla*$_{\text{NDM}}$-1 variants have been detected, the first variant was *bla*$_{\text{NDM}}$-2. This variant was recovered from *A. baunmanii* during the microbiological analysis of a specimen from a patient on hospital admission in Egypt, on transferred to Germany (Kaase *et al*. 2011). Since then different variant of *bla*$_{\text{NDM-3}}$ to *bla*$_{\text{NDM-7}}$ have equally been reported (Lahey, 2017).

2.9.7.3.4 *Bla*$_{\text{SIM-type}}$

Bla$_{\text{SIM}}$-1 was first detected in 2005 in Seoul Korea during a study to determine the prevalence of MBLs in *Pseudomonas aeroginosa* and *A. baunmanii* in a university hospital (Lee *et al.*, 2005). This gene identified was found to be chromosomally encoded and carried on class 1 integron, (Lee *et al.*,

2005; Perez *et al.*, 2007). However, *bla*$_{SIM}$-1 has been found to have similarity of 69% and 64% to *bla*$_{IMP}$-2 and *bla*$_{IMP}$-9 respectively (Perez *et al*. 2007). A study by Maspi *et al.*, (2016) in Iran have identified *bla*$_{SIM}$ has one of the MBL gene encoding resistance in *Acinetobacter baunmanii.*

2.9.7.3.5 *Bla*$_{GIM\text{-}type}$

Bla$_{GIM}$-1 was first detected in 2002, in five *Pseudomonas aeroginosa* isolates from five different patients in a medical centre at Dusseldorf, Germany (Castanheira *et al*., 2004). Although, *bla*$_{GIM}$-1 has been found to share 43.1%, 43.1% and 43.5% similarity with *bla*$_{IMP}$-1, *bla*$_{IMP}$-4 and *bla*$_{IMP\text{-}}$6 respectively and similarity of 31.2% with *bla*$_{VIM\text{-}}$7 and 28.8% similarity with *bla*$_{VIM}$-11, *bla*$_{VIM}$-4 and *bla*$_{VIM\text{-}5}$ have been reported (Rasmussen and Bush, 1997). Furthermore, a study by Maspi *et al.*, (2016) in Iran have identified *bla*$_{GIM}$ has one of the MBL gene encoding resistance in *Acinetobacter baunmanii* (Maspi *et al*., 2016).

2.9.8 Prevalence of metallo-β-lactamace producing *Acinetobacter4r4*

Several studies have expressed various concerns on the high rate of antibiotic resistance among *Acinotobacter* spp. in different countries. Between 1998-2004, the rate of resistance in Europe, North America, South America and Asia ranged between 0% - 40% (Perez *et al*. 2007). After 2005, the rate of resistance was found to be greater than 50%. In Iran, it was reported to be 63%, (Peymani *et al*. 2011), whilst, varying prevalence was found in Italy (62.5%), China (55.6%), Turkey (53.7%), Brazil (44%), Thailand (59%), Korea (51.1%) (Principe *et al*. 2009; Jin *et al.* 2009; Baran *et al*. 2008; Cereda *et al*., 2011; Tunyapanita *et al*., 2014; Lee *et al*. 2011).

In the United States, a report by Rhomberg and Jones, (2009) in a 10-years period of surveillance programme (1999-2008) from 15 medical centres revealed a 45.7% susceptibility rate for meropenem against 127 tested *Acinetobacte*r isolates. A regional South America study by Morfin-Otero *et al.* (2013) using the double disc diffusion method with meropenem and EDTA

to screen for MBL producers revealed 48% *Acinetobacter* isolates were resistant to either meropenem or imipenem or both. Of these, 83% (39 of 47) were MBL producers. Although an earlier surveillance study by Morfin-Otero *et al*.,(2012) in two Mexican referral hospital reported that more than 60% of *Acinetobacter* isolates were resistant to all antibiotics tested, a few were found to be imipenem-resistant (36.4%) and meropenem-resistant (37.4%).

In Iran, an earlier study by Feizabadi *et al*., (2008) reported 50.9% *A.baunmannii* resistance to imipenem, whilst, Karthika *et al*., (2009) and Peymani *et al*., (2011) reported MBL screen positive prevalence of 70.9% and 49% respectively. Karthika *et al*., (2009) also found 42% of the MBL strains harbouring bla_{IMP}. A recent study by Noori *et al*., (2014) reported MBL prevalence of 86.8%, of which 6.0% harboured bla_{IMP} gene. Noori *et al*., (2014) also revealed that the highest percentage of MBL producing *A. baumannii* were from respiratory tract (52.8%), urine (26.9%) and blood (7.4%) specimens. Another recent study in Iran reported *A. baunmannii* resistance of 51.2% to imipenem and 75.6% to meropenem of which 2 (2.3%), 13 (15.1%), 2 (2.3%), 4 (4.7%) and 2 (2.3%) isolates harboured bla_{VIM}, bla_{IMP}, bla_{SPM}, bla_{GIM} and bla_{SIM} genes respectively (Maspi *et al*., 2016).

In Pakistan, Irfan *et al*. (2008) and Kaleem *et al*., (2010) reported MBL prevalence of 96% and 84% in *A. baunmannii* respectively, whilst, Sinha *et al*., (2007) reported *Acinetobacter* species carbapenem resistance of 35% prevalence. Safari *et al*., (2013) reported resistance rate of 85%, 94%, 97%, 84%, 98% and 95% against imipenem, meropenem, ciprofloxacin, amikacin, cefotaxime. piperacillin/tazobactam.

In India, a study by Shareek *et al*., (2012) reported that 24.5% *Acinetobacter* isolates were susceptibile to meropenem, imipenem and amikacin, 28% to ceftazidime and ciprofloxacin, 12.2% to cefotaxime and cefuroxime, 22.8% to cotrimoxazole and 21% to

piperacillin\tazobactam. Whilst, Shivappa *et al*., (2013) reported a 70% prevalence of carbapenemase and a 6.7% MBL production among imipenem-resistant *A. baunmanii*. Furthermore, Kaur, (2015) study with Gram negative uropathogen reported carbapenem resistance of 13.89%, of these 35% were MBLs producers. Singla *et al*., (2013) reported 70% *Acinetobacter* spp, resistance to imipenem, of these, 55.7% were MBL producer. A recent study by Pourhajibagher *et al*., (2016) reported a 55% *A. baunmannii* resistance to imipenem, while, Rajput and Naik (2016) reported that 48.57% of *Acinetobacte*r spp were resistance to carbapenem, of these, 48.57% were MBL producers. In Iraq, Anoar *et al*., (2014) study with Gram negative bacterial isolates in burn patients reported that 24.5% of *Acinetobacter* spp were resistance to meropenem, of these, 56.6% were MBL producers with 10.73% and 2.8% harbouring bla_{IMP} and bla_{VIM} respectively. AL-Marjani *et al*., (2013) reported that 52.9% of *A.baunmaii* were. resistant to both meropenem and imipenem, of these, 42.8% were MBL producers and 42.8% encoding bla_{IMP}. In Korea, Lee *et al.*, (2003) have reported MBL prevalence of to be 14.2%, of these, 71.1% and 28.9% harboured bla_{VIM} and bla_{IMP} respectively. Limited studies have been carried on metallo-β-lactamase-producing *Acinetobacter* spp in West Africa, however, there are few report from North and South Africa. In a Moroccan hospital, a study carried out in 2005 reported 38% prevalence of MBL, while a follow up in 2010 in the same institution have reported 74% prevalence of MBL (Kabbaj *et al.*, 2013). In Egypt, Fattouh and Nasr-Eldin (2014) study reported 71.4% resistance to meropenem, imipenem, gentamicin and piperacillin, 60.7% to ceftazidime and amikacin, 57.1% to ciprofloxacin and 85.7% to ampicillin/sulbactam, of these, 86.7% were MBL producers. Similarly, El-din *et al*. (2014) study with foot ulcers reported that 34.61% of *A. baunmanii* were MBL producers, furthermore, 26% were found to be resistance to imipenem or meropenem of these, 23.07% were found to harbour

bla$_{VIM}$ and *bla*$_{IMP}$ with proportion of 15.38% and 7.69%.. In recent study by Uwingabiye *et al*., (2016) in Morrocco reported 86.03% and 76.19% resistance to ceftazidime and imipenem respectively. In South Africa, Ehlers *et al*., (2012) study reported that 45.0% of *A. baumannii* were MBL producers. In Sudan, Omer *et al*., (2015) study at the Royal Care International Hospital (RCIH) reported that 97% of *A. baunmani* was multidrug resistant.

2.9.9 Phenotypic detection of metallo-β-lactamase

Due to the propencity of MBL-producing bacteria to disperse from one region to another and to degrade several β-lactam agents, approprate detection of these resistance phenotypes in routine laboratories is a necessity in order to guide the initiation of restrictive antibiotic regimen and the implementation of proper infection control practices.

Modified Hodge test (MHT) (Lee *et al*., 2001) is recommended by CLSI (2012) for the detection of carbapenemase. However, several phenotypic detection methods have been proposed to detect and identify the presence of metallo-β-lactamase enzymes such as the MBL Etest (AB Biodisk, Solna, Sweden) (Walsh *et al*., 2005; Yong *et al*., 2002), the double-disk synergy tests (DDST) (Lee *et al*., 2001) and the combined disk test (CDT) (Yong *et al*., 2002). Majority of MBL detection tests relies on chelating agents such as EDTA and thiol-based compounds, to restrict MBL activity. But none of these MBL phenotypic methods is standardized. Therefore, the use of routine susceptibility tests to detect carbapenem resistance should be followed by phenotypic and genotypic confirmation. *Acinetobacter* spp. expressing resistance to meropenem or imipenem as recommended by CLSI (2015) (Table 2.3) are futher screened for MBL production.

2.9.9.1 Modified Hodge test (MHT)

Modified Hodge test MHT) is a simple and cheap procedure for the presumptive identification of carbapenemase activity amongst Gram-negative bacilli mostly *Escherichia coli* and *Klebsiella pneumoniae* (Lee *et al*., 2001). The Modified Hodge test cannot distinguish between MBL producing Gram negative bacilli and Non-MBL producing Gram negative bacilli. It has been found to have low sensitivity and low specificity for metalloenzymes (Girlich *et al*, 2012; Cuson *et al*., 2011; Nordamnn *et al*., 2011). The test is a first-line detection method of carbapenemase activity, where *E. coli* ATCC 25922 an indicator organism is swabbed on Mueller-Hinton agar plate (Oxoid, UK). Followed by placing an imipenem disc at the centre of the agar and streaking the carbapenem-resistance isolates from the edge of the imipenem disc to the edge of the agar plate. The growth of the indicator strain toward the impinem disc (coverleaf) is indicative of the presence of carbapenemase activity (Lee *et al*., 2001).

Table 2.3 Carbapenem breakpoint for *Acinetobacter* species

Antimicrobial agent	Disc content	Zone interpretative criteria (mm)			MIC interpretative criteria (µg/ml)		
		S	I	R	S	I	R
Imipenem (IPM)	10µg	≥22	19-21	≤18	≤2	4	≥8
Meropenem (MRP)	10µg	≥18	15-17	≤14	≤2	4	≥8

Clinical laboratory standard institute, (2015)

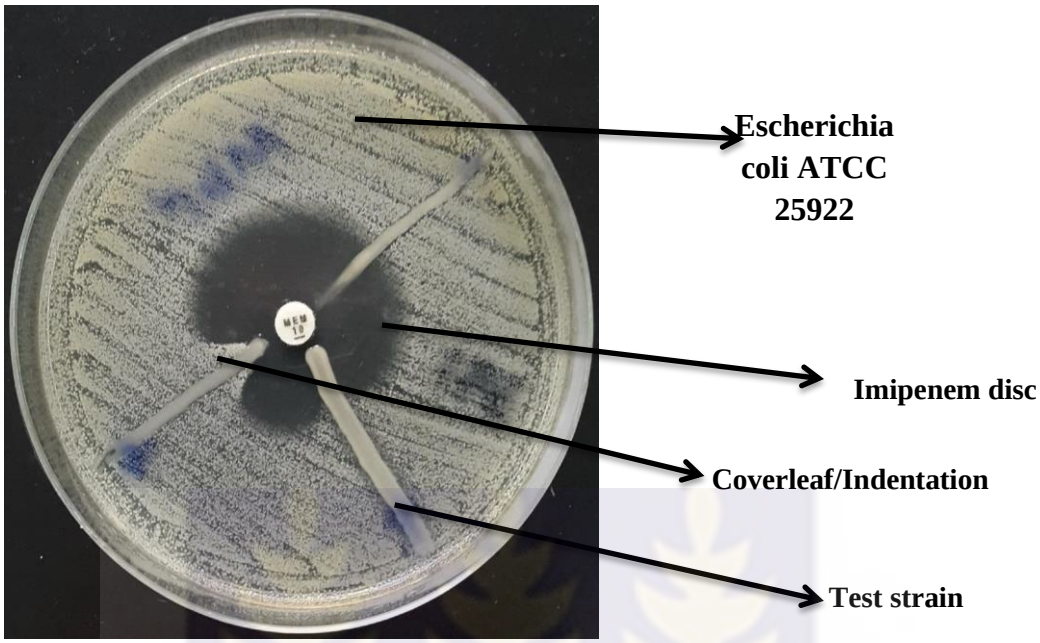

Figure 2.3 MODIFIED HODGE TEST

Source: Anwar *et al*., (2016)

2.9.9.2 Combined Disk Test (CDT)

Combined disk test employs an indicator antibiotics normally a β-lactam of carbapenem (meropenem or imipenem) or ceftazidime, in addition to a chelating agent such as an ethylene diamine tetra acetic acid (EDTA), mercaptopropionic acid (MPA), mercaptoacetic acid (MAC) (Yong *et al*., 2002). The combined disk tests are performed by following the CLSI recommendations for the disk diffusion method. A zone of inhibition $\geq 7mm$ produced between the carbapenem impregnated with EDTA compared to carbapenem alone is indicative of metallo-β-lactamase production (Yong *et al*., 2002). Due to its objective interpretation, this test has been considered a good phenotypic resource (Franklin *et al.*, 2006), it is cheap, easy to perform and could easily be integrated in routine susceptibility testings.

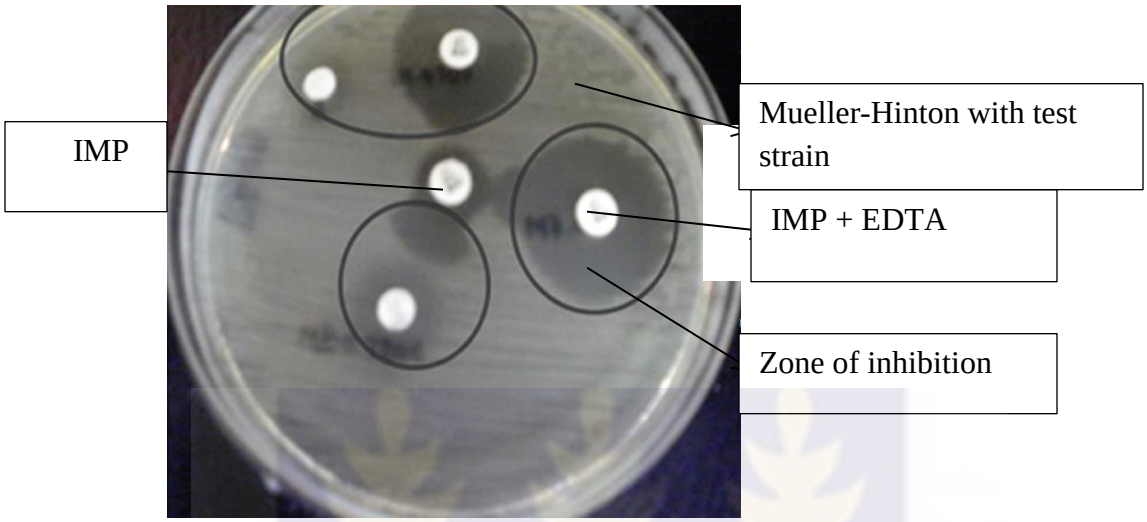

Figure 2.4 IMP-IMP+EDTA (Combined disc test)

Source: Anwar *et al*., (2016)

2.9.9.3 Double disk synergy test (DDST)

Imipenem-EDTA double-disk synergy test (DDST) has been found to be able differentiate metallo-β lactamase producing isolates from non-metallo-β–lactamase Gram-negative bacilli (Lee *et al.*, 2001). However, occasional some isolates demonstrate false negative outcome due to a deficiency or the lack of zinc within the isolate's active site (Yigit *et al.*, 2001). Some investigators demonstrated test can be improved by using an imipenem disc to which 10 µl of 50 mM zinc sulfate (140 µg/disk) has been added, to compensate for the lack of zinc or by using Mueller-Hinton agar to which zinc sulfate has been added to a final concentration of 70 µg.ml-1 (Yigit *et al*., 2001).

2.9.9.3 MBL Etest

Epsilometric (E-test) method (AB Biodisk, Sweden) is used to identify metallo-β-lactamase production by determining the minimum inhibitory concentration (MIC), A reduction in the MIC of imipenem of three or more 2-fold dilutions in the presence of EDTA is usually interpreted as a positive test

Indicative of MBL production. (Lee *et al*., 2005; Walsh *et al*., 2005). These tests are standardized for Enterobacteriaceae, although many investigators have extended its use to other Gram-negative bacilli.

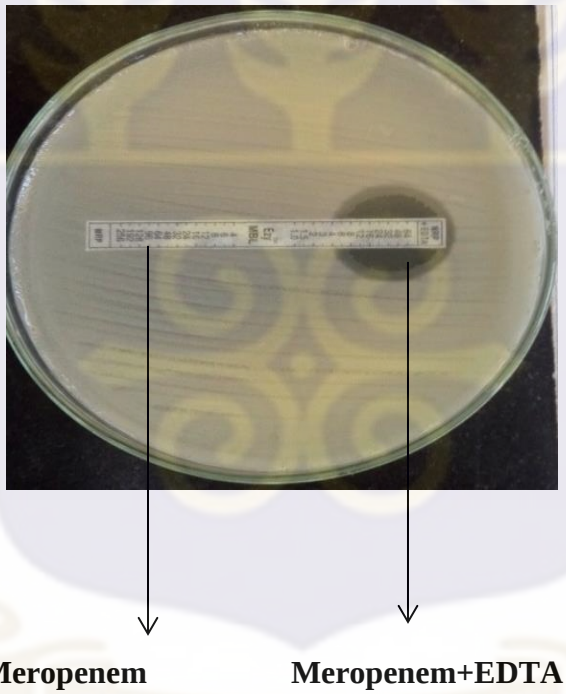

Figure 2.5 MBL E-Test

Source: Rajput & Naik, (2015)

2.9.9.4 Molecular detection of metallo-β-lactamase (Genotypic test)

Beside phenotypic detection of metallo-β-lactamase, molecular method has been described for the detection of metallo-β-lactamase encoding genes (Poirel *et al*, 2011; Ellington *et al*., 2007). The most commonly used method include polymerase chain reaction (PCR). Molecular method remains the gold standard for confirming the presence of carbapenemase encoding genes (Poirel *et al*, 2011). The common genotypic technique has been the PCR amplification with specific oligonucleotide primers of the gene family (Poirel *et al*, 2011). However, this does not distinguish between variants of metallo-β-lactamase type. Nucleotide sequencing remains the gold standard for determining specific variant of metallo-β-lactamase type present in bacterial isolates (Poirel *et al*, 2011). The disadvantage with molecular methods is the expensive nature to setup and that of reagents. Also, a trained technical personnel is required.

CHAPTER THREE

METHODOLOGY

3.0 Study Design

This was a retrospective study of routinely collected clinical isolates of *Acinetobacter* spp. from Korle-Bu Teaching Hospital (KBTH).

3.2 Study Site

Study site was the Korle-Bu Teaching Hospital (KBTH). KBTH is a one of the leading tertiary hospitals in Ghana. It has over 2000-bedded facility with an intensive care units that cater for surgical, medical and trauma emergencies (KBTH, 2016). It provides services to paediatric and adult population of over 3 million in the Greater Accra region as well as serve as a major referral health facility for an estimated population of over 24 million people within Ghana. It attracts medical tourisms from neighbouring West African countries. The bacteriology unit of the Central Laboratory of KBTH processes over 40,000 clinical cultures annually.

3.3 Sample

This study used clinical isolates of the genus *Acinetobacter* spp. recovered from clinical specimens of aspirates, eye swabs, eye swabs, wound swabs and urine submitted to the Central Microbiology Laboratory of KBTH from August 2014 to July 2015 consecutively. The routinely collected isolates were inoculated into brain heart infusion broth (Difco/BD Diagnostic systems, Sparks, Michigan, USA) supplemented with 30% glycerol and stored at - 80 $^{\circ}$C freezer at the Department of Medical Microbiology, University of Ghana Medical School.

3.4 Inclusion Criteria

This study investigated only *Acinetobacter* isolates that were fully identified as causative agents of the infection for which bacteriological examination were performed.

3.5 Exclusion Criteria

Multiple isolates per species from the same patient site that exhibited the same antimicrobial susceptibility pattern were considered to belong to the same organism and excluded from study to avoid duplication.

3.6 Sample Size Determination

Minimum sample size of *Acinetobacter* spp. to be screened for carbapenemase activiy were determined with the formula according to Daniel, (1999).

$$N = \frac{Z^2 (P) (1-P)}{E^2}$$

Z = z-score value for 95% percentile (1.96)

P = sample proportion of 7.2% (0.072), Prevalence of carbapenemase-producing *Acinetobacter baunmanii* by Codjoe. (2016) in Ghana

E = allowable error for the study (0.06)

Minimum sample size, $N = \frac{1.96^2 (0.072) (1-0.072)}{(0.06)^2}$

N = 72 *Acinetobacter* isolates.

87 *Acinetobacter i*solates were screened for carbapenemase activity.

3.7 Subculturing of routinely collected isolates

Work was carried out at the Department of Medical Laboratory Science, Microbiology Unit, School of Biomedical and Allied Health Sciences. Routinely collected clinical isolates of *Acinetobacter* spp. that were identified as causative agents of infections and stored at -80 °C freezer at the department of Medical Microbiology, UGMS, were streaked on nutrient agar (Difco/BD Diagnostic systems, Sparks, Michigan, USA), incubated aerobically at 37°C for 18-24hrs and then subcultured onto MacConkey agar (Difco/BD Diagnostic systems, Sparks, Michigan, USA) and incubated aerobically at 37°C for 18-24hrs.

3.8 Bacteriological Identification of Subcultured Isolates.

After overnight incubation, colony morphology on MacConkey agar was noted. Gram staining was then carried out on colonies that were 1.5-3 mm in size, smooth, convex and pale yellow to greyish-white in appearance. Bacterial isolates identified as Gram-negative cocobacilli were further identified by established bacteriological biochemical procedures that included oxidase, triple sugar iron (TSI), indole, urease, citrate, catalase and motility test (Koneman *et al*., 2006). *Acinetobacter* spp. were identified as been non-lactose fermentative, oxidase-negative, indole negative, urease negative, citrate positive, catalase-positve and non-motlie. Isolates suggestive for *Acinetobacter* spp. were maintained on nutrient agar slants in a 1.5 micro centrifuge tubes (Sigma, United Kingdom) and stored at 5°C until further use.

3.9 Antimicrobial Susceptibility Test for *Acinetobacter* species

Antibiotic susceptibility test were performed on all confirmed *Acinetobacter* isolates by the Kirby-Bauer disc diffusion method as recommended by the Clinical and laboratory and Standards Institute (CLSI, 2015). The procedure involved the preparation of an innoculum of 0.5

Mcfarland turbidity standards by transferring 2 to 3 colonies of an overnight culture of *Acinetobacter* isolate on MacConkey agar (Oxoid, UK) into a sterile saline. A sterile cotton swab was then dipped into the 0.5 Mcfarland standard prepared inoculum and used to inoculate the entire surface of Mueller-Hinton agar plates (Oxoid, UK). Then, using a sterile forcep, the following commercially available antibiotics were placed on the streaked Mueller Hinton agar plate. Amikacin (30µg), Gentamicin (10µg), Ampicillin (10µg), Ciprofloxacin (5µg), Levofloxacin (5µg), Meropenem (10µg), Imipenem (10µg), Amoxicillin/Clavulanate (30µg), Cefuroxime (30µg), Ceftazidime (30µg), Co-trimoxazole (25 µg) and for urinary isolates Nitrofurantoin (300µg) (Oxoid, UK) was included. The Mueller Hinton agar plates were thereafter incubated aerobically at 37ºC for 18-24hrs. The zone diameter of each of the antibiotics were measured with calipers and interpreted as per Clinical Laboratory and Standards Institute (CLSI 2015) recommendations. *Acinetobacter* isolates with reduced susceptibility to meropenem (10µg) antibiotic disk of ≤14mm were then selected for phenotypic detection of MBL production. Control strains of *Pseudomonas aeroginosa* ATCC 27853 and *Escheerhia coli* ATCC 25922 were used to assist in the evaluation of the performance of the test. According to the international standard definition for acquired resistance, and relative to the panel of antibiotics tested, multidrug-resistant (MDR) phenotype was defined as *in vitro* non-susceptibility to at least one agent in three or more categories of antimicrobial (Magiorakos *et al*., 2008)..

3.9.1 Phenotypic Carbapenemase Screening by Modified Hodge Test (MHT)

Modified Hodges Test (MHT) was performed as previously described by (Lee *et al*., (2001). Briefly, a control strain of *Escherichia coli* ATCC 25922 adjusted to 0.5 Mcfarland turbidity standard of innoculum was inoculated onto Mueller-Hinton agar (Oxoid, UK) as recommended

by (CLSI 2015), and the plates were allowed to dry for 5 mins, then one imipenem disk (10ug) (Oxoid, UK) was applied aseptically at the centre of the inoculated Mueller-Hinton agar plate, and a 0.5 Mcfarland standard inoculum of *Acinetobacter* isolates was streaked from the edge of the imipenem disk (10ug) to the edge of the Mueller-Hinton agar plate. After aerobic incubation at 37ºC for 18-24hrs. Mueller-Hinton agar plates was observed for cloverleaf effect or indentation (symbol that is typical of carbpenemase production) at the intersection of the test bacterium and the *E.coli* ATCC 29522 control strain within the inhibition zone of the imipenem disk (10µg) was read (Lee *et al*., (2001).

3.9.2 Phenotypic Detection of Metallo-β-lactamases (Combined disk test)

Combined disk test was performed as previously described by Yong *et al*., (2002). Test organism of carbapenem-resistant *Acinetobacter* with a turbidity of 0.5 Mcfarland standard was inoculated onto Mueller-Hinton agar plate (Oxoid, UK) as recommended by (CLSI, 2015). A 0.5 M EDTA solution was prepared by dissolving 18.61 g of disodium EDTA in 100 mls of distilled water and adjusting its pH to 8.0 by addition of NaOH and then autoclaved as described previously (Yong *et al*, 2002). Two 10µg imipenem discs (Oxoid, UK) was placed on the inoculated surface of the MH agar plate at 20mm apart from center to center and (5µl) of the prepared 0.5 M EDTA solution was added to one imipenem disc (Oxoid, UK) to obtain a desired concentration of 750µg. After 16-18 hrs of aerobic incubation at 37ºC, the inhibition zone displayed around imipenem (Oxoid, UK) and imipenem-EDTA was read and compared. A zone size difference of ≥7mm was taken as indicative of metallo-β-lactamase production as previously described by Yong *et al.*, (2002). This procedure was repeated to ensure reproducibility of result.

3.9.3 Molecular Detection of Metallo-β-lactamase (MBLs) encoding genes.

All isolates that screened positve for carbapenem-EDTA were characterized by PCR to confirm the presence of metallo-β-lactamases encoding gene.

3.9.3.1 DNA Extraction of Metallo-β-lactamase *Acinetobacter* Phenotypes

Bacterial DNA template for PCR assay was extracted by using the whole cell boiled lysate method previously described by Purohit *et al*., (2012). Briefly, four to five colonies of fresh bacterial culture on MacConkey agar was transferred into 500ul of sterile saline in an Eppendorf tube and vortexed briefly followed by heating at 100°C for 10 minutes. The suspension was centrifuged at 8000 rpm for 5 minutes the supernatant was stored at -20°C until required for polymerase chain reaction (PCR).

3.9.3.2 PCR Detection of Metallo-β-lactamase (MBLs) encoding genes

Polymerase chain reaction for MBL encoding genes was carried out using Eppendorf, master cycler epgradient S (BIORAD, USA). A typical 25µl PCR reaction mixture using primer set for the detection of MBL genes (bla_{IMP}, bla_{VIM} and bla_{NDM}) (Table 3.1) with their corresponding PCR conditions was carried out as previously described by Poirel *et al*., (2011).

Table 3.1 Primers to be used for the detection of MBL genes (Poirel *et al*., 2011)

Primer name	Primer Sequences	Genes	Size (bp)	Source
IMP-R	GGAATAGAGTGGCTTAACTCTC	***bla*$_{IMP}$**	**232**	(Poirel *et al*., 2011)
IMP-F	GTTTAACAAAACAACCACC			
VIM-R	TGGTGTTTGGTCGCAAT	***bla*$_{VIM}$**	**390**	(Poirel *et al*., 2011)
VIM-F	CGAATGCGCAGCACCAG			
NDM-R	CGGAATGGCTCATCACGATC	***bla*$_{NDM}$**	**621**	(Poirel *et al*., 2011)
NDM-F	GGTTTGGCGATCTGGTTTTC			

3.9.3.3 PCR Amplification of MBLs encoding genes

Multiplex PCR amplification was performed to detect MBL encoding genes listed above using the following conditions: For *bla*$_{IMP}$, *bla*$_{VIM}$ and *bla*$_{NDM}$ as follows, initial denaturation at 94^{o}C for 5 mins, followed by 33 cycles of denaturation at 94^{o}C for 30 sec, annealing at 53^{o}C for 30 sec, extension at 72^{o}C for 1 min and final extension at 72^{o}C for 7mins. Each reaction mixture consists 2.5 µl of 5 mM $MgCl_2$, 2µl of 25 pmol of each primer (IMP, VIM, NDM) (IDT). 0.125 µl of 1.0U Taq DNA polymerase (Biolab, New England, NEB MO237S), 2µl of 2 mM dNTPs , 2.5µl of 10 x PCR reaction buffer and 6.355 µl of nuclease free water in a total reaction volume of 25µl, including 2µl DNA templates were used. *Klebsiella pneumoniae* NCTC 13443 were used as *bla*$_{NDM}$ positive control, *Pseudomonas aeroginosa* NCTC 13437 as *bla*$_{VIM}$ positive control and nuclease free water as negative control. With the absence of a control strain for *bla*$_{IMP}$, its molecular size of 232 bp was relied upon by Poirel *et al*., (2011)

3.9.3.4 Agarose Gel Electrophoresis

Gel was prepared by using 1.5% agarose (Invitrogen, Carlsbad USA) in a 1x Tris-Acetate-EDTA (TAE) buffer .Prior to solidification of the gel, 2.5 µl of ethidium bromide was added. All PCR products were analysed by electrophoresis in the 1XTAE buffer. Amplicons were viewed with Ultraviolet light (Cleaver Scientific LTD). The length of various amplicons was then determined by comparing them with a 100 bp ladder as previously described by Poirel *et al*., (2011).

3.9.4 Quality Control

Quality control was performed to ascertain the quality of media and potency of antibiotics that was used. Controls strains of *Escherichia coli* ATCC 29522 and *Pseudomonas aeroginosa* ATCC 27853 were used to test for every batch of new antibiotics on Mueller-Hinton agar (Oxoid, UK) and the zone of inhibition interpreted as recommended in CLSI guidelines (CLSI, 2015). Stock cultures of control strains was stored frozen below -20^{o}C with 30% glycerol stabilizer. Working cultures was maintained on nutrient agar slants between 2-8^{o}C and always subcultured before use. Fresh slants were prepared every two weeks.

3.9.5 Statistical Analysis of Data

Data was entered into Microsoft excel (2010) database and analyzed with SPSS version 20.0 (SPSS Inc., Chicago, IL). Frequency tables, histograms and bar charts were used to display numbers, percentages of isolates, antibiotic responses and other variables. Chi square (X^2) was used to compare two proportions. P value ≤ 0.05 was taken as statistically significant.

CHAPTER FOUR

RESULTS

4.1 Demographic Data of *Acinetobacter* species

Between August 2014 to July 2015, a total of 2950 Gram-negative bacilli were isolated from about 16000 clinical samples processed at the Bacteriology Unit of the Central Laboratory of Korle-Bu Teaching Hospital. *Acinetobacter* isolates, comprised 3% (87) of the total number of Gram-negative bacilli isolated, and the majority of *Acinetobacter* isolates were from patient who were > 50 years of age. (Table 4.1). *Acinetobacter* spp. were obtained from the culture of aspirate, urine, ear, eye and wound swabs (Figure 4.1).

Table 4.1 Age group and Sex distribution of patients with *Acinetobacter* species

	Age categories/ Years							
SEX	< 1	1-10	11-20	21-30	31-40	41-50	> 50	TOTAL (%)
FEMALE	11	0	6	13	8	3	13	54 (62.1)
MALE	5	7	0	4	1	4	12	33 (37.9)
No.ofpatients(%)	16(18.4)	7 (8.1)	6 (6.9)	17 (19.5)	8 (10.3)	7 (8.1)	25(28.7)	87 (100)

4.2 Distribution of *Acinetobacter* spp. amongst clinical specimens

From a total of 87 *Acinetobacter* isolates, 45 (51.7%) were from wound swabs, 25 (28.7%) from urine. However, 3 (3.5%) were from aspirates.

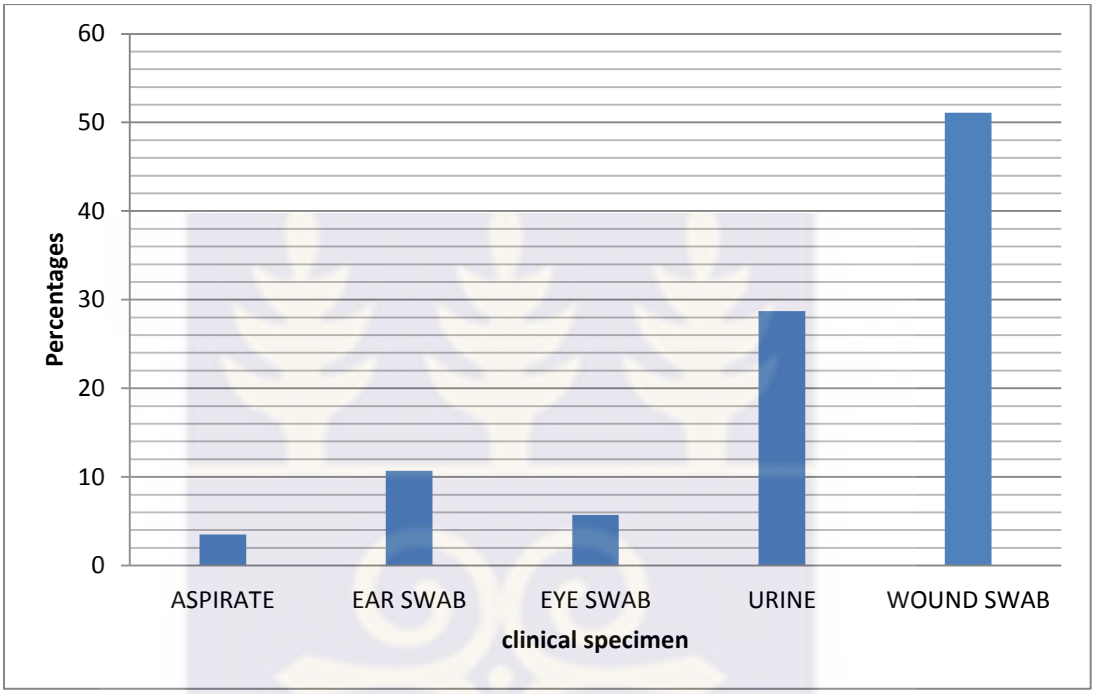

Figure 4.1 Distribution of *Acinetobacter* spp. amongst clinical specimens

4.3 Antimicrobial susceptibility pattern of *Acinotobacter* spp.

From total of 87 *Acinetobacter* isolates, 54 (62.1%) were multidrug-resistance and a high level of resistance were observed for ceftadizime (75.9 %), ciprofloxacin (64.4%), cefotaxime (90.8%), co-trimoxazole (70.1%) and meropenem (59.8%). However, low resistance was observed for amikacin (25.3 %) (Table 4.2).

Table 4.2 Antibiotic susceptibility pattern of *Acinetobacter* spp.

Antibiotics	No. % Resistant
Ampicillin	82 (94.3)
Amoxicillin-clavulanate	79 (90.8)
Amikacin	22 (25.3)
Cefuroxime	75 (86.2)
Cefotaxime	79 (90.8)
Ceftadixime	66 (75.9)
Co-trimoxazole	61 (70.1)
Ciprofloxacin	56 (64.4)
Gentamicin	63 (72.4)
Meropenem	52 (59.8)
Nitrofuratoin	23 (92.0)
Levofloxacin	59 (67.8)

4.4 Meropenem-resistant *Acinetobacter* spp. in relation with clinical specimens

From a total of 45 *Acinetobacter* isolates recovered from wound swabs, 30 (66.7%) were resistant to meropenem and 23 (92%) from urine were resistant to meropenem. However, none of the *Acinetobacter* isolates recovered from eye swabs was resistant to meropenem (Figure 4.2).

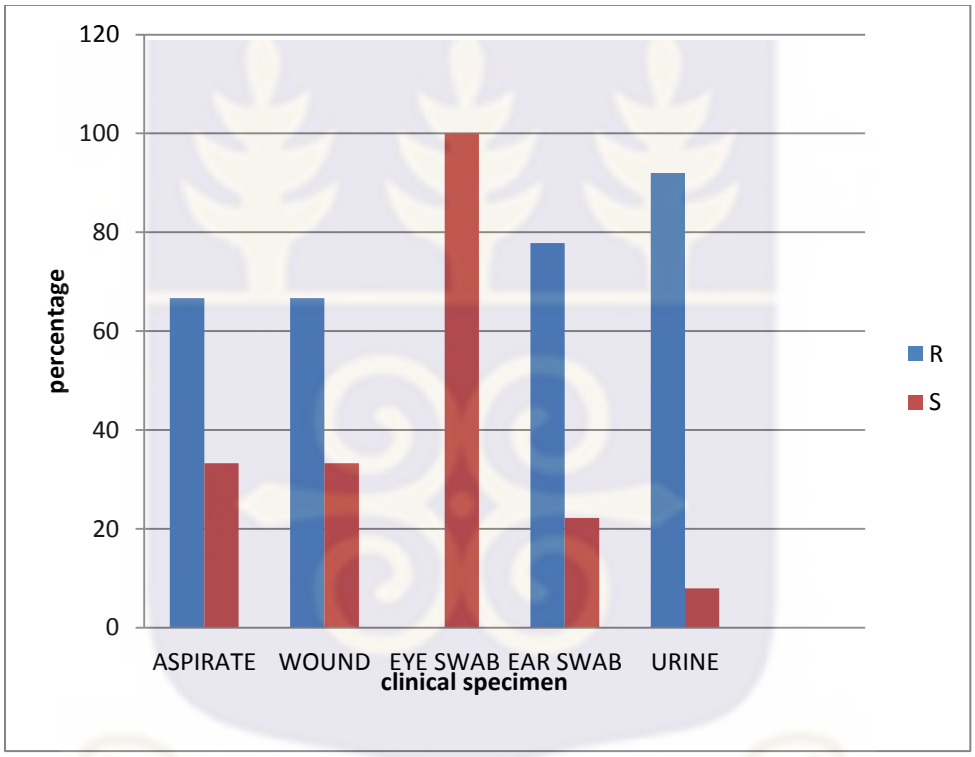

Figure 4.2 Meropenem-resistant *Acinetobacter* species in relation with clinical specimens

4.5 Meropenem resistance among age groups

Figure 4.3 shows the prevalence of meropenem-*Acinetobacter* isolates resistance among age categories.

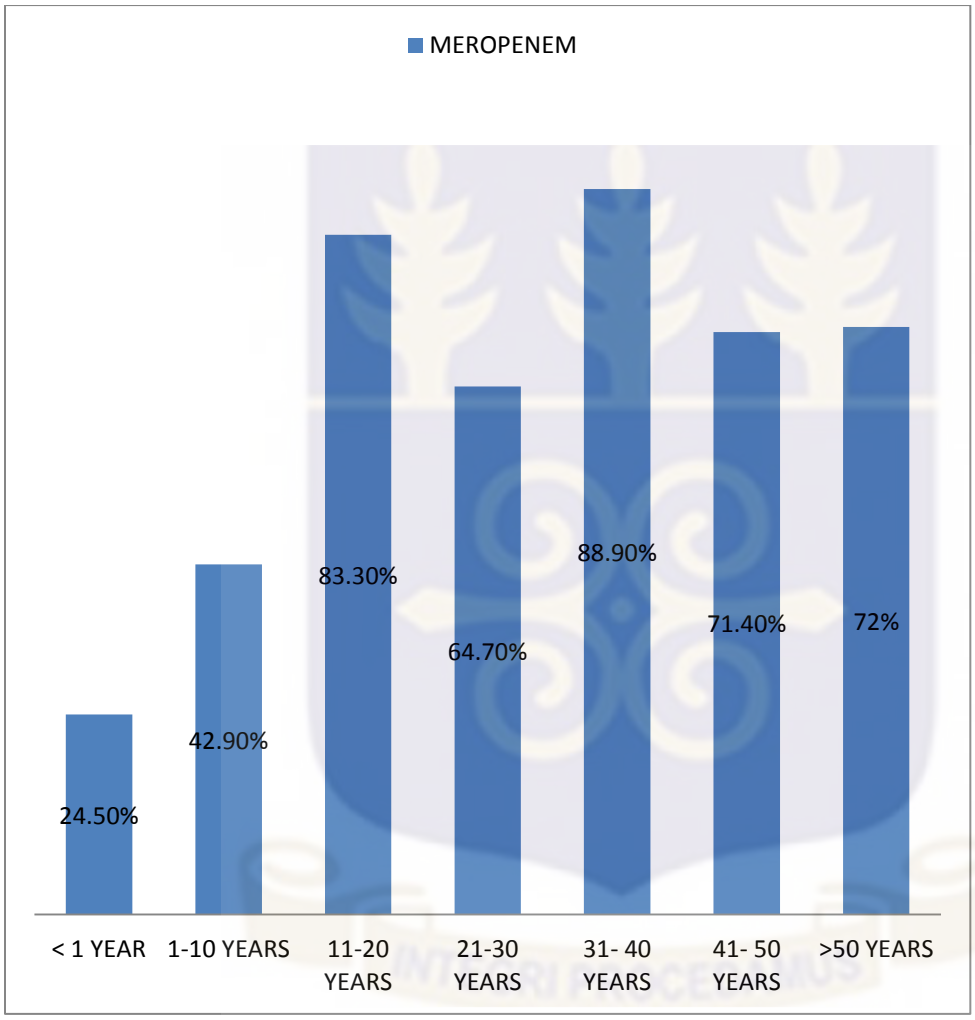

Figure 4.3 Prevalence of meropenem-*Acinetobacter* resistances among age categories.

4.6 Prevalence of carbapenem-EDTA test positve *Acinetobacter* spp.

Among the 52(59.8%) meropenem-resistant *Acinetobacter* isolates, carbapenemase activity was 5.8%, (3 of 52) were Modified Hodge test (MHT) positive isolates and 44.2%, (23 of 52) were carbapenem-EDTA (CDT) positive isolates. Carbapenem-EDTA test positive isolates were pedominant among patients, who were > 50years (39.12%), followed by 31-40 years (21.7%) and < 1years (13.0%) (Table 4.3). Isolates with carbapenem-EDTA test positive predominants female patients (62.2%). Between carbapenem-EDTA test positive and carbapenem-EDTA test negative isolates, gender distribution was not significant ($p > 0.05$). Carbapenem-EDTA positive test were mostly obtained from *Acinetobacter* isolates cultured from urine, wound and ear swabs. Among wound and urine isolates, the difference between carbapenem-EDTA test positive and carbapenem-EDTA test negative isolates were statistically significant ($p<0.05$).

Table 4.3 Distribution of carbapenem-EDTA test positive and carbapenem-EDTA test negative isolates, among sex, age and specimen

Variable	Carbapenem-EDTA positive test (n=23) No. (%)	Carbapenem-EDTA negative Test (n=29) No. (%)	P-value
Sex			
Female	15 (65.2)	15 (51.72)	0.4026
Male	8 (34.8)	14 (48.3)	0.4026
Specimen			
Aspirate	-	3(10.34)	0.2455
Eye swab	- -	- -	-
Ear swab	3 (13.04)	1 (3.44)	0.3101
Wound swab	6 (26.1)	16 (55.2)	0.0493*
Urine	14 (60.9)	9 (31.0)	0.0491*
Age group (years)			
< 1	3 (13.04)	3 (10.34)	1
1-10	2 (8.7)	4 (13.8)	0.6821
11-20	1 (4.4)	-	0.4423
21-30	3 (13.0)	7 (24.14)	0.4815
31-40	5 (21.7)	3 (10.34)	0.4411
41-50	-	2 (6.9)	0.497
> 50	9 (39.1)	10 (34.5)	0.778

4.7 Resistance patterns of carbapenem-EDTA test positive and carbapemen-EDTA test negative. The resistance pattern of carbapenem-EDTA test positive and carbapemen-EDTA test negative isolates is shown in (Table 4.4). Relatively high levels of antibiotic resistance were observed in both MBL screening test positive and MBL screening test negative *Acinetobacter* isolates ($p>0.05$).

Table 4.4 Resistance pattern of carbapenem-EDTA test positive and carbapemen-EDTA test negative.

Antibiotics	Carbapenem-EDTA test positive (23) (%)	Carbapenem-EDTA test negative (29) (%)	P-value
AMP	23 (100)	27 (93.1)	0.497
AMC	22 (95.7)	27 (93.1)	1.00
AMK	8 (34.8)	8 (27.6)	0.763
CTX	23 (100)	27 (93.1)	0.497
CXM	23 (100)	27 (93.1)	0.497
CAZ	23 (100)	29 (100)	1.00
COT	17 (73.9)	21 (72.4)	1.00
CIP	20 (87.0)	23 (79.3)	0.714
GEN	21 (95.7)	27 (93.1)	0.159
MER	23 (100)	29 (100)	1.00
NIT	14 (100)	7 (77.8)	0.261
LEV	22 (95.7)	24 (82.7)	0.210

4.8 MBL encoding gene among MBL-producers by PCR.

Figure 4.5 shows MBL encoding genes obtained by PCR screening. Among the 23 carbapenem-EDTA screen positive *Acinetobacter* isolates, bla_{NDM} was obtained in (8.1%) 7 of 87 isolates. No bla_{VIM} nor bla_{IMP} was detected in any of the MBL screen positive *Acinetobac*ter isolates.

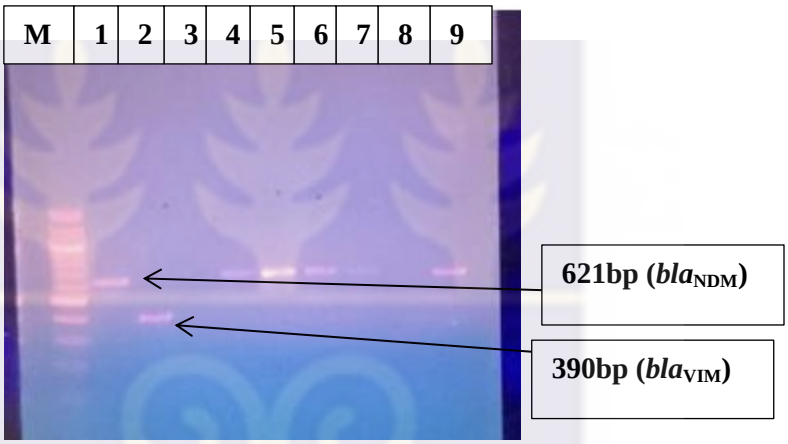

Figure 4.5 Ethidium bromide stained agarose gel of bla_{NDM} encoding *Acinetobacter* isolates identified by Multiple PCR. Lane 1: 100bp Molecular ladder, Lane 2: *Klebsiella pneumoniae* NCTC 13443 bla_{NDM} positive control, Lane 3: *Pseudomonas aeroginosa* NCTC 13437 bla_{VIM} positive control, Lane 3&8; negative control, Lane 4, 5, 6, 7 & 9 were positive for bla_{NDM} (621 bp).

4. 9 MBL encoding genes (*bla*$_{NDM}$) among specimen, gender and age group.

Table 4.6 shows the distribution of MBL encoding genes in relation to the phenotypic assays, *bla*$_{NDM}$ were obtained relatively more in female, wound swabs and among children less than 1 year old.

Table 4.5 Prevalence of MBL encoding gene (*bla*$_{NDM}$) among specimen, gender and age group.

	PHENOTYPIC TEST	GENOTYPIC TEST
	CDT	PCR
Variable	Carbapenem+EDTA	*bla*NDM
	No. (%)	No. (%)
Sex		
Female	15 (65.2)	4 (57.1)
Male	8 (34.8)	3 (42.9)
Specimen		_
Aspirate	-	_
Eye swab	-	_
Ear swab	3 (13.04)	1 (14.3)
Wound swab	6 (26.1)	4 (57.1)
Urine	14 (60.9)	2 (28.6)
Age group	(years)	
< 1	3 (13.04)	3 (42.9)
1-10	2 (8.7)	_
11-20	1 (4.4)	_
21-30	3 (13.0)	1 (14.3)
31-40	5 (21.7)	1 (14.3)
41-50	-	_
> 50	9 (39.1)	2 (28.6)

CHAPTER FIVE

DISCUSSION

This present study found a low prevalance of *Acinetobacter* isolates from routinely collected clinical specimens. This is in consistent with a study in Libya which reported 3.5-4.2% prevalence of *Acinetobacter* spp. (Ziglam *et al*., 2012). In contrast to this study, higher prevalance of *Acinetobacter baunmanii* 9.4% (155 of 1632) Gram-negative bacilli was reported at a tertiary hospital in India (Jaggi *et al*., 2012). Majority of *Acinotobacter* isolates were recovered from wound specimens. This is consistent with studies in Libya and Nigeria which reported 47.8% and 20.8% respectively (Mathlouthi *et al*., 2015; We *et al*., 2014). This may be due to the frequent isolation of *Acinetobacte*r spp. from wound cultures (Eveillard *et al*., 2013). In the current study, maximum *Acinetobacter* isolates were found in females patients. Similar studies in Benin and Brazil have reported *Acinetobacter* prevalence of 59.3% and 55.9% amongst females patients (Ahoyo *et al*, 2014; Hinrichen *et al*., 2014). Furthermore, majority of *Acinetobacter* isolates were recovered in patients who were >50 year old. This is similar to a study in Nigeria which reported a 36.0% prevalence amongst age group 51-60 years old (Odewale *et al*., 2015). In the United States, age group >55 years old have been identified as a risk factor for the colonization and infection with *Acinetobacter baunmanii* (Dent *et al*., 2010). The elderly may have suppressed immune system and may be susceptible to frequent nosocomial infections.

5.1 Antimicrobial susceptibility pattern of *Acinetobacter* spp

A high level of resistance to different classes of antibiotics were found among *Acinetobacter* isolates in the current study. Fifty four (62.1%) of these isolates were considered to be

multidrug-resistance (MDR), as defined by resistant to at least three or more antimicrobial agents (Magiokaros *et al*., 2008). This is consistent with report of multidrug–resistant prevalence in Italy (54%) and United States (72%) respectively (De Francesco *et al*., 2013; Dent *et al*., 2010). In contrast to our findings, higher prevalence were reported in Nepal (95.5%) and Sudan (97.0%) (Omer *et al*., 2015 & Rynga *et al*., 2015). The different prevalence in the various countries may be associated with varying dependency on antibiotics usage in the different countries (Bonnin *et al*, 2013).

Amongst third-generation cephalosporin in the present study, 75.9% of the isolates were resistant to ceftadizime. This is consistent with studies in China, India and Nepal which reported resistance levels of 83.98%, 84.0% and 80.0% respectively (Ren *et al*., 2016; Mohanty *et al*., 2013; Mishra *et al*., 2013). In contrast to the present study, higher level of resistance to ceftazidime have been reported in Benin (100%), Egypt (89.0%) and Sudan (96%) (Ahoyo *et al*., 2014; El-Den *et al*., 2014; Omer *et al*., 2015). Although *Acinetobacter* resistance to ceftazidime was 75.9% in this study, it was higher than the 58.8% reported in a study in Cameroun (Ebongue *et al*., 2016). In this study, *Acinetobacter* resistance to cefotaxime was 90.8%. This is comparable to the data reported in some studies in Pakistan (99.2%) and India (100%) (Sohail *et al*., 2016; Rynga *et al*., 2016). Generally, cephalosporins are broad spectrum β-lactams with high antibacterial activity and low toxicity. The high level of resistance observed amongst *Acinetobacter* spp. may be due to a high level of extended-spectrum-β-lactamase (ESBLs) induced by selective pressure from broad spectrum antimicrobial therapy (We *et al*., 2014).

In the present study, 64.4% of the isolates were resistant to ciprofloxacin. This is consistent with studies in India (64.0%) and South Africa (65%) (Mohanty *et al*., 2013; Kock *et al*., 2013). In contrast to our findings, higher prevalence of ciprofloxacin been reported in Brazil (80.0%) and

Egypt (88.8%) (Cereda *et al*., 2011; El-Den *et al.*, 2014). However, our resistance levels were higher than studies reported in Benin (16%), Cote d'Ivoire (35.2%) and Nigeria (40.3%) (Ahoyo *et al.*, 2014; Meiti *et al*., 2015; We *et al*., 2014). In the current study, 67.8% of *Acinetobacter* isolates were resistance to levofloxacin. In contrast to our finding, higher prevalence was reported in Mexico (78.1%) and China (82.5%) (Morfin-Otero *et al*., 2012; Ren *et al*., 2016). However, 50% resistance prevalence of levofloxacin to *Acinetobacter* isolates were reported in Cameroun (Ebongue *et al*., 2016). Opintan *et al*., (2015) in their antimicrobial resistance surveillance study reported a resistance prevalence of >50% to third generation cephalosporins and flouroquinolones throughout Ghana. In Ghana, the standard treatment guideline recommends the use of ciprofloxacin for the treatment of urinary tract and blood stream infections (MOH, 2010). The high level of resistance observed for flouroquinolones in the present study is quite worrisome and may be due to inappropriate and irrational use of these antibiotics (Okeke and Ojo, 2010).

A*cinetobacer* isolates were 70.1% resistant to co-trimoxazole. In contrast to finding in the present study, some studies in Nepal and India have reported a slightly higher resistance of 75.8% and 89% respectively (Mishra *et al*., 2013; Sanjeev *et al*., 2013). 72.4% of the isolates were resistant to gentamicin in the current study. This is comparable with studies in Benin (75%) and Kenya (68.8%) (Ahoyo *et al.*, 2014; Revathi *et al*., 2013). In contrast to this study, lower prevalence was reported in Cameroun (52.63%) and South Africa (58%) (Ebongue *et al*., 2016; Kock *et al*., 2013). The differences in prevalence may be due to antibiotic policy on the usage of co-trimoxazole as an empirical treatment.

In the current study, 25% of the isolates were resistant to amikacin.. This is similar with studies in Cameroun (24.3%) and China (25.24%) (Ebongue *et al*., 2016; Ren *et al*., 2016). However,

higher resistance prevalence to amikacin have been reported in Nigeria (43.1%) and Kenya (37.5%) (We *et al*., 2014; Revathi *et al*., 2013). Amikacin is often used as a combination therapy and the low usage of this antibiotic, since, it is an injectable may be the reason for the low resistance observed in this study (Somashekara *et al*., 2014).

Carbapenems have become the last selection for the treatment of *Acinetobacter* and other Gram-negative infections due to their wider spectrum of antibacterial activity and minimal side effect (Fonseca *et al.*, 2013). However, carbapenem-resistance to *Acinotobacter* spp. have been reported worldwide (Mendes *et al*., 2010; Peleg *et al*, 2008). The prevalence rate of carbapenem resistance in *Acinetobacter baunmannii* have been found to vary from one country to another (Bonnin *et al*, 2013).

In the present study, 59.8% (52 of 87) *Acinetobacter* isolates were resistant to carbapenem (meropenem). This is comparable with studies in Ghana (66%), Pakistan (58.9%) and Nigeria (63.6%) (Codjoe, 2016; Anwar *et al*., 2016; Odewale *et al.*, 2015). Whilst, studies by Hussein *et al*, (2013) and Ren *et al*., (2016) have reported carbapenem (imipenem) resistance of 58.26% in Iraq and 66% in China. Morfin-Otero *et al*., (2013) and Rajput & Naik, (2016) in Mexico and India have reported a lower carbaepenem (imipenem) prevalence of 48% and 48.57%. Furthermore, Fattouh & Nasr-Eldin, (2014) in Egypt have reported a higher carbapenem resistance of 71.4% to *Acinetobacter* spp. In a global study carried out between 2005 and 2009 from 146 hospitals in 32 countries, an overall imipenem and meropenem resistance of 45.7% and 48.5% was observered respectively (Mendes *et al*, 2010). The high resistance to meropenem may be due to the intrinsic ability of *Acinetobacter* to quickly utilize the efflux pumping mechanism or the capacity to acquire resistant determinants from the environment in response to selective pressure (Bonomo & Szabo, 2006; Coyne *et al*., 2011).

5.2 CDT screening for MBL expresssing *Acinetobacter* spp.

Among 52 meropenem resistant *Acinetobacter*, less than 6% (3 of 52) were positive for and presumably carbapenemase producers by the Modified Hodge test (MHT); Low sensitivity and specificity of MHT method in the detection of MBL- producing isolates have been reported by several studies (Doyle *et al*., 2012; Girlich *et al*., 2012; Nordmann *et al.*, 2011). Modified Hodge Test is a first line detection method for carbapenemase activity in carbapenem-resistant *Enterobacteriaceae* (CLSI, 2012). In the CLSI (2012) guidelines, there is no specific phenotypic method recommended for the screening and confirmation of *Acinetobacter* spp. Different investigators, therefore, employ various detection methods like imipenem-EDTA combined disc test, double disc synergy test and MBL E-Test (Fattouh and El-din, 2014; Lee *et al*., 2003; Yong *et al*, 2002).

The present study used the carbapenem-EDTA combined disc test (CDT) for MBL detection, This method is specific, sensitive and easy to perform in different range of laboratories (Yong *et al*, 2002). The phenotypic prevalence of CDT positive *Acinetobacter* isolates were 44.2% by the carbapenem-EDTA combined disc test (CDT). This is similar with studies in South Africa, Iran and India which reported CDT screen positive prevalence of 45%, 49% and 44.8% respectively (Ehler *et al*, 2012; Irfan *et al*, 2008; Moghadam *et al*, 2016). In contrast to findings in the current study, lower CDT prevalence of 25% and 34.3% were reported in some studies in India and Egypt (Gupta *et al*, 2016; El-Den *et al*, 2014). However, studies in India and Iran have reported higher CDT prevalence of 80.3% and 86.8% (Kauer *et al*, 2014; Noori *et al*, 2014). The varying prevalence of CDTs may be due to the different phenotypic detection methods employed by the various investigators in the different countries (Fattouh and El-din, 2014; Lee *et al*., 2003; Yong *et al*, 2002).

In the present study, MBL screen positive *Acinetobater* isolates showed high level of resistance to meropenem, cephalosporins, fluoroquinolones and gentamicin class of antibiotics. Similar findings have been reported in studies elsewhere (Al-Agamy *et al*, 2014; Reem & EI-Glil 2015). The high level of resistance may be due to betalactamase and efflux pump activities against routinely used antibiotics in our healthcare institution (Nordamnn *et al*., 2011).

In the present study, MBL screen positive *Acinetobacter* were predominant in urine specimen. Similar study in Nigeria have reported 30.4% carbapenemase in urine samples (Oduyebo *et al*, 2015). This may be due to carbapenemase-producing isolates association with fluid for growth and dissemination of resistance determinants in the environment. MBL screen positive *Acinetobacter* isolates were found in older age groups. In Pakistan, Anwar *et al*., (2016) found most neonates (25.8%) and infants (24.2%) harbouring MBL-producing *Acinetobacter baunmanii*. This may be due to the elderly and neonates frequent exposure to antimicrobial agents. MBL screen positive *Acinetobacter* isolates were detected in majority of female gender.

5.3 Prevalence of Metallo-beta-lactamase encoding genes.

Metallo-beta-lactamase producing *Acinetobacter* infections have become a public health concern due to few therapeutic choice for the treatment of such infections (Corniglia *et al*., 2011; Kempf *et al*., 2013). MBL-producing *Acinetobacter* possess intrinsic potential to acquire and maintain resistance genotypes to different classes of antibiotics (beta-lactams and non-beta-lactams) (Corniglia *et al*., 2013). Molecular based techniques are the gold standard for the identification and differentiation of carbapenemase genes (Poirel *et al.*, 2011; Takayama *et al.*, 2015). In the present study, multiplex polymerase chain reaction (PCR) was used to assay the presence of some MBL encoding genes (bla_{VIM}, bla_{IMP} and bla_{NDM}) (Poirel *et al*., 2011). Among the 23

(44.2%) CDT positive *Acinetobacter* isolates, 7 (8.1%) haboured MBL encoding gene (bla_{NDM}). However, no bla_{IMP} and bla_{VIM} was detected. This is comparable to a previous study in Ghana that reported 9 out of 31 carbapenem-resistant *Acinetobacter baunmannii* harbouring bla_{NDM} (Codjoe, 2016). Similar studies in Egypt, Kenya and China have equally reported the occurrence of bla_{NDM} in MBL-producing *Acinetobacter* (Chen *et al*., 2011; Reem and EI-Glil, 2015; Revathi *et al*., 2013). The non-detection of bla_{NDM} in 80 (91.9%) of the non-MBL producing *Acinetobacter* isolates in the present study, may be due to the presence of other resistance genes such as bla_{GIM} or bla_{SIM} (Queenam and Bush, 2007; Poirel *et al*., 2011), of which molecular analysis was not carried out. The MBL encoding gene (bla_{NDM}) was detected mostly from wound swabs and urine This is consistent with an earlier study in Ghana (Codjoe, 2016). The occurrence of bla_{NDM} gene may be suggestive that bla_{NDM} gene is one of the common carbapenemase gene circulation among *Acinetobacter* spp. and other related Gram negative bacilli in Ghanaian hospitals. Metallo-betalactamase encoding gene (bla_{NDM}) was first identified in a Swedish patient, who was hospitalized in India (Yong *et al*., 2009). The Indian and Pakistan regions have been found to be the primary reserviour for bla_{NDM} genes, since this discovery, bla_{NDM} genes have disseminated to over 40 countries including Kenya, South Africa, Morrocco, Algeria, Iraq, Kuwait, Oman, Israel, United Kingdom and United States (Johnson and Woodford, 2013: Nordamnn *et al*., 2011). Previously, dissemination of NDM genes was initially attributed to medical tourisms to the India subcontinent (Kumarasamy *et al*., 2011). However, recent finding have associated the presence of the bla_{NDM} gene to local spread in the environment (Yang *et al*, 2012; Rimrang *et al*., 2012). Metallo-beta-lactamase encoding bla_{NDM} gene presently is a public health menace and infections caused by bacteria carrying these genes are difficult to treat. Furthermore, they have a high propensity for horizontal transfer to neighboring Gram-negative

bacilli, hence, the presence of *bla*$_{NDM}$ gene in Ghanaian hospital is worrisome and calls for prompt detection, surveillance and strict infection control measures.

5.4 Conclusion

In this study, PCR analysis for *bla*$_{VIM}$, *bla*$_{IMP}$ and *bla*$_{NDM}$ showed that less than 9 % of 87 *Acinetobacter* spp. harboured NDM encoding genes. Also, high levels of resistance to multiple antibiotics were found amongst MBL-producing *Acinetobacter* isolates. The detection of *bla*$_{NDM}$ amongst MBL-producing-*Acinetobacter* is a cause for concern, therefore, strict antibiotics usage and infection control measures should be instituted to prevent the spread of these resistance genes.

Limitations

(I) The use of MHT for carbapenemase expression in *Acinetobacter* spp. is yet to be recommended.

(II) The in-house preparation of carbapenem-EDTA test severally limits its utilisation and reliability.

(III) The screening for the entire panel of MBL encoding genes may have revealed the occurrence of other resistant genes among MBL screen positive *Acinetobacter* isolates

5.6 Recommendation

There should be routine screening of MBL-producing *Acinetobacter* and related Gram-negative bacilli isolates to aid in judicious prescription of empirical therapy. Regular surveillance in tertiary care in Ghanaian hospital is required to know the MBL encoding genes circulation. The institution of strict infection control measures required to minimize the dissemination of resistance genes among Gram-negative bacilli.

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APPENDIX A

Demographic data, Meropenem susceptibility pattern, MHT and CDT.

S/N	PATH/N	SPM	SEX	AGE	MER	MHT	CDT	MBL
1	1A	ASP	F	3Days	R	-	-	NM
2	2A	ASP	M	27YRS	R	-	-	NM
3	3A	W/S	F	9Months	R	+	+	MP
4	4A	U	F	19YRS	R	-	+	MP
5	5A	U	F	33YRS	R	-	+	MP
6	6A	U	M	9YRRS	R	-	+	MP
7	7A	W/S	F	25YRS	R	-	-	NM
8	8A	W/S	M	70YRS	R		-	NM
9	9A	W/S	M	64YRS	R	-	-	NM
10	10A	W/S	F	51YRS	R	-	+	MP
11	11A	W/S	M	75YRS	R	-	+	MP
12	12A	U	F	27YRS	R	-	+	MP
13	13A	W/S	F	37YRS	R	-	-	NM
14	14A	W/S	M	43YRS	S			
15	15A	W/S	F	29YRS	S			
16	16A	W/S	F	28YRS	S			
17	17A	EYE/S	M	8Days	S			
18	18A	U	F	38YRS	R	-	+	MP
19	19A	W/S	F	21YRS	S			
20	20A	U	M	64YRS	R	-	+	MP
21	21A	U	M	65YRS	R			NM
22	22A	EYE/S	M	4Days	S			
23	23A	W/S	M	4DAYS	R			NM
24	24A	U	F	36YRS	R			NM
25	25A	U	F	26YRS	S			
26	26A	W/S	F	25YRS	S			
27	27A	U	F	23YRS	R	+	+	MP
28	28A	W/S	M	44YRS	S			

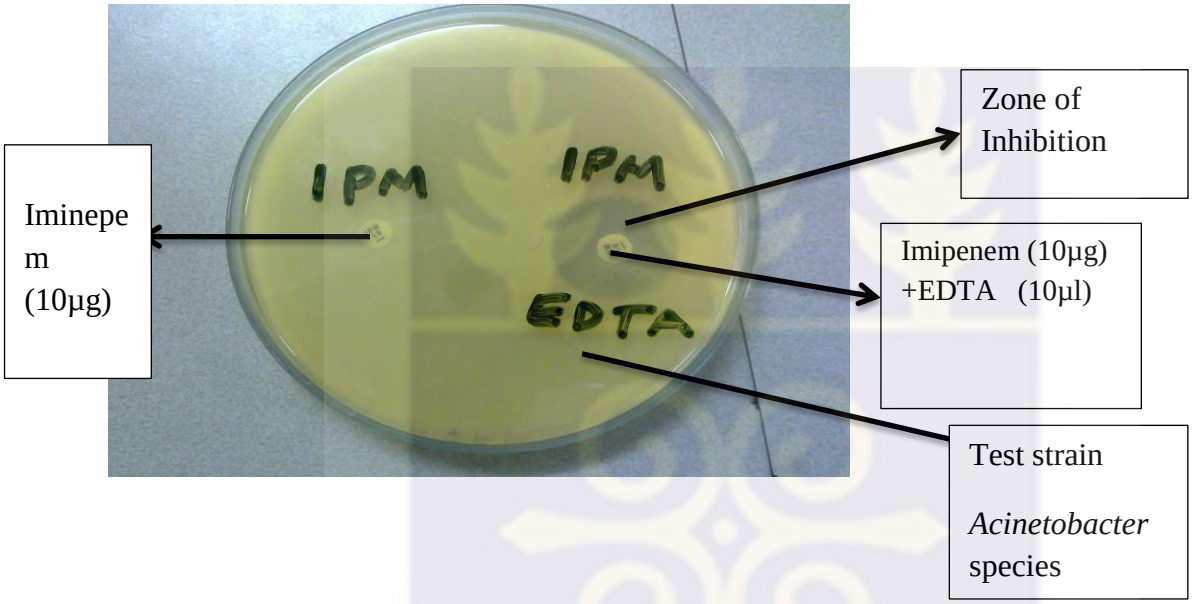
S/N	PATH/N	SPM	SEX	AGE	MER	MHT	CDT	MBL
29	29A	U	F	32YRS	R	-	+	MP
30	30A	W/S	F	55YRS	S			
31	31A	W/S	F	62YRS	R	-	-	NM
32	32A	EAR/S	F	28YRS	S			
33	33A	EAR/S	F	56YRS	R	-	+	MP
34	34A	W/S	F	18YRS	S			
35	35A	W/S	M	2YRS	S			
36	36A	U	F	83YRS	R	-	+	MP
37	37A	W/S	F	40YRS	R	-	-	NM
38	38A	W/S	M	31YRS	R	+	+	MP
39	39A	U	F	9Months	R			MP
40	40A	EYE/S	M	4Days	S			
41	41A	W/S	F	78YRS	S			
42	42A	U	M	56YRS	R	-	+	MP
43	43A	U	F	13YRS	S			
44	44A	W/S	F	53YRS	S			
45	45A	U	F	41YRS	R	-	-	NM
46	46A	U	F	56YRS	R	-	-	NM
47	47A	U	F	72YRS	R	-	-	NM
48	48A	W/S	F	61YRS	S			
49	49A	ASP	M	25YRS	S			
50	50A	W/S	M	27YRS	R	-	-	NM
51	51A	EAR/S	F	43YRS	S			
52	52A	W/S	F	2days	S			
53	53A	U	M	59YRS	R	-	-	NM
54	54A	EAR/S	F	9Days	S			
55	55A	W/S	F	11days	S			
56	56A	W/S	M	68YRS	S			
57	57A	W/S	F	53YRS	R	-	-	MP
58	59A	U	F	26YRS	R	-	+	MP
59	61A	U	F	52YRS	R	-	+	MP
60	63A	U	F	62YRS	R	-	+	NM

S/N	PATH/N	SPM	SEX	AGE	MER	MHT	CDT	MBL
61	64A	EAR/S	M	54YRS	R	-	+	MP
62	65A	EAR/S	F	7Days	R	-	-	NM
63	66A	W/S	F	17YRS	R	-	+	MP
64	67A	EAR/S	F	2Days	S			
65	68A	U	F	31YRS	R	-	-	NM
66	69A	W/S	M	43YRS	S			
67	70A	U	F	29YRS	R	-	-	NM
68	71A	W/S	M	72YRS	S			
69	72A	EYE/S	F	5Days	S			
70	73A	EYE/S	F	6Months	S			
71	74A	W/S	F	4DAYS	S			
72	75A	W/S	M	1Day	S			
73	76A	EAR/S	M	1Day	R	-	+	MP
74	77A	W/S	M	43YRS	R	-	-	NM
75	78A	W/S	M	10YRS	S			
75	78A	W/S	M	10YRS	S			
76	79A	U	F	35YRS	R	-	+	MP
77	80A	W/S	F	92YRS	S			
78	81A	EAR/S	F	7Months	S			
79	82A	W/S	F	20YRS	R	-	-	NM
80	83A	W/S	M	62YRS	R	-	-	NM
81	84A	W/S	M	60YRS	R	-	-	NM
82	85A	W/S	F	18YRS	R	-	-	NM
83	86A	W/S	M	2YRS	R	-	-	NM
84	87A	W/S	F	29YRS	R	-	-	NM
85	88A	W/S	M	4YRS	R	-	-	NM
86	89A	W/S	M	3YRS	R	-	-	NM
87	90A	W/S	M	7YRS	R	-	-	NM

MP: Metallo-beta-lactamase producer, NM: Non-Metallo-beta-lactamase producer

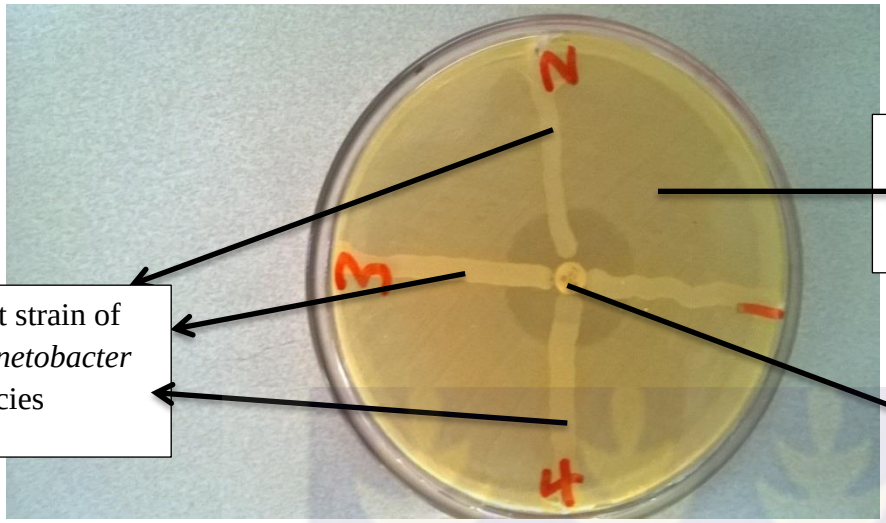
+: Positive, -: Negative. SPM: Specimen, U: Urine, W/S: Wound swab, MER: Meropenem, S: Susceptible, R: Resistant. MHT:Modified Hodge test, CDT:Combined disc test.

APPENDIX B



CARBAPENEM-EDTA DISC TEST

APPENDIX C



MODIFIED HODGE TEST